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Investigation on the influence of high protein concentrations on the thermal reaction behaviour of β-lactoglobulin by experimental and numerical analyses

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ABSTRACT

In this study, treatments at various temperature–time profiles were performed for β-lactoglobulin samples at different concentrations (50–70%) using a special rheometer as processing device. Rheological measurements, offline protein chemical analyses, and molecular dynamics analyses were performed to investigate the influence of high protein concentrations and treatment temperature on the denaturation and aggregation behaviour of β-lactoglobulin. Under these conditions, the degree of denaturation and aggregation decreased with increasing protein concentration. This corresponded to a strongly decreased diffusion and increased stability of exposed surface protein regions at high concentrations. Irreversible denaturation was observed for temperatures above 60 °C. Increasing thermal treatment intensity resulted in an increase of aggregation. Depending on the thermal treatment conditions, different protein–protein interactions were measured. By increasing the treatment temperature, the resulting aggregates were increasingly stabilised by covalent bonds. In addition to disulphide bonds, non-disulphide covalent cross-links were formed at temperatures above 100 °C.

1. Introduction

Whey proteins are widely used in the food industry because of their nutritional, technological, and functional properties. Depending on their native structure, proteins show diverse functionalities, such as the ability to form gels, increase the viscosity of solutions or dispersions, and stabilise emulsions (Dissanayake & Vasiljevic, 2009). The molecular structure of proteins, and, their functionality, can be modified by thermal, shear and/or pressure treatment (Bouaouina, Desrumaux, Loisel, & Legrand, 2006; Dissanayake, Liyanaarachchi, & Vasiljevic, 2012; Morr & Ha, 1993; Nicolai & Durand, 2013). Therefore, processes such as extrusion processing have been used to create protein-based products with improved functionality.

Extrusion processing has been applied to design protein-based products such as meat and dairy analogues, as well as protein-based emulsifiers and thickeners with specific functionalities (Camire, 1991; Cheftel, Kitagawa, & Quéguiner, 1992; Holay & Harper, 1982; Koch, Hummel, Schuchmann, & Emin, 2018; Onwulata, Konstance, Cooke, & Farrell, 2003; Osen, Toelstede, Wild, Eisner, & Schweiggert-Weisz, 2014; Pietsch, Emin, & Schuchmann, 2017; Qi & Onwulata, 2011; Queguiner, Dumay, Salou-Cavalier, & Cheftel, 1992; Wolz, Kastenhuber, & Kulozik, 2016). During extrusion processing, highly concentrated proteins are subjected to thermal and mechanical stresses simultaneously through the heated barrel and the rotating screws. Thermal and shear stresses can lead to changes in the protein conformation, such as protein denaturation. Proteins unfold and, thus, reactive sites become available and new protein-protein interactions are formed, leading to protein aggregation. The properties of aggregates (e.g., size, form, and intermolecular interactions) play a crucial role in the performance of the resulting products. Thus, the final product characteristics can only be controlled if the reaction behaviour and kinetics are known.

The reaction behaviour of proteins strongly depends on the environment the proteins are in (including protein concentration, pH and ionic strength) and on the processing conditions (temperature, shear rate, pressure). This has been well investigated for

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dilute whey protein solutions (protein concentration < 10%) especially under thermal stress and are well summarised by Brodkorb, Croguennec, Bouhallab, and Kehoe (2016), de La Fuente, Singh, and Hemar (2002), Foegeding, Davis, Doucet, and McGuffey (2002), Mulvihill and Donovan (1987) and Nicolai, Britten, and Schmitt (2011).

Thermal denaturation of whey proteins is often described as a two-step reaction (deWit & Klarenbeek, 1984; Mulvihill & Donovan, 1987). Heating the protein in solution increases the thermal motion of the structural elements in the polypeptide chain leading to protein unfolding, resulting in the rupture of inter- and intramolecular bonds (e.g., van der Waals interactions and hydrogen and disulphide bonds). Hydrophobic amino acid residues buried within the protein are exposed, which increases their reactivity. Through oxidation of cysteine (Cys) residues to disulphide groups, intermolecular disulphide bonds are formed, leading to irreversible aggregation of the protein (deWit & Klarenbeek, 1984; Mulvihill & Donovan, 1987). The main whey protein fractions are β-lactoglobulin (βLG) and α-lactalbumin (αLA). βLG is a globular protein with two intramolecular disulphide bonds and one free thiol group, which is hidden in the core of the native protein. αLA has four intramolecular disulphide bonds and no free thiol group. Other whey protein fractions include immunoglobulins, serum albumin and proteose peptone.

All protein fractions respond differently to heat, resulting in denaturation temperatures ranging from 62 °C to 78 °C (deWit & Klarenbeek, 1984; Kinsella & Whitehead, 1989), measured by DSC, also depending on the heating rate. Thus, the denaturation and aggregation of whey proteins represent the response of all the fractions involved. Furthermore, the different proteins may also interact with each other and with other whey components (i.e., lactose), which makes the investigations of the aggregation reactions more complex. For this reason, the thermal denaturation and aggregation of individual fractions (βLG and αLA, separately) has also been the subject of numerous studies. Thermal treatment at temperatures below 85 °C results in an increased denaturation/aggregation rate with increasing βLG concentration (from 2% up to 9%, w/w; Kehoe, Wang, Morris, & Brodkorb, 2011; Mudgal, Daubert, & Foegeding, 2011; Schokker, Singh, & Creamer, 2000). Although increasing the protein concentration can accelerate or retard the thermal denaturation of βLG in whey and milk systems (as discussed further below), it seems that, for systems only containing βLG, the denaturation and aggregation is accelerated.

Wolz and Kulozik (2015) studied the thermal denaturation kinetics of whey proteins up to concentrations of 40%. Elevated whey protein concentrations resulted in an enhanced denaturation rate. Extensions of this work to conditions in an extruder were limited to a protein concentration of 30%, since above 30%, it was not possible to control the resulting aggregate size and a continuous paste was generated, while the aim was to obtain microparticulated, functionalised whey protein aggregates. There is plenty of research focussing on thermal stress induced protein denaturation and aggregation via non-covalent (hydrophobic and electrostatic interactions) and covalent (disulphide) bonds for whey protein systems containing 30% (w/w) protein or less. To the best of our knowledge, only Koch, Emin, and Schuchmann (2017) investigated the reaction behaviour of whey protein isolate at a concentration of 72% (w/w) under defined thermal treatment conditions (temperatures > 80 °C). Nevertheless, information on the effect of protein concentration, especially in the concentration range interesting for extrusion processing (above 40%) on the thermal denaturation/aggregation is still missing. Furthermore, the reaction kinetics, molecular interactions and formation of bonds (e.g., disulphide and isopeptide bonds) remain unknown.

In dilute solutions, polymer chains are disentangled and behave as individual units (Maron, Nakajima, & Krieger, 1959). The configuration of the polymer chain depends on the space available for each molecule. As this space decreases with an increase in concentration, the polymer molecule is forced to reduce its effective volume by arranging itself into a coil. Above a critical concentration, all the polymer coils in solution effectively overlap, interpenetrate, and become entangled (Maron et al., 1959). This changes the rheological behaviour of the solution from mainly viscous to mainly elastic, with the viscosity of the matrix being governed by the mobility of the polymer molecules and the viscosity of the matrix increases exponentially on increasing the concentration of the polymer component (Krieger & Dougherty, 1959). Increasing the concentration further results in less volume available for all molecules and should very likely create a competitive situation between the protein molecules for the available water, especially when, like in this study, almost all dry matter was protein. Therefore, the randomness of the particle distribution in the concentrated polymer solutions decreases, which leads to an entropy decrease of the crowded solution (Ralston, 1990). Therefore, the free energy increases and results in an increase of the thermodynamic activity of polymer, which can affect the conformational stability and structural properties of biological macromolecules such as proteins (Eggers & Valentine, 2001a,b; Minton, 2000). This effect, also known as macromolecular crowding, has been demonstrated to affect the protein folding and unfolding (Eggers & Valentine, 2001a), binding of small molecules, enzymatic activity, protein-protein interactions, protein aggregation, and amyloid formation (Hatters, Minton, & Howlett, 2002).

For this reason, we expect that at protein concentrations above 50% (w/w), where the biopolymers occupy more than 51% of the total volume, molecular crowding occurs, resulting in an increased conformational stability of the proteins. This could lead to a decreased denaturation and aggregation due to an increase in activation energy with increasing the concentration of biopolymers. In contrast, we expect that this effect of the concentration on the reactions will decrease by increasing the temperature, as by increasing the temperature, the reactions are accelerated and the reaction rate increases. Therefore, the objective of this study was to investigate the effect of high protein concentrations and thermal treatment on the reaction behaviour of βLG systems, in some way representative for low moisture extrusion for the creation of structures from dairy protein powders. For this purpose, the degree of denaturation and aggregation as a function of temperature and protein concentration was analysed.

2. Material and methods

2.1. Material

Pure bovine βLG was produced at the Chair of Food and Bioprocess Engineering of the Technical University of Munich at Freising-Weihenstephan, Germany. βLG was isolated from whey protein solutions as described by Toro-Sierra, Tolkach, and Kulozik (2013). In brief, the isolation procedure comprises a selective thermal denaturation of αLA and other minor whey proteins with addition of citrate, membrane separation of the precipitated αLA

and the soluble βLG fractions with a combination of micro- and ultrafiltration, resolubilisation of the αLA fraction, and further purification with ultrafiltration. The βLG used was highly pure (<0.05% lactose, minerals < 0.7%), the moisture content was <4%, the degree of nativity was >90%, with 99% protein on dry matter, and the content of βLG in the dry matter was >98%.

2.2. Sample preparation

βLG was mixed with deionised water (Millipore Sigma, Burlington, USA) to the protein concentrations of 50%, 60% and 70% (w/w) and mixed with a Thermomix (Vorwerk, Wuppertal, Germany) for 3 min. To ensure a homogenous distribution of water, the samples were stored at 8 °C at least for three days.

2.3. Heat treatment and inline rheological analyses

Heat treatments and rheological measurements were performed by a closed cavity rheometer (RPA flex, TA Instruments, New Castle, Delaware, USA). A schematic illustration of the device is shown in Fig. 1. The test chamber consists of two cones in opposite. Both geometries are grooved to prevent slippage and thermoregulated by direct heating and forced air-cooling. For the experiments, 4.5 g of sample was placed between the cones. A polyamide film was used between the sample and both geometries to prevent fouling. The test chamber was then sealed and pressurised at 4.5 MPa to prevent water evaporation during the experiments. The lower cone oscillates with a defined frequency of 1 Hz and an amplitude of 1%. The torque response transmitted through the sample was measured by a transducer on the upper cone and used to calculate the dynamic rheological properties such as storage modulus G′, loss modulus G″ and complex modulus IG*I.

Temperature sweep analyses within a temperature range of 30–180 °C were carried out to determine reaction onset temperatures. The measurements were performed at a heating rate of 5 K min^{-1} on the LVE region (corresponding to a frequency and amplitude of 1 Hz and 1%, respectively). To investigate the influence of time and temperature on the reactions taking place, isothermal time sweep measurements were performed. Samples were treated at 60, 70, 80, 100, and 120 °C for 30 s, 60 s and 600 s. After the treatment, the samples were dried in a vacuum dryer (Heraeus, Hanau, Germany) at 40 °C and 80 mbar and milled to a particle size <500 μm. For further offline analyses, only the dried protein powders were used. All measurements were conducted in triplicate.

2.4. Degree of denaturation

To determine the degree of denaturation, the amount of native protein remaining in solution after the treatment was measured. For this, a reversed-phase (Jupiter, 150 × 4.6 mm, 4 μm particle size, Phenomenex, Torrance, USA) chromatography (RP-HPLC) was performed by using a Shimadzu HPLC System (Shimadzu, Kyoto, Japan). Samples (1 mg mL^{-1}) were solved in an acetate buffer (0.5 M, pH 4.6) to induce precipitation of denatured/aggregated proteins. After one hour, the denatured/aggregated proteins were removed by centrifugation at 4637 × *g* for 50 min, ensuring only soluble, and thus, native fractions remained in the supernatant. Mobile phases: solvent A and solvent B were filtered through 0.2 μm cellulose acetate filters (Sartorius, Göttingen, Germany) and degassed. The initial conditions were 74% solvent A [1%, v/v, trifluoroacetic acid (TFA; 99% extra pure, Thermo Fischer, Massachusetts, USA) in deionised water] and 26% solvent B [1%, v/v, TFA in acetonitrile (ACN; extra pure, Thermo Fischer)]. Elution was done under the following conditions: 0–30 min, linear gradient from 74% to 52% solvent A. The column was brought back to the starting conditions within 5 min. UV detection was done at 280 nm. Flow rate was set to 1 mL min^{-1}, oven temperature was 40 °C, and injection volume was 100 μL. The degree of denaturation (D_D; Equation (1)) was calculated as the ratio of the amount of native protein after (C_t) thermal treatment and before (C_0) thermal treatment.

$$D_D = \left(\left(\frac{C_0 - C_t}{C_0}\right) * 100\right) \quad (1)$$

The concentration of protein in samples, before and after treatment, was calculated from the peak areas of the chromatograms. The analyses were performed in triplicate for each sample.

2.5. Protein-protein interactions

To investigate which interactions between βLG molecules are responsible for the aggregation of the treated samples, solubility of the samples at various buffer solutions cleaving specific bonds was analysed using a modified version of the buffer systems previously described by Keim and Hinrichs (2004) and Shimada and Cheftel (1988). For the analysis of the participation of non-covalent (i.e., electrostatic interactions, hydrophobic and hydrogen bonds) and covalent bonds (i.e., disulphide bonds, isopeptide bonds) in the aggregation of βLG after thermal treatment, treated and untreated samples were dispersed in two 0.02 M phosphate buffers at pH 7.0. Phosphate buffer containing 0.05 M NaCl, 0.03 M SDS and 8 M Urea,

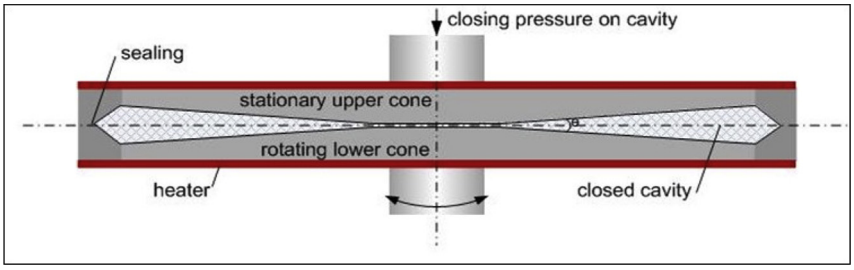

Fig. 1. Schematic illustration of the closed cavity rheometer (CCR).

was used to investigate the participation of covalent bonds, as the chemicals used are able to cleave or prevent the formation of new electrostatic interactions, hydrogen and hydrophobic bonds. Adding 0.03 M DTT to this buffer results in the cleavage of disulphide bonds, by which information on the participation of other covalent bonds beside disulphide bonds is gained. These extraction conditions are called non-reducing and reducing conditions, respectively. For the analysis, samples (1 mg mL^{-1}) were prepared with the two buffers. After 24 h of extraction on a rotary shaker at 200 rpm, insoluble proteins were removed by centrifugation at 4637×*g* for 50 min; ensuring only soluble proteins remained in the supernatant. Former aggregated proteins (insoluble proteins) formed through either covalent (e.g., disulphide and isopeptide bonds) or only disulphide bonds are de-aggregated and thus made soluble through cleaving the participating bonds and interactions. The supernatant was then analysed for protein solubility and changes in the molecular sizes of the soluble aggregates using UV–Vis spectroscopy and size-exclusion chromatography.

UV–Vis spectroscopy was performed to investigate the protein concentration remaining in the supernatant after the extraction under reducing and non-reducing conditions. Although the aggregation of whey proteins after thermal treatment is mainly described through the formation of non-covalent and disulphide bonds, we investigate the participation of other covalent bonds (i.e., isopeptide bonds) with extraction of the treated samples under reducing conditions. Absorption of the samples was measured by an Evolution 201 spectrophotometer (Waltham, MA, USA). For the measurements, the absorption of supernatant (1 mL) was measured at a wavelength of 280 nm. Untreated samples were also measured as a reference, and measurements were performed in triplicate for each sample. Using a calibration curve, the concentration in the supernatant was calculated from the measured absorption. The degree of covalent bond aggregation ($A_{C\text{-}B}$) (equation (2)) was calculated from the difference of the initial concentration of βLG with the residual βLG remaining in the supernatant after the extraction under non-reducing conditions. Similarly, the degree of non-disulphide covalent bond aggregation ($A_{nS\text{-}B}$) (equation (3)) was calculated from the difference of the initial concentration of βLG with the residual βLG remaining in the supernatant after the extraction under reducing conditions. The degree of disulphide bond aggregation ($A_{S\text{-}B}$) (equation (4)) was calculated from the difference between eqs. (2) and (3).

$$A_{C-B} = \left(\frac{C_0 - C_t}{C_0}\right) * 100 \Big| \text{non} - \text{reducing} \tag{2}$$

$$A_{nS-B} = \left(\frac{C_0 - C_t}{C_0}\right) * 100 \Big| \text{reducing} \tag{3}$$

$$A_{S-B} = A_{C-B} - A_{nS-B} \tag{4}$$

Size-exclusion chromatography (SEC) experiments were conducted to investigate changes in the molecular size distribution of the samples using a Shimadzu HPLC System (Shimadzu, Kyoto, Japan). As native proteins denature and aggregate, the native protein concentration decreases. Aggregation causes changes in the molecular weight distribution of the treated samples compared with the reference untreated sample. Under reducing conditions, disulphide bonds are cleaved and the aggregates stabilised by this bonds are made soluble. The peak area of the samples is proportional to the protein concentration in the supernatant. By cleaving the bonds or interactions that stabilise the aggregates, the peak area of the treated samples increases and can match the peak area of the untreated sample. If higher molecular aggregates are stabilised by disulphide bonds, it is possible that new peaks appear at lower elution times, as these aggregates now are soluble and can be measured using SEC. Aggregates which are stabilised by covalent bonds other than disulphide bonds (e.g., isopeptide bonds) are insoluble and not present in the supernatant. In this way, the concentration of the protein in the supernatant decreases and this result in a lower absorbance and a decreased total peak area compared with the untreated sample. The mobile phase, a 0.2 M phosphate buffer with 2 M urea (pH 7) was filtered through 0.2-μm cellulose acetate filters (Sartorius, Göttingen, Germany) and degassed. Finally, 100 μL of the supernatant was injected by an autosampler to the Bio SEC-5 column (Agilent Technologies, Santa Clara, CA, USA) with a fractionation range of 10–150 kDa. The measurements were conducted with a flow rate of 0.5 mL min^{-1} at 25 °C and an UV/VIS detector at 280 nm recorded the absorbance of the samples. The analyses were performed in triplicate for each sample.

2.6. Molecular dynamics simulation

Molecular dynamics (MD) simulations were performed to assess mechanisms involved in during concentration, pressurisation and heating of the proteins. All MD simulations and analysis were performed using the GROMACS software package version 5.1.1 (Berendsen, van der Spoel, & van Drunen, 1995; Lindahl, Hess, & van der Spoel, 2001; van der Spoel et al., 2005), using the OPLS-AA (all-atom) force field (Kaminski, Friesner, Tirado-Rives, & Jorgensen, 2001). The Visual Molecular Dynamics (VMD) program was used for the visualisations (Humphrey, Dalke, & Schulten, 1996). The simulated protein structures have been obtained from PDB (id 3PH5). For this study, the native and neutral dimeric state was used as a start structure; the single dimeric structure was first simulated at $T = 26.85$ °C (300 K) for 10 ns to obtain a steady state with the given force field and conditions. The herewith obtained single protein structure was then multiply placed at random positions and orientations within a triclinic box with periodic boundary conditions and size $(15 \times 15 \times 30)$ nm^3. The most dense possible packing without steric clashes and/or deformation of proteins led to an effective protein concentration of 20.2% (w/w), corresponding to 23 dimers in the box. Only a small distance of 0.4 nm of each single protein to any other periodic boundary protein (part) was added, to obtain a quasi-continuous protein solution (proteins shall interact with periodic counterparts). The system was energy minimised using the steepest descent algorithm (Cauchy, 1847) (approx. 7500 steps) to eliminate any overlap or clash between the atoms. Then it was equilibrated under an NVT ensemble ($T = 26.85$ °C) for 20 ps with position restraints on the heavy atoms of the protein leading to relaxation of the solvent molecules around it.

Protein concentrations higher than 20% (w/w) were obtained by iteratively performing further equilibration under NPT ensemble, removal of appropriate amounts of water, and re-equilibration (NPT). For (re-) equilibration, the following settings were used: bonds were not constrained to allow deformation due to compression; otherwise, the simulation of systems with protein densities above 50% (w/w) is not possible. The integration time step was set to 2 fs. The temperature was maintained to the reference value (300 K) using V-rescale (modified Berendsen thermostat) with coupling time constant $T = 0.1$ ps. The pressure was kept at 1 bar using Parrinello–Rahman barostat with coupling constant 2 ps. The isothermal compressibility value used for the pressure-coupling algorithm for water was 4.5×10^{-5} bar^{-1}. The long-range interactions were applied using the Particle Mesh Ewald (PME) method with a real space cut-off of 1 nm, a Fourier mesh spacing of 0.16 nm and fourth-order interpolation. The Lennard–Jones interactions were calculated using a cut-off of 1.0 nm. With decreasing amounts of water, the box sizes shrunk while maintaining pressure until a minimum box size of

($9.1 \times 9.1 \times 17.3$) nm^3 was reached for 90% (w/w) protein content. After the systems were well equilibrated, production MD was performed at variable temperatures, ranging from 300 K to 470 K in 10 K steps, at NVT ensemble, to mimic the heating of protein-water mixture in a closed cavity. Simulation time was at least 10 ns for all variants. Trajectory and energy analysis were done using GROMACS tools. Translatory diffusion coefficients were calculated by extracting centres of masses (CoM) for each protein, calculating the squared deviation of each CoM over time, averaging for all proteins in the box and computing the slope.

3. Results and discussion

3.1. Influence of temperature and protein concentration on the reaction behaviour and onset temperatures of β-lactoglobulin

The complex modulus IG*I is depicted in Fig. 2 as a function of temperature for three protein concentrations. For a βLG protein concentration of 60% (w/w), the complex modulus at first decreases, then increases and lastly decreases again with increasing temperature. It is known that the rheological properties of polymers are strongly influenced by temperature. According to the Arrhenius-Andrade Equation, a constant decrease in the viscosity is expected with increasing temperature. Polymeric materials can display a mixture of viscous and elastic (i.e., viscoelastic) behaviour. Using oscillatory rheology, complex functions (i.e., complex modulus and viscosity) describing the rheological behaviour can be measured. The complex modulus is used to describe the viscoelastic behaviour of a sample. The ratio of the complex modulus and the frequency describes the complex viscosity. Thus, a decrease in viscosity with increasing temperature is also expected for the complex modulus. Nevertheless, as observed in Fig. 2, the complex modulus did not constantly decrease and the slope of IG*I changed at certain temperatures.

As the rheological properties are a direct function of the structure of (bio-)-polymers (Berry & Fox, 1968), the results indicate that at temperatures, where the slope of the complex modulus changes, structural changes are taking place. Thus, these temperatures are defined as reaction-onset temperatures. For a βLG concentration of 60% (w/w), there are different regions, where the slope of IG*I changed. Around 60 °C, the slope of the complex modulus decreases further after its initial decrease. It is expected that, in this temperature range, the denaturation reaction is initiated, as it is known that, for βLG solutions heated at this temperature range, denaturation takes place as the non-covalent linked dimer unfolds to a reactive monomer. At a temperature of approx. 75 °C, IG*I increases. As an increase in molecular size or mass of polymers increases the viscosity (Berry & Fox, 1968), the increase in IG*I can be associated with structure-forming reactions (i.e., aggregation) (Emin, Quevedo, Wilhelm, & Karbstein, 2017; Koch et al., 2017; Madeka & Kokini, 1994). Therefore, the increase in the complex modulus at this temperature is assumed to show the onset temperature of aggregation. At 95 °C, the complex modulus reaches a plateau. Probably, at this temperature range, aggregation is still taking place, but the effect of temperature on the rheological properties dominates. Around 115 °C, the complex modulus begins to decrease, which indicates that the aggregation reactions are perhaps complete and/or the formed structures begin to break down (i.e., de-aggregation) (Emin et al., 2017; Madeka & Kokini, 1994; Pommet, Morel, Redl, & Guilbert, 2004).

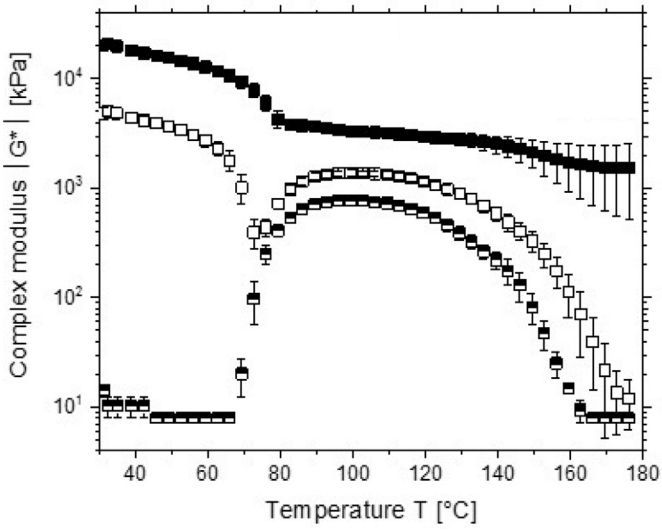

Fig. 2. Complex modulus IG*I as a function of temperature at 1 Hz and 1% for βLG concentrations of 50% (▬), 60% (□) and 70% (■) (w/w). Measurement were performed at a constant heating rate of 5 K min^{-1}.

Regarding the effect of the protein concentration on the reactions, it is clear that for a protein concentration of 50% (w/w), aggregation is taking place at temperatures below 70 °C, as at this temperature an increase in the complex modulus is observed. At this protein concentration, the denaturation onset-temperature cannot be defined, as the complex modulus cannot be measured at temperatures below 62 °C due to low viscosity, and therefore torque values are below the threshold levels that can be measured by the CCR. Nevertheless, compared with 60% βLG, where the aggregation reactions start at around 75 °C, this shows that the aggregation onset-temperature is concentration-dependent and increases with increasing protein concentration. At the same time, as observed in the complex modulus for 70% βLG, not only is the aggregation concentration-dependent, but also the denaturation. At 70%, the first change in the complex modulus occurs at around 70 °C, which implies that, at this temperature the denaturation reaction begins. Compared with 60% βLG, where the denaturation onsets at around 60 °C, an increase in the denaturation-onset temperature with increasing protein concentration was observed. In general, the results from temperature sweep analysis suggest that with increasing protein concentration, the reactions are hindered. Emin et al. (2017) also observed similar behaviour for vital gluten thermally and thermomechanically treated at high concentrations. They showed that the reaction onset temperature increased from 80 °C to 90 °C on increasing gluten concentration from 70% to 80% (w/w).

To investigate the influence of temperature and concentration on the reaction behaviour of βLG in more detail, isothermal time sweep analyses were performed. For this purpose, the temperature was kept constant at different values and the rheological properties, including the complex modulus, were measured as a function of time.

Fig. 3 shows the change in complex modulus for temperatures ranging from 60 °C up to 120 °C for a treatment time of 30 s and protein concentrations of 50%, 60% and 70% (w/w). In addition to a treatment time of 30 s, measurements for 60 s and 600 s were also performed and showed the same tendencies. As in Fig. 2, the complex modulus changed with time, implying changes in the molecular structure of βLG. If a (bio-)-polymer is treated below its reaction temperature, it remains unreactive, and it is expected that IG*I remains constant over time. An increase in the complex modulus over time at a defined temperature suggests that aggregation is taking place. Furthermore, at constant protein concentration, higher IG*I values at higher temperatures also indicate that the sample has higher molecular sizes because of aggregation. The

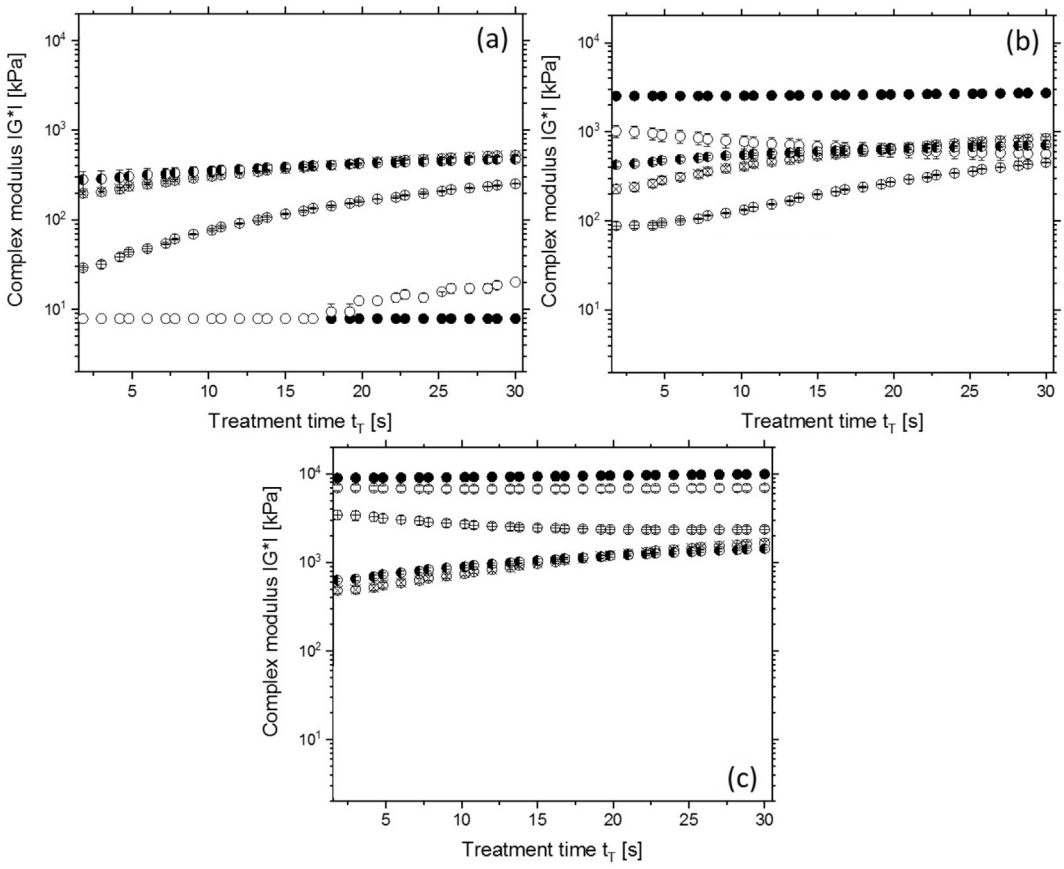

Fig. 3. Complex modulus as a function of treatment time at various temperatures of 60 °C (●), 70 °C (○), 80 °C (⊕), 100 °C (⊗), and 120 °C (◐) for βLG concentrations of (a) 50%, (b) 60%, and (c) 70% (w/w), respectively. The frequency and strain were kept constant at 1 Hz and 1%, respectively.

results in Fig. 3a show that the lowest value of the complex modulus results from the lowest treatment temperature, of 60 °C, and remained unchanged with time.

Increasing the treatment temperature appeared to increase the aggregation rate as not only higher initial values of the complex modulus were observed, but also an increase over time. Nevertheless, at 120 °C almost no changes in IG*I are observed, which suggests that, at this point, the aggregation appears to be complete. Compared with the case at 50%, the samples at 60% and 70% (w/w) protein showed a different behaviour. For both concentrations, increasing the temperature initially decreased the complex modulus, which can be related to the higher molecular mobility due to increased temperature.

These results support the findings from Fig. 2; i.e., aggregation is hindered at higher protein concentrations, as the values of IG*I start to increase over time at higher temperatures. At 50% βLG, aggregation starts at 70 °C, as an increase of IG*I was observed starting at this temperature over time. As shown in Fig. 3b and c, for 60% and 70% βLG, respectively, the aggregation begins at higher temperatures as an increase in the complex modulus is observed at 80 °C and 100 °C, respectively. With increasing protein concentration, the viscosity of the protein matrix also increases. This is shown in Fig. 3 by the higher initial values of IG*I with increasing concentration. The increased viscosity of the matrix causes a decreased mobility of the proteins at higher concentrations and thus the diffusion coefficient also decreases, according to the Stokes-Einstein-Equation. This possibly led to higher activation energies, thus resulting in lower reaction rates. The increased denaturation and aggregation onset temperatures support the hypothesis that the activation energy increases with increasing protein concentration.

3.2. Simulation-based determination of effective diffusion and protein disintegration

The impact of high molecular concentrations (molecular crowding) on effective diffusion of the βLG proteins was evaluated using molecular dynamics. A total amount of 23 βLG dimers were put into periodic boxes, with variable amount of water present in the box. The influence of temperature on the diffusion and protein stability was investigated in 10 K steps from 26.85 °C to 196.85 °C (300–470 K). The effective diffusion coefficients D were measured after 2.5 ns simulation time (Fig. 4), i.e., *before* strong aggregation occurred. This shows that, with increasing protein concentration, D decreased almost exponentially. A bilinear fit of the logarithmic apparent diffusion coefficient can be obtained according to Equation (5) with T denoting the temperature in K, c the protein concentration (w/w) in %, and the parameters approximated to $m_t = (9.0 \pm 0.54)\ 10^{-3}\ \mathrm{K}^{-1}$; $m_c = (-7.1 \pm 0.13)\ 10^{-2}$; $D_0 = (2.3 \pm 0.22)\ \mu\mathrm{m}^2\ \mathrm{s}^{-1}$; the corresponding surface is visualised in Fig. 4a.

$$D(T, c) \approx D_0 e^{m_t T + m_c c} \tag{5}$$

This observed relation leads to a qualitative change of diffusivity. While diluted βLG ($c < 0.2\%$ w/w) shows a diffusivity at $T = 26.85$ °C (300 K) of $D \approx 110\ \mu\mathrm{m}^2\ \mathrm{s}^{-1}$ and still approx. $D = 38\ \mu\mathrm{m}^2\ \mathrm{s}^{-1}$ for $c = 20\%$ (w/w), it deceases to less than $5\ \mu\mathrm{m}^2\ \mathrm{s}^{-1}$ at protein concentrations of more than 50% (w/w); this is seen by empty dots in Fig. 4a. For example, for a concentration of 60% (w/w), the highest measured diffusion at the highest analysed temperature $T \approx 190$ °C (470 K) was lower than with 40% (w/w) at $T \approx 20$ °C (see Fig. 4b).

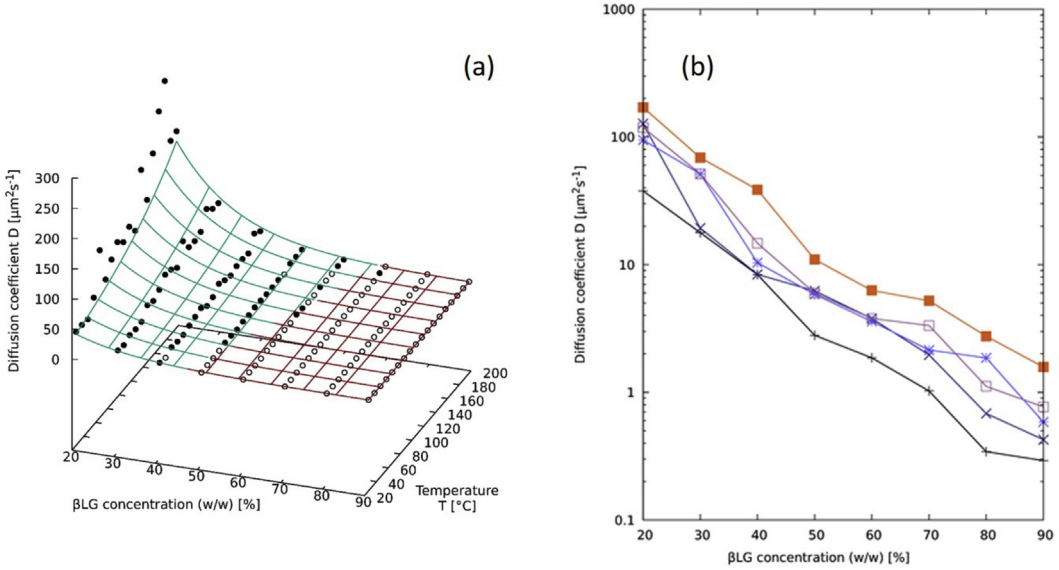

Fig. 4. Diffusion coefficient as a function of temperature and protein concentration, measuring time 2.5 ns: (a) linear surface plot and surface fit (see text for details), diffusion coefficients $D < 5\ \mu m^2\ s^{-1}$ are marked by empty dots and red coloured surface; (b) logarithmic plot of a subset of sampled temperatures (+, 300 K, 26.8 °C; ×, 340 K, 66.8 °C; ∗, 380 K, 106.8 °C; □, 420 K, 146.8 °C; ■, 460 K, 186.8 °C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Visually, protein concentrations of 70% and more appear almost solid. This observed drastic decrease of diffusion of the βLG proteins corresponds to a strong molecular crowding effect. For protein concentrations of more than approximately 50%, considerable deformation of the proteins occurred to achieve sufficient packing density. On the other hand, with increased temperature, simulated deformation also increased. However, the combination of high protein packing density and high temperature overall led to a relative stabilisation of proteins; this is especially visible for exposed loops (in the following, we use Ile_{18}-Gln_{21}; Thr_{65}-Glu_{67}; Asn_{79}; Ala_{102}-Asn_{104}; Asn_{125}-Glu_{130}).

This is visualised in Fig. 5; Fig. 5a shows an example of the displacement of an exposed amino acid (Pro_{129}), i.e., its root mean squared deviation (RMSD), depending on protein concentration and temperature, after a simulation time of 10 ns. This shows that, for low temperatures (below roughly 70 °C), low densities yield highest protein preservation, as expected. For temperatures above 80 °C, however, higher protein densities (between 50% and 60%, w/w, in this example) result in stabilisation of the structure. This relationship is further analysed for all protein residues and for a subset of exposed residues (Fig. 5b). The general trend again shows that, up to a temperature of approx. 60 °C, lower protein densities preserve structure best, while for temperatures above approximately 80 °C, higher densities preserve the structure best. This can be seen to some extent for all residues, but more clearly for the surface exposed loop residues: $c = (32 \pm 1.9)\%$ (w/w) for $T = 26.85$ °C (300 K) to $T = 56.85$ °C (330 K); $c = (50 \pm 2.5)\%$ (w/w) for $T = 106.85$ °C (380 K) to $T = 146.85$ (420 K); $p < 2 \cdot 10^{-5}$ according to a two-sided t-test. The corresponding amino acid positions are marked in Fig. 5c. This analysis showed that the combination of high protein density and elevated temperatures leads to a partial preservation of proteins at temperatures above approx. 80 °C and protein densities between 50% and 60% (w/w). For higher protein densities (i.e., 70%, w/w), this effect is observed when only N terminal loop residues (Ile_{18}-Gln_{21}) and/or longer simulation times are taken into account (data not shown).

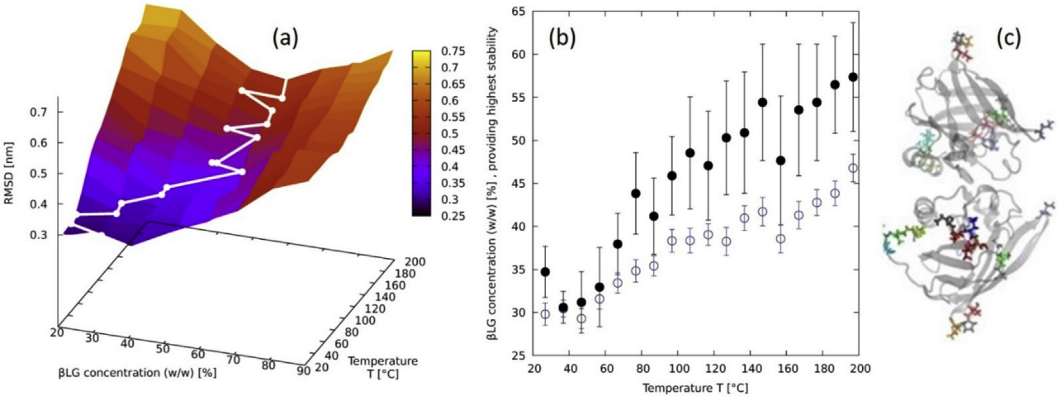

Fig. 5. Effect of temperature and protein density on simulated βLG stability at $t = 10$ ns simulation time. Panel a: visualisation of protein stability (root mean squared deviation with respect to native structure) for one example surface amino acid residue (Pro_{129}) and all temperatures and densities. Higher RMSD denotes stronger disintegration: For elevated temperatures, densities around 50–60% (w/w) preserve structure best (white path denoting minimum). Panel b: plot of best preserving protein densities, depending on temperature, for whole protein (○, all residues) and surface exposed loop residues (●); error bars denote 95% confidence intervals (assumed t-distribution). Panel c: visualisation of the same surface residues.

3.3. Influence of temperature and protein concentration on the structural changes of β-lactoglobulin

Fig. 6 shows the degree of denaturation for thermally treated samples, at temperatures ranging from 60 °C to 120 °C, for a treatment time of 30 s and protein concentration of 50%, 60% and 70% (w/w).

For a βLG concentration of 50% (w/w), the degree of denaturation increased with increasing treatment temperature. Thermal treatment at 60 °C and 70 °C results in approximately 20% and 40% denaturation, respectively. At treatment temperatures above 80 °C, complete denaturation was observed. Increasing the protein concentration to 60% and 70% (w/w) resulted in a similar trend: the degree of denaturation increased with increasing treatment temperature. This effect of concentration can be observed only for treatment temperatures below 80 °C, as treatment at higher temperatures lead to complete denaturation of proteins and the differences are no more visible.

For 70% βLG (w/w), complete denaturation is observed for temperatures above 100 °C. At 80 °C, approximately 80% of the sample is denatured. Treatment at lower temperatures for both concentrations resulted in a similar degree of denaturation, ranging from 1% up to approximately 20%, for 60 °C and 70 °C, respectively. Although the denaturation rate decreased with concentration, 30 s thermal treatment at 100 °C already achieved 100% denaturation of the proteins at all concentrations studied. These findings are in accordance with previous results (Anema, 2000; Hillier, Lyster, & Cheeseman, 1979). Anema (2000) studied the kinetics of βLG denaturation in reconstituted skim milk samples of various concentrations (9.6–38.4% total solids) over a wide temperature range (75–100 °C). At the lowest temperature (75 °C), the denaturation was retarded at higher milk solid concentrations. Increasing the temperature caused this effect to be less pronounced, so that, at 100 °C no effect of solids concentration on denaturation was observed. Hillier et al. (1979) showed that the rate of βLG denaturation in cheese whey, at a constant pH of 6.0, was retarded at higher whey concentrations and that this retardation was less pronounced at higher temperatures. It seems that the thermal motion at 100 °C is enough to denature βLG independent of the viscosity of the protein matrix and therefore no effect of the protein concentration on the reaction is observed.

To investigate which protein interactions are participating in the aggregation reactions, the extractability of proteins before and after thermal treatment was measured using two buffer systems, which are able to cleave non-covalent and disulphide bonds. The degree of aggregation through covalent bonds (e.g., disulphide bonds) as a function of treatment temperature for βLG concentrations of 50%, 60% and 70% (w/w) is depicted in Fig. 7. As with the degree of denaturation, aggregation also increased with increasing treatment temperature. Although, for protein concentration of 60% and 70% for temperatures ≤70 °C, a similar degree of denaturation was observed, taking the results from the temperature sweeps in consideration, different aggregation behaviour is expected. For 50% βLG, the covalent bond aggregation increases with increasing treatment temperature and a thermal treatment at 80 °C and 30 s results in approximately 100% aggregation through covalent bonds. Increasing the protein concentration to 60% and 70% results in less aggregation, which is in accordance with the results of the degree of denaturation.

Taking the results of the degree of denaturation for a βLG concentration of 70% (w/w) into consideration, a higher degree of aggregation as depicted in Fig. 7 was expected. For 70% (w/w) βLG, treatment at 70 °C and 80 °C resulted in approximately 20% and 90% denaturation, respectively. The degree of covalent bond aggregation measured at these temperatures was approximately 5% and 45%, respectively. This might be caused by the participation of not only covalent bonds but also non-covalent bonds in the formation of aggregates, which is in accordance with the findings of other authors. Thermal treatment of whey proteins at temperatures above 70 °C resulted in aggregation by covalent bonds (Anema, 2000; Galani & Apenten, 1999; Manderson, Hardman, & Creamer, 1998). Lower thermal treatment resulted in aggregates being stabilised by non-covalent interactions such as hydrophobic or electrostatic interactions (Iametti, Gregori, Vecchio, & Bonomi, 1996; Oldfield, Singh, Taylor, & Pearce, 1998; Schokker et al., 2000; Shimada & Cheftel, 1988; Wijayanti, Bansal, Sharma, & Deeth, 2014).

Although aggregation of whey proteins mainly takes place through the covalent bond cystine, which links two peptide chains through a disulphide (SS) bridge, depending on the treatment conditions other covalent bonds can also be formed. Under alkaline conditions, β-elimination of cystine occurs and leads to the

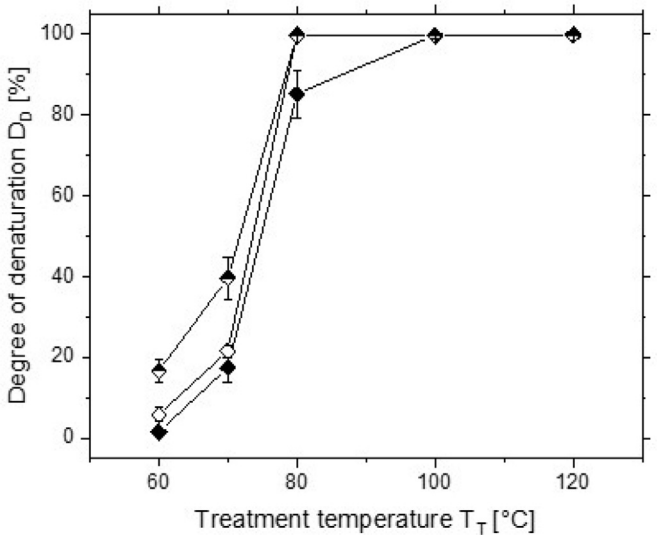

Fig. 6. Degree of denaturation as a function of temperature and βLG concentrations of 50% (◓), 60% (◇) and 70% (◆) (w/w) for a treatment time of 30 s. The frequency and strain were kept constant at 1 Hz and 1%, respectively.

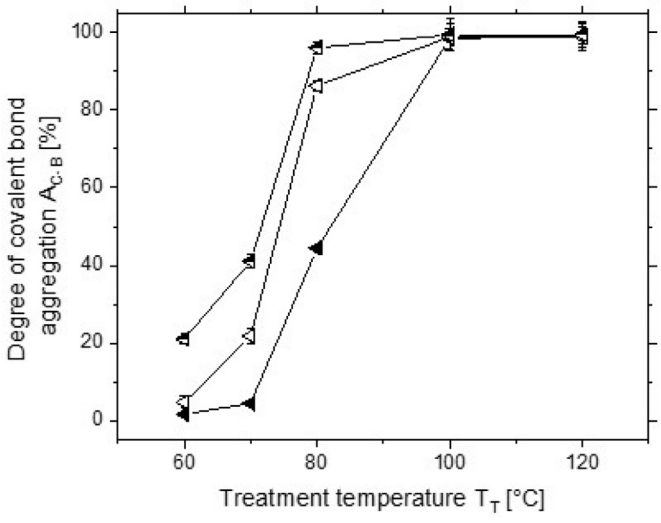

Fig. 7. Degree of covalent bond aggregation as a function of temperature and βLG concentrations of 50% (◂), 60% (◁) and 70% (◀) (w/w) for a treatment time of 30 s. The frequency and strain were kept constant at 1 Hz and 1%, respectively.

formation of intermediate dehydroalanine (DHA), which can then react with cysteine or lysine to form products stabilised by unreducible covalent cross-links such as lanthionine (LAN) or lysinolanine (LAL), respectively (Rombouts, Lagrain, Brijs, & Delcour, 2010). Reaction of the ε-amino group of lysine with the side-chain amide group of glutamine or asparagine results in the isopeptide bonds ε-(γ-glutamyl)lysine or ε-(β-aspartyl)lysine, respectively (Asquith, Otterburn, & Sinclair, 1974). To which degree disulphide bonds or other covalent bonds participate in the aggregation after thermal treatment is depicted in Figs. 8 and 9. The degree of disulphide bond aggregation is shown in Fig. 8. For all investigated protein concentrations, the degree of aggregation increased with increasing treatment temperature until a maximum was reached at 100 °C and then it decreased. The highest degree of aggregation was observed at this temperature for a βLG concentration of 70% (w/w). Although a decrease in the degree of aggregation was observed at 120 °C, at this concentration heating at temperatures ≥100 °C result in higher aggregation through disulphide bonds compared with 50% and 60% (w/w) βLG.

Thermal treatment of 50% and 60% (w/w) βLG resulted in a similar degree of disulphide bond aggregation at the temperatures investigated. Increasing the treatment temperature resulted in higher degrees of aggregation by disulphide bonds as, by increasing the temperature, the accessibility of the thiol group also increases, leading to an increase in the rate of aggregation via thiol/disulphide exchange reaction, and by this, by intermolecular disulphide bonds (Matsudomi, Oshita, Sasaki, & Kobayashi, 1992; Sawyer, 1968; Shimada & Cheftel, 1988). Sawyer (1968) observed that the disulphide bond aggregation is favoured during heating at 97.5 °C, whereas non-covalent aggregation is favoured at lower temperatures (below 85 °C). Increasing the temperature further (>100 °C) leads to changes in the protein structure measured by differential scanning calorimetry (DSC), which could indicate the breakdown of the aggregates stabilised by disulphide bonds (deWit & Klarenbeek, 1981; Holt et al., 1998; McPhail & Holt, 1999; Paulsson, Hegg, & Castberg, 1985). deWit and Klarenbeek (1981) postulated that this change in protein structure might also represent a second reaction process, which might be a secondary aggregation reaction. As thermal treatment at temperatures >100 °C resulted in a degree of denaturation of 100% for all investigated concentrations, similarly, a complete aggregation was expected. However, as depicted in Fig. 8,

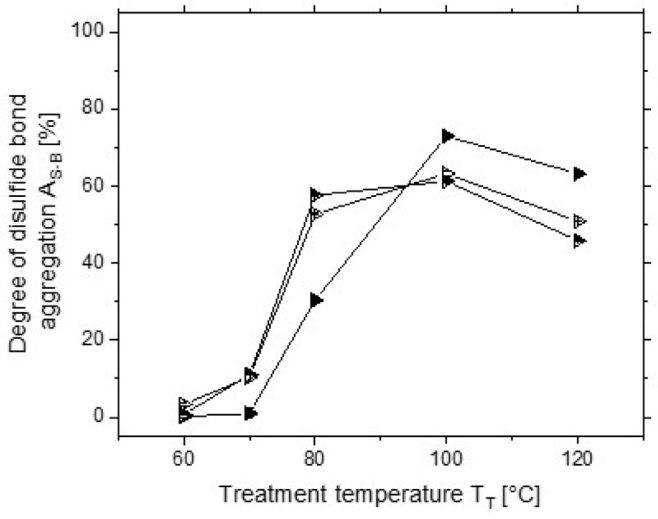

Fig. 8. Degree of disulphide bond aggregation as a function of temperature and βLG concentrations of 50% (▶), 60% (▷) and 70% (▶) (w/w) for a treatment time of 30 s. The frequency and strain were kept constant at 1 Hz and 1%, respectively.

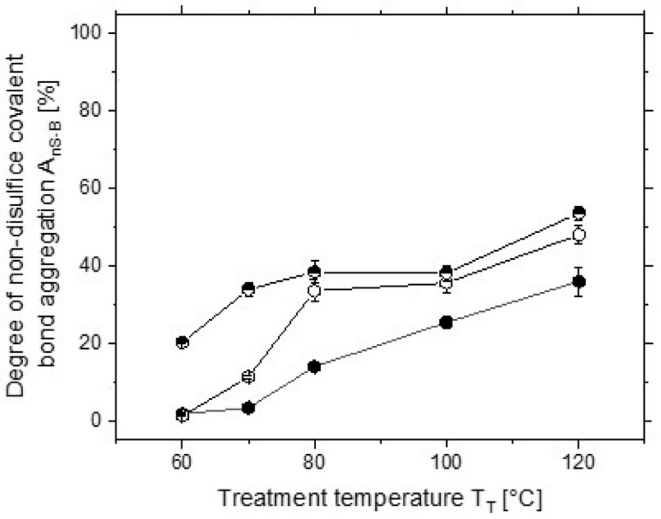

Fig. 9. Degree of non-disulphide covalent bond aggregation as a function of temperature and βLG concentrations of 50% (◓), 60% (○) and 70% (●) (w/w) for a treatment time of 30 s. The frequency and strain were kept constant at 1 Hz and 1%, respectively.

the degree of disulphide bond aggregation is not 100% for none of the investigated protein concentrations at temperatures above 100 °C. This indicates that other covalent bonds are expected to participate in the aggregation reactions. The degree of non-disulphide covalent bond aggregation is shown in Fig. 9. Increasing the treatment temperature, results in a higher degree of non-disulphide covalent bond aggregation for all βLG concentrations investigated. A thermal treatment at 60 °C and 30 s for 50% (w/w) βLG results in a degree of aggregation of approximately 20%. Increasing the temperature to 120 °C leads to an increase in non-disulphide covalent bond aggregation, to almost 60%. Thermal treatment at 120 °C for 60% (w/w) βLG results in an equal aggregation via disulphide and non-disulphide covalent bonds. At lower temperatures, the aggregation takes place mainly via disulphide bonds. Increasing the concentration up to 70% (w/w) βLG resulted in aggregates mainly stabilised by disulphide bonds, as the highest value of degree of non-disulphide bond aggregation is 40%.

Nevertheless, aggregation via other covalent bonds was observed for all investigated concentrations at treatment temperatures above 80 °C. At lower temperatures, βLG remains native and is not aggregated or aggregates via non-covalent or disulphide bonds, depending on the concentration. Furthermore, it was also shown that both, the treatment temperature and protein concentration influenced the degree of aggregation and protein interactions stabilising the aggregates. Although disulphide and other covalent bonds stabilise the aggregates, disulphide bonds are mainly responsible for the stabilisation of aggregates at higher protein concentrations, which is also supported by the results from the SEC.

Fig. 10 shows the elution profiles of thermally treated and untreated βLG at different concentrations under reducing conditions (non-covalent and disulphide bonds are cleaved). For all investigated protein concentrations (Fig. 10a–c), a decrease in the total peak area was observed with increasing treatment temperature, which indicates that insoluble non-reducible aggregation products are being formed. Similarly, as the degree of non-disulphide covalent bond increased with decreasing protein concentration (70–50% βLG), the concentration of protein in the supernatant (i.e., peak area) also decreased. This is in accordance with the results discussed above. Overall, it was shown that the degree of non-disulphide covalent bond aggregation increases with increasing

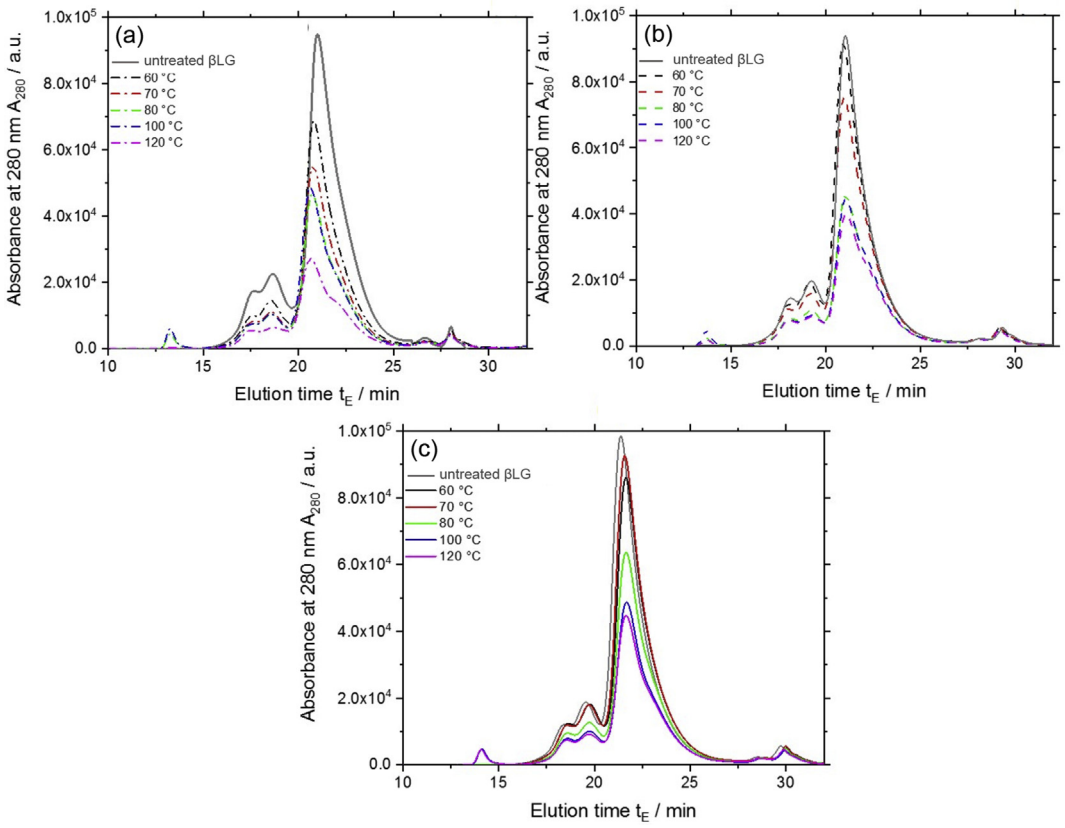

Fig. 10. Size exclusion chromatogram of untreated (grey trace) and treated samples under reducing conditions at various temperatures of 60 °C (black trace), 70 °C (red trace), 80 °C (green trace), 100 °C (blue trace), and 120 °C (purple trace) for a treatment time of 30 s for βLG concentrations of (a) 50%, (b) 60%, and (c) 70% (w/w), respectively. The frequency and strain were kept constant at 1 Hz and 1%, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatment temperature and decreasing protein concentration, possibly due to an increase in the aggregation rate. At 50% βLG and temperatures above 100 °C non-disulphide covalent bond aggregation outweighs aggregation via disulphide bonds. At higher βLG concentrations (60% and 70%), this trend was no longer observed, as even the highest thermal treatment result in aggregation via non-disulphide covalent bonds under 50%. Although it was shown that besides disulphide bonds other covalent bonds stabilise the aggregates, with the methods used the formation of non-disulphide covalent bonds, whether isopeptide bonds or other non-disulphide covalent bonds (such as LAN or LAL), cannot be identified, as they all are non-reducible in DTT-containing systems. Since these type of covalent bond are usually formed at much higher pH values (pH > 10) and not at pH values between 6.5 and 7.0, as was the case in this study, which type of covalent bond is indeed being formed, should be further investigated.

4. Conclusion

The objective of this study was to examine the influence of protein concentration on the reaction behaviour and structural changes of highly concentrated β-Lactoglobulin solutions, resulting from defined heat treatment. Therefore, the samples were analysed inline by rheological measurements and offline by reversed-phase and size-exclusion chromatography and spectrophotometry. From the inline measurements, it was observed that, for a protein concentration of 50% (w/w), aggregation is taking place at temperatures below 70 °C, as at this temperature the complex modulus increases. At higher concentrations, the denaturation onset temperature increased to 60 °C and 70 °C for 60% and 70% βLG, respectively. Similarly, the aggregation onset temperature increased to 75 °C and 80 °C for both concentrations. Furthermore, rheological measurements showed a dramatic increase in the viscosity of the protein matrix at higher protein concentrations, which caused a decrease in the molecular diffusion reducing the rate of reactions taking place. The results from molecular modelling simulations confirmed these expectations and showed that the effective diffusion coefficient decreased almost exponentially with increasing protein concentration, which is also in accordance with the offline structural analysis.

Overall, the results show that the denaturation and aggregation behaviour strongly depends on the protein concentration and thermal treatment conditions, increasing the protein concentration far beyond the level investigated in studies so far leads to a lower degree of denaturation and aggregation, as the reaction rate decreases. For all investigated protein concentrations (50–70%) and treatment times (30–600 s), the degree of denaturation and aggregation increases with increasing treatment temperature and a complete denaturation was observed at temperatures ≥100 °C. The analysis of the interactions between the proteins revealed that depending on the protein concentration and heat treatment, non-covalent or covalent bonds are responsible for the formation of aggregates. With increasing the aggregation rate, not only disulphide bonds but also other bonds including in DTT non-reducible covalent bonds are formed.

This study forms the basis for a better understanding of the thermal denaturation and aggregation of highly concentrated whey protein systems (protein concentration > 40%). To get a better

insight in the reactions occurring during process such as extrusion processing, an extension of this study to include the effect of additional stresses and a more complex protein matrix including other components such as lactose or other protein fractions (i.e., αLA) on the denaturation/aggregation behaviour is needed.

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