Pressure-Induced Conformational Changes of β-Lactoglobulin by Variable-Pressure Fourier Transform Infrared Spectroscopy

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Pressure-induced conformational changes in D_2O solutions of the two genetic variants of β-lactoglobulin A (β-lg A) and β-lactoglobulin B (β-lg B) and an equal mixture of both variants (β-lg A+B) were studied by employing variable-pressure Fourier transform infrared (VP-FTIR) spectroscopy. Changes in the secondary structure of β-lg A were observed at lower pressure compared to β-lg B, indicating that β-lg A had a more flexible structure. During the decompression cycle β-lg A showed protein aggregation, accompanied by an increase in α-helical conformation. The changes in the secondary structure of β-lg B with the pressure were minor and for the most part reversible. Upon decompression no aggregation in β-lg B was observed. Increasing the pressure from 0.01 to 12.0 kbar of a solution containing β-lg A+B resulted in substantial broadening of all major amide I bands. This effect was partially reversed by decreasing the hydrostatic pressure. β-lg A+B underwent less aggregate formation than β-lg A, possibly as a result of protein−protein interactions between β-lg A and β-lg B. Hence, it is likely that the functional or biological attributes of β-lg proteins may be affected in different ways by hydrostatic pressure.

Keywords: *Fourier transform infrared spectroscopy; β-lactoglobulin; pressure; secondary structure*

INTRODUCTION

Proteins play a major role in determining the sensory, textural, and nutritional characteristics of various food products (Kinsella, 1997). Increasing interest in these functional characteristics of proteins in foods has heightened the need for assessment of the stability of proteins as determined by their conformation. The native conformation of proteins can be converted to a denatured state by changing the chemical or physical condition of the medium via changes in pH, temperature, or pressure (Casal et al., 1988). The suscetibility of a protein to denaturation is dependent upon its primary and secondary structures (McSwiney, 1994; Marshall, 1982).

Whey proteins, which comprise 20% of milk proteins, are extensively used in food products due to their functional properties such as solubility, gelation, and coagulation (Pour-El, 1981). β-Lactoglobulin (β-lg) is the dominant whey protein in milk and is largely responsible for the physicochemical characteristics of whey proteins (Hambling et al., 1992). Under physiological conditions, β-lg exists as a dimer because of electrostatic interaction between aspartate (Asp) 130 and glutamate (Glu) 134 of one monomer with lysyl residues of another monomer (Marshall, 1982). Circular dichroism (CD) and infrared studies indicate that β-lg consists of an α-helical content of 10−15%, a β-structure content of 50% with turns accounting for 20%, and the remaining 15% representing amino acid residues in a random nonrepetitive arrangement without well-defined structure (Casal et al., 1988; Timasheff et al., 1966; Susi and Byler, 1986).

To date, seven genetic variants of β-lg have been characterized. The major genetic variants of β-lg present in bovine milk are β-lg A and β-lg B. Their primary structures differ only at residues 64 (β-lg A/β-lg B, Asp/Gly) and 118 (β-lg A/β-lg B, Val/Ala) (Swaisgood, 1982). A number of studies (Huang et al., 1994a,b; McSwiney et al., 1994) have reported that minor differences in the primary structures of β-lg A and β-lg B resulted in a significant difference in gelation temperature and rheological properties of the gel formation. β-lg has five cysteine residues that are disulfide-bonded and one (Cys 121) which remains single and free (Somero, 1992). Aggregation of β-lg is known to be contingent upon the propagation of a sulfhydryl−disulfide interchange, resulting from the interfacial denaturation by heat or high pressure (Monahadan, 1995; Funtenberger, 1997). Tanaka et al. (1996) showed that the reactivity of the SH group of β-lg B, which is buried in the protein, increased with increasing pressure as a result of exposure of the SH group to the protein surface. Tanaka et al. concluded that inter- and intramolecular reactions of the SH group with increasing pressure may be the main causes for irreversible pressure-induced denaturation of β-lg. β-lg B has a greater number of Glu residues in the less defined or unordered structures than β-lg A (Eigel et al., 1984; Papiz et al., 1986). An assessment of aggregates and gel formation after hydrolysis of β-lg with Glu- and Asp-specific proteases indicated that (a) the greater number of residues in β-lg B versus β-lg A impeded aggregate formation and (b) electrostatic interactions between amino groups and carboxylic acids of Glu residues play an essential role in aggregate

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formation (Otte et al., 1997). The differential susceptibility of genetic variants of β-lg to unfolding and aggregation under high pressure, however, has not been studied.

In recent years, pressure treatment of food has received interest because of its advantage as an alternative to heat treatment for sterilization or gel formation (Tucker and Clark, 1990; Camp and Huyghebaert, 1995; Lagoueyte and Lagaunde, 1995). Previous work has demonstrated that pressure treatment can induce dissociation and denaturation of oligometric proteins by (a) introducing water in the void space between individual protein subunits and (b) causing a decrease in the volume of the protein that is accompanied by the hydration of the subunits (Weber, 1987; Wong et al., 1989). Fluorescence and CD spectroscopy have demonstrated that the bovine proteins α-lactalbumin (α-lc) and β-lg showed different responses to pressure, probably reflecting the degree of compactness of their pressure-perturbed structures (Tanaka et al., 1996). It is not known, however, whether minor differences in the primary structures of the two main genetic variants of β-lg influence the impact of high-pressure treatment on the secondary structure of the protein.

As a part of our ongoing series of Fourier transform infrared (FTIR) spectroscopy studies on the effect of changes in physicochemical conditions on the secondary structure of whey proteins, the present study describes the effect of increasing hydrostatic pressure (up to 12.0 kbar) on the secondary structure in the amide I band (1600−1700 cm^{-1}) in D_2O solutions of the two major genetic variants of β-lg A and β-lg B and a 50% mixture of the two variants (β-lg A+B).

MATERIALS AND METHODS

β-lg A, β-lg B, and a 1:1 mixture of the two variants, β-lg A+B, were purchased from Sigma Chemical Co. (St. Louis, MO). D_2O was obtained from CDN Isotopes Co. (Pointe Claire, PQ, Canada). Solutions of each protein (17% w/v) were prepared <1 h prior to the start of the infrared measurements. All of the variable-pressure studies were carried out in triplicate. A small amount of the protein solution (∼1 μL) was placed together with α-quartz powder in the 0.5 mm diameter hole of a 0.20 mm thick stainless steel gasket mounted on a diamond anvil cell (High-Pressure Diamond Optics, Inc., Tucson, AZ). The infrared spectra were recorded on a Nicolet 510 FTIR spectrometer equipped with a liquid-nitrogen-cooled mercury−cadmium−telluride (MCT) detector. The infrared spectra were recorded at a resolution of 4 cm^{-1} and encoding interval of 2 cm^{-1}. A total of 512 scans were co-added. The band assignments in the amide I area (1600−1700 cm^{-1}) in D_2O solution were based on band assignments from the previous FTIR and variable-pressure/FTIR studies (Susi and Byler, 1986; Wong et al., 1985; Susi et al., 1967).

The pressure was calculated from quartz band frequencies according to the equation

$$P\ (\text{kbar}) = \alpha_1 \Delta\nu + \alpha_2 \Delta\nu^2$$

where $\alpha_1 = 1.2062$, $\alpha_2 = 0.1516$, and $\Delta\nu$ is the measured frequency shift with respect to the α-quartz band measured at atmospheric pressure (Wong et al., 1985; Wong and Hermans, 1988). After the highest pressure of ∼12.0 kbar at 25 °C was attained, the pressure was lowered gradually to the starting point. Fourier self-deconvolution using a bandwidth of 13 cm^{-1} and an enhancement factor of 1.8 was carried out prior to analysis of the amide I band (Casal et al., 1988).

RESULTS

The relatively strong amide I band in the infrared spectra of polypeptides and proteins is widely used for the determination of secondary structure (Susi et al., 1967; Parker, 1971). This band is located in the frequency region between 1600 and 1700 cm^{-1} and is due to the in-plane C═O stretching vibration weakly coupled with C−N stretching and in-plane N−H bending (Susi, 1969). The amide I band usually appears as a broad band consisting of several maxima that can be attributed to various specific types of secondary substructures of globular proteins (Wong and Hermans, 1988). The principal components of the amide I band observed after Fourier self-deconvolution of the spectra of β-lg A, β-lg B, and β-lg A+B before pressurization or after decompression are shown in Tables 1−3. The band assignments in these tables are based on previous studies of β-lg by infrared spectroscopy at ambient pressure (Casal et al., 1988; Susi, 1969; Krimm and Bandekar, 1986).

Table 1. Frequencies (cm^{-1}) and Band Assignments of β-lg A in the Amide I (1600−1700 cm^{-1}) Region before Pressure Treatment and after Decompression in D_2O

before pressure treatment	after decompression	band assignment[a]
1690	1689	antiparallel β-turns
1680	1680	antiparallel β-sheet
1669	*b*	antiparallel β-sheet
1657	1657	α-helix
1649	1649	α-helix and random coil
1635	1637	antiparallel β-sheet
1625	1626	β-sheet
1613	1612	β-sheet

[a] Casal et al. (1988); Susi and Byler (1988); Boye et al. (1996); Krimm and Bandekar (19860. [b] Not observed.

Table 2. Frequencies (cm^{-1}) and Band Assignments of β-lg B in the Amide I (1600−1700 cm^{-1}) Region before Pressure Treatment and after Decompression in D_2O

before pressure treatment	after decompression	band assignment[a]
1692	1692	
1681	1683	antiparallel β-sheet
1646	1648	unordered and α-helix
	1658[b]	α-helix
1652	1648	α-helix and random coil
1635	1634	β-sheet
1625	1625	β-sheet
1614	1612	β-sheet

[a] Casal et al. (1988); Susi and Byler (1988); Boye et al. (1996); Krimm and Bandekar (1986). [b] Structure appeared.

Table 3. Frequencies (cm^{-1}) and Band Assignments of β-lg A+B in the Amide I (1600−1700 cm^{-1}) Region before Pressure Treatment and after Decompression in D_2O

before pressure treatment	after decompression	band assignment[a]
1692	*b*	turns
1680	1679	antiparallel β-sheet
1650	1649	α-helix and random coil
1635	1634	antiparallel β-sheet
1625	1626	β-sheet
1614	1612	β-sheet

[a] Casal et al. (1988); Susi and Byler (1988); Boye et al. (1996); Krimm and Bandekar (1986). [b] Not observed.

Effect of Pressure on β-lg A. *Compression Phase.* Examination of the amide I band in the infrared spectrum of β-lg A (Figure 1a) at 0.9 kbar pressure in the diamond anvil cell reveals seven bands. Increasing the hydrostatic pressure from 0.09 to 2.0 kbar results in changes in the relative intensities of these bands. The bands at 1635 and 1680 cm^{-1}, attributed to antiparallel

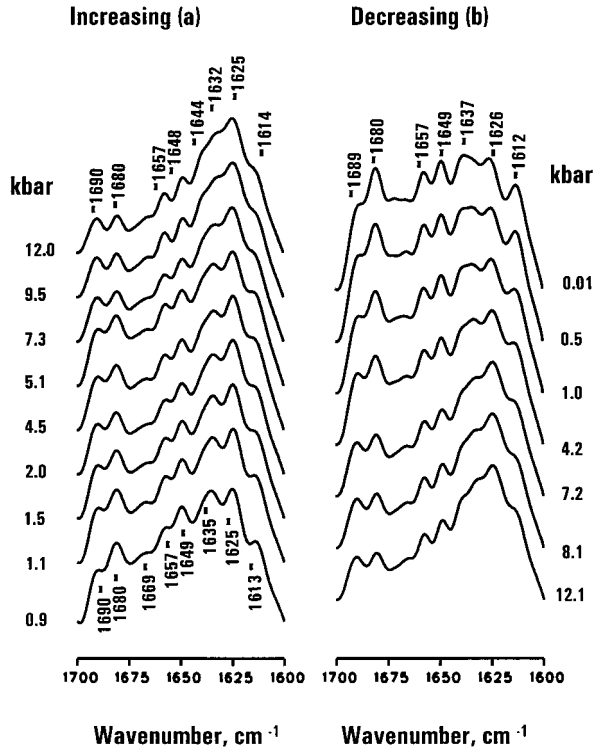

Figure 1. Infrared spectra of β-lg A (17% w/v) in D_2O in the amide I region at the indicated pressures. The spectra were recorded upon compression (a) and decompression (b) and are resolution enhanced by Fourier self-deconvolution with a band-narrowing factor of 1.8 and a half-bandwidth of 13 cm^{-1}.

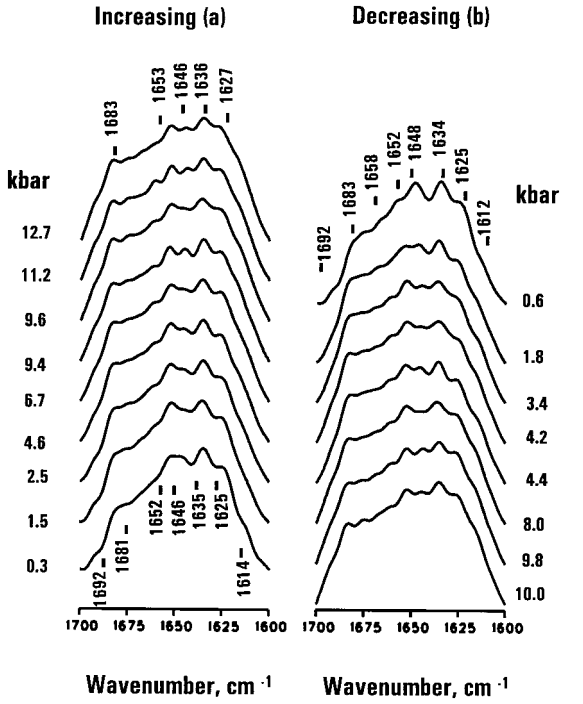

Figure 2. Infrared spectra of β-lg B (17% w/v) in D_2O in the amide I region at the indicated pressures. The spectra were recorded upon compression (a) and decompression (b) and are resolution enhanced by Fourier self-deconvolution with a band-narrowing factor of 1.8 and a half-bandwidth of 13 cm^{-1}.

β-sheet structure (Surewicz and Mantsch, 1988; Susi and Byler, 1983, 1988; Arrondo, 1988; Casal et al., 1988), decrease in intensity relative to the band at 1625 cm^{-1}, assigned to extended β-sheet structure. The change in the relative intensities of the 1635 and 1625 cm^{-1} bands has been associated with a dimer−monomer transition by Casal et al. (1988), who examined the effect of pH on the dissociation of β-lg B by FTIR spectroscopy. The band at 1649 cm^{-1}, assigned to α-helical structure, remains mostly unchanged in intensity with increasing pressure; however, a band at 1657 cm^{-1}, which is assigned to another α-helical structure (Casal et al., 1988; Surewicz and Mantsch, 1988; Susi and Byler, 1983), increases in intensity with increasing pressure. An increase in pressure to 7.3 kbar results in a decrease of the band intensity at 1614 cm^{-1} attributed to protein aggregation. Above 7.3 kbar the band at 1635 and 1690 cm^{-1} becomes broader and decrease in intensity relative to the 1625 cm^{-1} band. This trend is accompanied by the appearance of a shoulder at 1644 cm^{-1} attributed to unordered structure.

Decompression Phase. Figure 1b shows a plot of the amide I band of β-lg A in the amide I region recorded during the decompression of the sample. A major change observed with decreasing pressure is a shift of the 1635 cm^{-1} band to 1637 cm^{-1} and the dramatic increase relative to the 1626 cm^{-1} with decreasing pressure, indicative of the re-formation of the dimer. The 1614 cm^{-1} band shifts to 1612 cm^{-1} after decompression and increases in intensity relative to the 1626 cm^{-1} band, indicating a net increase in the aggregation of the protein during and after depressurization (Clark et al., 1981). The observed shifts of the 1635 and 1614 cm^{-1} bands are accompanied with increases in the broadness and intensity of the related bands relative to 1626 cm^{-1} band, which indicates real changes in the structure of the protein. The 1657 cm^{-1} band, attributed to α-helical components, also shows a net increase after decompression. The 1690 cm^{-1} band after decompression shifts to 1689 cm^{-1} with the same intensity, indicative of H−D exchange.

Effect of Pressure on β-lg B. *Compression Phase.* Figure 2a shows a plot of the amide I band of β-lg B as a function of increasing hydrostatic pressure. Five bands are seen at 0.3 kbar pressure, with the most prominent band at 1635 cm^{-1} attributable to β-sheet structure. Because the obtained spectrum is underdiamond anvil cell and at 0.3 kbar, the spectrum we obtained is to some extent different from what Casal et al. (1988) obtained in ambient pressure. Increasing the hydrostatic pressure to 2.5 kbar results in minor changes in the amide I band profile. A further increase in the pressure to 9.4 kbar results in (a) the better resolution of the band at 1646 cm^{-1}, which is attributed to unordered structures, and (b) an increase in the intensity of the 1625 cm^{-1} band relative to the intensity of the band at 1635 cm^{-1}. An additional increase in the pressure to 12.7 kbar results in a further decrease of the 1635 cm^{-1} band relative to the intensities of the 1625 and 1646 cm^{-1} bands. There is a complete absence of the 1614 cm^{-1} band with increasing hydrostatic pressure, indicating that no aggregate formation had taken place. The band at 1614 cm^{-1} appears as a weak shoulder and disappears by increasing the pressure to 1.5 kbar.

Decompression Phase. Figure 2b shows a stacked plot of the amide I band of β-lg B as a function of decreasing hydrostatic pressure. Decreasing the pressure results in a major reversal of the changes observed with increasing pressure with the notable exception of the 1652 cm^{-1} band. The 1652 and 1646 cm^{-1} bands shift with decreasing pressure, and the resultant broad band

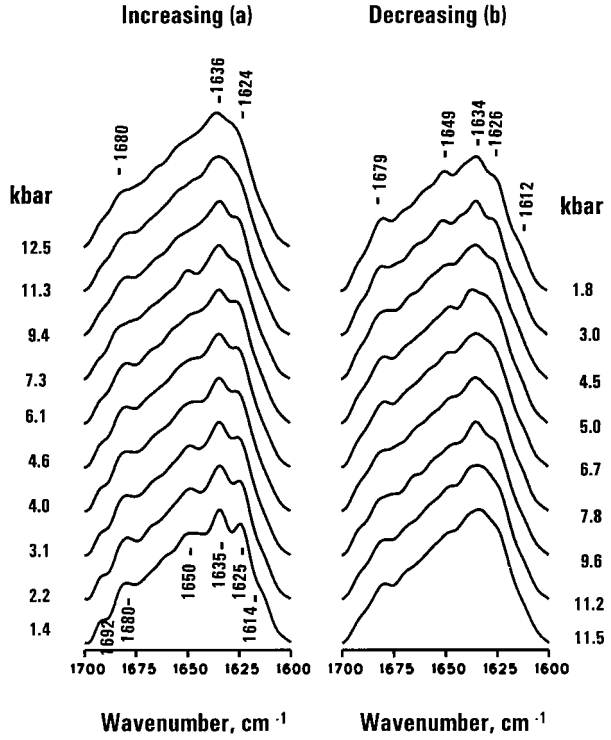

Figure 3. Infrared spectra of β-lg A+B (17% w/v) in D_2O in the amide I region at the indicated pressures. The spectra were recorded upon compression (a) and decompression (b) and are resolution enhanced by Fourier self-deconvolution with a band-narrowing factor of 1.8 and a half-bandwidth of 13 cm^{-1}.

appears at 1648 cm^{-1} after the decompression. The 1692 cm^{-1} band that appeared in the form of a weak shoulder prior to pressurization reappears as a weaker shoulder after the pressure cycle is released, indicating that pressure cannot reach buried parts of the molecule. No evidence of aggregate formation is observed with decreasing pressure.

Effect of Pressure on β-lg A+B. Figure 3a shows the amide I region in the spectrum of a 1:1 mixture of β-lg A and β-lg B (β-lg A+B) as a function of increasing hydrostatic pressure. The amide I band consists of five main bands and multiple shoulders. The relative intensities of the bands can be accounted by the sum of the absorbance contributions from β-lg A and β-lg B.

Compression Phase. Increasing the hydrostatic pressure from ambient pressure to 7.3 kbar results in (a) a broadening of the bands at 1650 and 1625 cm^{-1} and (b) a decrease in the intensity of the band at 1692 cm^{-1} relative to the bands at 1635 and 1680 cm^{-1}. Above 9.0 kbar, all of the major amide I bands of β-lg A+B begin to broaden substantially. The intensity of the 1625 cm^{-1} band increases relative to the 1625 cm^{-1} band, whereas at pressures <7.0 kbar the 1635 cm^{-1} band is significantly higher in intensity than the 1625 cm^{-1} band.

It is of interest to note that the 1614 cm^{-1} band, which appeared as a weak shoulder at 1.4 kbar and is associated with oligomer formation and/or aggregation, does not increase in intensity with increasing hydrostatic pressure. Furthermore, the intensity of the band at 1692 cm^{-1}, which is insensitive to pressure in β-lgA, becomes broader and possibly weaker with increasing hydrostatic pressure, whereas the band at 1682 cm^{-1} increases in intensity.

Decompression Phase. Reducing the pressure from 11.5 to 5.0 kbar has little effect on the amide I band profile. Below 5.0 kbar the amide I bands become sharper and the 1635 cm^{-1} band becomes more prominent relative to the 1625 cm^{-1} band. The bands at 1650, 1680, and 1614 cm^{-1} reappear at 1649, 1679, and 1612 cm^{-1}, respectively, and return to their original intensity. No change in the intensity of the shoulder at 1614 cm^{-1} is observed during the complete decompression cycle. The 1625 cm^{-1} band, which shifts to 1626 and 1692 cm^{-1}, is not well resolved after decompression of the sample.

DISCUSSION

Comparison of the amide I band profile of β-lg A at ambient pressure with that of β-lg B at the same pressure reveals that the amide I bands in the spectrum of β-lg B have much broader bandwidths. The relative intensities of the amide bands are also different, indicating that the minor differences in the amino acid sequences of the two proteins result in significant differences in the secondary structure.

The decrease in the intensity of the 1635 and 1682 cm^{-1} bands relative to the 1625 cm^{-1} band observed in the three protein solutions with increasing pressure suggests that the antiparallel β-sheet structure is sensitive to changes in volume. The decompression of β-lg A from 12.1 kbar to ambient pressure is accompanied by an increased intensity of the 1683 and 1614 cm^{-1} bands attributed to intermolecular hydrogen-bonded β-sheet (Clark et al., 1981; Ismail et al., 1992). In the β-lg A and β-lg A+B solutions the 1635 cm^{-1} band broadens irreversibly with increasing pressure, indicating that conformational drift takes place. In contrast, the 1635 cm^{-1} band in β-lg B does not undergo band broadening with increasing pressure or after depressurization of the sample. The greater extent of the conformational drift of the β-sheet structures seen in β-lg A relative to β-lg B may allow some of the peptide segments to associate with each other to form aggregated species. Aggregate formation in β-lg A is indicated after the pressure cycle by the increase in the intensity of the 1614 cm^{-1} band.

The primary structure of β-lg A protein has an additional Asp, and thus β-lg A is likely to form dimers through salt-bridge formation. Hence, as hydrostatic pressure has been shown to be effective in the disruption of salt-bridges (Ismail et al., 1992; Weber, 1987), this may account for the greater sensitivity of β-lg A structure to pressure, resulting in the reversal of the intensity of the 1635 and 1625 cm^{-1} bands with increasing pressure.

Pressure-induced aggregation in β-lg A during the pressure cycle is consistent with work by Funtenberger et al. (1997), who showed a progressive increase in S−S-bonded oligomers and aggregates in β-lg A+B isolates exposed to pressure up to 4.5 kbar (Funtenberger et al., 1995, 1997). In the studies by Funtenberger et al. (1995, 1997), however, the influence of genetic variants on pressure-induced changes in β-lg was not looked at. In the present study, β-lg B did not unfold under pressure, indicating a more stable structure. The resistance of β-lg B to unfolding would hinder the exposure and activation of the buried free sulfhydryl groups, which are necessary for the sulfhydryl−disulfide interchange reaction (Damodaran and Anand, 1997). This was supported by the findings of McSwiney et al. (1994), who showed that loss of native structure and formation of disulfide-linked aggregates occurred under thermal conditions at a

slower rate for β-lg B than for β-lg A. The greater stability of β-lg B could also be due to the location and higher number of Glu residues in β-lg B compared to β-lg A, which have been indicated to impede aggregation (Otte et al., 1997).

A sharpening of the 1692 cm^{-1} band along with the decrease in its intensity in β-lg A is observed with increasing pressure. After release of the pressure, the band at 1692 cm^{-1} shifts to lower wavenumber and returns to its original intensity, showing that pressure has no effect on the structure associated with the 1692 cm^{-1} band. Boye et al. (1996) have proposed that the band at 1692 cm^{-1} may be assigned to β-type structures buried deep within the protein that are inaccessible to solvent. These workers also reported that this structure was sensitive to temperature; heating β-lg B to 45 °C resulted in exposure of the hidden structures to solvent, and the 1692 cm^{-1} band completely disappeared and a new band at 1648 cm^{-1} assigned to H−D exchange (β-type structure) appeared. In the present work, an increase in the hydrostatic pressure results in a very minor change in the 1692 cm^{-1} band, indicating that pressure did not cause sufficient change in β-lg A and β-lg B to allow solvent to enter the buried regions of the protein. The band at 1657 cm^{-1} in β-lg A, which appears at higher pressures, persists after depressurization of the sample. This band is assigned to turns or an α-helical structure with amide groups that are involved in a weak H-bonding arrangement.

CONCLUSION

High pressure caused both reversible and irreversible changes in the conformation of β-lg proteins. The three types of protein samples showed different behaviors and susceptibilities to compression and decompression. β-lg A showed the most susceptibility to structural changes under high hydrostatic pressure. β-lg B was resistant to pressure-induced denaturation and aggregation even at pressures approaching 12.0 kbar. Most of the secondary structural changes of β-lg A+B induced under high pressure were reversible. This latter finding may have been due to differences in hydrophobic interactions and/or disulfide bonds in the secondary structures of β-lg B compared to β-lg A. Hence, it is likely that the functional or biological attributes of β-lg may be differentially affected by a single application of hydrostatic pressure depending upon the genetic variant of β-lg. Our findings indicate that β-lg A+B underwent less aggregate formation than β-lg A, possibly as a result of protein−protein interactions between β-lg A and β-lg B. These results indicate that subtle changes in the primary structure of β-lg protein can result in significant differences in the behavior of the protein introduced under pressure treatment.

ABBREVIATIONS USED

FTIR, Fourier transform infrared spectroscopy; β-lg, β-lactoglobulin; β-lg A, β-lactoglobulin A; β-lg B, β-lactoglobulin B; β-lg A+B, β-lactoglobulin A + β-lactoglobulin B.

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