Heat induced β-lactoglobulin polymerization: role of the change in medium permittivity

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Abstract

The decrease in medium permittivity due to temperature rise could play an important role in the denaturation/polymerization of heat-treated β-lactoglobulin. The permittivity of a β-lactoglobulin suspension was decreased, either by heat treatment or by adding organic solvent, and the rate of loss of the native β-lactoglobulin was estimated by SDS-PAGE analysis. Setting the permittivity to 63.8 by addition of organic solvent, induced β-lactoglobulin polymerization, but it differed from that induced by change in permittivity upon thermal treatment itself (heating to 70 °C). To explain the protein polymerization process induced by heating, other physical parameters should be taken into account, such as change in water organization around the protein.
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Keywords: β-Lactoglobulin; Polymerization; Permittivity; Thermal treatment; Water structure

1. Introduction

Because almost all the dairy industry uses heating, a greater understanding of the effect of heat on whey proteins in foods is important. Whey proteins are used as ingredients because of their good nutritional value and interesting physicochemical properties (De Wit, 1990, Kinsella & Whitehead, 1989). β-lactoglobulin is the main protein in whey, constituting about 50% (mol/mol) of the total whey proteins in bovine milk. It is a well-characterized globular protein (molecular weight 18.4 kDa) containing two disulfide bonds and one thiol group (McKenzie, Ralston, & Shaw, 1972). At room temperature and at physiological pH, β-lactoglobulin exists mainly as non-covalently linked bi-units.

According to literature reports (Bauer, Hansen, & Ogendal, 1998; Hoffmann, & Van Mil, 1997; Hoffmann, Olieman, & Dekruif, 1997; Holt et al., 1998; Manderson, Hardman, & Creamer, 1998; Prabakaran & Damodaran, 1997; Relkin, 1998; Roefs & De Kruif, 1994; Verheul, Roefs, & DeKruif, 1998; Xiong, Dawson, & Wan, 1993), the effect of heating

on β-lactoglobulin is a two-step process. Firstly, upon heating, β-lactoglobulin undergoes conformational changes and unfolding. This first step is reversible. The unfolded structure returns to the original state when the protein is cooled. This leads to a molten-globule-like structure (Iametti, DeGregorio, Vecchio, & Bonomi, 1996) with increased exposure of both the previously buried inner hydrophobic groups and the sulfhydryl group. At pH > 6 this exposed thiolate group can induce thiol/disulfide exchange reactions which produce disulfide-linked aggregates. The formation of β-lactoglobulin polymers corresponds to the second step of the heating process. Regarding molecular interpretation, the production of β-lactoglobulin polymers is consecutive to the modification of the tertiary structure of the protein, which depends on physicochemical interactions between functional groups such as charge-charge, charge-dipole and dipole-dipole interactions and between functional groups and water.

The energy of the physicochemical interactions which structure the protein, is a function of the medium permittivity.

For charge-charge interactions (e.g.), the interaction energy is:

$$w = q_1 q_2 / 4\pi\varepsilon_0 \varepsilon d$$

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where q_1 and q_2 are the two charges separated by a distance d in a uniform medium, ε_0 is the permittivity of free space and ε is the permittivity of the medium (Chottard, Depezay, & Leroux, 1987). Consequently, it could be assumed that any modification of the medium permittivity could lead to changes in the energy of the physicochemical forces responsible for the protein structure and consequently in the protein unfolding and polymerization mechanisms.

Regarding the process of heating itself, the heating of a solution induces: (1) an increase in molecular agitation, (2) a change of molecular solvation and (3) a decrease in the medium permittivity (Fig. 1) according to the following expression (1) (Pauling, 1988, Tanford, 1980):

$$\text{Log}\varepsilon = c - dT \qquad (1)$$

where c and d are empirical constants and T is absolute temperature.

We have hypothesized that the decrease in medium permittivity induced by heating leads to changes of physicochemical forces structuring the protein and hence contributes to protein unfolding, reorganization of structure and consequently to protein polymerization. The aim of this study is to report and compare the effects, on β-lactoglobulin denaturation and polymerisation, of a decrease in medium permittivity due to either addition of organic solvent or heating of the dispersing medium. The disulfide exchange occurs by nucleophilic substitution after the attack of a thiolate on a disulfide. The pK of small molecular weight thiols is 8. At pH > 6 (nearby pK value of the protein thiol), no disulfide bond exchange occurs without a heating process. At temperature below the thermodenaturation threshold, the thiolate group is not accessible to a disulfide bond. When temperature exceeds 65 °C, at pH > 7, the polymerization of β-lactoglobulin occurs (Prabakaran and Demodaran, 1997). The medium permittivity is also decreased, at 25 °C, using organic solvents chosen both for their miscibility with water and permittivity value, (lower than that of the water).
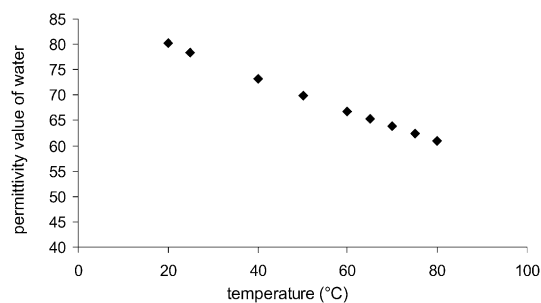
2. Materials and methods

2.1. Isolation of β-lactoglobulin

Reconstituted skim milk was made by dispersing 365 g of ultra low heat skim milk powder, as described by Fairise, Cayot, and Lorient (1999) in 1 l deionized water. The composition of milk powder is given in Table 1. β-Lactoglobulin was then purified following a method previously described (Kinekawa & Kitabatake, 1996). Whey proteins, obtained by casein precipitation (pH = 4.6), were treated with pepsin [protein/enzyme ratio 200:1 (wt/wt)]. The hydrolyzed whey was diafiltered (cut threshold 10 kDa) against a 20 mM phosphate buffer (Na_2HPO_4/KH_2PO_4), pH = 7, until reaching this pH value. Then, the permeat was concentrated and freeze-dried. The purity of β-lactoglobulin was controlled by SDS-PAGE (data not shown). The powder was stored at −20 °C. β-Lactoglobulin polymers were considered as an indication of a sufficiently advanced denaturation, allowing exposure of the thiolate group. The analysis by SDS-PAGE, under non reducing conditions, was consequently chosen as a method to identify protein denaturation and to measure the proportion of remaining protein monomers.

2.2. Organic solvents

Organic solvents, such as 1-4 dioxane, 1-propanol, ethanol, 1-2 propanediol, glycerol, dimethylsulfoxyde (DMSO), were purchased from Prolabo (Nogent sur Marne, France). They were chosen for the range of their permittivity values lower than that of the water and their good miscibility with water. At 25 °C the values of permittivity of these pure solvents and of water are respectively : 2.2, 20.1, 24.3, 32, 42.5, 45 and 78.3.

2.3. Heat-treated β-lactoglobulin

To determine the temperature at which β-lactoglobulin polymerization occurs under the experimantal conditions, 32.5 mg of protein powder were dispersed in 5 ml of heated deionised water. The protein dispersion was kept at 50, 60, 70 and 80 °C, for 2 h, in tightly closed tubes to prevent evaporation during heating. Then, aliquots (0.5 ml) of the protein dispersion were withdrawn and immediately

Fig. 1. Permittivity value of water as a function of temperature (data from literature).

Table 1
Composition (g/kg) of the skim milk powder

Dry extract	TN	NPN	NCN	Ash	Lactose
949.86	365	21.0	80.1	79.28	nd

TN is the amount of total nitrogen components; NPN is the amount of non protein nitrogen components; and NCN is the amount of non caseinic nitrogen components. nd: not determined.

mixed with equal volume of sodium dodecyl sulphate electrophoresis buffer [0.5 M tris–HCl buffer, pH 6.8, glycerol 20% (v/v), bromophenol blue and SDS 10% (w/v)] at 20 °C. Samples were immediately cooled in an ice bath and then stored at −18 °C until the analysis of the polymerization level by SDS-PAGE. As previously reported (Anema & McKenna, 1996; Prabakaran & Damodaran, 1997; Roefs & De Kruif, 1994), we observed that the formation of dimers occured only when the β-lactoglobulin solution was heated for 2 h above 60 °C (data not shown). Below this temperature, no polymerization was observed. So, based on this result, the following thermal treatments were conducted at 70 °C. Kinetics of β-lactoglobulin polymerization at 70 °C were studied as follows: 32.5 mg of β-lactoglobulin powder were dispersed in 5 ml of 70 °C-heated deionized water and immediately transferred to a 70 °C oil bath. Tubes were closed tightly to prevent evaporation during heating for 96 h. Aliquots (0.5 ml) of the protein solution were withdrawn at various time intervals (0, 1, 3, 6, 12 h), immediately mixed with equal volume of SDS-PAGE buffer sample at 20 °C and then cooled in a ice bath and stored at −18 °C until the analysis of the polymerization level by SDS-PAGE. No cryodenaturation of the protein was observed (data not shown). The time at which the powder was dispersed in heated water was chosen as time zero ($t = 0$).

2.4. Organic solvent-treated β-lactoglobulin

To study the effect of organic solvent on β-lactoglobulin polymerization, 32.5 mg of β-lactoglobulin were dispersed in 5 ml water/solvent mixture. The mole fractions of ethanol, 1-propanol, 1,2-propanediol, glycerol, DMSO and 1,4-dioxane in the organic solvent/water mixture were, respectively, 0.14, 0.25, 0.31, 0.40, 0.43 and 0.19, in order to obtain, at 20 °C, the same permittivity as heated water at 70 °C, i.e. 63.8. Permittivity (ε) of organic solvent-water mixture was calculated from the additive law as below (2):

$$\varepsilon_{final} = x_{solvent} \times \varepsilon_{solvent} + (1 - x_{solvent})\varepsilon_{eau} \qquad (2)$$

where x is the mole fraction of organic solvent in the aqueous solution.

After addition of organic solvent, pH values of β-lactoglobulin suspension were 6.93, 9.55, 6.36, 7.67, 7.83, 7.55 and 7.02 for, respectively, hot-water, DMSO, 1,4-dioxane, 1,2-propanediol, ethanol and 1-propanol. Vials were closed and kept at 20 °C for 96 h. Aliquots (0.5 mℓ) of the protein suspension were withdrawn at various time intervals (0, 3, 12 h) and immediately mixed with equal volume of SDS-PAGE buffer sample. The time at which the powder was dispersed in organic solvent/water mixture was chosen as time zero (t 0).

2.5. Protein polymerization analysis

SDS-PAGE of both heat and organic solvent-treated β-lactoglobulin were performed using a 12% separating gel and a 4.5% stacking gel. The electrophoreses were run under non-reducing conditions to avoid cleavage of interproteic disulfide bonds formed upon heating or organic-solvent treatment. Protein bands were fixed in 12% (w/w) trichloro-acetic acid for 30 min, stained in 0.05% (w/v) R 250 Coomassie Brilliant Blue and destained in a solution of 5% (v/v) methanol and 7.5% (v/v) acetic acid. The relative loss of monomers, as a function of both heating and organic solvent incubating time, were estimated by densitometry (densitométre CS-9000, Shimadzu Roucaire, France). The loss of monomers was expressed as the evolution of the $\beta lg_t / \beta lg_0$ ratio as a function of time (with βlg_0 the concentration of native protein at time *0* and βlg_t the concentration of native protein at time *t*).

2.6. Statistical analysis

All experiments were performed in triplicate. Statistical analyses were performed by using the statistical package Stat view (version 5, Sas Institute Inc). To assess a possible difference between solvents or between a solvent and the heated water, we compared $\beta lg_t / \beta lg_0$ ratio with an ANOVA, followed by a post hoc comparison with the Tukey-Kramer test when appropriate ($P < 0.05$).

3. Results and discussion

3.1. General

As shown in Fig. 2, upon heating at 70 °C, the colour intensity of electrophoresis bands corresponding to the β-lactoglobulin monomer decreased, suggesting that the concentration of monomers gradually decreased with heating time. Concurrently, it should be noted that the concentration of dimers gradually increased with heat-
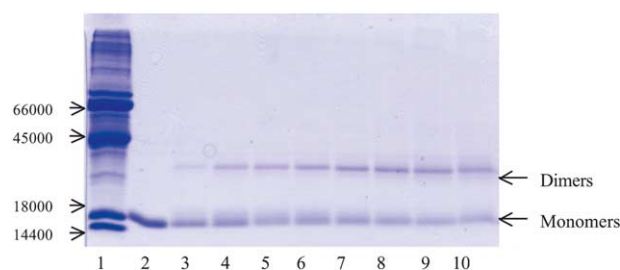

Fig. 2. The SDS-PAGE pattern of β-lactoglobulin dispersion heated at 70 °C. Lane 1: molecular mass marker protein ; lanes 2–10 represent samples heated for 0, 1, 3, 6, 12, 24, 48, 72 and 96 h, respectively.

ing time up to 12 h. As seen in Fig. 3, where (βlg$_t$/βlg$_0$) was plotted against t, the observed curve suggested that the β-lactoglobulin polymerization occurred during heating up to 12 h, and then stabilized thereafter. Although we tried to evaluate the order of the reaction, it was not possible because of the too low square correlation coefficient obtained (Table 2). This implied that the process of β-lactoglobulin polymerisation, which could be interpreted in terms of the sum of various sub-processes, such as the denaturation step (unfolding of molecules) and polymerization, was a complex one. Nevertheless, Anema and McKenna (1996), Prabakaran and Damodaran (1997) and Roefs and De Kruif (1994) observed a reaction order of 1.5 for thermal treatment of β-lactoglobulin and found rate constants $k_{1.5}$ 6.94×10^{-9} s^{-1}, $k_{1.5}$ 6.94×10^{-10} s^{-1} and $k_{1.5}$ 3.47×10^{-9} s^{-1} respectively. As demonstrated by Hoffmann and Van Mil (1997), Qi, Brownlow, Holt, and Sellers (1995) and Xiong et al. (1993), the rate of β-lactoglobulin denaturation upon heating strongly depends on ionic strength, type of ions, pH and β-lactoglobulin concentration. According to Hoffmann and Van Mil (1997), pH strongly affected the rate of polymerisation reaction and the size of aggregates formed at 65 °C. The authors have shown that, at 65 °C, the β-lactoglobulin polymerization reaction accelerated as the pH value increased (range 6.0–8.0); this increase can be ascribed to an increase in reactivity of the thiol group with increasing pH. Park and Lund (1984), by measuring

rate constants for thermal denaturation of β-lactoglobulin at 82 °C, have shown that the rate of denaturation depended on pH and that β-lactoglobulin was more stable in the pH range 5.0 to 7.0 and more sensitive at pH 4.0.

In addition, in order to achieve short heating and cooling times, we used conditions which were very different from those typically encountered in previous studies. Indeed, whereas we dispersed β-lactoglobulin in hot-water, other authors previously cited dispersed it at room-temperature in water and then put it in a hot-oil bath. They considered, as time zero, the time when the temperature reached the target value. They did not take into account the role of the increase in temperature. Moreover, in the present study, we used a medium which contained only phosphate salt and β-lactoglobulin as protein, but not all the milk proteins Anema and McKenna (1996) as did. Some of the proteins of whole milk, such as κ-casein, may influence the rate of polymerization of β-lactoglobulin.

Because we chose to conduct thermal experiment at 70 °C, experiments with different mixed organic solvent solutions were conducted at 25 °C, and at a constant permittivity of 63.8, value corresponding to the value for water at 70 °C.

Table 3 shows the (βlg$_{t1}$/βlg$_{t0}$) ratio, obtained at time 1H, for the various mixtures used. The (βlg$_{t1}$/βlg$_{t0}$) ratio varied, depending on the organic solvent used. No significant difference was observed between various mixtures used, except the 1,4-dioxane/water mixture for which the result appeared significantly different from those of 1,2-propanediol, hot-water, DMSO and glycerol/water mixture, respectively. Among all the organic solvents used, 1,4-dioxane would be the cosolvent leading to the most important β-lactoglobulin polymerization. At time 1h, compared to heat treatment (which was the reference sample), two trends could be described: (1) the use of 1,2-propanediol, 1-propanol, ethanol and 1,4-dioxane led to a decrease in (βlg$_{t1}$/βlg$_{t0}$) ratio and (2) the use of DMSO and glycerol led to a increase in (βlg$_{t1}$/βlg$_{t0}$) ratio. Table 3 lists the (βlg$_{t12}$/βlg$_{t0}$) ratios, obtained at time 12h, for the various mixtures used. Compared to values obtained at time 1h, the (βlg$_{t12}$/βlg$_{t0}$) ratio tended to be lower, except for both glycerol and 1,4-dioxane used as cosolvents. At time 12h, the highest decrease in (βlg$_{t12}$/βlg$_{t0}$) ratio was obtained for, respectively, DMSO and ethanol used as cosolvents. Conversely glycerol addition did not induce any polymerization.

Based on these results, it can be assumed that the decrease in permittivity value of the dispersing medium by organic colvent addition imperfectly follows the effect of heating. So, as the decrease in the medium permittivity value would not be the only factor to be considered, there must be other parameters of the dispersing medium associated with the nature of the cosolvents (Table 4).

Table 2
square correlation coefficient (r^2) as a function of kinetic order tested applied to fit the loss of β-lactoglobulin monomer upon thermal treatment (duration = 12 h; 70 °C)

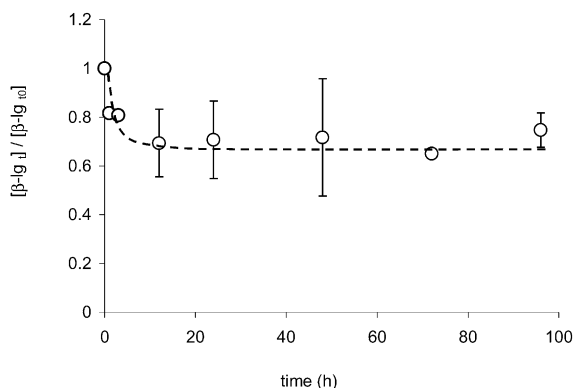|	Kinetic order tested				
	0	1	1.5	2	3
r^2	0.58	0.10	0.07	0.17	0.27

Fig. 3. Evolution of the βlg$_t$/βlg$_0$ ratio as a function of time for β-lactoglobulin suspension in 70 °C-water. Medium permittivity was 63.8. Error bars, represent the mean standard error between the measurements.

3.2. Case where the use of cosolvent led to a decrease in the ($\beta lg_{t1}/\beta lg_{t0}$) ratio (t = 1, h = t1)

As seen in Table 3, the use of 1,2-propanediol, 1-propanol, ethanol and 1,4-dioxane as cosolvents, led to a decrease in the ($\beta lg_{t1}/\beta lg_{t0}$) ratio, suggesting that the β-lactoglobulin polymerization was enhanced, relatively to hot-water or to the other cosolvents.

According to the literature, (Narizhneva & Uversky, 1998; Uversky, Narizhneva, Kirschstein, Winter, & Löber, 1997; Lehrman, Tuls, & Lund, 1990), β-lactoglobulin underwent at least two consecutive conformational transitions with a decrease only in the medium permittivity. The first transition corresponded to the disruption of rigid tertiary structure in protein, while the second led to the formation of an expanded helical conformation, typical of protein. This meant that decrease in permittivity provoked the formation of a denatured intermediate state with secondary structure and native-like compactness: the molten globule state. In the first state of denaturation (conformation transition), the accessibility of the sulfhydryl group should be low. On the contrary, the molten globule state, obtained after the second conformation transition, should allow exposive of the sulfhydryl group and ipso facto induce the polymerization of β-lactoglobulin by disulfur bond exchange, but only if the medium pH value is favourable.

As seen in Table 4, by using alcohols (1,2-propanediol, 1-propanol and ethanol) as cosolvents, the medium pH values were higher than that of a hot-water medium. Moreover, the electic dipole moment values of alcohol would be close to that of water. The increase in pH may induce an increase in the proportion of reactive species (thiolate, R-S⁻) in alcohol/water mixtures com-

paratively to hot-water. So, as soon as the β-lactoglobulin becomes unfolded and the thiolate group exposed, increase in pH could explain the β-lactoglobulin polymerization enhancement ($v = k \cdot [R\text{-}S^-]^a \cdot [\text{-Prot-S-S-}]^b$). By applying the mass action law ($pH = pK + \log[R\text{-}S^-]/[R\text{-}SH]$), it would be possible to evaluate the increase factor of the thiolate number into alcohol/water mixtures in comparison with that into hot-water. As the pK value of β-lactoglobulin thiol was established to 9.35 (Kella and Kinsella, 1988), $[R\text{-}S^-] = C.10^{pH-9.35}/[1 + 10^{pH-9.35}]$, where C is a constant equal to ([R-S⁻] + [R-SH]). Consequently, the quantity of [R-S⁻] obtained in mixtures containing 1-propanol, 1,2-propanediol, and ethanol was respectively 4.1, 5.7 and 7.7 times higher than that in hot-water sample. Comparatively to hot-water, this great increase of reactive species number could explain the enhancement of β-lactoglobulin polymerization when alcohols were used as cosolvent.

In our study, it was interesting to note that comparison of alcohols with the same number of hydrocarbon group but different numbers of hydrophilic hydroxyl (OH) groups, showed that the hydroxyl groups would contribute negatively to the alcohol capacity to induce a polymerization. Our observations are in agreement with data by Hirota-Nakaota and Goto (1999) whose far-UV circular dichroism analysis showed that alkanediols have a profoundly weaker potential than alkanols in inducing denaturation of β-lactoglobulin.

By using 1,4-dioxane as cosolvent, the suspension pH value was the lowest (Table 4), suggesting that the [R-S⁻]/[RSH] ratio would not be high enough to facilitate the substitution of a thiolate for a disulfide. Moreover, as compared to alcohols, the electric dipole moment of 1,4-dioxane was the lowest. Plotting ($\beta lg_{t1}/\beta lg_{t0}$) ratio

Table 3
Value of the $\beta lg_t/\beta lg_0$ ratio as function of mixtures used at, respectively, time 1 h and 12 h, at 25 °C (medium permittivity value was 63.8)

	1,4-Dioxane	Ethanol	1-Propanol	1,2-Propanediol	Hot-water	DMSO	Glycerol
1 h	0.524a	0.711ab	0.754ab	0.887b	0.907b	0.94b	0.96b
	±0.013	±0.123	±0.242	±0.098	±0.103	±0.07	±0.06
12 h	0.550AB	0.359A	0.612AB	0.786BC	0.696ABC	0.646AB	0.971C
	±0.114	±0.202	±0.326	±0.106	±0.197	±0.051	±0.249

Mean value with different letters were significantly different ($P < 0.05$).

Table 4
Various parameters characterising the dispersing medium used as β-lactoglobulin suspension medium

	1,4-dioxane	Ethanol	1-Propanol	1,2-Propanediol	Hot-water	DMSO	Glycerol
Mole fraction[a]	0.19	0.14	0.25	0.31		0.43	0.40
pH[b]	6.3±0.6	7.8±0.0	7.5±0.0	7.7±0.0	6.9±0.0	9.5±0.0	7.0±0.0
μ[c] (Debye)	0	1.69	1.68	2.2	1.85	3.63	2.6

[a] Mole fraction of organic solvent to obtain the permittivity value 63.8.
[b] pH of protein suspension was measured at 25 °C using a Mettler Toledo MP220 pH meter.
[c] Dipole moment (μ) of pure organic solvent was reported as described in *Handbook of Chemistry and Physics*.

as a function of the electric dipole moment value of various organic solvents used ($r^2 = 0.90$ Fig. 4), under the employed conditions, a linear correlation was obtained (Fig. 4), suggesting that the lower is the dipole moment of organic solvent, the lower the ($\beta lg_{t1}/\beta lg_{t0}$) ratio. This result leads us to conclude that, in addition to the change in the medium dielectric constant, a decrease in the electric dipole moment value of the cosolvent favours the protein denaturation/polymerization process. The role of the pH value of dispersing medium intervenes as soon as the protein becomes unfolded, exposing reactive thiolate species. As compared to alcohol/water mixtures, the pH value of 1,4-dioxane/water mixture was lower, suggesting that the number of reactive thiolate species is lower too. Although the pH value was not favourable for the nucleophilic attack of a thiolate on a disulfide, protein polymerization would occur, suggesting that even if the pH value were one of the major factors in the polymerization process, it would not be the only deciding factor.

3.3. Case where the use of cosolvent led to an increase in ($\beta lg_{t1}/\beta lg_{t0}$) ratio

As seen in Table 3, compared to the hot-water sample, the use of glycerol and dimethylsulfoxide (DMSO) as cosolvent leads to an increase in ($\beta lg_{t1}/\beta lg_{t0}$) ratio, meaning that β-lactoglobulin polymerization was prevented. The results for glycerol, are in agreement with the literature since glycerol is conventionally used as a cosolvent for stabilizing the protein native structure.

Analysis of the various parameters characteristing the dispersing medium (Table 4) may explain why β-lactoglobulin polymerization is limited when glycerol or DMSO are used as cosolvents. As a first observation (Table 4), the mole fraction of these cosolvents, in order to obtain the permittivity value of 63.8, was more important than for previous experiments done with all

the other cosolvents. So, the proportion of water available to solvate the protein decreased. As a second observation, the electric dipole moment of these cosolvents was the highest. Considering the above observations and results, it seems that the impediment to β-lactoglobulin polymerization is due to modification of the solvation of the protein. A possible explanation of the decrease in polymerization upon DMSO or glycerol addition is that these cosolvents are preferentially excluded from protein in the native state and are totally inert toward chemical groups on the protein surface. Because of the exclusion of these cosolvents from the space where the protein exists, the hydration of the protein is favoured, so that it is protected from denaturation and hence, from polymerization. This is in agreement with Jarabak, Seeds and Talalay (1966) and Ruwart and Suelter (1971) who proposed that the absence of protein denaturation upon glycerol addition is due to the formation of a hydration layer around the protein. This hydration layer stabilizes the structure of the protein.

The role of water molecules in the denaturation/polymerization processes should be emphasized. Consequently, we have attempted to find a relationship between the water activity (A_w) value of various cosolvent/water mixtures and the β-lactoglobulin polymerization state. As an index of the state of water in solutions, the A_w for various cosolvent/water mixtures was calculated from the mole fraction of the cosolvent by the following Eq. (3):

$$A_w = X_w = 1 - X_c \qquad (3)$$

where X_w and X_c are mole fraction of water and mole fraction of cosolvent respectively.

As shown in Fig. 5, where ($\beta lg_{t1}/\beta lg_{t0}$) is shown as a function of the calculated A_w for various cosolvent/water mixtures, a linear correlation was observed ($r^2 = 0.89$), suggesting that higher the A_w of cosolvent/
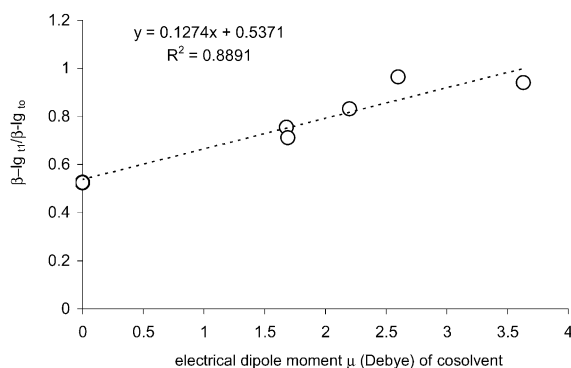
Fig. 4. Evolution of the $\beta lg_{t1}/\beta lg_0$ ratio as a function of the electrical dipole moment value (μ, Debye) of the various organic solvents used as cosolvents. $\beta - lg_0$ and $\beta - lg_{t1}$ are concentration of β-lactoglobulin at time 0 and 1 h respectively. Mixture permittivity was 63.8. Temperature: 20 °C.

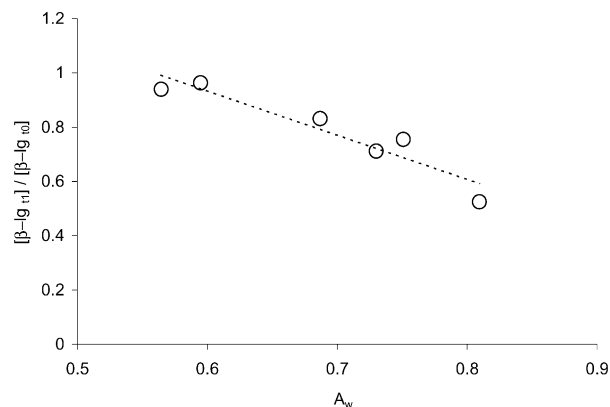Fig. 5. Evolution of the $\beta lg_{t1}/\beta lg_{t0}$ ratio as a function of A_w of the various β-lactoglobulin mixtures. $\beta - lg_{t0}$ and $\beta - lg_{t1}$ are concentration of β-lactoglobulin at time 0 and 1 h respectively. Mixture permittivity was 63.8. Temperature: 20 °C.

water mixture, lower is the ($\beta lg_{t1}/\beta lg_{t0}$) ratio. In others words, the lower the hydration of the protein, the higher the denaturation/polymerisation process.

The pH value of DMSO/water mixture being the highest, the potential reactivity of thiolate species is the highest too. But, as the protein was not unfolded, despite the strong reactivity of thiolate, the protein polymerization did not occur. Consequently, we can conclude that, at time 1h, the use of high pH values did not offset the effect of stabilizing water structure around the protein.

As seen in Table 3, at time 12H, the effects of cosolvents on protein polymerization were either similar (1,4-dioxane, 1-propanol, 1,2-propanediol and glycerol) or enhanced (ethanol and DMSO), relatively to results obtained at time 1h.

Regarding the "*stabilizing effect*" of glycerol, we can suppose that the hydratation layer around the protein, due to glycerol addition, does not evolve with time. On the other hand, the "*stabilizing effect*" of DMSO, observed at time 1H, was changed to a "*destabilizing effect*" at time 12H, suggesting a "temporary stabilizing effect" of DMSO under the employed conditions. Because of the high pH value of the DMSO/water mixture, as a function of time, intramolecular electrostatic repulsion would increase, favouring protein unfolding. Moreover, the reactivity of thiolate species would increase too, leading to an enhancement of the β-lactoglobulin polymerization.

As a result of the "*destabilizing effect*" due to 1,4-dioxane addition, it would be maximal from time 1h, and would not change thereafter. So, the use of 1,4-dioxan as cosolvent leads to optimum protein unfolding at time 1h.

5. Conclusion

For all solvents except glycerol, setting the permittivity to 63.8 by addition of organic solvent induced either an enhancement or an impediment of β-lactoglobulin polymerization, in comparison with result obtained when the permittivity was changed by thermal treatment (heating to 70 °C). Making medium permittivity decrease by addition of organic solvent did not simulate the modifications occurring during heating, because it was impossible to modify the permittivity value without changing other medium parameters, such as pH value or water organization. Consequently, even if addition of organic solvent decreased the permittivity of the medium as increase temperature did, the addition of cosolvents in aqueous suspension should also change the structure and distribution of water around the protein. This change of interaction between water and protein would be a major factor, in addition to the change in medium dielectric constant, in the protein denaturation

process. Medium pH plays a role in the protein polymerization process as soon as the protein becomes unfolded and reactive species we exposed. Moreover, as a function of time, the medium pH could also play a role in the protein denaturation process but only at high values.

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