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Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of β-lactoglobulin B

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Abstract

Heat and pressure treatment of β-lactoglobulin B (β-LG) causes it to partially unfold and aggregate. β-LG solutions at pH 7.2 were heat treated at temperatures between 40 and 93 °C for 12 min or were pressure treated at pressures between 50 and 800 MPa for 30 min. Another set of samples also contained myristic acid (MA) or conjugated linoleic acid (CLA) in a molar ratio of 1:1.1 protein:ligand. All the treated samples were analysed using polyacrylamide gel electrophoresis (PAGE) and near- and far-UV circular dichroism (CD) spectroscopy. Native- and sodium dodecyl sulfate (SDS)-PAGE showed that, at temperatures above about 63 °C, bands of lower mobility were produced. As the treatment temperature increased, the quantity of native protein decreased steadily and the quantity of higher molecular weight aggregates increased. Thus β-LG could be native (Stage I T) or non-native disulfide-bonded monomers and polymers (and their stable hydrophobic adducts) (Stage II T). Pressure treatments of 50 and 100 MPa had no discernible effect. At pressures between 150 and 350 MPa, PAGE analysis showed that only non-native and dimer β-LG were produced but that hydrophobic adducts were apparent in the native-PAGE patterns. At pressures above 500 MPa, the whole range of polymers (higher molecular weight aggregates) was discernible. Therefore three stages have been proposed for the pressure denaturation of β-LG. Thus β-LG could be native (Stage I P), non-native disulfide-bonded monomers, intermediates in the aggregation process and dimers (and their stable hydrophobic adducts) (Stage II P) or larger polymers (Stage III P). This model is consistent with the near- and far-UV CD data and with reported 8-anilino-1-naphthalenesulfonate probe data. The addition of MA or CLA to β-LG followed by heat or pressure treatment shifted the transition of native β-LG to non-native monomer or dimer by stabilizing the native structure (Stage I T or Stage I P). Pressurizing similar samples at higher pressures showed that only CLA had the ability to inhibit the transition from Stage II P to Stage III P.

Keywords: Heat; Pressure; β-Lactoglobulin B; Myristic acid; Conjugated linoleic acid

1. Introduction

Whey proteins are used as functional ingredients in many food products and their functionality can be affected by either heat treatment (Mulvihill & Donovan, 1987) or pressure treatment (Patel, Singh, Havea, Considine, & Creamer, 2005). β-Lactoglobulin (β-LG), the most abundant whey protein in milk (Farrell et al., 2004), governs the overall process-induced aggregation and gelation of whey protein products (Van Camp & Huyghebaert, 1995; Zasypkin, Dumay, & Cheftel, 1996). Heat-induced denaturation of β-LG has been reported in detail at the molecular level (Belloque & Smith, 1998; Edwards, Jameson,

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Palmano, & Creamer, 2002). The free thiol of Cys121 of native β-LG can react with the Cys106:Cys119 disulfide bond to give a free Cys119 and Cys121:Cys106. The free Cys119 reacts with the other β-LG disulfide bond, Cys66:Cys160, leading to the formation of Cys119:Cys66 and a free Cys160, which has the potential to react with the disulfide bonds of other β-LG molecules if present (Creamer et al., 2004a) to give a wide range of β-LG polymers.

The results of pressure studies (in situ) at 130 MPa led Panick, Malessa, and Winter (1999) to conclude that hydrophobic interactions are absent and that a single aggregation mechanism is operative. Considine, Singh, Patel, and Creamer (2005a) suggested that pressures below approximately 150 MPa at neutral pH and 22 °C do not allow exposure of CysH121 and therefore disulfide bond reaction cannot occur. Pressures between 150 and 250 MPa allow the reaction of Cys121 with Cys106:Cys119 to occur. Pressures greater than 250 MPa allow this exchange and the further reaction to release Cys160.

The effect of some ligands on thermal unfolding and aggregation has been examined recently (Considine, Patel, Singh, & Creamer, 2005b) and a similar study using high pressure showed some significant differences in aggregate formation (Considine et al., 2005a). The differences found explain, to some extent, the macroscopic results of the two different treatment types. It has also been shown (Swaisgood, Wang, & Allen, 2001) that a number of bioactive hydrophobic molecules, e.g. retinol and other vitamins, can bind to β-LG, which consequently increases the resistance of the protein to proteolytic degradation (Creamer et al., 2004b; Puyol, Pérez, Mata, Ena, & Calvo, 1993), thermal denaturation (Considine et al., 2005b; Puyol, Pérez, Peiro, & Calvo, 1994), pressure-induced aggregation (Considine et al., 2005a) and unfolding in urea solutions (Creamer, 1995). This has been receiving increased interest, not only as a means of controlling the denaturation pathway of β-LG but also as a method of ligand transport (Swaisgood et al., 2001). All the fatty acids were probably bound in the central calyx of β-LG (Qin, Creamer, Baker, & Jameson, 1998; Wu, Pérez, Puyol, & Sawyer, 1999).

Pérez et al. (1989) found that fatty acids were bound to β-LG in milk and that palmitic (31–35%), oleic (22–23%) and myristic (14–17%) acids predominated. The apparent binding constants of a number of saturated and unsaturated fatty acids to β-LG (Frapin, Dufour, & Haertlé, 1993; Pérez et al., 1992) were in the range of 10^{-7} M at neutral pH.

One of the most interesting groups of fatty acids, from a health and nutritional point of view, is the C18 unsaturated fatty acids. Conjugated linoleic acid (CLA) is a collective term for a mixture of positional and geometric isomers of octadecadienoic acid (18:2) in which the double bonds are conjugated, in comparison with the double bonds of linoleic acid, which are separated by a methylene group. The configuration of the double bonds may exhibit several possible positions, but they are found mainly at 9 and 11 or 10 and 12 (Ha, Grimm, & Pariza, 1987). Of the individual isomers of CLA, *cis*-9, *trans*-11-octadecadienoic acid has been implicated as the most biologically active because it is the predominant isomer that is incorporated into the phospholipids of cell membranes (Ip, Singh, Thompson, & Scimeca, 1994). Various studies have discussed the potential health claims associated with CLA (Kritchevsky, 2000; O'Shea, Devery, Lawless, Murphy, & Stanton, 2000; Devery, Miller, & Stanton, 2001; Haugen & Alexander, 2004; Wang & Jones, 2004).

Relatively little information on the binding of CLA to β-LG is available. The binding constants (K'_d) for linoleic acid (1.43×10^{-7} M), CLA (1.86×10^{-7} M) and CLA's methyl ester (CLAME) (1.88×10^{-7} M) were determined by changes in the tryptophan fluorescence of β-LG (Swaisgood et al., 2001). As the effect of the binding of CLA to β-LG on its aggregation behaviour during processing treatments is largely unknown, this study aims to highlight its potential role in delaying denaturation and further dairy protein aggregation.

In previous studies, we have examined the influence of a range of ligands on the early stages of heat-induced (Considine et al., 2005b) and pressure-induced (Considine et al., 2005a) changes to native β-LG and its subsequent aggregation. This study investigates the effect of two fatty acids, myristic acid (MA) and CLA, on the heat- and pressure-induced denaturation of the native state and the subsequent aggregation pathways of β-LG at pH 7.2. MA was used to further the information we have already obtained on the effect of a range of ligands (Considine et al., 2005a, 2005b). The differences in chain length (18 carbon atoms for CLA and 14 carbon atoms for MA), plus the more rigid structural properties of the molecules, may offer some insight into why these ligands can offer β-LG protection against thermal or pressure treatment.

2. Materials and methods

β-LG B was prepared as described by Manderson, Hardman, and Creamer (1998). Octadecadienoic acid, conjugated (CLA; which is a mixture of *cis*- and *trans*-9, 11- and 10,12-octadecadienoic acids) and butylated hydroxytoluene were obtained from Sigma Chemical Co., St. Louis, MO, USA. MA was obtained from Fluka Chemie AG, CH-9470 Buchs, Switzerland. All other chemicals were AnalaR grade and were from BDH Laboratory Supplies, Poole, England; Coomassie Blue R250 and the polyacrylamide gel electrophoresis (PAGE) chemicals were obtained from BioRad Laboratories, Hercules, CA, USA; Amido black 10B was obtained from Merck, Darmstadt, Germany. The water was from an artesian bore and was purified by reverse osmosis followed by ion exchange and carbon treatment using a Milli-Q system (Millipore Corp., Bedford, MA, USA). The conductivity of the water was checked routinely.

β-LG (1.5 mg/ml) was mixed with 26 mM sodium phosphate buffer, pH 7.2, containing 68 mM sodium chloride. MA and CLA (1 mg/ml) were prepared in degassed ethanol and an equimolar quantity of butylated hydroxytoluene was included in the CLA solution. Duplicate experiments were carried out but only one data set is shown.

Aliquots of MA and CLA solutions were added to β-LG (1.5 mg/ml) solutions at a molar ratio of 1:1.1 protein:ligand, based on a molecular weight of 18,300 Da for β-LG. Aliquots (4 ml) of the β-LG mixtures were put into glass vials, which were closed tightly to prevent evaporation during the heat treatment, and were heated for 12 min at temperatures between 40 and 93 °C in a temperature-controlled water bath. After the heating period, each tube was immediately placed in an ice/water mixture for 5 min and then allowed to stand for 2 h at room temperature. Possible loss of sample, or change of concentration, was checked by weighing the vials before heating and after cooling (Considine et al., 2005b).

Aliquots (approximately 6 ml) of the β-LG mixtures were transferred into Beckman Polyallomer Quick-Seal™ centrifuge tubes (13 mm internal diameter, 51 mm high, Beckman Instruments, Inc., Palo Alto, CA, USA), which were heat sealed and transferred to the pressure chamber of a high-pressure unit ('Food-Lab' food processor, Model S-FL-085-9-W, Stansted Fluid Power Ltd, Essex, UK). Samples were individually treated at pressures from 50 to 800 MPa for 30 min at 20 °C. A homogenized emulsion, consisting of a mixture of 10% vegetable-oil-in-water with surfactant and preservative (potassium sorbate, water, vegetable oil, Tween 80 and Span 60), was used as the pressurizing fluid. The dimensions of the chamber of the high-pressure rig were 17 mm × 132 mm. The pressurization and depressurization rates were 5 and 14.5 MPa per second respectively. The average adiabatic heating during compression was 1.4 °C per 100 MPa and the cooling during decompression was 1.3 °C per 100 MPa. After the pressurizing treatment, each tube was immediately placed in an ice/water mixture for 5 min and then allowed to stand for 2 h at room temperature. Possible loss of sample, or change of concentration, was checked by weighing the tubes before and after treatment.

2.1. PAGE

PAGE (both native- and sodium dodecyl sulfate (SDS) (non-reduced)-PAGE) gels were prepared and run as outlined by Anema (2000). After electrophoresis, the gels were stained using 0.1% (wt/vol) Amido black 10B in 10% acetic acid. After staining, the gels were destained using a 10% acetic acid solution until a clear background was achieved. Stained gels were scanned using a Molecular Dynamics model PD-SI computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA). The scanned images were processed using ImageQuant software, version 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) to obtain quantitative data.

2.2. Near- and far-UV circular dichroism (CD)

The β-LG solutions were scanned from 250 to 400 nm in 10 mm quartz cells with a Jasco Model J-720 spectropolarimeter (Jasco, Hachioji City, Tokyo, Japan) to obtain near-UV CD spectra. The samples were scanned at 50 nm/min, using a 2 s time constant, a 0.2 nm step resolution, a 1 nm bandwidth and a sensitivity of 10 millidegrees. Five scans were accumulated and the average spectrum was saved. The solution was diluted 10-fold with water and scanned using a 0.5 mm cell from 185 to 250 nm and 10 scans were averaged and saved as the far-UV spectrum. The sample compartment of the instrument was flushed with oxygen-free dry nitrogen, prior to measurement.

3. Results

3.1. PAGE

The SDS-PAGE gel patterns of the proteins in the heat- and pressure-treated β-LG samples are shown in Fig. 1a and b, respectively. The patterns of the heat- and pressure-treated β-LG solutions are in agreement with the electrophoretic patterns shown by Considine et al. (2005a, 2005b). The measured quantities of monomeric β-LG in the SDS-PAGE gels were plotted as a function of temperature (Fig. 2a) and pressure (Fig. 2b). As there was a decrease in the intensity of the monomer band (Figs. 1a, 2a, 1b and 2b), a corresponding increase in the intensity of the dimer band (Figs. 1a and b, 2c and d) occurred.

The native-PAGE patterns of the heat-treated samples are shown in Fig. 3a. Similar to SDS-PAGE, the major band in the protein solutions heated up to approximately 52 °C was native monomeric β-LG (results not shown) as well as very small quantities of non-native monomer, which decreased in intensity after 72 °C. Comparable results were reported by Considine et al. (2005b). The native-PAGE patterns of the pressure-treated samples are shown in Fig. 3b. β-LG was the major band present after pressure treatments up to 100 MPa (Fig. 4b). Both non-native monomer and dimer bands increased with increasing pressure, but then decreased after 600 MPa (Figs. 3b and 4d).

3.1.1. Effect of ligands

SDS-PAGE analysis of the heat- and pressure-treated solutions of β-LG plus ligand showed that, although there were monomers, dimers and ranges of less mobile bands with patterns similar to those present in the control solution (Fig. 1a and b), mixtures containing CLA and MA had more monomer protein than the control under all conditions used (Fig. 1a and b).

The heat-treated samples showed that there was little difference in the disappearance of β-LG monomer with time between the samples containing CLA or MA. In comparison, the pressure-treated samples containing ligands displayed similar behaviour at pressures up to 250 MPa (Fig. 2b and d). However, samples treated at higher pres-

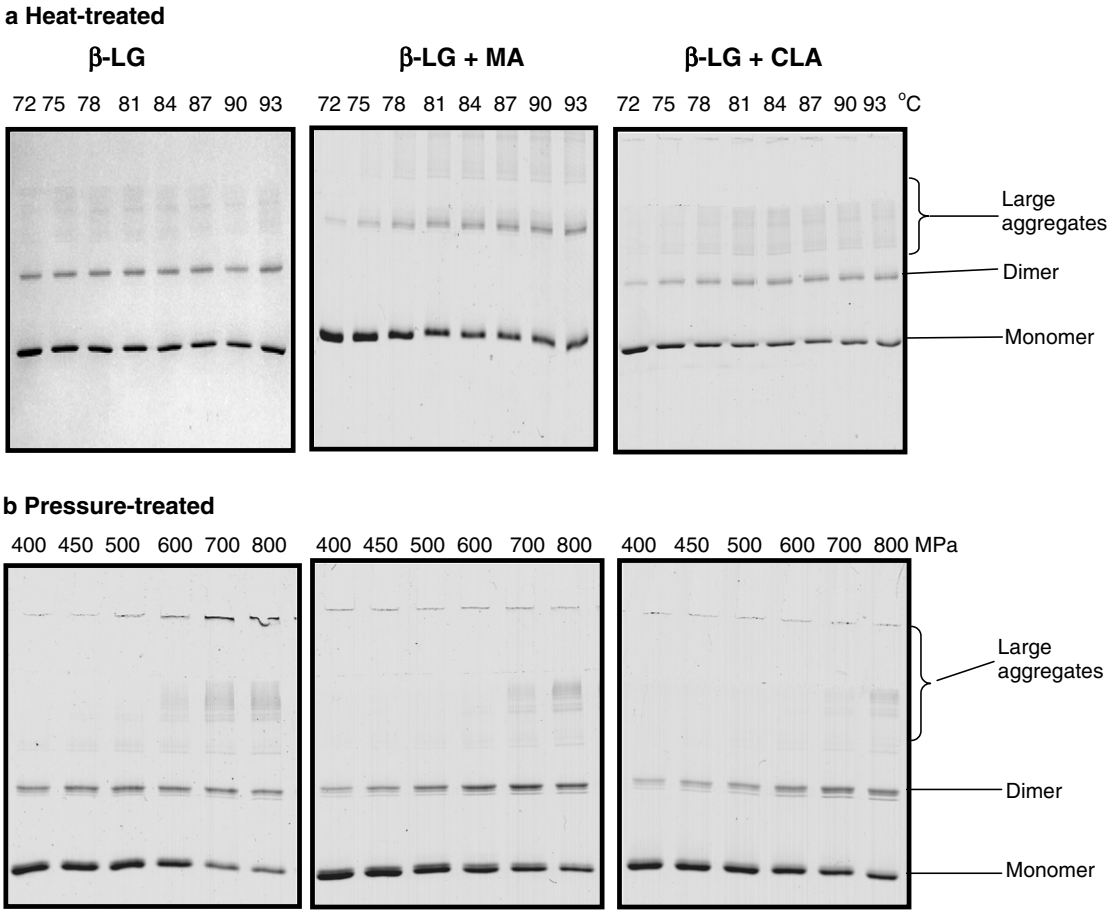

Fig. 1. SDS-PAGE of β-lactoglobulin B (β-LG), β-LG with myristic acid (MA) and β-LG with conjugated linoleic acid (CLA) that had been (a) heat treated between 72 and 93 °C for 12 min and (b) pressurized between 400 and 800 MPa for 30 min.

sures resulted in the stabilizing power of the ligands following the order CLA > MA > control (Figs. 1b and 2b). Similar findings were evident for dimer formation in the pressure-treated samples. However, the heat-treated samples containing ligands had similar monomer levels throughout the temperature profile, whereas dimer formation followed the stabilizing order MA > CLA > control from approximately 72 °C upwards (Fig. 2c). Similar profiles were obtained using native-PAGE (Figs. 3a and b and 4a–d).

3.1.2. Hydrophobically bonded protein polymers

The difference between the concentrations of monomers in native- and SDS-PAGE is partly caused by SDS dissociating hydrophobic aggregates. Thus, the β-LG in a native-PAGE pattern is the β-LG that is native monomer at a pH of approximately 8.5, whereas the β-LG monomer in an SDS-PAGE pattern contains all the various monomers that have a protein mass of approximately 18,300 Da, regardless of conformation, and that can be dissociated from other protein aggregates. Consequently, the numeric difference between the SDS-PAGE monomer band and the native-PAGE monomer band gives a guide as to the sum of the various non-native monomers (i.e. SDS-PAGE intensities–native-PAGE intensities). The difference in the results for native-PAGE and SDS-PAGE has been discussed by Considine et al. (2005a) and is shown in Fig. 5. The heat- and pressure-treated samples displayed very different profiles. For β-LG, the non-native monomer concentration was low and remained relatively constant up to 60 °C, but higher temperatures caused an increase in the proportion of non-native monomer. Addition of both ligands resulted in a delay in the increase of this hydrophobically bonded protein (approximately 66 °C). As with low temperatures, for β-LG, low pressures yielded a constant low level of non-native monomer up to 100 MPa and there was a moderate level up to 250 MPa. Above this pressure, the levels of non-native monomer decreased. Addition of ligands hindered the formation of non-native monomer until pressures of about 200 MPa were reached. Interestingly, higher levels of non-native monomer were achieved by β-LG with added MA than by β-LG with added CLA. β-LG with MA maintained a higher level of the non-native protein up to 500 MPa before the level decreased, whereas, for β-LG with CLA, the level of non-native protein began to decline at approximately 400 MPa (Fig. 5). Given the variability of the band intensities, which was compounded by

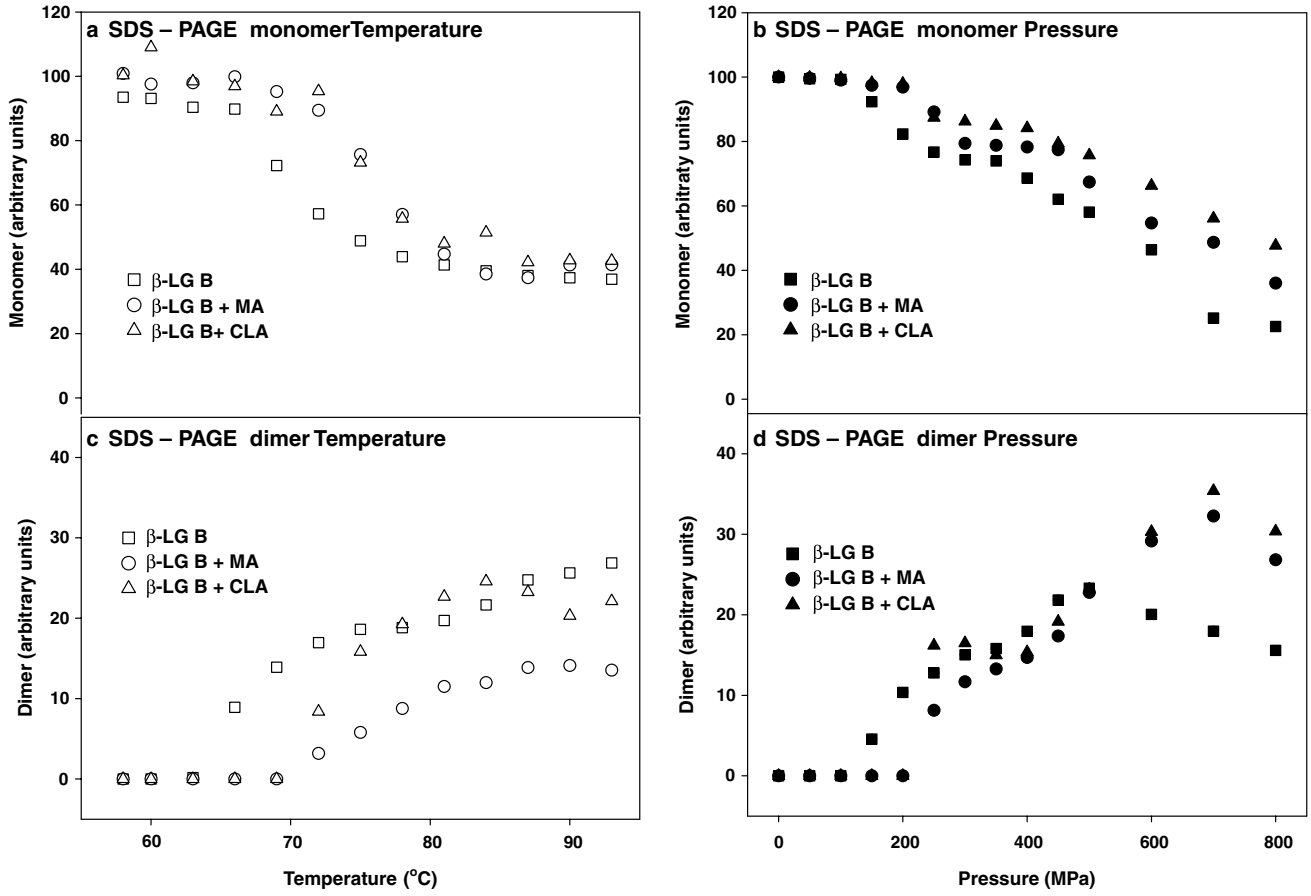

Fig. 2. Plots of SDS-PAGE band intensities of β-lactoglobulin B (β-LG), β-LG with myristic acid (MA) and β-LG with conjugated linoleic acid (CLA) that had been (a, c) heat treated between 58 and 93 °C for 12 min and (b, d) pressurized between 0.1 and 800 MPa for 30 min.

subtraction, the general trends were still noteworthy. Overall, it appears that heat treatment increased the quantity of non-native monomer but pressure treatment led to a maximum quantity of non-native monomer at about 400 MPa. The binding of ligands to β-LG delayed the peak to higher pressure (Fig. 5b) and delayed the transition from small to larger quantities of non-native β-LG (Fig. 5a).

3.2. Near-UV CD

The near-UV CD spectra of heat- and pressure-treated mixtures of β-LG with CLA are shown in Fig. 6a and b, respectively. Even after heating or pressurizing at the highest level, some of the protein appeared to retain an ordered structure. These near-UV CD spectra were similar to those previously reported by Considine et al. (2005a, 2005b). The effect of heat and pressure treatments on the intensity of the 293 nm trough of β-LG and β-LG with added ligands is shown in Fig. 6c and d. For β-LG, both treatments showed a similar trend, i.e. a decrease in the 293 nm signal with increasing temperature and pressure. The pressure and the temperature at which the decrease began were higher for samples containing ligands than for the control (Fig. 6c and d).

3.3. Far-UV CD

The far-UV CD spectra of native β-LG with added CLA are shown in Fig. 7a and b. The trough at 216 nm gradually broadened and deepened with increasing temperature (Fig. 7a) and pressure (Fig. 7b) and the trough minimum shifted to a lower wavelength (approximately 208 nm). These results were consistent with earlier studies on the effect of heat (Considine et al., 2005b; Manderson, Creamer, & Hardman, 1999a) and pressure (Considine et al., 2005a; Ikeuchi et al., 2001; Yang, Dunker, Powers, Clark, & Swanson, 2001) on β-LG. The changes in CD intensity at 200 nm with temperature and pressure are shown in Fig. 7c and d, respectively. All samples showed an increase in intensity with increasing treatment. However, when MA or CLA was added to β-LG, the increase in intensity at 200 nm occurred at a higher temperature or pressure than for the control.

4. Discussion

It is evident from this work and from our previous studies (Considine et al., 2005a, 2005b) that β-LG responds differently to heat and pressure treatments. Analysis of native-PAGE patterns showed that heating β-LG solutions

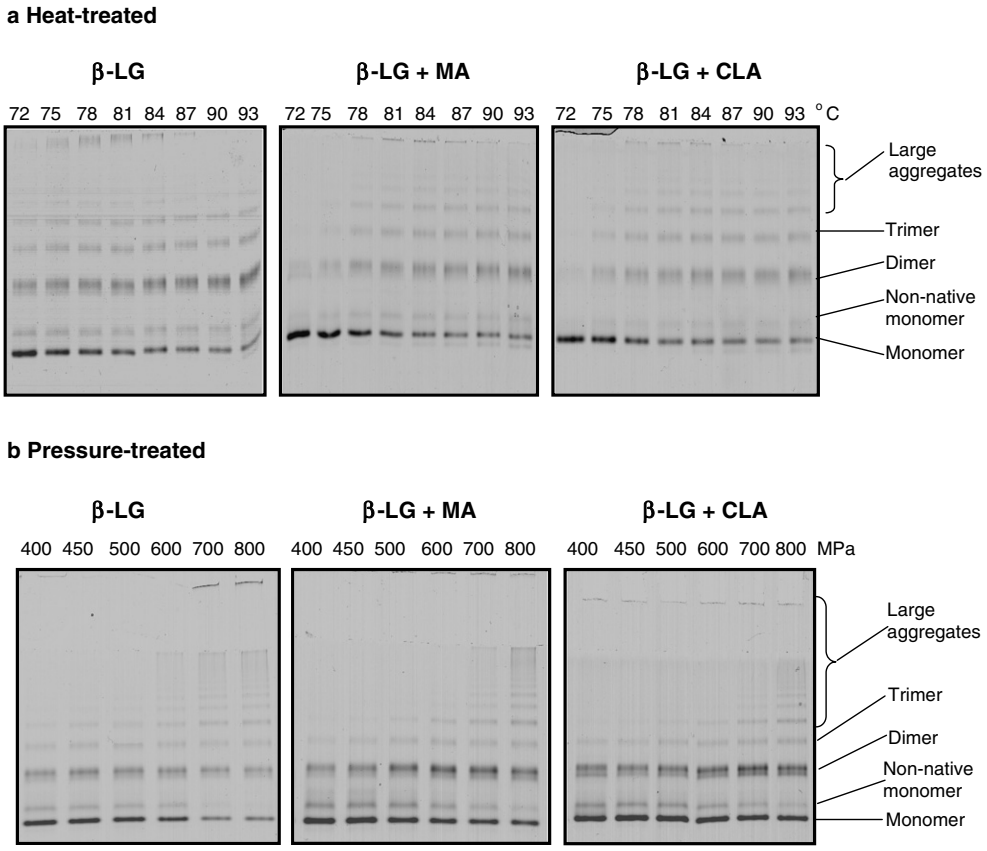

Fig. 3. Native-PAGE of β-lactoglobulin B (β-LG), β-LG with myristic acid (MA) and β-LG with conjugated linoleic acid (CLA) that had been (a) heat treated between 72 and 93 °C for 12 min and (b) pressurized between 400 and 800 MPa for 30 min.

at temperatures up to approximately 66 °C produced complete dissociation of β-LG dimers into monomers (see Fig. 2B in Considine et al., 2005b) but heating at temperatures greater than about 75 °C produced a wide range of polymers from dimers to large aggregates. Relkin (1998) monitored conformation changes through reversible heat-induced denaturation of β-LG using differential scanning calorimetry (DSC). Although higher concentrations of β-LG were used by Relkin, similar observations with respect to the type of interactions, namely hydrophobic and disulfide bonds were observed. Relkin's model suggested that the dissociation of dimers to monomers, and exposure of the free thiol and some of the hydrophobic groups occur at temperatures greater than 70 °C, which is in close agreement with the results presented here. Almost all of the results obtained using different methods (e.g. 293 nm CD spectra) can be represented as two-state processes and we propose to call these Stage I T (native protein) and Stage II T (heat-denatured protein) as defined by the absence of native disulfide bonding after the heat treatment (Fig. 8). For simplicity, Stage I T signifies at least 90% native structure and the β-LG in Stage II T may include up to 20% native protein. A two-stage denaturation model has previously been discussed by several authors (Aymard, Durand, & Nicolai, 1996; Gimel, Durand, & Nicolai, 1994; Le Bon, Nicolai, & Durand, 1999), who used dynamic and static light scattering techniques. Considine et al. (2005b), using PAGE, CD spectroscopy and 8-anilino-1-naphthalenesulfonate (ANS) fluorescence, also suggested a two-stage denaturation and aggregation model for β-LG. Sava, Van der Plancken, Claeys, and Hendrickx (2005) suggested that the kinetics of heat-induced structural changes of β-LG, as measured by changes in solubility, followed a two-step process, with an unfolding step between 70 and 75 °C and an aggregation step between 78 and 82.5 °C. The study by Sava et al. (2005) and this present study show similar results although different heating times were monitored, 45 min versus 12 min.

In contrast, for pressure treatment of β-LG between 0.1 and 150 MPa (see Figs. 2b, 4b, 1 and 2 in Considine et al., 2005a), only monomeric β-LG was evident after pressure treatment. Pressure treatments for 30 min between 200 and 500 MPa produced non-native monomers, dimers, trimers and tetramers (Fig. 3b), and pressures > 500 MPa produced a wider range of higher molecular weight aggregates (Fig. 3b). The formation of the non-native monomer was more evident in the pressurized samples (Fig. 3b) than in the heat-treated samples (Fig. 3a). This result suggested that non-native monomer and dimer β-LG accumulated in samples pressure treated at 150 or 200 MPa whereas the heat-treated samples progressed to larger polymers (Fig. 3a). Considine et al. (2005a) called these three categories Stages

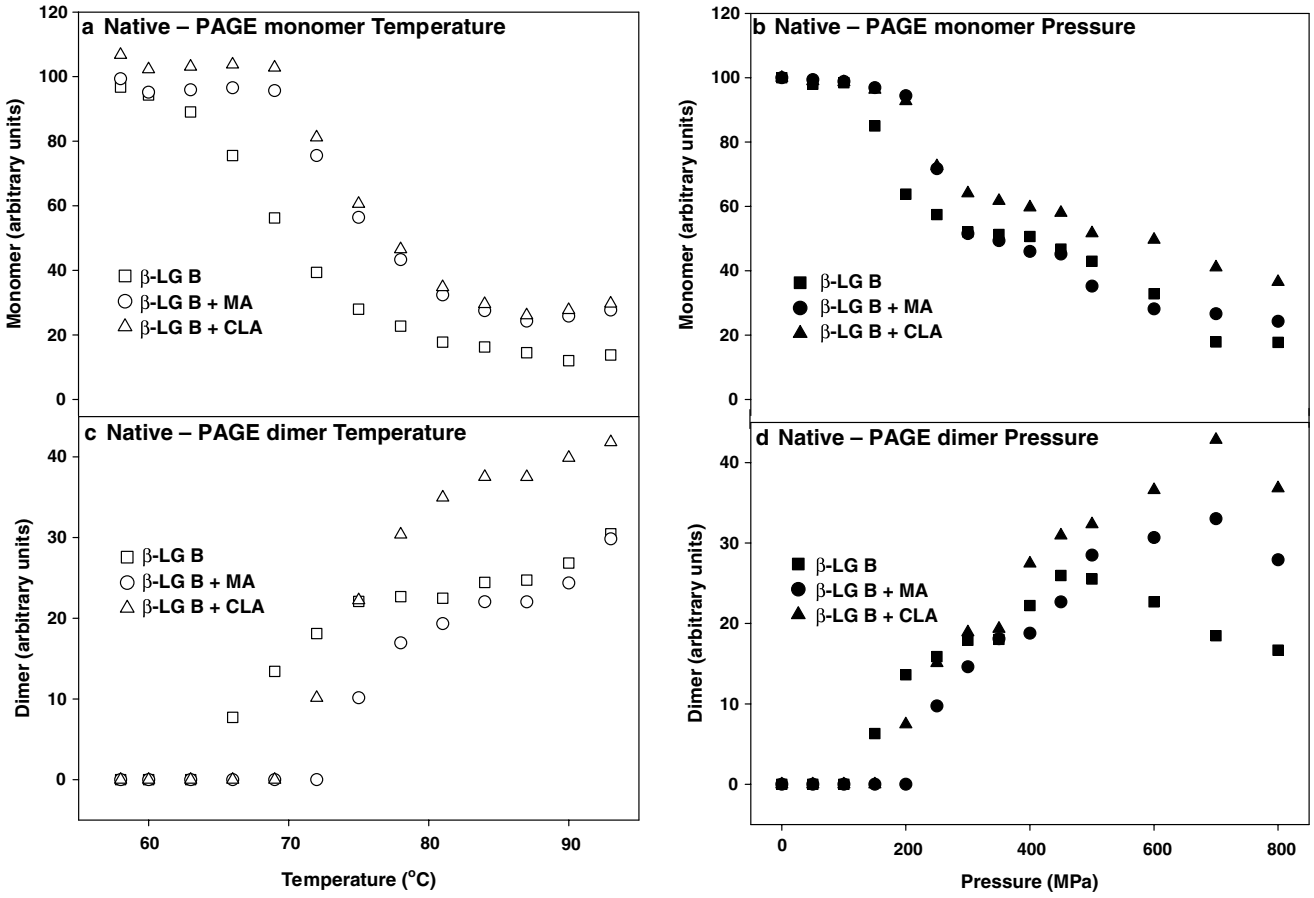

Fig. 4. Plots of native-PAGE band intensities of β-lactoglobulin B (β-LG), β-LG with myristic acid (MA) and β-LG with conjugated linoleic acid (CLA) that had been (a, c) heat treated between 58 and 93 °C for 12 min and (b, d) pressurized between 0.1 and 800 MPa for 30 min.

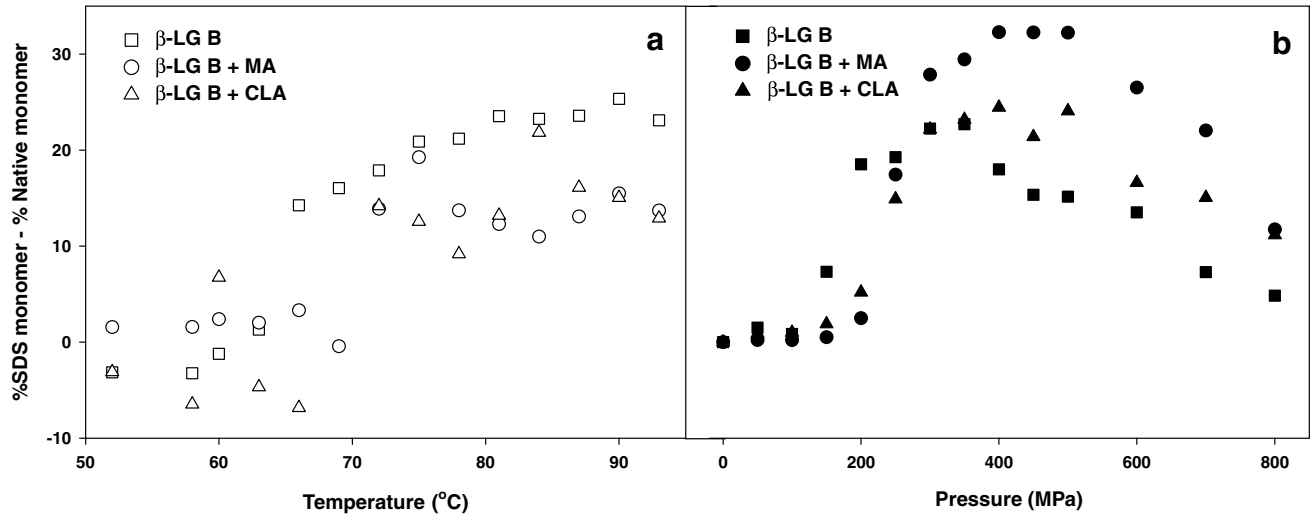

Fig. 5. Effect of (a) temperature and (b) pressure on the concentration of non-native monomer (difference between SDS monomer and native monomer, shown in Figs. 2 and 4) for β-lactoglobulin B (β-LG) solutions with and without added ligands.

I P, II P and III P (Fig. 8). Stages II P and III P were loosely encompassed by Stage II T in the heated systems, indicating that some of the interchange reactions that occur in systems heated at lower Stage II T temperatures, e.g. 75 °C, do not occur at intermediate pressures (e.g. 250 MPa).

An examination of some of the available data in the light of these models is valuable because many of the structural probes examine only a single feature, e.g. β-LG Trp19 fluorescence or CD spectroscopy. Comparisons of the near-UV CD data of heat and pressure treatment (Fig. 6a, c and

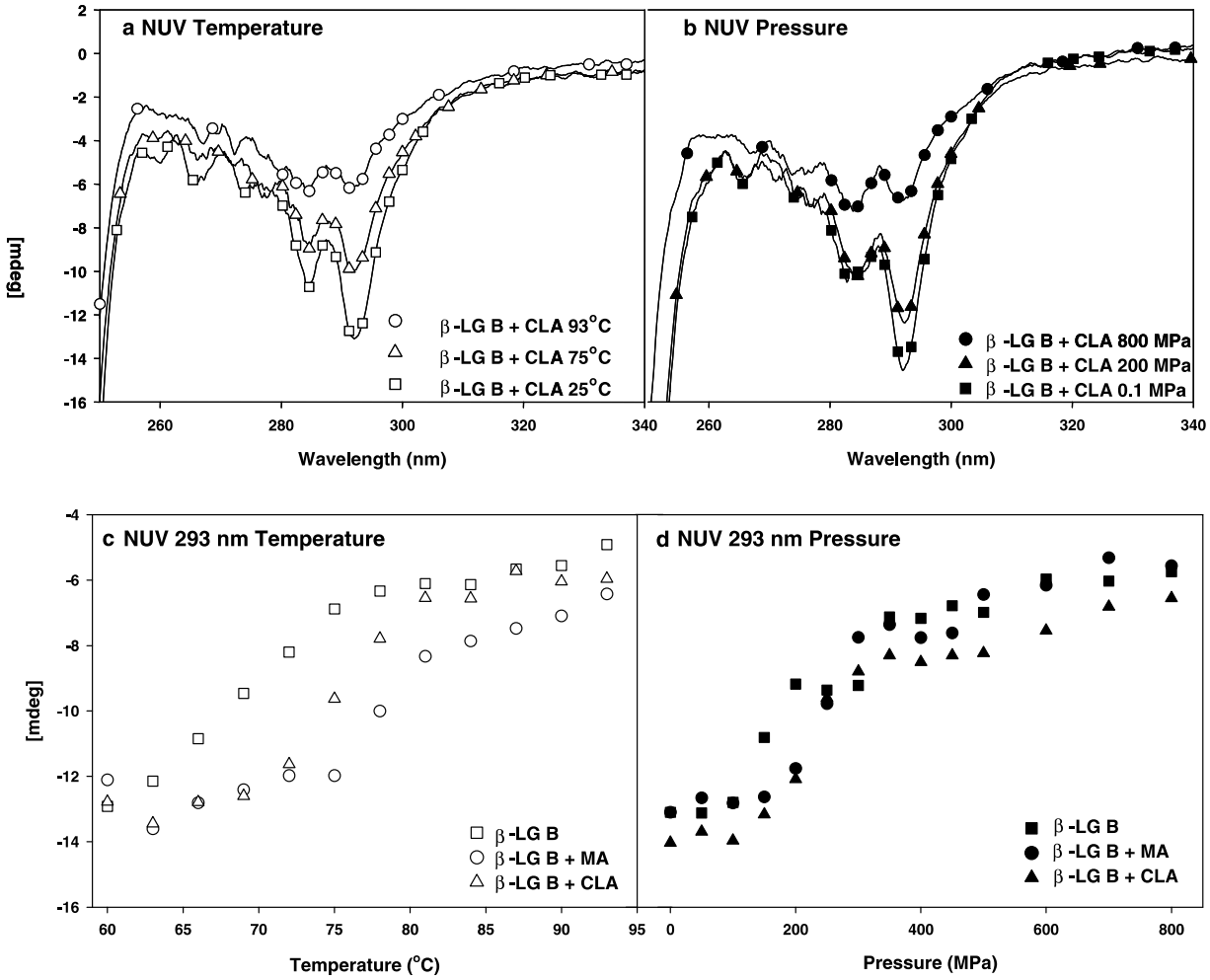

Fig. 6. Near UV CD spectra of (a) heat-treated or (b) pressure treated β-lactoglobulin B (β-LG) with conjugated linoleic acid (CLA). Plot of the CD intensity at 293 nm of (c) heat-treated or (d) pressure-treated β-LG, β-LG with myristic acid (MA) and β-LG with CLA.

b and d, respectively) give simple curves with midpoints of 69 °C and 175 MPa. A previous study (Manderson et al., 1999a) showed a strong correlation between the CD signal at 293 nm and the content of native β-LG in a heated sample, indicating that the loss of chirality is an index of the availability of a free thiol (Manderson, Hardman, & Creamer, 1999b) and is probably linked to the Cys121 interaction with Cys106:Cys119 (Creamer et al., 2004a; Croguennec, Bouhallab, Molle, O'Kennedy, & Mehra, 2003).

Conversely, the interpretation of the shifts in the far-UV CD spectra (Fig. 7), which are the summation of the signals generated by several secondary structures, is not as clear-cut, partly because the signal-to-noise ratio is quite low and partly because the differences between the various structures are moderately small. But there was a continuing change as each amino acid and its peptide bond moved between several different structures as a consequence of the heat or pressure treatments. Nevertheless, there was a continuous decrease in the native secondary structure.

ANS has been used as a structural probe for β-LG and binds to some of the differently folded states of β-LG (Mills & Creamer, 1975); in our recent studies (Considine et al., 2005a, 2005b), it altered the transition points. Nevertheless, its action as a probe showed that temperature effects indicated two stages (native and denatured) with a midpoint heating temperature of about 67 °C (Fig. 8B in Considine et al., 2005b) but that pressure effects indicated two transitions with midpoint pressures at about 175 and 550 MPa (Considine et al., 2005a). This three-stage model for the pressure treatment of β-LG is in good agreement with a recent model proposed by Considine et al. (2005a) and also consistent with that proposed by Stapelfeldt and Skibsted (1999). Although both heat treatment at temperatures above 80 °C and pressure treatment at pressures above 500 MPa lead to similar aggregation products, pressure treatments of 300–400 MPa give a different product mix.

The addition of ligands, either MA or CLA, prior to heat or pressure treatment delayed the formation of higher molecular weight aggregates, in comparison with the control. At low (60–72 °C) and high (>84 °C) temperatures, both ligands delayed the aggregation process (Figs. 1a, 2a, 2c, 3a, 4a, 4c, 5a, 6a, 6c, 7a and 7c). Similar results were

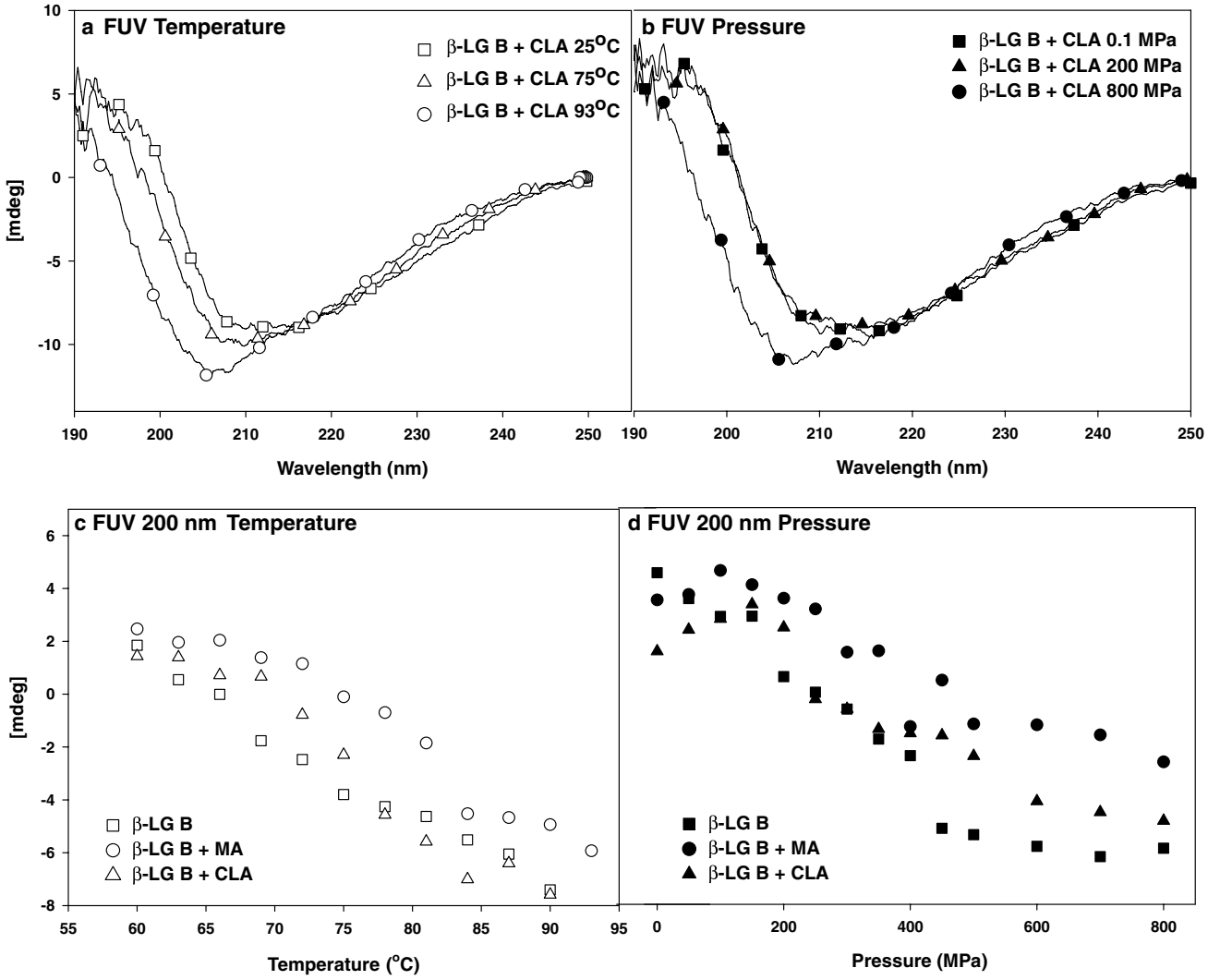

Fig. 7. Far-UV CD spectra of (a) heat-treated or (b) pressure treated β-lactoglobulin B (β-LG) with conjugated linoleic acid (CLA). Plot of the CD intensity at 200 nm of (c) heat-treated or (d) pressure-treated β-LG, β-LG with myristic acid (MA) and β-LG with CLA.

found at low pressures (Figs. 1b, 2b, 2d, 3b, 4b, 4d, 5b, 6b, 6d, 7b and 7d). However, at pressures > 250 MPa, CLA offered greater protection (CLA > MA > control). Comparison with other ligands (SDS, retinol and ANS from heat and pressure studies by Considine et al., 2005a, 2005b) showed that ligands that protected β-LG from early denaturation and aggregation followed the order SDS > retinol > ANS ≈ control. From these results and structural studies (e.g. Kontopidis, Holt, & Sawyer, 2004; Qin et al., 1998; Wu et al., 1999), it may be speculated that the binding pocket of β-LG best accommodates a molecule of 12–18 carbon atoms in order to protect β-LG from low heat or pressure (i.e. 60–70 °C or 0.1–150 MPa) treatment. When higher temperatures or pressures are applied (i.e. >70 °C or between 150 and 300 MPa), molecules that have 16 or 18 carbon atoms offer better protection than a molecule with 14 carbon atoms. On increasing the pressure to between 300 and 800 MPa, the order of protection appears to be ligands with 18 > 14 > 16 ≈ 12 carbon atoms. Therefore, these results would suggest that the best ligand to use to delay the pressure denaturation and aggregation of β-LG is CLA as it is effective at both low and high pressures.

The extent of protection of β-LG against heat or pressure denaturation that is provided by the ligands may be related to the affinity of these ligands for β-LG. The binding constants of a range of ligands have been widely reported (Considine et al., 2005b; Sawyer, Brownlow, Polikarpov, & Wu, 1998), although there appear to be some discrepancies in these values, probably because of the different methods and conditions used for their determination. Fatty acids bind strongly to β-LG (16 > 18 > 14 > 20 > 12 carbon atoms; Frapin et al., 1993). Frapin et al. (1993) reported a K'_d of 3.3×10^{-7} M for MA and of 1.9×10^{-7} M for linoleic acid (C18:2 Δ9,12). This is in good agreement with those reported for CLA (1.86×10^{-7} M) and CLAME (1.88×10^{-7} M) by Swaisgood et al. (2001). The effect of ligands on the different stages in heat-induced denaturation and aggregation show that both MA and CLA stabilized Stage I T and the transition from Stage I T to Stage II T. Both CLA and MA had the ability to inhi-

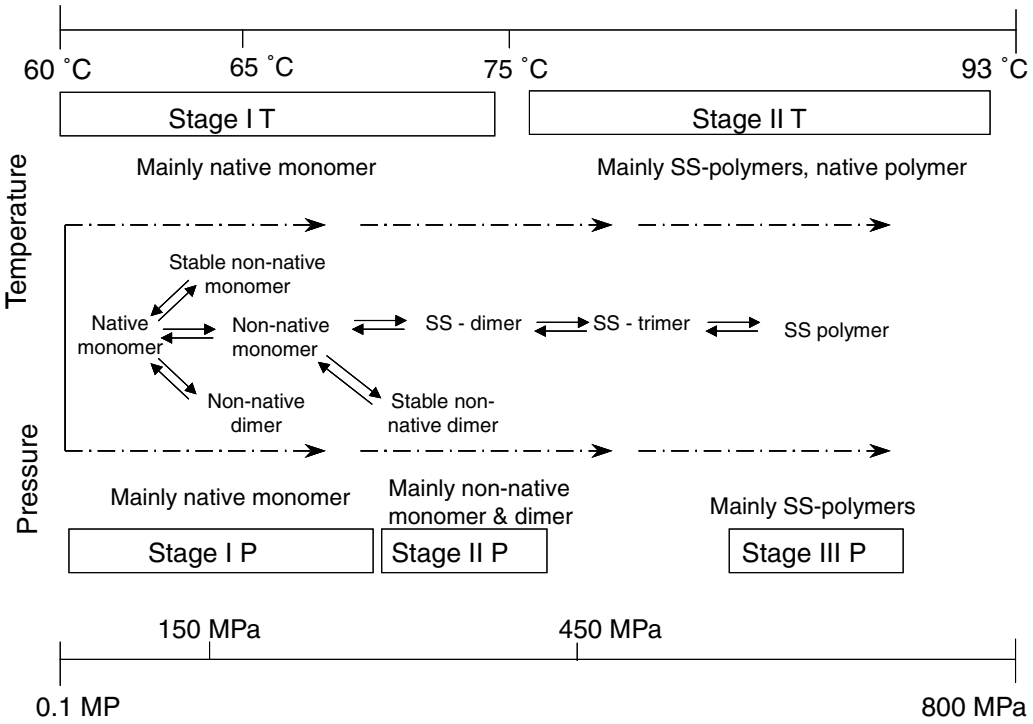

Fig. 8. Proposed model of the heat and pressure denaturation of βl-actoglobulin B (β-LG). *T*, temperature; *P*, pressure.

bit the conversion of Stage I P to Stage II P but only CLA had the ability to delay the subsequent conversion to Stage III P.

5. Conclusions

The present study shows that both heat treatment and pressure treatment can unfold and denature β-LG to give the same range of polymeric disulfide-bonded species. At pressures between 150 and 350 MPa, only the first stage, namely the formation of a non-native monomer and a disulfide-bonded dimer, was observed in the SDS-PAGE patterns. Trimers and dimers were evident in the native-PAGE patterns, suggesting that these were hydrophobically linked and thus absent in SDS-PAGE. However, at pressures above 500 MPa, a much larger range of aggregates was produced. In contrast, at every heating temperature above 72 °C, a large range of polymers was produced.

Addition of MA or CLA to β-LG prior to either treatment diminished the transition of native β-LG to non-native monomer or dimer by stabilizing the native structure, i.e. they stabilized Stage I P or Stage I T and the subsequent conversion to Stage II P or Stage II T respectively. However, only CLA had the ability to inhibit the conversion of Stage II P to Stage III P.

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