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Comparison of Changes in the Secondary Structure of Unheated, Heated, and High-Pressure-Treated β-Lactoglobulin and Ovalbumin Proteins Using Fourier Transform Raman Spectroscopy and Self-Deconvolution

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Changes in protein secondary structure and conformation of ovalbumin and β-lactoglobulin (15% protein w/w) were investigated by Fourier transform Raman spectroscopy and self-deconvolution. The amounts of α-helix, β-sheets, random coil, and β-turns in native β-lactoglobulin were 15, 54, 6, and 25%, respectively, and those for ovalbumin (41, 34, 13, and 12%) compared well with published values obtained by X-ray crystallography. The proteins were heated at 90 °C for 30 min and high-pressure-treated at 600 MPa for 20 min. Heating increased β-sheet structures in both proteins at the expense of α-helix; for β-lactoglobulin β-sheet structures increased from 54 to 70% and for ovalbumin, from 34 to 54%. Random coil increased from 6% in the native protein to 30% in high-pressure-treated β-lactoglobulin. However, for ovalbumin, the contribution from β-turns doubled in high-pressure-treated samples, with little change in random coil. Further examination of the deconvoluted amide I band in heated samples revealed several component bands. Bands at 1626 and 1682 cm^{-1} for ovalbumin and at 1625 and 1680 cm^{-1} for β-lactoglobulin were observed and are associated with aggregated, intermolecular β-sheet (β-aggregation), indicative of heat denaturation. The band seen at 1632−1640 cm^{-1} corresponded to intramolecular β-sheet structures, whereas the band at 1625 cm^{-1} is associated with exposed β-sheets (for example, β-strands with strong hydrogen bonding that are not part of the core of β-sheets). In high-pressure-treated samples bands were also observed at 1628 and 1680 cm^{-1} for ovalbumin and at 1626 and 1684 cm^{-1} for β-lactoglobulin, suggesting involvement of β-sheet structures in protein aggregation. Raman bands were observed at 1665−1670 cm^{-1} for ovalbumin and at 1663−1675 cm^{-1} for β-lactoglobulin due to random coil structures. The bands at 1650−1660 cm^{-1} due to α-helices were observed in both heated and high-pressure-treated samples. In addition, in heated samples of both ovalbumin and β-lactoglobulin, peak intensity increased for β-sheet in the amide III region, 980−990 cm^{-1}, and decreased for helix structures (900−960 cm^{-1}). In contrast, there was no peak at 1240 cm^{-1} (amide III β-sheet structures) in either high-pressure-treated ovalbumin or β-lactoglobulin, suggesting that high-pressure denaturation at 600 MPa for 20 min is less extensive than heat denaturation at 90 °C for 30 min.

KEYWORDS: Ovalbumin; β-lactoglobulin; Raman spectroscopy; high pressure; heat; secondary structure; amide I band

INTRODUCTION

Protein function plays an important role in the development of new food products; this is strongly determined by the protein structure. During processing changes occur that alter protein structure and hence functionality (*1*−*3*). Unfortunately, measurement of the actual changes in secondary structure in foods is sometimes difficult due to the requirements of sample preparation for certain methods, including medium conditions (pH, presence of minerals, concentration, type of solvent). Moreover, there is a lack of reliable methodologies for observing protein structural changes in real food samples.

Secondary structures are characterized by periodic motifs such helices, β-sheets, turns, and disordered structures. The gold standard method is X-ray crystallography, which requires the molecules to form well-ordered crystals. This is not always possible for all proteins and, indeed, most proteins used in the food industry cannot be crystallized as the desired functionality for the molecule most likely exists in solution, gel, or a colloidal

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state. In addition, X-ray crystallography requires that a suitable heavy metal be incorporated without distorting the crystal (*4*).

An alternative to X-ray crystallography is multidimensional nuclear magnetic resonance (NMR) spectroscopy, which allows structure determination in solution. However, the interpretation of the NMR spectra of large proteins is very complex and limited to small proteins (15−25 kDa), and the technique is very costly and time-consuming. These limitations have spurred the development of alternative methods that are not able to generate structures at atomic resolution but provide structural information on proteins (especially on secondary structures) at a molecular level. These methods include circular dichroism (CD) and vibrational (infrared and Raman) spectroscopy. The CD technique measures the difference in absorbance between left and right circular polarized light in the far-ultraviolet region, mainly in 230−185 nm (wavelength dependence on ellipticity). CD bands give rise to either positive or negative ellipticity produced by optically active peptide bonds for each of the standard conformational states (α-helix, *β*-sheet, and random coil). However, a limitation of the CD method is that measurements are made on highly dilute, optically clean solutions (any scattering components will affect results). Although CD is better at following changes in the α-helix region, nonperiodic, *β*-turn structures cannot be distinguished by this method (*3*, *5*).

Vibrational techniques such as Fourier transform infrared (FTIR) and Raman spectroscopies may be used in the study of food proteins that are turbid or particulate either in nature or as a result of processing. The two techniques are complementary as infrared absorption requires a change in the intrinsic dipole moment with molecular vibrations and Raman scattering depends on changes in polarizability of the molecule (*6*). Polar functional groups such as C═O and O−H have strong infrared stretching vibrations, whereas intense Raman lines are associated with nonpolar groups such as C═C and S−S.

The use of FTIR analysis in the estimation of protein secondary structure in lyophilized samples or D_2O solutions is limited by the formation of possible artifacts and errors (*7*−*9*). Water has a strong infrared absorption band (1650 cm^{-1}) that interferes in the infrared spectrum (amide I band region) of aqueous protein samples. Therefore, specialized sampling techniques and careful baseline subtraction are required. In contrast, water is a poor Raman scatterer, and therefore direct analysis of aqueous samples and foods is feasible (*10*).

The use of Raman spectroscopy in the determination of secondary structures is based on the premise that protein and peptide structures contain specific absorption bands, particularly in the amide I region (1600−1700 cm^{-1}), which are due to contributions from C═O stretching vibration of the amide group, coupled with the in-plane N−H bending and C−N stretching vibration (*11*). The exact frequency of the amide I band depends on the nature of hydrogen bonding between C═O and N−H moieties. In proteins, each of the amide groups is involved in a secondary structure of some type, either a helix, *β*-sheet, or random structure. Because each of these secondary structural motifs is associated with a characteristic hydrogen-bonding pattern between amide C═O and N−H groups, it follows that each type of secondary structure will give rise to characteristic amide I absorptions. It is this separation of amide I absorptions that underlies the determination of protein secondary structure by Raman spectroscopy.

The relationship between the position of the amide I band and the type of secondary structure may be best observed from the infrared spectra of homopolypeptides that adopt well-defined and often highly homogeneous secondary structures (*12*, *13*). However, in heteropolypeptides and in real food protein systems, there are a variety of domains containing polypeptide fragments in different conformations (*8*). Therefore, the amide I band contours of proteins are usually complex composites that consist of a number of overlapping component bands representing α-helices, *β*-sheets, turns, and random structures.

Resolution-enhancement techniques by band narrowing allow the identification of these otherwise hidden component bands. Such analysis requires that the correspondence between the resolved component of the amide I band and specific polypeptide structures be established. A basis for this has been provided by the analysis of X-ray crystallography data and corresponding infrared spectra of proteins of known three-dimensional structure, making the assignment of band conformation possible (*14*). Previous studies (*15*−*18*) have revealed that the composite amide I band can be broken down into a number of individual components. Several mathematical procedures such as Fourier self-deconvolution (FSD) and derivatization have been developed to allow visualization of overlapping bands following manipulation of spectra (*19*, *20*).

FSD is used together with curve-fitting analysis, which enables not only resolution of individual component bands but calculation of the area of each of the bands. Although in many cases the deconvoluted amide I bands have been used to determine secondary structure by curve fitting, it should be recognized that resolution-enhanced spectra, especially derivative spectra, do not reproduce true band intensities and relative component fractions cannot be obtained directly from them. Despite these shortcomings, both methods are extremely useful for identifying component frequencies in complex spectra.

The effect of different conditions (salt concentration, pH, effect of pressure, disulfide bond exchange) on the secondary structure of *β*-lactoglobulin during gel formation has been reported using circular dichroism (*21*) and infrared spectroscopy during pressure and temperature treatment (*22*−*24*). For ovalbumin, changes in secondary structure using different methods (*25*) reported an increase in the intermolecular *β*-sheet structure during thermal aggregation using Raman spectroscopy. Clark et al. (*26*) reported a similar observation by an infrared and laser Raman spectroscopic study during heat-induced gelation of a number of globular proteins. However, these findings are based on qualitative data, and more recent studies (*27*, *28*) used CD spectroscopy to quantitatively estimate various secondary fractions. Limited irreversible changes in secondary structure during pressure denaturation of ovalbumin using CD and FTIR spectroscopy have been reported (*29*).

Although deconvolution studies of the CH-stretch region for heated *β*-lactoglobulin and lysozyme have been reported (*1*), no studies to date exist on the use of Raman spectroscopy to analyze the secondary structure by deconvolution of the amide I band of whey and egg albumen proteins using temperature and pressure denaturation. In this study, quantitative changes in the secondary structure of *β*-lactoglobulin and ovalbumin were investigated through the deconvolution of the amide I band, whereas the overall secondary structure of both *β*-lactoglobulin and ovalbumin has been established by X-ray crystallography (*30*, *31*). The objectives of this study were to establish the role of Raman spectroscopy in the quantitative determination of changes in protein secondary structure during processing.

MATERIALS AND METHODS

Materials. Deuterium oxide (D, 4561, 99.9% atom D), ovalbumin, and *β*-lactoglobulin were obtained from Sigma-Aldrich Chemical Co., Poole, Dorset, U.K.

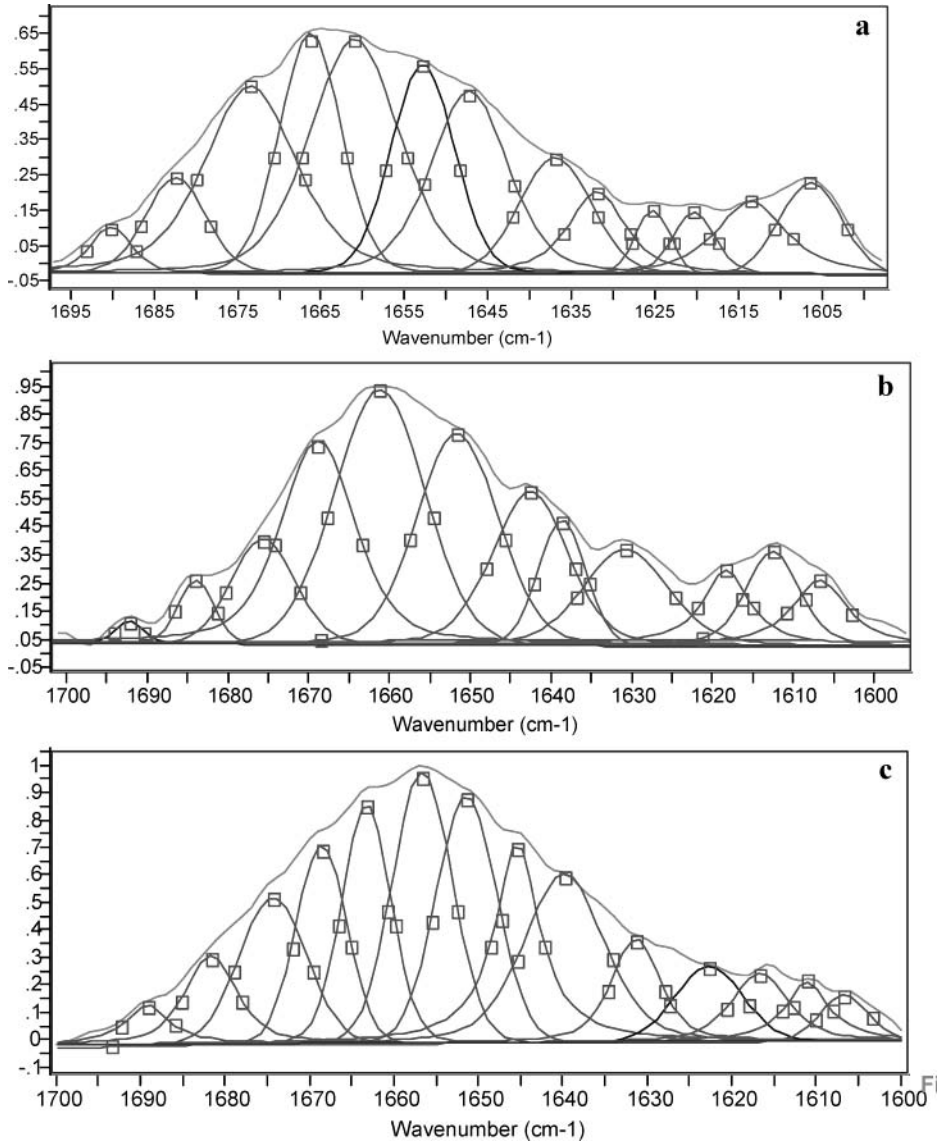

Figure 1. FT Raman spectra, amide I region (1600–1700 cm^{-1}), of (**a**) native, (**b**) heated, and (**c**) high-pressure-treated (600 MPa for 20 min) ovalbumin (15% w/w) dissolved in D_2O; the peaks were fitted after deconvolution.

Methods. *Sample Preparation.* Solutions of ovalbumin and β-lactoglobulin 15% (w/w) were prepared in D_2O. Heat-treated gels of the above protein solutions were made as follows. Protein solution (7 mL) was poured into stainless steel tubes 50 mm long and 15 mm in diameter and heated at 90 °C for 30 min to form gels. The gels were stored overnight at 4 °C to promote network formation (*32*). Unheated and heated samples were placed in NMR tubes (5 mm diameter, precision grade, Aldrich Chemical Co., Milwaukee, WI) and analyzed by FT Raman spectroscopy.

Pressure-Treated Samples. The solutions for high-pressure-treated gels were prepared according to the same method as heat-treated gels except the samples were placed in Visking tubing tied at both ends and heat sealed in a high-pressure polythene bag (Crayovac, W. R. Grace Limited, Cambridge, U.K.). The samples were introduced into the working chamber (300 mL capacity) of a Stansted Food Lab high-pressure rig (Stansted Fluid Power, Ltd., Stansted, Essex, U.K.) containing low-compressibility fluid (2:8 mixture of castor oil/ethanol). The equipment was installed in an air-conditioned laboratory at 20 °C. The temperature in the pressurization chamber increased during compression, reaching a maximum after 2 min. The maximum temperature achieved in all treatments was 30 °C, corresponding to the treatment at 400, 500, and 600 MPa), then decreased to 20 °C in 0.5 min, and remained stable at 20 °C until the end of the cycle. The compression and decompression times were about 1−2 and 0.5 min, respectively. The samples were stored at 4 °C similarly to the heat-treated ones. For samples of <10 mL, the Stansted Micro Food-Lab, model S-FL-085-9W, high-pressure rig was used.

FT Raman Spectroscopy, Data Collection, and Analysis. Raman spectra were recorded at 4 °C on a Perkin-Elmer 2000 FT Raman spectrometer (Beaconsfield, Buckinghamshire, U.K.). Spectral resolution was set at 4 cm^{-1} and laser power at 1600 mW, and the data presented are based on 128 co-added spectra. Frequency calibration of the instrument was undertaken using the sulfur line at 217 cm^{-1}. The spectra were analyzed using Grams 32 software (Galactic Industries Corp., Salem, NH). The protein spectra obtained were baselined, and the intensity was normalized using the phenylalanine peak at 1004 cm^{-1} (*6*, *33*). The major bands in the spectra, related to vibrational motions of various side chains or polypeptide backbone, were assigned by comparison with Raman spectra of proteins that have been reported in the literature (*2*, *33*). To calculate the secondary structure components, the amide I region (1600−1700 cm^{-1}) was truncated and deconvoluted using a nonlinear least-squares curve-fitting subroutine, which included mixed Gaussian and Lorenztian components. The percentage of each secondary structure component, that is, α-helix, β-sheet, β-turns, and random coil (unspecified), was determined as follows (*34*):

$$\%\ \text{of secondary structure} = \frac{\text{area of secondary structure}}{\text{area of amide I band}}$$

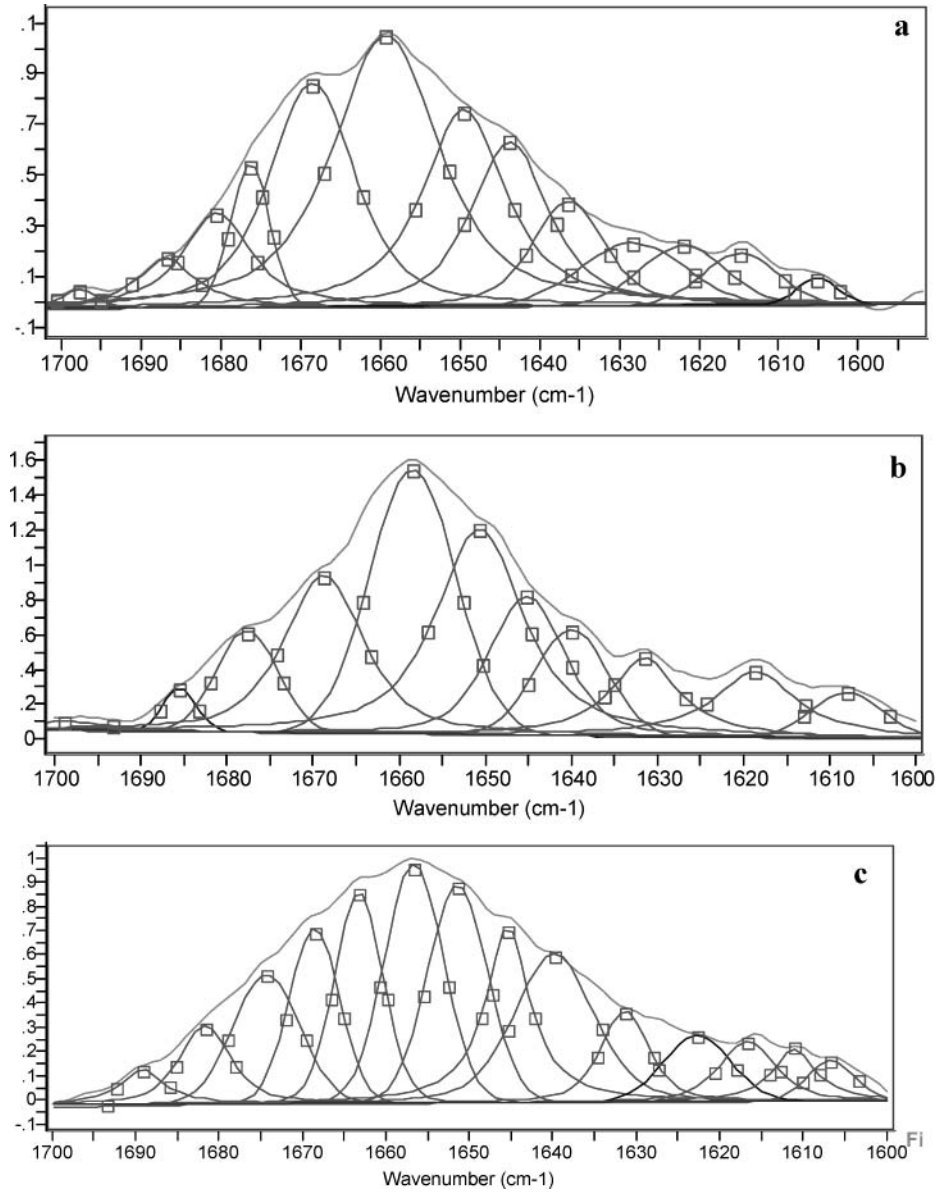

Figure 2. FT Raman spectra, amide I region (1600–1700 cm^{-1}), of (**a**) native, (**b**) heated, and (**c**) high-pressure-treated (600 MPa for 20 min) β-lactoglobulin (15% w/w) dissolved in D_2O; the peaks were fitted after deconvolution.

RESULTS AND DISCUSSION

The results of deconvolution of the amide I bands of unheated, heated, and high-pressure-treated (600 MPa for 20 min) ovalbumin are shown in **Figures 1** and **2** for β-lactoglobulin, respectively. There are marked differences in the secondary structures of the proteins subjected to various treatments. The spectra of unheated ovalbumin exhibited 12 major bands related to secondary structure; the assignments of the bands were made on the basis of previous infrared studies for over 50 proteins in water solutions (*35*, *36*). In ovalbumin the bands at 1636, 1632, 1625, and 1620 cm^{-1} can be assigned to low-frequency β-sheets and those as 1682 and 1673 cm^{-1} to β-turns, whereas the band at 1690 cm^{-1} is associated with high-frequency β-turns. These bands are clearly discernible as shoulders. The presence of the 1625 cm^{-1} band has been reported by several workers (*37*, *38*). The α-helix band was centered at 1648 and 1652 cm^{-1}. The two low-frequency bands near 1605 and 1614 cm^{-1} are caused by aromatic side chains of the individual amino acids (*39*) and do not contribute to the secondary structure.

Quantitative analysis revealed that unheated ovalbumin had a composition corresponding to 41% α-helix, 34% β-sheet, 12% β-turns, and 13% random coil. These results are consistent with values determined by X-ray crystallography showing 35% α-helix and 45% β-sheet (*29*, *31*, *40*); FITR in D_2O by factor analysis indicated 35% α-helix and 44% β-sheet, and CD studies showed that native ovalbumin is made up of 40−49% α-helix, 30% β-sheet, and 28% unordered structure (*27*, *28*).

β-Lactoglobulin also exhibited bands at 1643, 1637, 1632, 1626, and 1622 cm^{-1} assigned to low-frequency β-sheets and at 1682 and 1673 cm^{-1} assigned to β-sheets. The band at 1690 cm^{-1} is associated with high-frequency β-turns. Other studies (*15*, *39*) support these findings. Quantitaive analysis of unheated β-lactoglobulin showed 15% α-helix, 54% β-sheet, 25% turns, and 6% random coil. This is consistent with previous Raman studies [$\sim$54% β-sheet, $\sim$10% α-helix, and 32% unspecified (*15*, *41*)]. CD and infrared studies suggest an α-helix content of 10−15% and a β-sheet content of $\sim$50%, with turns accounting for 20% and the remaining 15% representing amino acid residues in a random nonrepetitive arrangement without a

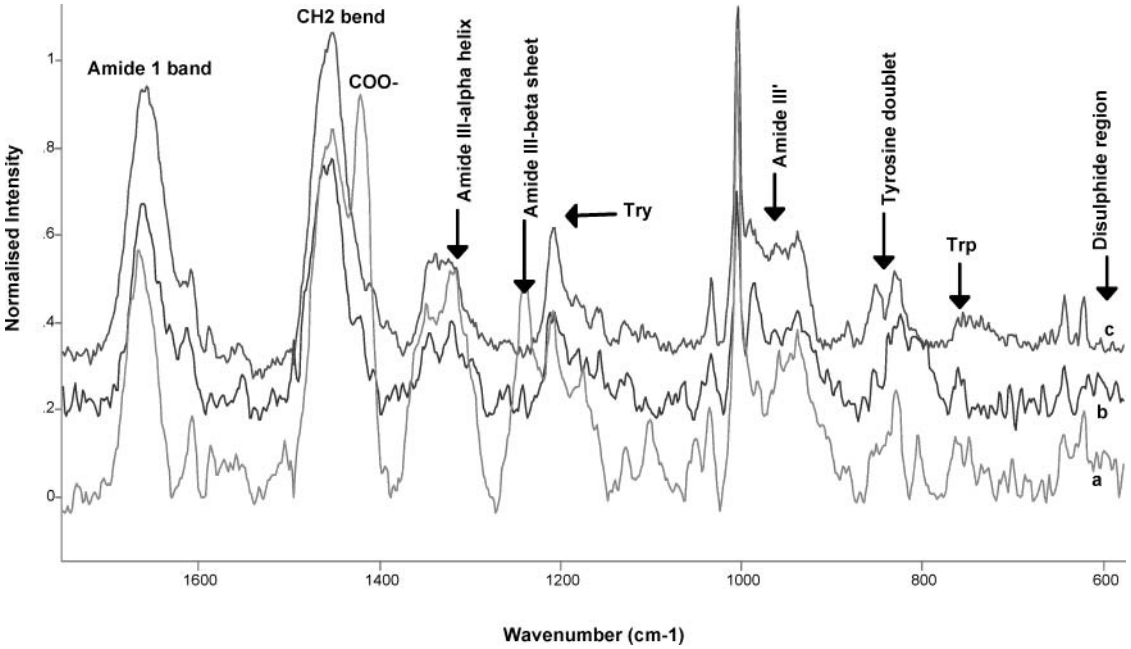

Figure 3. FT Raman spectra (500–1800 cm^{-1}) of (**a**) native, (**b**) heated, and (**c**) high-pressure-treated (600 MPa for 20 min) ovalbumin (15% w/w) dissolved in D_2O. The spectra were baselined and normalized to the phenylalanine peak at 1004 cm^{-1}.

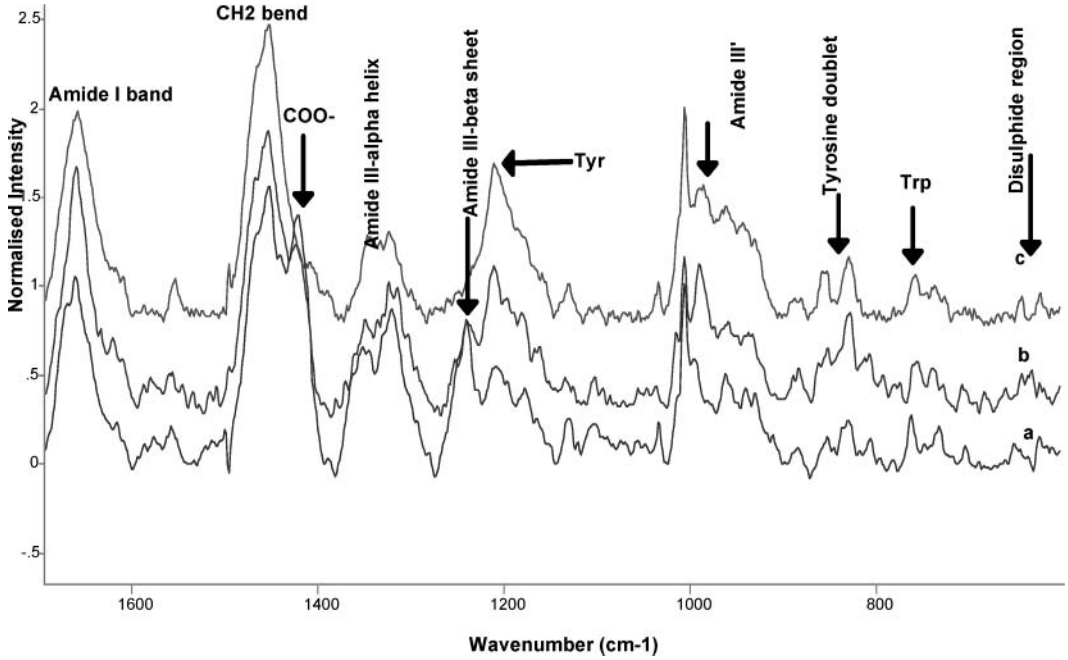

Figure 4. FT Raman spectra (500–1800 cm^{-1}) of (**a**) native, (**b**) heated, and (**c**) high-pressure-treated (600 MPa for 20 min) β-lactoglobulin (15% w/w) dissolved in D_2O. The spectra were baselined and normalized to the phenylalanine peak at 1004 cm^{-1}.

well-defined structure (*15*, *23*, *42*, *43*). These results indicate that the secondary structure of β-lactoglobulin is dominated by a β-sheet structure, whereas ovalbumin is dominated by an α-helix secondary structure.

The FT Raman spectra for native, heated (90 °C for 30 min), and high-pressure-treated (600 MPa for 20 min) ovalbumin and β-lactoglobulin are shown in **Figures 3** and **4**. The high helical content in unheated proteins is also reflected in the amide III′ region, 900−970 cm^{-1}, in both ovalbumin and β-lactoglobulin. In addition, the amide III′ α-helix band at 1318 cm^{-1} had a higher peak intensity in both ovalbumin and β-lactoglobulin, whereas the peak at 1239 cm^{-1} amide III′ β-sheet band had a higher peak intensity in β-lactoglobulin (0.79) compared to ovalbumin (0.13) ($p < 0.05$). The high peak intensity at 1239 cm^{-1} of the amide III′ β-sheet band supports the observation above that β-lactoglobulin has high β-sheet content; our previous work on heated whey isolate and egg albumen is consistent with these findings (*44*).

In this study, major changes were observed in the heated samples of ovalbumin and β-lactoglobulin, especially a decrease in the band at 1648−1658 cm^{-1}, which is attributed to the α-helical structure (**Table 2**). Previous studies reported similar findings (*23*, *33*, *39*). For heated ovalbumin the composition was as follows: 16% α-helix, 51% β-sheet, 20% turns, and 13% random structure. Heated β-lactoglobulin had 10% α-helix, 70% β-sheet, 14% β-turns, and 6% random coil. This decrease in

Table 1. Tentative Peak Assignments of Deconvoluted Amide I Components

structure	amide I wavenumber[a] (cm^{-1})
aggregated strands	1610–1620
β-sheet (low frequency)	1620–1640
β-sheet (high frequency)	1670–1680
β-turns	1658–1670
	1680–1699
α-helix	1650–1660
random coil	1660–1670

[a] Data adapted from refs *5*, *23*, and *39*.

Table 2. Percentage Breakdown of Different Fractions of Secondary Structures in Unheated, Heated, and High-Pressure-Treated Ovalbumin and β-Lactoglobulin Proteins

	% secondary structures			
	α-helix	β-sheet	β-turns	random coil
ovalbumin				
native	41	34	12	13
heated	16	51	20	13
high-pressure-treated	34	30	25	11
β-lactoglobulin				
native	15	54	25	6
heated	10	70	14	6
high-pressure-treated	10	37	23	30

helical structures has been observed by other workers (*27*, *33*, *45*−*47*). There was a relatively weak band in β-lactoglobulin in the amide III′ at 940 cm^{-1}. This may be due to the low α-helix content of this protein; $\sim^1/_{10}$ of the total residues are involved in α-helix structures (*31*). There was a substantial increase in the 1674 cm^{-1} band attributed to β-sheet structures by some workers (*39*) and to turns by others (*14*).

In addition, there was an increase in protein aggregation bands at 1684 and 1618 cm^{-1} attributed to the intermolecular hydrogen-bonded β-sheet structures (*37*, *48*). The presence of the band at 1618−1620 cm^{-1} in both heated ovalbumin and β-lactoglobulin indicated the disruption of hydrogen bonds within some secondary structure and formation of new stronger hydrogen bonds that are associated with aggregation, which is the formation of intermolecular hydrogen bonds (*49*). The development of such a band has been observed with the formation of gels in some proteins such as serum albumin (*26*) and in glycinin (*50*), indicating the unfolding of the compact structure and the formation of β-aggregation. The presence of the 1680 cm^{-1} band suggests that these aggregates consist of antiparallel β-strands. There was a decrease in the peak intensity of the 1318 cm^{-1} amide III band that corresponds to α-helix structure in both ovalbumin and β-lactoglobulin; a similar trend was observed in all heated proteins in our previous study (*44*). The absence of the band at 1240 cm^{-1} (amide III β-sheet) in both high-pressure-treated ovalbumin and β-lactoglobulin indicates that subtle differences in protein conformation may be responsible for the presence of aggregation bands (*51*).

In this study, comparison of native, heated, and high-pressure-treated ovalbumin and β-lactoglobulin shows major differences. Quantitative analysis of high-pressure-treated ovalbumin indicated 34% α-helix, 30% β-sheet, 25% β-turns, and 11% random structure; these data differ from those for native ovalbumin above (41% α-helix, 34% β-sheet, 12% β-turns, and 13% random coil). There is twice the number of β-turns in the high-pressure-treated ovalbumin compared to the native form.

Compared to unheated ovalbumin, there was an increase in β-sheet structure from 41 to 51% in heated ovalbumin and an increase in random coil from 13% in native ovalbumin to 20% in heated ovalbumin. In addition, there was some difference in the α-helix content of native (41%) and high-pressure-treated ovalbumin (34%). Some reports have indicated that the spectrum of high-pressure-denatured protein has more features of the native protein than that of the temperature-denatured protein (*51*). This suggests that the unfolding for pressure denaturation for treatments tested (400−800 MPa) is less extensive than unfolding for temperature denaturation (90 °C for 30 min). Pressure-induced denaturation with gel formation has been observed in a number of studies (*52*−*54*). Questions remain as to whether the gel formation is also accompanied by the appearance of bands indicative of intermolecular hydrogen bonds as in the case of temperature denaturation.

Comparison of native β-lactoglobulin with high-pressure-treated samples showed that pressure induced more changes in β-lactoglobulin compared to ovalbumin. High-pressure-treated β-lactoglobulin had 10% α-helix, 40% β-sheet, 23% β-turns, and 30% random structure compared with 15% α-helix, 54% β-sheet, 25% turns, and 6% random coil in the native form. The increase in random structure in high-pressure-treated β-lactoglobulin at the expense of β-sheet and α-helix has been reported in the literature (*24*). However, heat denaturation indicated 10% α-helix, 70% β-sheet, 14% β-turns, and 6% random coil ($p < 0.05$). This indicates that heat denaturation results in the increased formation of β-sheet structures compared to 37% in high-pressure-treated β-lactoglobulin. In the heated amide I band of β-lactoglobulin the shoulder at 1645 cm^{-1} assigned to β-sheet is more pronounced than in the high-pressure-treated one.

In conclusion, in this study we have shown by using Fourier transform Raman spectroscopy and self-deconvolution that the amide I band of unheated, heated, and high-pressure-treated ovalbumin and β-lactoglobulin can be resolved into component bands. The results showed some similarities in the conformation of the amide I band; the bands around 1636 cm^{-1} (low-frequency β-sheets) were present in both heated and pressure-treated ovalbumin and β-lactoglobulin. Interestingly, like most proteins, there was more than one β-sheet component; in the 1680−1690 cm^{-1} region (high-frequency β-sheets) indicative of protein aggregation and in hydrogen bonding between β-strands. Heat-induced gelation produced a greater increase in β-sheet structure in both heated ovalbumin and β-lactoglobulin compared to high-pressure-treated samples. In addition, the absence of the amide III′ β-sheet structure at 1239 cm^{-1} in both high-pressure-treated ovalbumin and β-lactoglobulin samples gave further evidence that the involvement of β-sheet structures is less intense in high-pressure-treated proteins gels, which may explain why pressure-treated gels were found to be weaker in previous studies (*44*). There was a trend toward an increase in β-sheet structure at the expense of α-helix structures in all of the heated samples, whereas in high-pressure-treated samples, changes in secondary structures showed an increase in random structure in β-lactoglobulin and a decrease in the α-helix. On the other hand, ovalbumin showed an increase in β-turns without much change in α-helix and β-sheets; these differences in ovalbumin and β-lactoglobulin may reflect the differences in the way the two proteins respond to pressure treatment. This study has highlighted that heat and pressure unfolding and denaturation differed with respect to secondary structure changes.

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