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Heat- and cold-setting gels of β-lactoglobulin solutions. A DSC and TEM study[1]

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Abstract

We extracted data from the differential scanning calorimetric signals obtained with two different solutions of β-lactoglobulin at pH 3.5 and 7 and at a concentration which favours protein–protein interactions. The transition peaks were partially reversible only with solutions at pH 3.5. The temperatures corresponding to the first increase in heat flow, Θ_{onset}, and to the amplitude of maximum deviation, Θ_{max}, and the calorimetric enthalpy changes of heat reaction, $\Delta_r H$(cal) were higher at pH 3.5 than at pH 7. These differences indicated higher conformational stability at pH 3.5 than at pH 7: the heat transitions from the initial to the final conformational states could process through the reversible 'two-state model' at pH 3.5, while stable intermediate species might be created at pH 7. Isothermal treatments at $\Theta_{onset}<\Theta<\Theta_{max}+5$ K, applied to the study of the sol–gel transition showed that solutions which denatured reversibly formed *cold-setting gels*, while solutions which denatured irreversibly formed *heat-setting gels*. Furthermore, the curve (Θ–t) which demarcated the diagrams of the sol–gel states intersected at Θ approx. equal to Θ_{max} and the gel structures observed by transmission electronic microscope showed that cold-setting gels were constituted by linear aggregates ('string of beads') while heat-setting gels were constituted by very large clumped aggregates. All these results are discussed in terms of protein–protein interaction forces. © 1998 Elsevier Science B.V.

Keywords: DSC; Heat denaturation; β-lactoglobulin; Sol–gel transition; TEM

1. Introduction

β-lactoglobulin (β-lg) is the major bovin whey protein. It is a well characterized globular protein. When prepared from salt supersaturated solutions, this protein crystallizes in the dimeric form [1]. In an aqueous medium it displays a dynamic association/dissociation balance [2,3], which is coupled with a conformational transition [4,5] at pH 7.5 and which involves the free sulfydryl group reactivity in the basic pH range [6].

For many years, this globular protein has been chosen as a model system to study the denaturation mechanism [7,8] and attention has been paid to its heat-aggregation mechanism and to the microstructure

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of their gels [9–14]. The heat-induced molecular events have been shown to be dependent on the physicochemical environment. Particularly, previous studies by differential scanning calorimetry (DSC), have shown that the heat behaviour (denaturation+aggregation) of β-lg solutions is dependent on protein concentration, genetic variants, heating rate, pH, nature and concentration of buffer salt [15–24]. Other studies by scanning or transmission electronic microscope have shown that the microstructure (as with visual aspect) of heat-induced gels of β-lg was also salt, pH and heating rate dependent [9–11,14]. Recently, other globular proteins, used in food technology, were shown to form transparent heat-induced gels when salt was added to pre-heated solutions [25,26].

The aim of this present study was to combine DSC and TEM techniques to study the denaturation and aggregation behaviours of solutions of β-lactoglobulin molecules in the presence of electrolyte ions (0.1 M NaCl) and two pH values where the protein molecules were oppositely charged.

2. Experimental

2.1. Sample preparation

The β-lactoglobulin sample was kindly given by J. Fauquant from the 'Laboratoire de Recherche de Technologie Laitière' of INRA-Rennes. It was obtained by membrane micro and diafiltration on whey which had been previously separated from micellar casein of skim milk [27]. The β-lg content in the spray dried powder was ≈95% of dry matter. The purity of the sample was checked by reducing SDS-PAGE stained with Coomassie blue. We used a Phastsystem apparatus (Pharmacia-France) following the instructions of the manufacturer. In the electrophoregraph pattern, only β-lg trace was detected with a densitometer based on GS 370 v 2.3 software (Hoefer Scientific Instruments, San Francisco-USA). The sample was dispersed in distilled water and extensively dialyzed against NaCl (0.1 M). The protein concentration was determined with Folin–Lowry's method [28] with bovin serum albumin as a standard, and then adjusted to 4.5% and pH 7 with NaOH (1 M) or pH 3.5, with HCl (1 M). All the chemicals (Prolabo-France) were of reagent grade.

Zeta-potential measurements were performed by using a Malvern-ZetamasterS instruments (UK-model ZEM 5002) and a rectangular quartz capillary cell containing 2 ml of β-lg solutions in 0.1 M NaCl.

2.2. Calorimetric measurements

The conformational changes of β-lg molecules against heat treatment were monitored by DSC (DSC7, Perkin–Elmer) following the methodology developed elsewhere [29]. The first heating scans in the range 293–378 K, at various heating rates (2.5–10 K min^{-1}) were followed by a quenching step to 293 K and then by a re-heating step. Calorimetric data were extracted from DSC signals from which the sample baseline had been substracted. All the results were mean values obtained from three different samples. Experimental uncertainties were ±0.5 K for temperatures and ±20 kJ mol^{-1} for calorimetric enthalpy changes. The temperatures corresponding to the first increase, Θ_{onset}, and to the maximum deviation, Θ_{max}, of heat flow were corrected from the furnace+sample thermal-lag [29].

2.3. Aggregation study

The heat-aggregation process was determined by a simple tilting test: protein solutions (1 ml) were heated in sealed tubes for various lengths of time and then cooled in water bath, held at 277 K overnight and then tilted. The sol–gel transition state was reached when there was no deformation of the meniscus upon tilting. The experimental values of time, t, needed to the sol–gel transition at temperature, Θ, were used to draw the sol–gel diagrams of states.

The reactivity of total free thiol groups in pre-heated solutions or gels was determined by the Ellman's method [30]. Amounts of covalently bound β-lg molecules were calculated from non-reducing SDS-PAGE stained with Coomassie blue. The intensity of individual protein bands was quantified through the analysis of peaks obtained with the densitometer.

Using transmission electronic microscope (Philips EM-300) at an accelerated voltage of 60 kV, we observed the superstructure of heat-induced gels. Small cubes of gels were cut and fixed in 2% v/v

glutaraldehyde in 0.1 M NaCl (pH 7) for 60 min. After rinsing, the samples were postfixed in 2% v/v O_sO_4 in 0.1 M NaCl (pH 7) for 120 min. It was rinsed again and dehydrated for 10 min in ethanol series at different concentrations. After transferring to propylene oxide, samples were embedded in oven for two days at 333 K and thin sections were then cut with a diamond knife and stained with lead citrate (3–4 mg cm^{-3}) and uranyl acetate.

3. Results and discussion

3.1. DSC measurements

In Fig. 1, we have reported examples of DSC thermograms obtained at 10 K min^{-1} with protein solutions at 4.5 wt% in 0.1 M NaCl and pH 7 or 3.5. Solutions at pH 7, gave a signal with small and gradual changes in heat flow from temperature around 333 K (Fig. 1(a)). At acid pH, heating leaded to a more symmetrical and a sharper transition which began around 343 K (Fig. 1(b)). Peak temperature, Θ_{max}, were lower with pH 7 solutions (348±0.5 K) than with pH 3.5 (357±0.5 K). They increased with heating rates in the range 2.5–10 K min^{-1}, as observed in previous study [16]. These temperature values which correspond to the maximum rate of the main heat process, were in agreement with the published values obtained with β-lg at pH 3.5 and 7 [15–24] with some differences which were probably due to differences in calorimeters operating with small (our results) or large volume samples (High-Sensitive DSC) and to salt composition of buffer or traces of other proteins. Another difference appeared between those two types of solutions: the transition peak at pH 3.5 was partially reversible, while no signal was observed by re-heating the solution at pH 7. It can be seen in Fig. 1(c) that the DSC re-heating curve is obtained after the first heating program, followed by quenching to 277 K. The degree of reversibility, R%, calculated from the ratio $\Delta_r H(\text{cal})_2/\Delta_r H(\text{cal})_1$ determined through the second and first heating scan, respectively, was dependent on the heating rate applied in the first heating program (Fig. 2(a)). Similar partial reversible peak had also been observed in our previous studies [31,32] with a β-lg sample (5 wt%) containing 2% salt and dispersed in distilled water. Total reversible peak has been observed by Griko and Privalov [33] with β-lg dispersed (0.1 wt%) in KCl/HCl or in sodium phosphate buffers

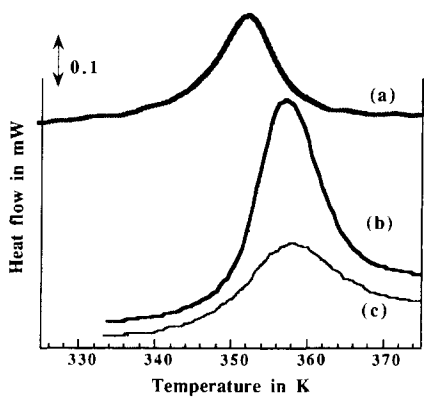

Fig. 1. Examples of DSC curves obtained with β-lactoglobulin solutions heated in the range 293–378 K at 10 K min^{-1}: (a) first heating at pH 7, (b) first heating at pH 3.5, (c) re-heating at pH 3.5.

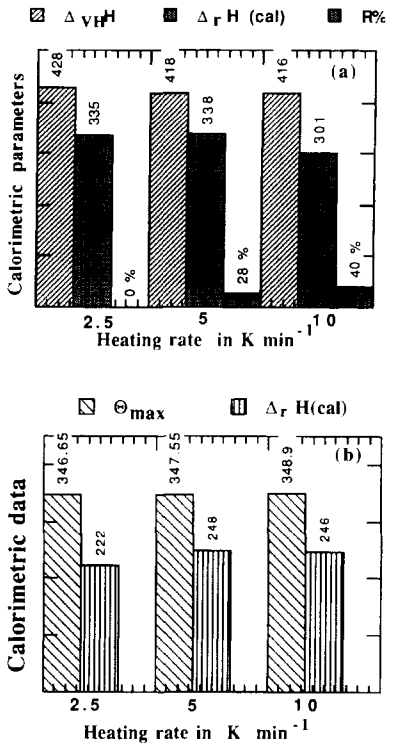

Fig. 2. Calorimetric parameters of thermal transition of β-lactoglobulin as a function of heating rate. (a) non-previously heated solutions at pH 3.5. (b) non-previously heated solutions at pH 7.

at pH 2 and by Azuaga et al. [34] with β-lgB (0.6%) in 20 mM phosphate buffer at pH in the range 1.5–3. It is evident that the difference in the degree of reversibility between these results was due to the fact that our protein solutions are ≈50 times more concentrated. The values of the calorimetric heat of reaction, ΔH(cal), corresponding to the first scan (Fig. 2(a)), did not depend on the heating rate for pH 3.5 and they were higher (310±20 kJ mol^{-1}) than at pH 7. At pH 7 (Fig. 2(b)) they did not vary significantly (247±20 kJ mol^{-1}) when $\beta \geq 5$ K min^{-1}. However, when heating rate was lower, we observed no peak on re-heating the solution at pH 3.5 (Fig. 2(a)) and $\Delta_r H$(cal) values decreased significantly with solution at pH 7 (Fig. 2(b)). The value of $\Delta_r H$(cal) corresponded to dissociation and denaturation (endothermic) processes which could be superimposed to an aggregation process (exothermic) during the time scale of DSC measurement. From our results, it could seem that the exothermic reaction (aggregation) occurring simultaneously with denaturation (endothermic), was more enhanced with solutions at pH 7 than at pH 3.5, and also when exposure to heating was increased (low scan rate).

We analyzed the partial reversible peak transition of the first heating program of solutions at pH 3.5 by using the expression of van't Hoff enthalpy change:

$$\Delta_{VH}H = 4R\Theta_{max^2}h_{max}/A\beta$$

where R (J mol^{-1} K^{-1}) is the gas constant, h_{max} (mW) the maximum deviation of heat flow, A (mJ) the area under the peak transition and β the heating rate (K s^{-1}). We observed (Fig. 2(a)) that $\Delta_{VH}H$ values were ≈25% higher than the $\Delta_r H$(cal). This result could indicate that the reversible two-state process of heat denaturation was followed by an irreversible phenomenon at pH 3.5. However, at pH 7 the complete lack of reversible peak transition and the decrease in $\Delta_r H$(cal) with heating rate could be due to the formation of aggregated species during the DSC scan, implying the non-relevance of the 'two-state model' of heat denaturation.

3.2. Aggregation properties

The ability of protein solutions to form a gel, was determined by the simple tilting test. We observed, in our experimental conditions, that the solutions at pH 7 formed gels upon heating (heat-setting gels) while solutions at pH 3.5 became viscous and formed gels only after cooling (cold-setting gels). The time, t, of heat treatment needed to the formation of the gel state was dependent on the temperature of the pre-heating, Θ. The values of time–temperature experimental points were fitted (Fig. 3) to two straight lines, intersecting at 358.7±1.5 K (pH 3.5) or 349.5±1.5 (pH 7). These intersecting temperatures were, within the experimental uncertainties, very close to the peak temperatures measured by DSC (Fig. 1). This correlation could indicate that at $\Theta < \Theta_{max}$, heat-denaturation process is the rate-limiting step. In other words, there was a change in the rate-limiting step reaction when the maximum rate of heat denaturation (meaning of peak denaturation) was reached.

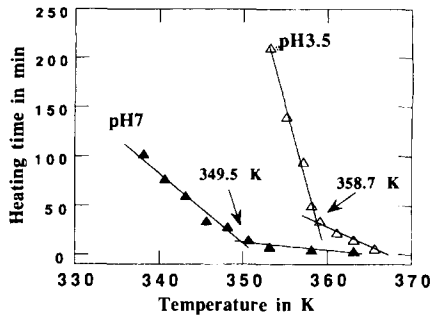

Fig. 3. Evolution of time (min) needed for sol–gel transition with β-lactoglobulin solutions at pH 7 and 3.5, as a function of pre-heating temperature (K).

3.3. Reactivity of free thiol groups

Fig. 4 shows that the reactivity of total free SH groups decreased with the length of the pre-heating time at $\Theta > \Theta_{max}$ (353 K for pH 7 and 363 K for pH 3.5). At pH 7 after pre-heating during 15 min, about 30% of total free SH groups of β-lg molecules were no more exposed to the DTNB probe, in comparison with unheated molecules. At pH 3.5, the reactivity of SH groups did not vary significantly with treatment at 363 K for 60 min. The trend of variation of SH groups in solutions at pH 7 could be explained through the SDS-PAGE patterns (results not shown): after heating for 5 min at 353 K, new covalently bound dimers and trimers were formed. For a shorter heating time, the monomer band dominated and for a longer time, very high molecular weight species were observed only

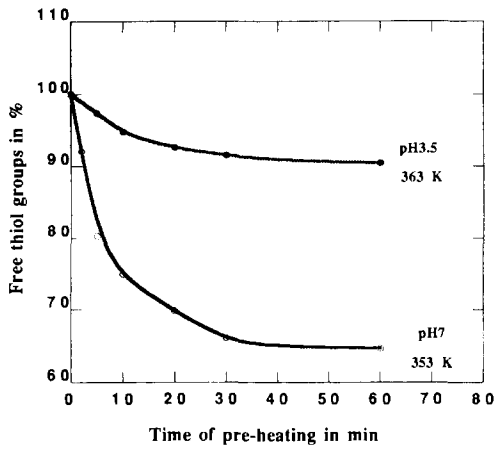

Fig. 4. Evolution of the total reactivity of the free thiol groups as a function of heating time of β-lactoglobulin solutions at pH 3.5 (Θ=363 K) and pH 7 (Θ =353 K).

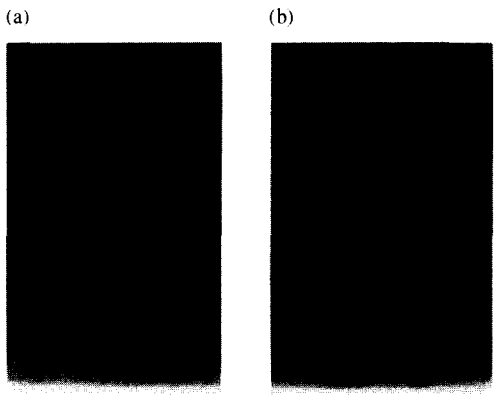

Fig. 5. Transmission electron micrographs. (a) Cold-setting gel from a β-lactoglobulin solution at pH 3.5 which was pre-heated at 363 K for 30 min (1 cm=80 nm) (b) Heat-setting gel from a β-lactoglobulin solution at pH 7 which was pre-heated at 353 K for 15 min (1 cm=80 nm).

with solutions at pH 7, in agreement with most of the published results [35,36].

Thus, the lack (or the presence) of transition peaks in the re-heating curve of solutions at pH 7 (or 3.5), respectively, may be explained by the involvement or not of disulfur covalent protein–protein linkage, at least at $\Theta \geq \Theta_{max}$. Examining the reversibility of re-heating after a first isothermal heating at θ=343 K for 1.6 min, gave 50% (pH 7) and 100% (pH 3.5) reversibility. No covalently bond species were detected in the corresponding heated solutions (results not shown).

3.4. Electron microscopy

We observed by TEM the structure of the clear gels formed at 363 K (pH 3.5) and opaque gels formed at 353 K (pH 7). Photographs of thin sections are shown in Fig. 5(a) (pH 3.5) and Fig. 5(b) (pH 7). At pH 3.5 the protein molecules were joined together to form strands having different lengths in the range ca. 100–150 nm long and a thickness of ca. 3–5 nm. Similar structures were also observed in previous studies on β-lg at pH<pI [9,11] and at pH 8 [10], but in the absence of added salt. Solutions at pH 7, provided microstructures formed by much larger strands (ca. 100 nm) which were constituted by numerous aggregated molecules and connected together with an overall impression of spacial inhomogeneity. A similar inhomogeneity in the density of aggregates [14] was also observed with β-lg (13 wt%) at pH 7.5 when heating was performed at a much lower rate ($\leq$0.1 K min^{-1}).

Based on the results obtained for the reactivity of total free SH groups (Fig. 4) and bands observed in non-reducing SDS-PAGE pattern (results not shown), we suppose that at pH 7, the large aggregates observed by TEM could be caused by S–S linkages due to SH/S–S intermolecular interchange reactions. On another hand, zeta-potential measurements with β-lg solutions in 0.1 M NaCl showed that the net surface charge of molecules at pH 7 was more shielded (Z=−3.3 mV) than at pH 3.5 (+15.3 mV), causing more protein–protein electrostatic attractions. At pH 3.5, the fine linear aggregates observed seemed to be composed of native-like monomers or dimers (3–5 nm diameter). The lower screening effects of surface charges by electrolyte ions and the lack of formation of S–S linked polymers, comparatively to pH 7 solutions, was reflected by less attractive forces and therefore could result in gel network formed by linear aggregates.

4. Conclusion

The DSC study has shown that β-lg molecules at pH 3.5 have higher conformational stability than at pH 7. This result was reflected by higher values of Θ_{max}, and $\Delta_r H$(cal) and by the presence (pH 3.5) or absence (pH 7) of a reversible peak transition. The analysis of the

DSC signals, using van't Hoff equation showed that long heat treatment leads to the enhancement of intermolecular protein–protein interactions, particularly with solutions at pH 7.

At pH 7, the reactivity of total free SH groups was correlated with the development of S–S linked species and with the formation of the opaque heat-setting gels composed of large aggregated microstructures. At pH 3.5, the lack of newly created S–S bonds, the low screening effects of protein surface charges by electrolyte ions may be correlated with the reversibility of DSC peak transition and with the formation of clear cold-setting gels, composed of linear aggregates of native-like monomers or dimers ('string of beads').

The diagrams of sol–gel heat-induced transitions with both solutions at pH 7 and 3.5, showed that when heat treatment was performed at $\Theta > \Theta_{max}$ the attractive forces could overcome the repulsive ones, thus enhancing the aggregation phenomenon.

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References

[1] S.G. Hambling, A.S. McAlpine, L. Sawyer, in: P.F. Fox (Ed.), Advance Dairy Chemistry-1: Proteins, Elsevier Applied Science, London, 1992, p. 141.
[2] M. Dupont, Compte-rendu Académie. Sci. Paris 257 (1963) 3495.
[3] J.K. Zimmerman, G.H. Barlow, I.M. Klotz, Arch. Biochem. Biophys. 138 (1970) 101.
[4] D. Pantaloni, Compt. Rendu. 252 (1961) 2459.
[5] C. Tanford, V.G. Taggart, J. Am. Chem. Soc. 83 (1961) 164.
[6] H.W. Sawyer, R.S. Norton, L.W. Nichol, G.A. McKenzie, Biochim. Biophys. Acta 243 (1971) 19.
[7] O.E. Mills, Biochim. Biophys. Acta 434 (1976) 324.
[8] H.L. Casal, U. Köhler, H.H. Manstsh, Biochim. Biophys. Acta 957 (1988) 11.
[9] V.R. Harwalkar, M. Kalab, Milchwinssenschaft 40 (1985) 31.
[10] D.M. Mulvihill, J.E. Kinsella, J. Food Sci. 53 (1988) 231.
[11] M. Langton, A.M. Hermansson, Food Hydrocolloids 6 (1992) 523.
[12] D. Renard, M. Axelos, J. Lefebvre, Food macromolecules and colloids, in: E. Dickinson, D. Lorient (Eds), The Royal Society of Chemistry, Cambridge, 1995, p. 390.
[13] J.-C. Gimel, D. Durand, T. Nicolai, Macromolecules 27 (1994) 583.
[14] M. Stading, M. Langton, A.-M. Hermansson, Food Hydrocolloids 5 (1992) 455.
[15] J.N. De Wit, G.A.M. Swinkels, Biochim. Biophys. Acta 624 (1980) 40.
[16] P. Relkin, B. Launay, Food Hydrocolloids 4 (1990) 19.
[17] X.L. Qi, S. Brownlow, C. Holt, P. Sellers, Biochim. Biophys. Acta 1248 (1995) 43.
[18] P.O. Hegg, Acta Agric. Scand. 30 (1980) 401.
[19] J.N. De Wit, G.J. Klarenbeek, Dairy Research. 48 (1981) 293.
[20] M. Paulsson, P. Djemek, J. Dairy Sc. 73 (1990) 45.
[21] S.M. Gotham, P.J. Freire, A.M. Pritchard, Int. J. Food Sci. Technol. 27 (1992) 313.
[22] V.R. Harwalkar, C.Y. Ma, in: H. Visser H. (Ed.), Protein Interactions, VCH Weinheim, 1992, p. 359.
[23] T.-X. Liu, PhD. Thesis, ENSIA-France, 1993, pp. 148.
[24] M.E. Huang, E.A. Foegeding, H.E. Swaisgood, J. Agric. Food Chem. 42 (1994) 1064.
[25] E. Doi, Trends Food Sci. Technolol. 4 (1993) 1.
[26] D.J. McClement, M.K. Keogh, J. Sci. Food Agric. 69 (1995) 7.
[27] J. Fauquant, J.L. Maubois, A. Pierre, Technol. Laitière 1028 (1988) 21.
[28] O.H. Lowry, N.J. Rosbrough, A.L. Farr, J. Randall, J. Biol. Chem. 193 (1951) 265.
[29] P. Relkin, Critical Rev. Food Sci. Nutrition. 36 (1996) 565.
[30] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[31] P. Relkin, B. Launay, J. Thermal. Anal. 37 (1991) 1887.
[32] P. Relkin, L. Eynard, B. Launay, Thermochim. Acta 204 (1992) 111.
[33] Y.V. Griko, P.L. Privalov, Biochemistry 31 (1992) 8810.
[34] A.I. Azuaga, M.L. Galisteo, O.L. Mayorga, M. Cortijo, P.L. Mateo, FEBS Letters 309 (1992) 258.
[35] K. Shimada, J.C. Cheftel, J. Agric. Food Chem. 37 (1989) 161.
[36] F. Xiong, J.E. Kinsella, J. Agric. Food Chem. 38 (1990) 1887.