Heat Treatment of β-Lactoglobulin: Structural Changes Studied by Partitioning and Fluorescence

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Functional properties of whey protein concentrates (WPC) are primarily dependent on the degree of denaturation of β-lactoglobulin (β-LG), the major globular whey protein. Irreversible modifications in the tertiary structure and association state of β-LG after heat treatment were studied by partition in aqueous two-phase systems and fluorescence quenching. Partitioning of preheated β-LG in two-phase systems containing 5% (w/w) poly(ethylene glycol) and 7% (w/w) dextran, between pH 6.0 and7.0, are appropriately related with the intensity of heat treatment. An increase in the partition coefficient of β-LG was observed with increasing temperature of heat treatment. On the other hand, fluorescence quenching of β-LG by acrylamide was used to study the conformational flexibility of the protein at pH values between 4.0 and 9.0. The values of bimolecular quenching rate constant (k_q) obtained showed that β-LG appears to be more flexible at high pH values, while at low pH the protein assumes a more compact form. The efficiency of acrylamide quenching on preheated β-LG was substantially more pronounced than for the untreated protein. This difference can be ascribed to the presence of unfolded monomers and aggregates of denatured molecules formed after heat treatment, whose tryptophanyl residues are more exposed to the solvent. In conclusion, the results suggest that partition studies in aqueous two-phase systems and fluorescence quenching are very useful tools to detect changes in conformation and aggregation of β-LG induced by heat treatment.

Keywords: *β-Lactoglobulin; heat treatment; aqueous two-phase systems; fluorescence quenching*

INTRODUCTION

Because of its high nutritional value and functionality, whey protein concentrates (WPC) obtained from dairy processing has become an important source of functional ingredients used in many formulate foods, including processed meat, bakery, and dairy products (Kinsella and Whitehead, 1989). At present, WPC are extracted, on an industrial scale, by ultra/diafiltration or by ion exchange chromatography. Although these processes give large yields of protein, they may cause irreversible conformational changes with undesired effects such as loss of protein solubility, and functionality and flocculation. β-lactoglobulin (β-LG) is the major protein in whey and therefore tends to dominate the behavior of the total whey protein system. Because of this dominant role, a great deal of attention has been given to the properties of β-LG under various experimental conditions (Relkin, 1996; Wong et al., 1996). Notwithstanding this, a complete picture is lacking.

β-LG is a globular protein with a monomer molecular weight of about 18,300 and exists in various oligomeric states (dimers and octamers) as a function of pH, temperature, concentration, ionic strength, and genetic variant (Wong et al., 1996). The equilibrium between the monomeric and oligomeric forms of β-LG is shifted to the monomeric form when the ionic strength is decreased and/or the temperature is increased. (Aymard et al., 1996; Renard et al. 1998). β-LG denatures at temperatures above 65 °C. On mild heating, the loss of the compact globular conformation may be reversible, but on more severe heating β-LG tend to become associated through disulfide linkage and hydrophobic interactions (De Wit, 1990; Regester et al., 1992).

At low concentrations (5−15%), poly(ethylene glycol) (PEG) mixed with other polymers, such as dextran (Dx), forms two aqueous immiscible phases, in which the proteins can be partitioned. Previous works (Axelsson, 1978) have suggested that partition studies are a very useful tool to detect changes in conformation and aggregation and to characterize the corresponding hydrophobic surface properties of a protein. In this study, we have investigated the temperature-dependent conformational and aggregation changes of β-LG by partition in aqueous two-phase polimeric systems.

On the other hand, fluorescence is a useful technique to study the structure and dynamics of protein molecules in solution. The intrinsic fluorescence of Trp residues is particularly sensitive to their microenvironments and provides an appropriate method to perform this kind of study. The degree of exposure of Trp residues in the β-LG molecule can be evaluated by following the external quenching of the intrinsic protein fluorescence by added solutes (Eftink and Ghiron, 1981; Busti et al., 1998). To complement and extend the partitioning studies of the heat-induced species of β-LG, we used the intrinsic fluorescence quenching by acrylamide to examine the conformational states of the protein.

MATERIALS AND METHODS

Materials. β-LG AB, PEG 8000, Dx T 500, and acrylamide were purchased from Sigma and used without further purification. All other chemicals were of analytical grade.

Heat Treatment of β-LG. A stock 3 mM β-LG solution was prepared in 20 mM phosphate buffer at pH 6.8. Aliquots of

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this solution were placed in small stoppered glass tubes and heated for 60 min in a thermostated water bath at 65 or 85 °C. Once removed, the samples were cooled to room temperature and analyzed after about 5 h of heat treatment.

Phase System. Two-phase systems containing 5% (w/w) PEG and 7% (w/w) Dx were prepared from 40% (w/w) and 20% (w/w) aqueous stock solutions of the respective polymers and the respective buffer. The following buffers (ionic strength 25 mM) were used: acetate (4.0−5.0), phosphate (6.0−6.8), Tris HCl (8.0), and NaOH/glycine (8.0−9.0). Both phases in equilibrium were separated after centrifugation at low speed.

Measurement of Partition Coefficient. One gram of each of the preformed two phases was mixed together with protein (10 to 50 μL of 3 mM β-LG). After mixing by inversion during 4 h, the systems were centrifuged for 10 min at 1000*g* with a Rolco centrifuge for the separation of the two phases. Samples were withdrawn from the separated phases, and after dilution, the protein concentration in each phase was determined by measurement of light absorption at 280 nm. The partition coefficient was obtained from the slope of the plot, which relates the protein concentration in the top PEG phase against the concentration in the bottom Dx phase. The temperature of partition was controlled at 22 or 37 °C.

Quenching of the Intrinsic Fluorescence of β-LG by Acrylamide. Solutions of 20 μM β-LG (native or preheated) in the respective buffer were placed in the 10 mm square quartz cell of a Jasco FP-770 spectrofluorimeter, and the fluorescence intensity at 337 nm using excitation at 295 nm was measured (F_o). Aliquots of 5 M acrylamide were mixed into the cell content, and the fluorescence intensity was redetermined (F). Final acrylamide concentrations ranged from 0 to 0.7 M. A correction factor (C) was applied to F because of the absorbance of acrylamide at the exciting wavelength. C was calculated as

$$C = \frac{A_t}{A_f}\left(\frac{1 - 10^{-A_f}}{1 - 10^{-A_t}}\right)$$

where A_f and A_t are the absorbances at the exciting wavelength for the fluorophore and total solution, respectively (Lloyd, 1981).

An appropriate form of the Stern−Volmer equation for the quenching of heterogeneously fluorescent protein is

$$\frac{F_o}{F} = \left[\sum_{i=1}^{n} \frac{f_i}{(1 + K_i[l])e^{V_i[l]}}\right]^{-1}$$

where F_o and F are the fluorescence in the absence and presence of quencher Q, respectively (Eftink and Ghiron, 1981). K_i and V_i are the dynamic and static quenching constants for fluorescent component i and f_i is the fractional contribution of component i to the total fluorescence.

The quenching of preheated β-LG by acrylamide was also carried out in the presence of PEG. All the quenching experiments were performed at 22 °C.

Electrophoresis. SDS−PAGE of native and preheated β-LG was performed as described by Laemmli (1970) using a 12% slab gel. The gels were run under nonreducing conditions to avoid cleavage of intermolecular disulfide bonds formed during the heat treatment. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250. The gels were scanned using a Hewlett-Packard ScanJet 5p connected to a computer. To quantify the relative intensities of the stained protein bands, the pixel densities of digitized images were analyzed using software developed by our group. The molecular weight of each protein band was checked with known protein standards.

RESULTS AND DISCUSSION

Partitioning of β-LG as a Function of pH. The partition curves in Figure 1 are very similar to those obtained by Axelsson (1978) for the partition of β-LG in a zero-potential phase system. In this earlier work the changes observed in the partition with pH were attributed to changes in conformation and/or aggregation of the protein. β-LG generally exists as a dimer which dissociates into its monomers below pH 3.5 and above pH 6.5. In the phase system this was seen as an increase in ln K near these pH values due to a higher partition coefficient for the monomer. Figure 1 shows a dip in the partition curve around pH 4.8. The β-LG used in this work contains equal amounts of β-LG A and β-LG B. The dip reflects the known association of β-LG A into aggregates larger than dimers (octamers) at low temperatures and high concentrations (Pessen et al., 1985; Relkin, 1996). Figure 1 shows that this local effect tends to disappear at higher temperatures due to the dissociation of the oligomers. The behavior observed around pH 7.5 is caused probably by the Tanford transition (Tanford et al., 1959). This conformational change, which is reversible, involves only a certain region of the molecule and is accompanied by the release of a single buried carboxyl group per monomer. This reversible unfolding is followed by slow changes which become increasingly irreversible with increasing pH. These changes promote an increase in ln K above pH 8.0.

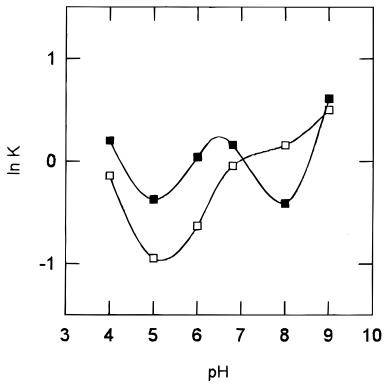

Figure 1. The partition of β-LG as a function of pH at two different temperatures: (□) 22 °C and (■) 37 °C. All points were the mean of three measurements.

Conformational Flexibility of β-LG Studied by Fluorescence Quenching. We study the degree of exposure of tryptophanyl residues in the β-LG molecule following the quenching of intrinsic protein fluorescence by acrylamide (Eftink and Ghiron, 1981; Creamer, 1995; Busti et al., 1998). Figure 2 shows the Stern−Volmer plots for the quenching of β-LG with acrylamide, with different conditions assayed. To obtain the quenching parameters, the Stern−Volmer equation was directly fitted to the quenching data making the following assumptions:

(a) Bovine β-LG contains two Trp residues, Trp-19 and Trp-61, which are in quite different environments. The lattice Z crystal structure (Qin et al., 1998a,b) shows that Trp-19 is in an apolar environment within the cavity of β-LG, whereas Trp-61 protrudes beyond the surface of the molecule and is quite close to the Cys-66−Cys-160 disulfide bridge, which can be an effective Trp fluorescence quencher (Cowgill, 1967; Hennecke et al., 1997; Chen and Barkley, 1998). The intrinsic fluorescence of the protein is almost exclusively attributed to Trp-19 (Cho et al., 1994, Manderson et al., 1999). For these reasons, we have assumed that $f_{19} = 1$ and $f_{61} = 0$ in the Stern−Volmer equation for the untreated β-LG.

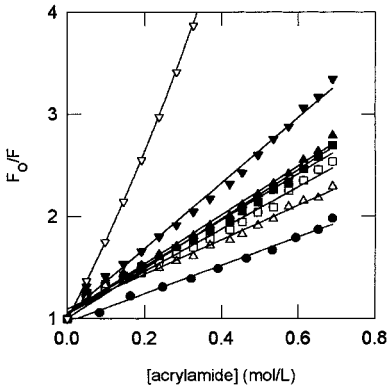

Figure 2. Stern−Volmer plots of β-LG fluorescence quenching by acrylamide at various pH values: (●) pH 3.0, (○) pH 4.0, (■) pH 5.0, (□) pH 6.0, (△) pH 6.8, (▲) pH 8.0, (▼) pH 9.0, and (▽) at pH 6.8 in 8 M urea solution. All points were the mean of three measurements. Temperature, 22 °C; protein concentration, 10 μM.

Table 1. Acrylamide Quenching Data for β-LG[a]

pH	K (M^{-1})	$k_q \times 10^{-9}$ (M^{-1} s^{-1})
3.0	1.37 ± 0.04	0.98
4.0	2.42 ± 0.07	1.73
5.0	2.26 ± 0.05	1.61
6.0	2.07 ± 0.05	1.48
6.8	1.68 ± 0.04	1.20
8.0	2.39 ± 0.05	1.71
9.0	3.20 ± 0.05	2.99
6.8 (denatured)[b]	K_{19} = 6.57 ± 0.47	nd[c]
	K_{61} = 6.57 ± 0.47	nd

[a] Temperature, 22 °C; λ_{em}, 295 nm; λ_{exc}, 337 nm; protein concentration, 20 μM [b] Protein in 8 M urea solution. [c] Not determined

(b) The alteration in β-LG fluorescence emission upon its denaturation with urea followed the trend observed for other globular proteins. In other words, there was a red shift of the emission spectrum induced by the denaturation process due to the major exposure of the two tryptophanyl residues to the aqueous solvent. However, the quantum yield increases when β-LG is denatured in urea solution, presumably because the decrease in quenching of Trp-61, which becomes more distant from the Cys-66−Cys-160 disulfide bond (Creamer, 1995; Manderson et al., 1999). The major exposure of tryptophanyl residues to the aqueous solvent promotes an increase in the fluorescence quenching of denatured β-LG by acrylamide (Figure 2). The unfolding with urea 8 M "normalizes" the environment and exposes the two tryptophanyl residues of β-LG. Thus, a good fitting of the experimental Stern−Volmer plot for β-LG in the presence of 8 M urea solution was achieved considering equal contribution of each of the two tryptophanyl residues present in the denatured β-LG molecule ($f_{19} = f_{61} = 0.50$).

(c) Eftink and Ghiron (1981) found that the K_i and V_i values are related. For the quenching of several proteins with acrylamide, V_i was found to be approximately $^1/_{10}$ of K_i. This value was applied to the analysis of our experimental data.

Table 1 shows the values of the Stern−Volmer constant (K_{SV}) calculated from the curves in Figure 2 using these assumptions. For untreated β-LG, the exponential factor in the Stern−Volmer equation is near unity at acrylamide concentrations up to 0.7 M. For this reason, the Stern−Volmer plots for the quenching of untreated β-LG by acrylamide appear to be linear at all the pH assayed (Figure 2). As demonstrated by Eftink and Ghiron (1981), if a protein possesses two fluorophores having quenching constants nearly identical, plots of F_o/F versus quencher concentration may appear upward curving. This was the case for the Stern−Volmer plot for the quenching of denatured β-LG by acrylamide (Figure 2).

K_{SV} for the collisional quenching process is equal to $k_q\tau_o$, where k_q is the bimolecular quenching rate constant and τ_o is the fluorescence lifetime of the fluorophore in the absence of quencher. In fact, k_q is the parameter which provides specific information concerning the degree of exposure of a particular fluorophore and/or its microenvironment. Between pH 3.0 and 9.0 the position of the fluorescence emission maximum remained constant, suggesting that the hydrophobic environment of the fluorophores remained unchanged (Renard et al., 1998). The fact that the retinol binding to the central hydrophobic pocket of β-LG, where Trp-19 is located, does not show a pH dependence (Fugate and Song, 1980) is consistent with this supposition. These authors have determined that the fluorescence lifetime of β-LG (Trp) is not influenced by pH and it is equal to 1.40 ns. These observations allow us to estimate k_q values (Table 1), thus providing information concerning the exposure and microenvironment of Trp-19 at the different pH assayed.

Previous studies have shown that for a series of single-tryptophan-containing proteins, k_q values for acrylamide quenching were found to range from less than 5×10^7 M^{-1} s^{-1} (no detectable quenching) for the buried tryptophan of azurin to 4×10^9 M^{-1} s^{-1} for the fully exposed residues in glucagon and adenocorticotrophin (Eftink and Ghiron, 1981). The relatively high k_q values shown in Table 1 suggest that the quenching of the buried residue (Trp-19) could be facilitated by the stochastic structural fluctuations of the protein matrix that increase the inward diffusion of acrylamide (Eftink and Ghiron, 1981). In conclusion, β-LG appears to be more flexible at high pH values, while a major rigidity was observed at low pH.

Calhoun et al. (1983), using oxygen quenching of tryptophanyl phosphorescence, criticized the use of acrylamide quenching to determine protein mobility, though subsequently Ghiron and Eftink (1984) pointed out that the mechanism of phosphorescence quenching is significantly different from fluorescence quenching. The spectral overlap between protein fluorescence and acrylamide is small and the acrylamide absorption is also small; hence, the mechanism of acrylamide singlet quenching is more likely to be an electron transfer than an electronic energy transfer (Evans et al., 1978). Chen et al. (1987) proposed that the quenching process is an electron transfer between the acrylamide bound to the surface of the protein and a tryptophanyl residue located beneath the surface of the molecule. These authors suggested that the quenching simply senses the distance of closest approach of the acrylamide to the tryptophan. On the basis of these remarks, it seems that the acrylamide quenching technique could be used to detect conformational changes which modify the referred distance. In that sense, the shortest distance between Trp-19 and the acrylamide bound to the surface of β-LG occurs at pH 9.0, where the higher quenching efficiency was obtained. Hence, at high pH, the structure of β-LG becomes more open and the interior Trp-19 becomes more exposed to the aqueous solvent.

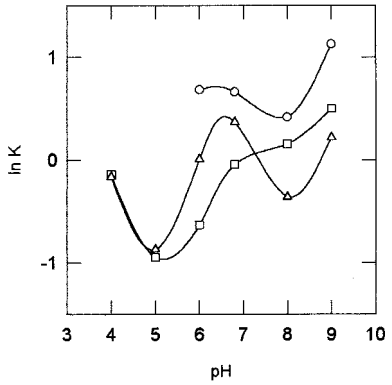

Figure 3. Effect of heat treatment on the partition of β-LG: (□) untreated β-LG, (△) β-LG preheated at 65 °C for 1 h, and (○) β-LG preheated at 85 °C for 1 h. All points were the mean of three measurements. Temperature, 22 °C.

Partitioning Behavior of Preheated β-LG. At 40 °C β-LG undergoes some small, reversible conformational changes, whereas on further heating the protein (partially) unfolds, resulting in a molten-globule-like structure (Iametti et al.; 1996), with increased exposure of the previously buried inner hydrophobic groups and the reactive Cys-121. The thiol group in the modified monomer can induce thiol/disulfide exchange reactions, leading to the formation of disulfide-linked aggregates. In addition to this chemical aggregation, also noncovalent inteactions (ionic, van der Waals, hydrophobic) are involved in the heat-induced aggregation and gelation of β-LG (Shimada and Cheftel, 1989; McSwiney et al., 1994a,b; Hoffmann and Van Mil, 1997; Qi et al., 1997).

The presence of several protein species must be considered when heated β-LG solutions are cooled to room temperature. The composition of the product formed is governed, to a large extent, by the intensity of heat treatment, protein concentration, pH, genetic variant, and small ion concentrations. The results obtained by Manderson et al. (1998, 1999) showed that dilute β-LG solutions, which have been preheated, are mixtures of native β-LG, non-native monomers, and aggregates of denatured β-LG molecules that are cross-linked into dimers, trimers, etc. On the other hand, a gel is formed when the protein concentration exceeds some critical level, because the protein forms a three-dimensional network of aggregated or entangled molecules that fills the entire volume of the container (Bryant and McClements, 1998).

Figure 3 shows the partition coefficients for the native and preheated β-LG at 65 and 85 °C as a function of pH. At pH values above the isoelectric point of β-LG (pH 5.2), in the absence of electrolyte and at the protein concentration used in this work, the unfolded species remain in solution due to the strong electrostatic repulsion. However, we observed that the denatured molecules undergo extensive aggregation promoted by noncovalent interactions near the isoelectric point. This fact makes the determination of partitioning of β-LG preheated at 85 °C impossible at pH values lower than 6.0.

As can be seen in Figure 3, the values of the partition coefficients at pH levels ranging from 6.0 to 7.0 are appropriately related with the intensity of heat treatment. An increase in the partition coefficient of β-LG was observed with increasing temperature of heat treatment. In this range of pH, partitioning of β-LG reflects principally the degree of denaturation of the protein induced by heat treatment. The composition analysis of native and preheated β-LG solutions (pH 6.8) by SDS−PAGE, under nonreducing conditions, is shown in Figure 4. In samples heated at 65 °C of 1 h, the monomeric forms (native and denatured) were the predominant species. In samples heated at 85 °C for 1 h, the prevailing species were aggregates of more than three denatured β-LG molecules cross-linked by intermolecular disulfide bonds that could not penetrate the separating gel (Figure 4).

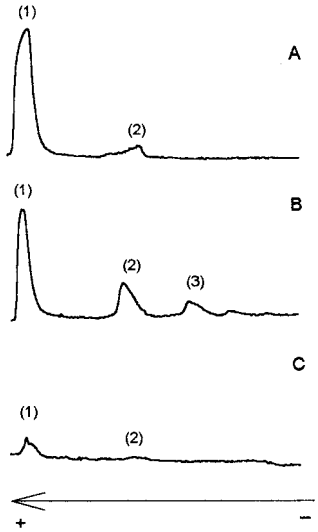

Figure 4. Electrophoretic patterns of β-LG heated at pH 6.8. SDS−PAGE: A, no heating; B, 65 °C for 1 h; 85 °C for 1 h. Peak numbers: (1) monomer; (2) dimer; (3) trimer.

At pH values higher than 7.0 other unknown components may be taken into account in the partitioning behavior of preheated β-LG. Notwithstanding this, the higher values of the partition coefficient were obtained for β-LG preheated at 85 °C at all the pH values assayed.

Fluorescence Quenching of Preheated β-LG. The heat treatment of β-LG results in a number of irreversible, linked, spectrally detectable changes to the structure of the protein. The structural changes result in the loss of the hydrophobicity of the Trp-19 environment, variations in thiol group availability, and ANS binding (Creamer, 1995; Manderson et al., 1999). After the protein has been treated, the wavelength of the maximum of the intrinsic fluorescent emission is shifted from 332 nm to longer wavelengths. This red shift indicates that the major fluorophore (Trp-19) has moved from an apolar environment to a more polar region. Moreover, the quantum yield of β-LG increases when the protein is denatured by heat treatment, presumably because the decrease in quenching of Trp-61, which becomes more distant from the Cys-66−Cys-160 disulfide bond. Consequently, in the unfolded state, the solvent exposure of both Trp-19 and Trp-61 could be approximately the same. Therefore, both tryptophanyl residues could be similarly quenched by acrylamide with a higher efficiency than in the native state of the protein. In conclusion, the more intense the heat treatment, the higher is the degree of denaturation and more pronounced should be the Stern−Volmer plot. This trend was observed in the quenching of preheated β-LG fluorescence by acrylamide, at pH 6.8 (Figure 5). The slight upward curvature of the Stern−Volmer plots in Figure 4 can be attributed to the presence in solution of β-LG denatured species which possess nearly identi-

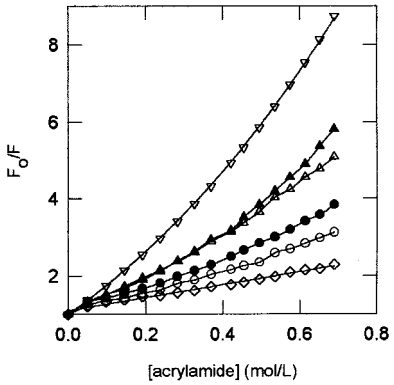

Figure 5. Stern−Volmer plots of β-LG fluorescence quenching by acrylamide at pH 6.8. In the absence of PEG: (○) β-LG preheated at 65 °C for 1 h and (△) β-LG preheated at 85 °C for 1 h. In the presence of PEG 10% (w/w): (●) β-LG preheated at 65 °C for 1 h and (▲) β-LG preheated at 85 °C for 1 h. This figure includes the following Stern−Volmer plots: (◇) untreated β-LG and (▽) β-LG in 8 M urea. All points were the mean of three measurements. Temperature, 22 °C, protein concentration, 10 μM.

cal quenching constants for Trp-19 and Trp-61 (Eftink and Ghiron, 1981).

On the other hand, the red shift induced by heat treatment is 10 nm less than that of a fully exposed Trp residue, e.g., that of β-LG in urea solution (Creamer, 1995; Manderson el al., 1999). SDS−PAGE experiments show that the predominant species present in a preheated β-LG solution at 85 °C are aggregated of denatured molecules of relative high molecular weight. However, the effect of acrylamide on the fluorescence of these β-LG unfolded species was substantially less than for β-LG denatured by urea (Figure 5). These results point to the presence in the denatured cross-linked species of a residual structure that hinders the fully Trp accessibility to the acrylamide.

In the unfolding process, the protein surface becomes more hydrophobic because of a transfer of apolar amino acids from the protein "inside" to its surface. PEG is hydrophobic in nature and may interact favorably with the apolar side chains exposed upon unfolding. This hydrophobic interaction promoted an increase of the partition coefficient of untreated β-LG at basic pH (Figure 1) and of preheated β-LG at pH 6.8 (Figure 3), conditions where the conformational flexibility of the protein is high. Moreover, this conformational flexibility was increased by the presence of PEG. Figure 5 shows that for the same experimental conditions, the Stern−Volmer plot was more pronounced if PEG was present in the system.

CONCLUSIONS

The demand from industry to predict the functionality of WPC has not yet been met. The relationship between heating process and whey protein denaturation needs to be well-established for WPC to be used as a source of functional ingredients in formulated foods. We focused our attention on β-LG, the major whey protein that dominates WPC behavior. Our results showed that aqueous two-phase systems and fluorescence quenching proved to be useful techniques for studying the thermal denaturation of β-LG. Partitioning of preheated β-LG in two-phase systems containing PEG and Dx, between pH 6.0 and 7.0, depends on the degree of denaturation of the protein promoted by heat treatment. In addition, the intrinsic fluorescence quenching of β-LG by acrylamide may be used to verify the conformational flexibility of the protein at pH values between 4.0 and 9.0.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; WPC, whey protein concentrates; PEG, poly(ethylene glycol); Dx, dextran; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Trp, tryptophan.

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