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Multi-step thermally induced transitions of β-lactoglobulin − An in situ spectroscopy approach

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A B S T R A C T

An in-situ approach based in multiple spectroscopic techniques and benchmarked with DSC was used to characterise β-Lg thermally-induced transitions. The methodology applied overcomes previously reported limitations by ensuring similar experimental conditions in different determinations, non-aggregation conditions and allowing differentiation between fluorescent variations due to collisional quenching and structural modifications. These experimental improvements along with the correlation of complementary data from the assessment of several unfolding-related events, allowed a real time, precise and detailed description of the unfolding/refolding pathways of β-Lg. The existence of a complex multi-step unfolding mechanism was confirmed, with a focus on the reversible conformational changes. The elusive unfolding intermediates were characterised in terms of structural swelling, hydrophobic sites accessibility and tryptophan exposure. This approach allowed establishing a clear order of events during thermally-induced structural changes, representing a step forward in the understanding of protein stability and interactions, useful, e.g., when establishing heat treatments of dairy products.

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1. Introduction

β-Lactoglobulin (β-Lg) is the most abundant protein in whey, dominating its functional properties (Ramos et al., 2015). This small globular protein with 162 amino acids holds a central calix and has the ability in to bind small hydrophobic molecules (Considine, Patel, Singh, & Creamer, 2005). Due to its industrial relevance, it has been the focus of many studies regarding conformation and thermal stability. Therefore β-Lg is a well-studied protein and often referred to as a model for globular protein behaviour, either on molecular stability and conformation as in aggregation and supramolecular structure formation (Kontopidis, Holt, & Sawyer, 2004).

Extensive studies deal with β-Lg interactions with other proteins, fatty acids, biopolymers and micronutrients; additionally its self-interactions are of major importance and result on the

formation of diverse structures as self-supporting gels and several types of aggregates as nano and micro spherical aggregates and fibrillary amyloid-like structures (Kontopidis et al., 2004; Loveday, Anema, & Singh, 2017; Nicolai, Britten, & Schmitt, 2011). β-Lg behaviour and stability change significantly with environmental conditions such as temperature, pH and salt content. Most reports refer to pH values close to neutral and relatively low ionic strength as these are the most physiologically relevant conditions (Bhattacharjee & Das, 2000). Its thermal denaturation involves multiple steps, comprehending a sequence of changes in tertiary and secondary structure. These phenomena may not take place simultaneously but rather sequentially or triggering each other (Burgos, Dassie, Villarreal, & Fidelio, 2012; Loveday, 2016; Seelig & Schönfeld, 2016).

It is generally recognised that β-Lg undergoes structural changes above 60 °C becoming reactive to polymerisation and that these changes can become irreversible between 70 and 140 °C (Iametti, De Gregori, Vecchio, & Bonomi, 1996; Tolkach & Kulozik, 2007). Furthermore, the existence of reversible structural changes occurring in β-Lg below 60 °C have been reported in previous studies

(Bhattacharjee & Das, 2000; Cairoli, Iametti, & Bonomi, 1994; Tolkach & Kulozik, 2007), although these reports are still scarce, providing few details about these transitions.

Despite the extensive literature on β-Lg thermal denaturation, the sequence and extent of thermally induced structural changes and their impact on β-Lg functionality are often inconsistent. One of the reasons for these inconsistencies arises from the fact that different reports use different sets of characterisation techniques and experimental conditions, evaluating different aspects and effects of the thermally induced transition. Furthermore, in dairy science, protein denaturation is often evaluated indirectly by measuring consequences of the structural changes (i.e., reactivity of disulphide groups, surface hydrophobicity, change in elution time, solubility loss, aggregation) and not by evaluating the in situ unfolding events (Loveday, 2016). This is particularly critical for the study of reversible structural changes, where limitations in real-time monitoring and operation under non-equilibrium conditions impaired an accurate characterisation of these events.

Indeed, the temperature effects on protein structure result from several phenomena and they can only be characterised by a systematic series of studies using a set of complementary techniques. The use of "online" techniques, characterising the events as they occur is fundamental to disclose the complex unfolding pathways and reversible events. The biophysical characterisation of a protein as function of the temperatures is crucial to understand and control its conformation, biological activities and functional properties (Privalov & Gill, 1988; Thirumalai, Liu, O'Brien, & Reddy, 2013).

Protein unfolding results in a rearrangement of the tertiary and secondary structure causing a protein to undergo a transition from a well-defined native state conformation to a partially or completely unfolded state (Rees & Robertson, 2001). In its simplest form, proteins' thermally induced transition is addressed by a two-step model in which the protein assumes only the folded and the unfolded states. In this model the temperature at which 50% of the protein molecules undergo unfolding is referred to as the melting temperature (T_m) (Ibarra-Molero, Naganathan, Sanchez-Ruiz, & Muñoz, 2016; Rees & Robertson, 2001). More recently, various multi-step models for protein unfolding have been suggested, as for many proteins thermal unfolding occurs through a progressive sequence of partially unfolded states (Bhattacharyya & Varadarajan, 2013; Seelig & Schönfeld, 2016).

The intermediates that occur during protein folding—unfolding equilibrium can provide valuable information about the complex structural transition pathways. Techniques as differential scanning calorimetry (DSC) and spectroscopic methods, such as circular dichroism (CD), fluorescence spectroscopy and light scattering can be used to follow in real time the unfolding mechanism of proteins. Despite the advantages of real time monitoring, adjacent phenomenon associated with temperature increase and protein unfolding may affect the results obtained and their interpretation. In particular, protein aggregation occurring simultaneously to the unfolding process may either impair the protein refolding upon cooling and/or interfere with the protein's fluorescence by affecting the quenching mechanisms (Delahaije, Gruppen, Van Eijk-Van Boxtel, Cornacchia, & Wierenga, 2016; Eftink, 1994). During thermal-scan fluorescence spectroscopy especially, the increase of the collisional quenching must not be overlooked. This technique is commonly used because it is highly sensitive to small conformational changes; however, the quantum yield decrease associated with the temperature and entropy increase results in the relative reduction of the florescence intensity. This must be differentiated from fluorescence variations resultant from conformational changes.

The aim of this work was to study β-Lg thermally induced transitions using a new approach by monitoring in real time the conformational and structural transitions involved during temperature increase. Data from thermodynamic and structural stability as obtained from DSC and CD were correlated with autofluorescence (AF), fluorescent probe binding and static light scattering (SLS). It was expected that this approach would provide a novel strategy to establish a more complete picture of the thermally induced events occurring even below denaturation temperature, thus allowing to observe early and reversible conformational changes as they occur and to establish a more detailed sequence of events during β-Lg unfolding. The obtained results may contribute to the improved knowledge on the thermal unfolding of β-Lg that impacts technological processes involving milk and whey processing by affecting stability or structural modifications of purified or enriched β-Lg fractions.

2. Material and methods

2.1. Protein preparation

β-Lg was purified from commercial whey protein isolate (Lacprodan DI-9212, Arla Foods, Viby, Denmark). The purification was performed by salting out method using the procedure of Maté and Krochta (1994) adapted according to Konrad, Lieske, and Faber (2000) and finally freeze dried. This method was chosen for being suited for the recovering of high purity fractions of β-Lg in its native form. The recovery yield was of 25% of the estimated total amount of β-Lg in the isolate and the reaming fractions, correspondent to denatured and aggregated fractions, discarded during the purification process. The final product was freeze-dried and stored at −20 °C until further use. The purified proteins were benchmarked with commercial β-Lg from Sigma−Aldrich (lyophilised powder ≥90% PAGE) and against literature data. The assessment of structure and conformation was performed by using HPLC, native and SDS-PAGE and CD, matching it in all criteria, thus ensuring purity and conformation.

β-Lg solutions were prepared in 25 mM phosphate buffer at pH 7.0. The solutions were kept under stirring for 2 h to ensure full solubilisation. The pH was then readjusted if needed with 1 M hydrochloric acid or sodium hydroxide. For the DSC experiments, β-Lg solutions of different concentrations were prepared in the same buffer just prior to the respective experiment and used immediately after full solubilisation. For the spectroscopic techniques, β-Lg concentration was maintained at 10 μM (0.184 mg mL^{-1}). The protein concentration chosen was substantially lower than the critical association concentration reported for β-Lg (Baussay, Bon, Nicolai, Durand, & Busnel, 2004; Mehalebi, Nicolai, & Durand, 2008), in an attempt to reduce association and aggregation to a neglectable extent. This allowed focusing on conformational and structural changes in mostly non-aggregated proteins, otherwise hindered by association phenomena.

All chemicals were of analytical grade and purchased from Sigma−Aldrich (Steinheim, Germany). The water used for buffer preparation was purified through a Millipore system.

2.2. Differential scanning calorimetry

After β-Lg solution preparation, protein concentration was confirmed by UV-absorbance (absorbance at 280 nm, absorbance coefficient ε = 17,600 M^{-1} cm^{-1}) and then transferred to the DSC cell after degassing for 15 min in the ThermoVac (Microcal/Malvern). DSC analysis was performed in a MicroCal VP-DSC microcalorimeter from Malvern (Worcestershire, UK). Blank experiments with phosphate buffer in both cells were performed overnight prior to sample loading, for subsequent blank correction. Samples were run against the buffer in the reference cell, performing two

successive heating and cooling scans for each sample, at a scanning rate of 1.5 °C min^{-1}, over the temperature range 20−110 °C. To ascertain possible association/dissociation events, denaturation scans were performed at different concentrations, namely 498, 157 and 47 μM. All procedures regarding sample preparation and handling (lag time at low temperature, temperature scan rate, gain and filter period) were kept constant in all experiments, to ensure that all samples had the same thermal history. In all cases, the reported DSC curves are corrected for the respective blank experiment.

2.3. Circular dichroism measurements

CD measurements were performed on a Chirascan plus circular dichroism detector (Applied Photophysics, Leatherhead, UK). The samples were placed in a quartz cuvette with 1 cm light path, the temperature control was ensured by the Peltier element on the cuvette holder and magnetically stirred at 200 rpm to ensure homogeneity. CD spectra were obtained in the far-UV range (260−200 nm) and the CD signal at a fixed wavelength (208 nm) was collected as a function of temperature from 20 to 90 °C. Here and in all spectroscopic determinations, a scanning rate of 1 °C min^{-1} was used during the spectroscopic determinations. That scanning rate is a standard for low gradient scans, allowing the direct comparison of these results with those published in several literature reports. Furthermore, it has been reported that for β-Lg the T_m obtained at this scanning rate (and below) is almost constant and close to the T_m value obtained when extrapolating the modelled results to scanning rates asymptotically approaching 0 °C min^{-1}, thus eliminating kinetic effects (de Jongh, Gröneveld, & de Groot, 2001; Relkin & Mulvihill, 1996).

2.4. Steady-state fluorescence spectroscopy and static light scattering

All measurements were conducted on a PTi fluorescence RTC 2000 spectrometer (Photon Technology International, Ontario, Canada) with a T-configuration, using a 75-W Xenon arc lamp coupled to a monochromator. The samples were placed in a quartz cuvette with 1 cm light path and magnetically stirred at 200 rpm to ensure homogeneity. Illumination power was set at 5.5 μW for all determinations except where otherwise mentioned. Thermal effects on β-Lg were followed on the RTC 2000 spectrometer using the Peltier element as a temperature control device and a water bath as heat sink. Temperature was scanned from cycles from 20 °C to 90 °C at a heating/cooling rate of 1 °C min^{-1}. This procedure was adopted for all the determinations described below.

Time-based fluorescent emission kinetic traces (emission fixed at 350 nm) were obtained during the thermal ramps at a continuous 295 nm excitation. Before and after each thermal ramp session, excitation and emission spectra were acquired for both the protein (excitation 295 nm, emission 350 nm) and for the aromatic amino acids photoproducts (excitation 320 and 360 nm, emission 405 and 435 nm) (Correia, Neves-Petersen, Jeppesen, Gregersen, & Petersen, 2012).

8-Anilino-1-naphthalenesulfonic acid (ANS) was used as a conformational probe. ANS stock solution (1.2 mM) was prepared in methanol and stored in the dark at 4 °C. Final concentration of ANS used in the protein solution was 50 μM and after excitation at 370 nm the fluorescence emission at 480 nm was recorded.

Static light scattering (SLS) experiments were performed by illuminating the sample at 532 nm, and collecting the light scattered at the same wavelength at 90° with respect to the light source.

2.5. Data analysis

All data analysis, plotting and fitting procedures were performed on Origin 8.1 software (OriginLab Corporation, Northampton, MA, USA). In the case of DSC, Origin 7 with the dedicated DSC module was used in data analysis. All spectroscopic determinations were obtained in triplicate and presented as average of the three accumulations. All intensity profiles were smoothed using a 10 points adjacent averaging filter and normalised by dividing each data point by the initial intensity value of the fresh sample.

For the DSC experiments, the protein scan was corrected to the buffer−buffer scan, the data normalised to the protein concentration in each case, and data curves treated with different models.

3. Results and discussion

3.1. Differential scanning calorimetry

DSC directly measures the heat absorbed during conformational transitions and is therefore a central method for a complete thermodynamic analysis of the unfolding process. The DSC curve for the experiment performed at 498 μM is shown in Fig. 1. It is clear from the asymmetry and width of the curve that the protein does not denature through a simple 2-state transition, nor is it a highly cooperative transition. In fact, attempts to adjust a 2-state model gave a very poor representation of the denaturation curve. Therefore, models considering multiple transitions state were tried. These multi-step models involve the increase in flexibility of the protein chains, followed by the collapse of some groups, culminating on energetically significant structural transitions (Bhattacharjee & Das, 2000; Semisotnov et al., 1991). The best results were obtained for the model that considers that protein denaturation proceeds via three transitions (four-state unfolding). The deconvolution of the original curve into three Gaussian curves (grey lines) can be seen in Fig. 1 with an excellent agreement between the original curve (black line) and the total fitted curve (grey dotted line).

The experimental curve was integrated to retrieve the calorimetric enthalpy, leading to a value of 525 kJ mol^{-1}. The parameters for the four-state unfolding are $T_1 = 61.0$ °C, $\Delta H_1 = 101$ kJ mol^{-1}, $T_2 = 72.7$ °C, $\Delta H_2 = 201$ kJ mol^{-1} and $T_3 = 81.0$ °C, $\Delta H_3 = 223$ kJ mol^{-1}. It should be noted that although the four-state
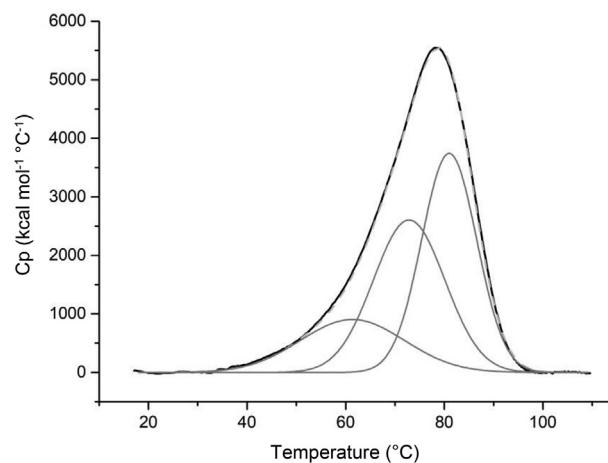

Fig. 1. DSC profile (black line) for the denaturation of β-Lg (498 μM in 25 mM phosphate buffer at pH 7.0); three curves resulting from the deconvolution to a three-state transition (grey lines) and the total fitted curve (grey dotted line).

model did provide the best results, the fitting to a 3-state unfolding (2 transitions) also resulted on a rather acceptable fitting of the DSC results (results not shown). In any case, our results show that β-Lg does not follow a single cooperative unfolding − rather different segments of the molecule may melt independently of one another.

To assess possible concentration effects that point to association/dissociation events during the thermal scans, DSC experiments were repeated at lower concentrations (i.e., 157 and 47 μM) to corroborate the obtained results and check if the transition temperatures changed with concentration. The denaturation profiles were similar at the three studied concentrations, and only the fitting to a 4-state unfolding model (three transitions) provided good results in all cases. Only the transition temperatures changed, increasing somewhat with the decrease in protein concentration. This effect has been previously reported in similar conditions (Qi, Brownlow, Holt, & Sellers, 1995). Further DSC studies and refined models that cope with association upon denaturation are needed to fully resolve this concentration dependence.

3.2. Circular dichroism

CD spectroscopy on the far-UV range was used to follow the structural changes of β-Lg upon heating. Fig. 2A shows the spectra of the protein, plotted between 260 and 200 nm, for 20 °C, 90 °C and after cooling back to 20 °C. Significant structural changes can be noticed during the heating cycle as the decrease in ellipticity, especially below 210 nm, can be interpreted as the collapse of the secondary structure of β-Lg at 90 °C (Griffin, Griffin, Martin, & Price, 1993). As expected, the spectra collected at 20 °C before and after heating to 90 °C are different, showing that heating to 90 °C causes irreversible denaturation of at least part of the protein. The difference between the spectra at 20 °C after cooling from 90 °C from the one collected at 90 °C indicates that the protein suffered a partial refolding during the cooling down process. It is well established that many proteins do not unfold completely after Tm, retaining a significant fraction of secondary structure and conformation or partially refolding into a different conformation once the temperature is decreased. The occurrence of these partially unfolded/refolded species with loss of tertiary structure, maintaining significant fractions of secondary structure, are often referred to as "molten globules" (Bhattacharyya & Varadarajan, 2013; Semisotnov et al., 1991).

Considering the changes in the CD spectra of β-Lg, the changes in ellipticity at 208 nm were tracked during the heating cycle and used as an indicator of the structural changes occurring during the process. From Fig. 2B it is clear that a transition takes place, particularly between 60 °C and 80 °C, which can be related with the

secondary structure transition of the protein (Greenfield, 2007). Taking the derivative of the CD signal, the middle point of the structural transition can be determined, given by the temperature value at the inflexion point. This transition takes place at 73.8 °C (marked on the figure by the vertical dashed line).

3.3. Assessment of conformational changes

β-Lg auto fluorescence (AF) intensity is mostly resultant from the two Trp residues and therefore in this study, β-Lg fluorescence was obtained in conditions specific to follow Trp exposure (excitation 295 nm, emission 350 nm). It is generally accepted that the microenvironment of Trp on the protein affects its relative fluorescence, thus factors resulting from conformational modifications such as solvent exposure or changes in the distance to neighbouring amino acids result in fluorescence changes (Busti, Scarpeci, Gatti, & Delorenzi, 2002). Protein association and aggregation are possible at temperatures above 60 °C also can contribute to fluorescence intensity modifications. However, these phenomena were prevented by carrying the experiments significantly below the reported critical association concentration and their absence was corroborated by SLS data, presented further on this section.

Several studies identified fluorescence intensity reduction during the thermal increase of protein solution, attributing these changes to the occurrence of structural modifications on the protein structure (Bhattacharjee & Das, 2000; Cairoli et al., 1994; Fessas, Iametti, Schiraldi, & Bonomi, 2001). Fluorescence emission is expected to decay with temperature increase due to collisional quenching (Lakowicz, 2006). This is the result of entropy increase, causing an increase in collisions between excited state fluorophores and other molecules in solution leading to their deactivation. The fluorescence of pure Trp solution was followed in the same conditions (i.e., molar ratio, buffer, heating rate and light intensity) as for β-Lg. This allowed to establish a base-line for the fluorescence decay resulting from collisional quenching and differentiate proteins' structural changes from collisional quenching phenomenon. The comparison between the fluorescence scan of Trp solutions and β-Lg can be seen in Fig. 3A. The fluorescence scan of β-Lg during temperature increase and decrease for samples for a series of heating cycles between 20 and 50, 60, 70 or 90 °C is shown in Fig. 3B.

A single exponential decay model (Eq. (1)) was fitted to the data of Trp fluorescence intensity profile as function of temperature, where the fluorescence intensity (F) is described by the pre-exponential factor A_0, the decay constant τ, and the temperature T.
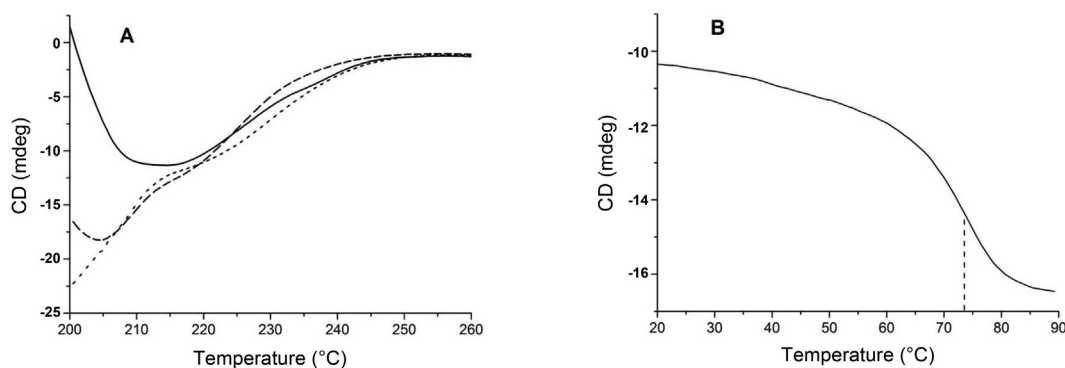
$$F = A_0.e^{(-\tau.T)} \tag{1}$$

Fig. 2. CD spectra (A) of β-Lg at 20 °C (continuous line), at 90 °C (dotted line) and cooled down back to 20 °C (dashed line), and CD signal (B) at 208 nm during heating until 90 °C (vertical line indicates the middle point of the transition given by the first derivative of the curve).
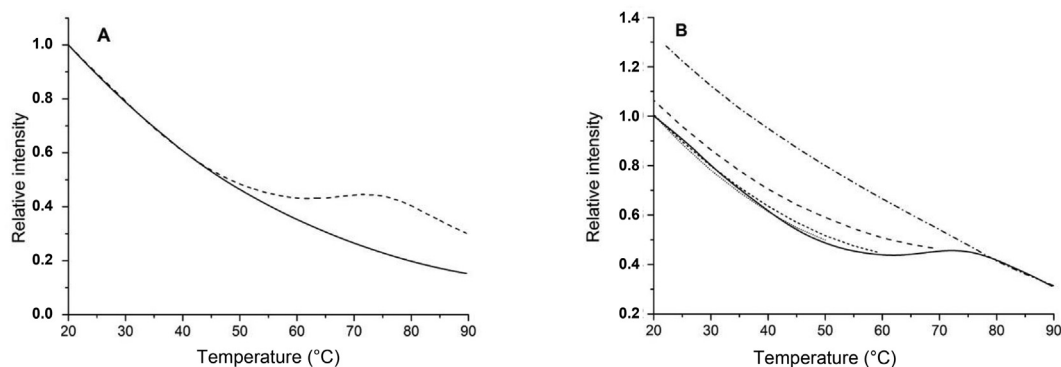
Fig. 3. Fluorescence intensity (A) of Trp (continuous line) and β-Lg (dashed line) at exitation of 295 nm and emission of 350 nm, as function of temperature and fluorescent intensity profiles (B) for heating up to 90 °C (black line) and cooling from 50 °C, 60 °C, 70 °C and 90 °C in the different dashed lines.

The values obtained from this model have shown an excellent fit (Adjust RMS > 0.999). This is in agreement with the described model for fluorescence decay involved in collisional quenching (Gratton, Jameson, Weber, & Alpert, 1984; Lakowicz, 2006). Adjusting the same model to the fluorescence data of β-Lg only, resulted in a satisfactory fit for the temperature range 20–50 °C; above this temperature the fluorescence intensity drifts form the Trp collisional quenching profile. Fluorescence intensity may suffer variations due to a) conformational changes resulting in Trp repositioning, b) changes in neighbouring amino acids or c) modified solvent exposure (Bhattacharjee & Das, 2000). Therefore, in the 50–75 °C interval the changes observed in the fluorescence profile are caused by structural changes associated with a partial loss of tertiary structure. At the temperature of 75 °C a second shift in the fluorescence profile is observed, and until 90 °C the fluorescence decays again on a profile consistent with collisional quenching.

Trp scans were performed cyclically, from 20 °C up to four different temperatures and back to 20 °C, namely: 20 °C → 90 °C → 20 °C, 20 °C → 70 °C → 20 °C, 20 °C → 60 °C → 20 °C and 20 °C → 50 °C → 20 °C (see Fig. 3B). For all the cases, the ascending temperature scans overlapped perfectly, whereas for the descending scans they diverged according with the denaturation degree and reversible/irreversible nature of the structural changes induced by the treatment. In the cycle reaching 90 °C, the heating and cooling fluorescence scans overlap above ≈78 °C. This behaviour is consistent with collisional quenching of the Trp signal by water molecules and suggests that no further significant changes on the Trp microenvironment take place in this temperature range, thus the transition is complete above 78 °C. As the temperature descends to 20 °C again, the fluorescence intensity is substantially higher than the initial value, indicating large modifications in the protein structure. On the scans descending from 70 °C to 20 °C the fluorescence profile immediately diverges from the corresponding heating scan, also suggesting the irreversibility of the modifications in the protein's conformation. The fluorescence scanning descending from 60 °C to 20 °C is slightly different from the corresponding heating scan, eventually merging for temperatures below ≈35 °C. This indicates that the changes occurred until 60 °C are reversible. The fluorescence profiles obtained during the temperature cycling from 20 °C to 50 °C and back to 20 °C overlap – and so does the pure Trp scan – indicating that only collisional quenching is occurring. This means that no rearrangement on Trp positioning and thus no significant structural changes took place within this temperature interval.

Despite the occurrence of reversible structural changes in β-Lg below 60 °C having already been pointed out in previous studies (Bhattacharjee & Das, 2000; Cairoli et al., 1994; Tolkach & Kulozik,

2007), to our understanding this study is unique in exploring these phenomena in situ, during the thermal scans and with a series of heating cycles between different temperatures. Further, the existing studies did not consider the effects of collisional quenching and often attribute the decrease of fluorescence to structural changes or self-quenching or even reversible structural modifications, while in some of the cases collisional quenching could be the main reason for the observed behaviour. With our methodology, we were able to differentiate these phenomena occurring simultaneously and precisely pinpoint the thermally-induced transitions.

The use of the extrinsic fluorescent probe ANS offers additional possibilities for protein characterisation and the determination of thermal transitions. This extrinsic dye interacts with proteins mainly via non-specific hydrophobic interactions, (although electrostatic interactions can also be involved) and are sensitive to conformational changes and to accessibility of hydrophobic pockets (Hawe, Sutter, & Jiskoot, 2008). ANS binds to β-Lg in its native form by interacting mainly with the opening of the hydrophobic calix, involving low energy values (i.e., −26 to −23 kJ mol^{-1}) (Collini et al., 2003; Collini, D'Alfonso, & Baldini, 2000). Due to the non-specific interaction nature of ANS binding and the dynamics of the protein structure, changes in the protein conformation will impact its affinity to ANS (D'Alfonso, Collini, & Baldini, 1999).

Fig. 4 shows the fluorescence profile of ANS in the presence of β-Lg for the thermal cycles from 20 °C to either 90 °C, 70 °C, 60 °C or 50 °C and back to 20 °C, as described above. The binding of ANS to
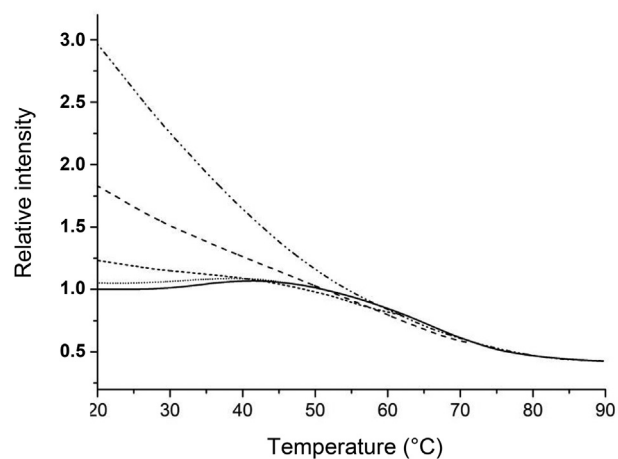

Fig. 4. Fluorescence intensity of hydrophobic dye ANS during heating to 90 °C (continuous line), and cooling from 50 °C, 60 °C, 70 °C and 90 °C in the different dashed lines.

native β-Lg was confirmed through the control sample performed without the protein (ANS in buffer solution). The fluorescence intensity in the presence of β-Lg increased about 5 times and a significant blue shift was observed (data not shown). ANS fluorescence is constant between 20 °C and 30 °C suggesting that the binding to β-Lg and the microenvironment is unchanged. From 30 °C to 45 °C, an increase of 10% in ANS fluorescence was observed, possibly caused by the increase of the protein structural dynamics (i.e., side-chains and loops flexibility increase), thus facilitating the access of the probe to the opening of the calix (Collini et al., 2000).

It is expected that the exposure of the hydrophobic sites occurs during heating, as conformational and structural changes take place. This exposure would result in an increased binding of ANS to the protein, however the ANS fluorescence decreases above 45 °C, attenuating around 75 °C and remaining almost constant above this temperature. Despite of the occurrence of structural changes and the exposure of potential ANS binding sites, the probe is actually detaching from the protein. Due to the low energy involved in ANS binding and the entropy increase during heating, the binding equilibrium must be limited by free energy associated with the entropy increase, overcoming the affinity between the protein and ANS. During the cooling stage of the thermal cycles, the fluorescence profiles partially follow the inverse path of the heating profiles until a point where the profiles diverge. These diverging points must coincide with the temperature where the probe affinity overcomes the entropy of the system and ANS binds again to the protein. In all the thermal cycles, with exception of the one reaching 50 °C, the fluorescence intensity increases substantially with the temperature reduction.

The points at which the fluorescence profiles during cooling diverge from the fluorescence profiles during heating are positively correlated with the maximum temperature attained in each cycle. This suggests that the increase in exposure temperature - and the consequent structural changes - results in the creation of more binding sites and/or in a concomitant higher affinity to ANS. It is interesting to notice that contrasting with the AF determinations, the increased binding of ANS to β-Lg exposed to 60 °C suggests the occurrence of irreversible conformational changes. This either supports that ANS is more sensitive to transient states in protein conformation (Hawe et al., 2008) or that it impairs the refolding process by disturbing the hydrophobic interactions. For the fluorescence scanning cycling between 20 °C and 50 °C and back to 20 °C again, the ANS fluorescence remains at levels near the maximum observed at 45 °C, suggesting that besides the increase in the access to the calix, no further binding sites were created nor the affinity increased by exposure at 50 °C.

The progressive increase in flexibility and disorder of the protein structure caused by thermal stress also results in the unpacking of the structure and increase of the radius of the hydrated protein, thus allowing this process to be followed by light scattering techniques (Xu, 2015). The scattering profile of β-Lg solution at 532 nm during the heating and cooling cycle between 20 and 90 °C is shown in Fig. 5. It is clear that the hydrodynamic radius of β-Lg increases in response to the temperature increase.

The observations agree with the multistep model for protein unfolding, where the increase in flexibility of the peptide chain and loss of tertiary and secondary structures, lead to the unpacking of protein conformation. This process culminates with protein melting, reaching a maximum hydration radius. In the temperature range 30−40 °C the SLS intensity starts to increase, correlating with the observation of an increase in ANS fluorescence. This corroborates the hypothesis of the initial expansion in this temperature range, facilitating access of ANS to the central calix. The plateau reached during 75 °C−90 °C corresponds to a structural stabilisation after unfolding took place. In our opinion, this is the
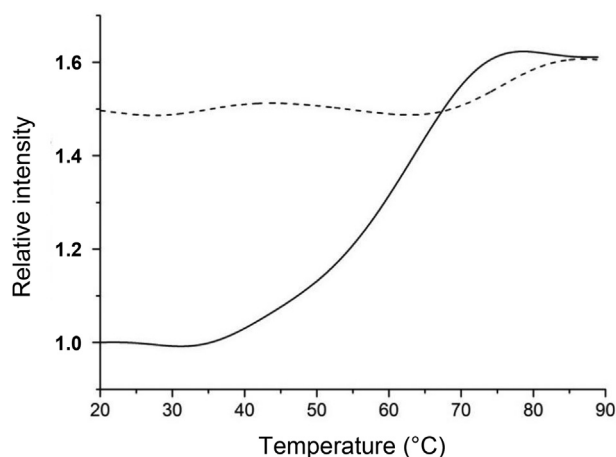

Fig. 5. Scattering intensity of β-Lg during heating (continuous line) and cooling (dashed line).

confirmation that after the transition culminating at 75 °C, no further significant changes occur in the protein conformation in the interval of temperatures tested. The stabilisation of the scattering intensity after denaturation also supports the inexistence of association phenomena, as the scattering intensity would substantially increase if higher molecular weight species were formed. By following the cooling processes, a decrease in SLS intensity between 90 °C and 70 °C is indicative of a partial refolding, corroborating the observation in the CD spectra presented above.

3.4. Unfolding mechanism

The use of different "online" techniques has shown to be productive, as all of them allowed obtaining information about structural features and thermally induced effects and transitions. Correlating the data obtained from the different determinations allows establishing the sequence and a clear understanding of the events involved in β-Lg thermal unfolding. The midpoint transition temperatures obtained by each technique were: DSC, 61.0, 72.7 and 81.0; CD, 73.8; Fluorescence (AF), 67.5 and 83.7; ANS, 34.9 and 66.3; SLS, 66.5.

It is generally accepted a multistep mechanism in β-Lg thermal unfolding, (de Wit, 2009; Fessas et al., 2001; Loveday, 2016). Classically protein-unfolding events are characterized by the energetic and structural transitions obtained by DSC and CD analysis. The CD midpoint transition (73.8 °C) is concordant with the second of the three discreet transitions observed by DSC (72.7 °C) and it would be safe to consider this as the denaturation temperature of the protein, in the context of a two state unfolding model. Nonetheless the other two transitions detected by DSC (61.0 °C and 81.0 °C) and the complementary data from AF (67.5 °C and 83.7 °C), ANS binding (34.9 °C and 66.3 °C) and SLS (66.5 °C) provide additional information about other structural and local conformational events resulting from the temperature increase and denaturation.

The methodology adopted in this study not only confirmed the existence of reversible conformational changes occurring above 35 °C and irreversible changes above 60 °C, as it allowed to stablish a more detailed sequence of events during the protein's unfolding. The earlier event during thermal increase is an expansion of the protein's hydrodynamic radius detected by SLS and coincident with an increase of ANS florescence. Above 50 °C the Trp fluorescence shift suggests changes in local positioning of Trp and neighbour amino acids and over 60 °C these changes become irreversible. These events are consistent with the fist energetic transition

detected by DSC which corroborates the permanent character of the structural changes above this temperature. Between 66.3 and 67.5 °C are positions the midpoint of the AF, ANS and SLS transitions, implying significant structural changes affecting not only the proteins hydrodynamic radius, but also Trp solvent exposure and ANS affinity. Some authors have reported that the partial loss of secondary structure, particularly α-helical content, occurs between 60 and 70 °C (de Wit, 2009; Qi et al., 1997). This would contribute to the disruption of the globular conformation, increase the hydrodynamic radius of the protein and increase the exposure of Trp and hydrophobic sites, being consistent with the observations reported here. DSC and AF detect a later transition above 80 °C and above this temperature the profiles obtained by CD, auto-fluorescence, ANS and SLS remain unchanged. This can be interpreted as a stabilisation of the remaining protein's fold and the culmination of the thermal induced effects in the temperature range studied.

β-Lg presents a highly dynamic structural behaviour as a function of factors such as pH or ionic strength, allowing the protein to undergo several transitions and assume different conformations. These structural dynamics are associated with the presence of a central hydrophobic cavity instead of a compact hydrophobic core (Gutiérrez-Magdaleno, Bello, Portillo-Téllez, Rodríguez-Romero, & García-Hernández, 2013). The free-energy associated with this is compensated by a rigid β-barrel forming the core of the structure and by the entropy associated to changes in the flexible loops at the barrel extremities (Jameson, Adams, & Creamer, 2002). The observations made in this study reflect the dynamic structural properties as a function of temperature. The multi-step thermal transition corroborated by several techniques confirms the complexity of the thermally induced effect on the protein's structure. The reversible structural changes observed below the first thermal transition (35–60 °C) are a clear sign of a highly dynamic behaviour of β-Lg, usually not addressed in research, nor considered in industrial applications. The methodology used allowed a more precise and detailed description of the unfolding/refolding pathways of β-Lg. This contributes to a better understanding of these complex phenomena and represents one more step towards improving and controlling protein functionality.

4. Conclusion

The interpretation of complementary information obtained by calorimetric and in-situ spectroscopic allowed the characterization of the very complex structural dynamics during β-Lg thermal transitions. The sequence of unfolding events was characterized in terms of energetic transitions, Trp positioning, accessibility of hydrophobic sites and structure unpacking. This strategy allowed to confirm, and better characterise, the reversible conformational changes occurring at low temperatures (i.e., <60 °C); these involve structural expansion and increased hydrophobic sites accessibility above 35 °C, and Trp repositioning from 50 °C to 78 °C.

These findings and the implemented characterisation strategy can certainly contribute to the development of technological applications of purified or enriched β-Lg fractions, as well as to tune their functional aspects. Despite the described structural dynamics may be influenced by composition in more complex systems (e.g., milk or whey) the perception of such complex dynamics and its implication in stability, interactions and aggregation of β-Lg may contribute to the improvement of processing and storage of dairy products.

Acknowledgements

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