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Study on β-lactoglobulin glycosylation with dextran: effect on solubility and heat stability

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Abstract

In this paper, the characteristics of purified conjugates formed between β-lactoglobulin (β-lg) and a high molecular weight dextran under different dry heating conditions were investigated. SDS–PAGE analyses under non-reducing and reducing conditions showed that glycation of β-lg led to the formation of high molecular weight complexes and induced polymerization of the protein by disulfide bonds. The fluorescence emission spectra did not show changes in λ_{max}, which was indicative of a similar conformation around Trp residues. The conjugate formed at 60 °C, 0.44 a_w and 2:1 weight ratio of dextran to β-lg (conjugate 1) exhibited a fluorescence intensity very similar to that of the native protein and was selected to study the influence of glycosylation on the solubility and heat-stability properties. Solubility of conjugate 1 was higher than that of the dry-heated β-lg in the pH range from 3 to 9 and, particularly, around the isoelectric point of the protein. As compared to the native protein, the solubility of the conjugate decreased at pH 4. The glycated β-lg presented lower stability to heating at pH 7.0 than native β-lg, but its thermal stability was higher at pH 5.0 at temperatures above 85 °C.
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Keywords: β-Lactoglobulin; Dextran; Glycosylation; Solubility; Heat stability

1. Introduction

Whey proteins, available in large amounts due to the great production of milk and cheese, can be used in the formulation of nutritious, high-protein food ingredients, due to their functional qualities, solubility being the main characteristic. Important molecular parameters, such as charge, mass, hydrophobicity and conformation, affect in a great extent their functional properties (Kinsella, 1976; Persson & Gekas, 1994). Milk protein manufacturers are interested in treatments to produce proteins with improved functionality. In particular, glycosylation using the Maillard reaction seems to be an efficient method for this purpose as it increases the amphipathicity of proteins without the need for toxic chemical reagents (Aoki, Fukumoto, Kimura, Kato, & Matsuda, 1994; Kato, Aoki, Kato, Nakamura, & Matsuda, 1995; Morales, Dill, & Landmann, 1976).

β-lactoglobulin (β-lg), which constitutes a major secondary product of the cheese industry, is responsible, in great part, for the properties of whey proteins (Zayas, 1997). The drawbacks of β-lg as an emulsifier lay in its poor stability to acid and heating. Evidence for conjugate formation of β-lg with mono- and disaccharides via Maillard reaction has been reported (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001b; Fenaille, Morgan, Parisod, Tabet, & Guy, 2003; Matsuda, Kato, & Nakamura, 1991; Morgan, Léonil, Mollé, & Bouhallab, 1999) and the conjugates exhibited better solubility and emulsifying properties than the non-glycosylated protein (Nacka, Chobert, Burova, Léonil, & Haertlé, 1998).

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In general, it has been shown that polysaccharides may enhance the stability and functional properties of proteins to a higher extent than mono-, di- and oligosaccharides, because their larger size and net charge lead to important structural changes (Dickinson & Galazka, 1991). In fact, ovalbumin–dextran conjugates obtained by the Maillard reaction showed higher heat stability than ovalbumin and three times better emulsifying properties than ovalbumin–glucose conjugates (Kato, Sasaki, Furuta, & Kobayashi, 1990). Lysozyme–dextran conjugates acted as emulsifiers with improved activity against Gram-negative bacteria and enhanced heat stability (Nakamura, Kato, & Kobayashi, 1991). Other polysaccharides, such as galactomannan, were also successfully used to improve the functionality of lysozyme (Nakamura, Kato, & Kobayashi, 1992), dried egg white protein (Kato, Minaki, & Kobayashi, 1993; Matsudomi, Nakano, Soma, & Ochi, 2002), ovalbumin (Nakamura & Kato, 2000), bovine serum albumin (Kim, Choi, Shin, & Moon, 2003), protamine (Matsudomi, Tsujimoto, Kato, & Kobayashi, 1994), plasma (Matsudomi, Inoue, Nakashima, Kato, & Kobayashi, 1995) and soy proteins (Babiker et al., 1998). The emulsifying properties were maintained in high-salt and acidic pH conditions and improved by heating the conjugates, which is favourable for pasteurization and food applications. The emulsifying properties of the conjugates increased in proportion to the length of the polysaccharide and the binding number, while the heat stability was enhanced regardless of its molecular size (Shu, Sahara, Nakamura, & Kato, 1996).

There are fewer data on functional improvement of β-lg through conjugation with polysaccharides. A soluble complex of β-lg and dextran exhibited enhanced emulsifying behaviour, while the same dry-heat treatment (60 °C, 35–40% relative humidity) applied to β-lg alone led to a loss of emulsifying capacity (Dickinson & Galazka, 1991). In a previous paper, we studied the effect of different dry-heating conditions, such as water activity (a_w), temperature, time and weight ratio (WR) of polysaccharide to protein on the conjugation between β-lg and dextran (43,000 Da) (Jiménez-Castaño, Villamiel, Martín-Alvarez, Olano, & López-Fandiño, 2005). It was found that 60 °C, 0.44 a_w and 2:1 WR of dextran to β-lg and 50 °C, 0.65 a_w and 6:1 WR of dextran to β-lg led to a similar degree of glycosylation, as measured by the formation of the Amadori compound. However, high a_w and dextran concentration (0.65 a_w and 6:1 WR) promoted brown colour development and protein polymerization. In the present paper, we study more in depth the characteristics of the conjugates formed between β-lg and dextran under the different conditions and their influence on primary properties, such as solubility and thermal stability.

2. Materials and methods

2.1. Preparation of β-lg–dextran conjugates

β-lactoglobulin AB (β-lg) and dextran (average molecular weight 43,000 Da), produced by *Leuconostoc mesenteroides* strain No. 232-677-5, were purchased from Sigma Chemical Co. (St. Louis, MO.). Dextran was dialyzed (Spectra/Por® Biotech Dialysis Membranes, 6500 Da MW cut-off, Spectrum Europe, Breda, The Netherlands) versus deionized water to remove low molecular weight oligosaccharides. All other reagents were of analytical grade.

Mixtures of dextran and β-lg were dissolved in 0.1 M phosphate buffer (pH 7.0) in the weight ratios (WR) 2:1 and 6:1 and lyophilized. The freeze-dried mixture at WR 2:1 was incubated at 60 °C and a_w 0.44 (conjugate 1) and the mixture at weight ratio 6:1 at 55 °C and a_w 0.65 (conjugate 2), in dessicators containing saturated K_2CO_3 and KI solutions, respectively. Samples were taken for analysis after 4 and 10 days of dry-heating. After incubation, the powders were reconstituted in distilled water to a protein concentration of 1 mg/ml. As control, β-lactoglobulin was individually heated in a similar manner.

In order to separate conjugates 1 and 2 from unbound β-lg and dextran, aliquots (2.0 ml) were ultrafiltered through a hydrophilic 50,000 Da cut-off membrane (Centriprep, Amicon Inc, Beverly, MA, USA) by centrifugation at 15,000*g* for 15 min. The retentates were washed three times by the addition of Milli-Q water up to 2.0 ml and ultrafiltered by centrifugation after each wash. The final retentates from ultrafiltration were reconstituted to a protein concentration of 1 mg/ml. Optimization of the ultrafiltration conditions was initially performed on solutions of native or dry-heated β-lg (1 mg/ml) and dextran (6 mg/ml). The volume of the ultrafiltration permeates was measured and the amounts of dextran and β-lg were determined by measuring the absorbance at 490 nm for dextran after colour development with the phenol-sulfuric acid reactive (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and 280 nm for β-lg.

2.2. Characterization of β-lg–dextran conjugates

For SDS–PAGE analyses, protein solutions were mixed either with 10 mM Tris–HCl buffer, pH 8.0 (non-reducing conditions), or in the same buffer containing 2.5% SDS, 10 mM EDTA, and 5.0% β-mercaptoethanol (reducing conditions), and heated at 100 °C for 10 min. SDS–PAGE was conducted with the PhastSystem Electrophoresis apparatus, precast PhastGels Homogeneous 20%, and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). Electrophoretic conditions and silver staining followed the procedures of the manufacturer.

Fluorescence was measured in a Shimadzu RF- 1501 fluorescence spectrophotometer (Kyoto, Japan). The intrinsic fluorescence of aqueous solutions of the purified conjugates (1 mg/ml protein concentration) was measured under an excitation wavelength of 280 nm.

2.3. Solubility

Solubility of native, heated and ultrafiltered glycated β-lg (1 mg/ml) was measured by adjusting the pH to 3, 4, 5, 7 and 9 using 1 M HCl or NaOH. After 30 min of stirring at room temperature, the samples were centrifuged for 15 min at 4 °C and 3500*g*. Solubility was expressed as the percentage of the initial β-lg concentration, determined by measuring the absorbance at 280 nm of the supernatants and using a standard curve of β-lg (0.3–2 mg/ml). In all cases, before absorbance was measured, β-lg solutions were diluted (1:1) with 8 M urea (Sigma Chemical Co., St. Louis, MO) to prevent the interference of non-soluble solids (Moreno, López-Fandiño, & Olano, 2002). Analyses were done in duplicate.

2.4. Thermal stability

Native, heated and ultrafiltered glycated β-lg (1 mg/ml) at pH 5 and 7 were heated at different temperatures (50–95 °C) for 15 min. Samples were then cooled to room temperature and centrifuged for 5 min at 4 °C and 3500*g*, to precipitate aggregates. Finally, the β-lg content of the supernatants was measured as indicated above, and was compared with that of the corresponding untreated samples. Analyses were done in duplicate.

3. Results and discussion

3.1. Isolation of β-lg–dextran conjugates and study on structural changes

Ultrafiltration through a membrane of 50,000 Da molecular weight cut-off was attempted to purify the conjugates formed between β-lg and dextran under the assayed conditions of temperature, a_w and reactants WR after 4 and 10 days. Results from triplicate experiments on the behaviour of solutions of dextran (6 mg/ml) and native β-lg (1 mg/ml) showed that free dextran was found in proportions around 50%, 41% and 9% in the three first permeates, while β-lg was completely recovered in the first permeate. In the case of β-lg dry-heated for 10 days at 60 °C and 0.44 a_w or at 55 °C and 0.65 a_w, approximately 72% and 28% of protein were recovered in the first and second ultrafiltration permeates, respectively, which demonstrated the aggregation of β-lg under the dry-heating conditions studied. These results revealed that it was possible to remove free dextran and either native or heat-denatured β-lg by using a membrane with 50,000 Da cut-off. Regarding glycosylated β-lg, both conjugates 1 and 2, produced after 4 and 10 days of dry heating, were obtained in the retentates with 100% recovery on the basis of the amount of β-lg used.

Fig. 1 shows the SDS–PAGE gels of native, dry-heated and glycosylated β-lg, in the absence or presence of β-mercaptoethanol. The SDS–PAGE pattern obtained under non-reducing conditions of β-lg dry heated in the absence of dextran at 60 °C and 0.44 a_w and at 55

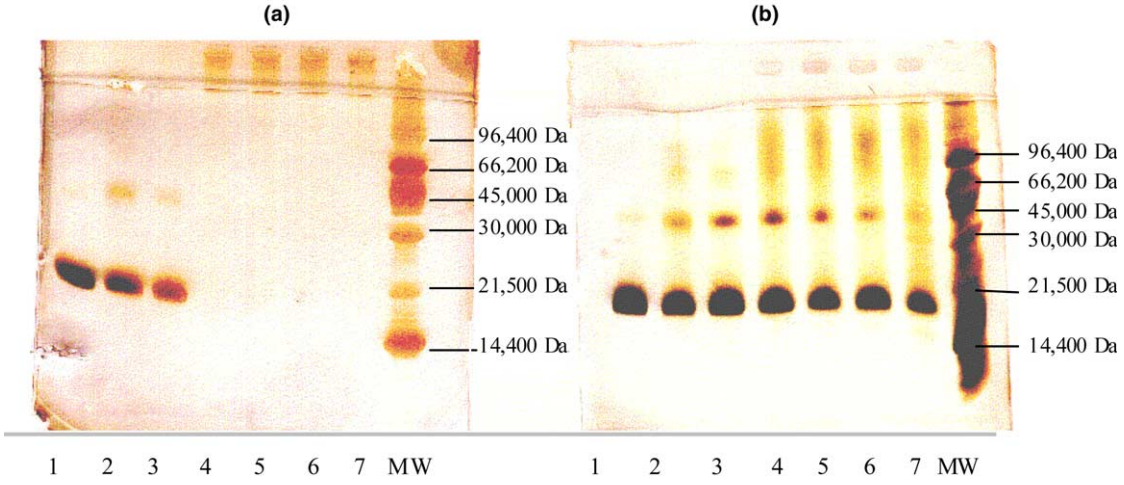

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns in the absence of β-mercaptoethanol (a) and in the presence of β-mercaptoethanol (b) of native β-lg (Lane 1); β-lg dry-heated at 60 °C, 0.44 a_w, 10 days (Lane 2); β-lg dry-heated at 55 °C, 0.65 a_w, 10 days (Lane 3); Conjugate 1, 4 days (Lane 4); Conjugate 2, 4 days (Lane 5); Conjugate 1, 10 days (Lane 6); Conjugate 2, 10 days (Lane 7). Molecular weight standards: α-Lactalbumin, 14 kDa; Trypsin inhibitor, 21 kDa; Carbonic anhydrase, 30 kDa; Ovalbumin, 45 kDa; Bovine serum albumin, 66 kDa; Phosphorylase b, 96 kDa.

°C and 0.65 a_w (Fig. 1a) presented, in addition to the band of monomer β-lg, a band corresponding to the dimeric protein (about 36 kDa) that could not be reduced by β-mercaptoethanol (Fig. 1b). This was already found in heated β-lg by other authors (Chevalier, Chobert, Dalgalarrondo, & Haertlé, 2001a; Dickinson & Galazka, 1991) and can be attributed either to an incomplete reduction of the disulfide bond (Morgan et al., 1999) or to a portion of dimer β-lg being maintained by non-reductible forces.

Evidence for the polymerization of β-lg due to the dry heat treatment with dextran was obtained using SDS–PAGE under non-reducing conditions. After 4 and 10 days of dry heating of β-lg in the presence of dextran, the patterns of purified conjugates 1 and 2 obtained without β-mercaptoethanol did not show bands corresponding to native and dimer β-lg. In the gel there were bands retained in the stacking gel and in the boundary between the stacking and resolving gel, that indicated covalent aggregation of β-lg in the presence of dextran to form high molecular weight complexes. Unlike that which was previously observed by SE-HPLC (Jiménez-Castaño et al., 2005), no qualitative differences in the aggregation behaviour were observed by SDS–PAGE analysis among conjugates 1 and 2 at 4 and 10 days of dry-heating.

Such high molecular weight aggregates were partially reduced with β-mercaptoethanol. The SDS–PAGE patterns of conjugates 1 and 2 obtained under reducing conditions showed bands corresponding to native and dimer β-lg as well as to ill-resolved broad bands of high molecular weight that could correspond to heterogeneous glycoforms of β-lg and dextran. A smaller amount of glycosylated β-lg was still retained in the stacking gel. This showed that dry-heating with dextran enhanced polymerization of β-lg molecules alone, mainly by disulfide exchange in addition to conjugation with the polysaccharide. This observation contrasts with previous information regarding glycosylation with monosaccharides, which indicated that when β-lg is heated in the presence of glucose, polymerization occurs essentially due to non-S-S bonds (Chevalier et al., 2001a).

The fluorescence emission spectra of native, dry-heated and glycosylated β-lg were examined (Fig. 2). When excited at 280 nm, native β-lg exhibited a fluorescence emission maximum (λ_{max}) at 328 nm. The polarity of the environment surrounding Trp residues affects the λ_{max}. Native β-lg and β-lg glycosylated with dextran exhibited the same λ_{max}, whereas in β-lg dry-heated without dextran, a slight red shift of the maximum emission was observed, probably due to conformational changes around the Trp residues (Trp^{19} and Trp^{61}) (Hattori, Ametani, Katakura, Shimizu, & Kaminogawa, 1993; Kaminogawa et al., 1989). The fluorescence intensity increased in β-lg dry heated in the absence of dextran at 60 °C and 0.44 a_w and at 55 °C and 0.65 a_w, which might indicate structural changes due to heat denaturation. Heating of β-lg above its denaturation temperature has been reported to increase the fluorescence intensity and this was associated with protein aggregation (Renard, Lefebvre, Griffin, & Griffin, 1998). We had already found that the SE-HPLC elution profiles of β-lg dry-heated without dextran under these conditions provided evidence for the progressive appearance of β-lg forms with an apparent molecular mass larger than that of the dimer (Jiménez-Castaño et al., 2005).

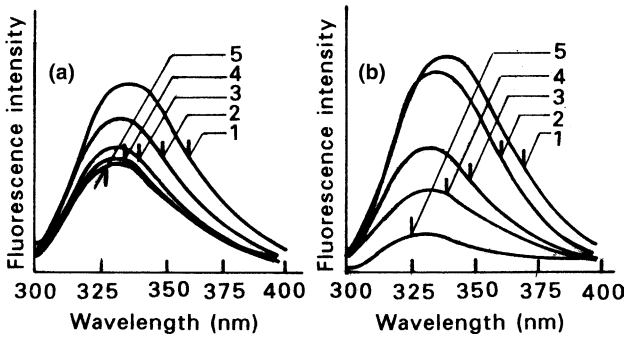

Fig. 2. Fluorescence emission spectra of β-lg dry-heated at 60 °C and 0.44 a_w (a) and at 55 °C and 0.65 a_w (b). (1) β-lg 10 days, (2) β-lg 4 days, (3) native β-lg, (4) β-lg–dextran 4 days, (5) β-lg–dextran 10 days.

In the case of the conjugates, the fluorescence intensity of conjugate 1 was slightly lower than that of native β-lg. Hattori, Nagasawa, and Ametani (1994) Hattori, Numamoto, Kobayashi, and Takahashi (2000) also found that the fluorescence intensity of β-lg–carboxymethyl dextran and β-lg–carboxymethyl cyclodextrin conjugates was lower than that of the native protein and this was attributed to a shielding effect of the polysaccharide chain bound to β-lg. Fluorescence intensity was the lowest for conjugate 2 and it decreased with the time of dry heating. Considering the blocking of Lys residues calculated from the furosine values, similar glycosylation levels that corresponded to, approximately, 0.5 blocked Lys residues per β-lg molecule were obtained after 4 and 10 days of dry-heating during formation of conjugates 1 and 2, respectively (Jiménez-Castaño et al., 2005). This suggests that the differences found in Trp fluorescence between conjugates 1 and 2 at different times of dry heating cannot be attributed to differences in the degree of glycosylation but rather to other changes in protein conformation arising from polymerization and progress of the Maillard reaction. Such changes, as previously reported, were more pronounced during the formation of conjugate 2 (Jiménez-Castaño et al., 2005). Moreaux and Birlouez-Aragon (1997) reported that intense advanced Maillard reactions in milk reduce relative fluorescence due to Trp degradation in the protein.

3.2. Solubility and thermal stability of β-lg–dextran conjugates

The solubility and thermal stability of β-lg–dextran conjugates obtained under the conditions leading to less structural modification of the protein (60 °C, 0.44 a_w, 2:1 WR, 4 days, conjugate 1) were studied. Solubility of native, dry-heated and β-lg glycosylated for 4 days, measured as a function of pH, is shown in Fig. 3. According to Nacka et al. (1998), native β-lg (1 mg/ml) was soluble in the pH range 3–8, with a decrease (minimum 80% solubility) in the region of its isoelectric point (p*I*). However, our results resemble those of Chevalier et al. (2001a, 2001b) who reported that native β-lg (2 mg/ml) was soluble over the whole pH range 2–10. After heating β-lg for 4 days at 60 °C and 0.44 a_w a 30–40% decrease in solubility was observed in the pH range 4.5–5.5, with a minimum at 5.0 (near the isoelectric point, pI, of the protein).

In general terms, β-lg modified with dextran exhibited higher solubility than the dry-heated β-lg over the whole pH range studied. Therefore, a protective effect of glycosylation towards the decrease of solubility due to dry-heating was observed. As compared with the native protein, the solubility of the conjugate decreased at pH 4. This could be due to a pI shift of the modified protein, as it is known that glycosylation of β-lg lowers its pI, possibly due to a reduction in the number of positive charges and an increase in the net negative charge. This causes the minimum solubility to shift to lower pH and explains why conjugate 1 showed an increased solubility at pH 5.0 as compared with dry-heated β-lg (Chevalier, Chobert, Dalgalarrondo, Choiset, & Haertlé, 2002).

Results on the effect of glycosylation on the heat stability of β-lg at pH 7 and 5 are shown in Fig. 4. Native β-lg was almost 100% soluble after being heated up to 90 °C at pH 7.0, but its solubility began to decrease steadily above 85 °C at pH 5.0. Dry-heated β-lg was less soluble than the native protein after heating at both pHs, but particularly at pH 5.0. At pH 7.0 the heat-stability of glycosylated β-lg followed a similar trend to dry-heated β-lg over the range of temperatures studied, decreasing approximately by 30% after heating at 95 °C. However, at pH 5.0 the glycosylated protein presented a higher thermal stability than native β-lg above 85 °C and was far more stable at all temperatures than β-lg heated without the polysaccharide. Chevalier et al. (2001a) found that modification of β-lg with different monosaccharides and lactose improved its thermal properties at acidic pH.

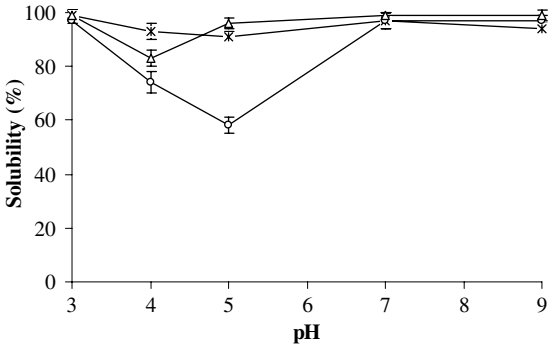

Fig. 3. Solubility of native β-lg (*), β-lg dry-heated at 60 °C, 0.44 a_w, for 4 days (○) and β-lg glycosylated with dextran at 60 °C, 0.44 a_w, 2:1 WR and 4 days (△) as a function of pH. Results are means of two independent experiments ± standard deviation.

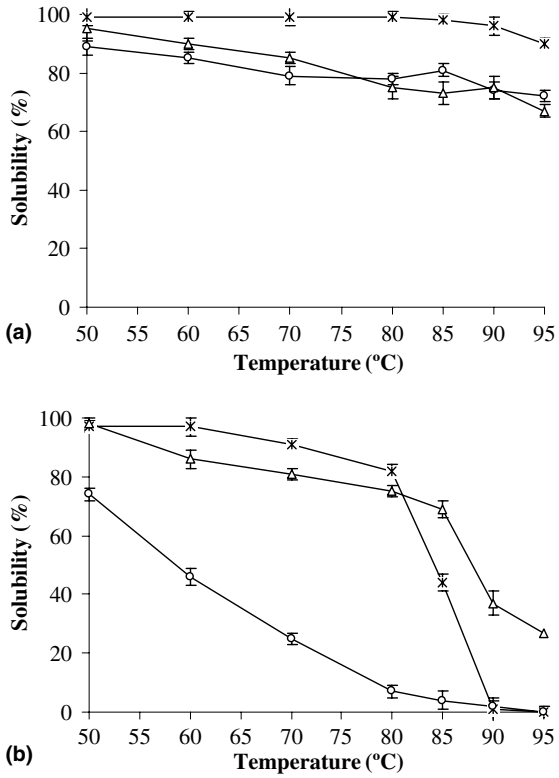

Fig. 4. Heat stability at pH 7 (a) and at pH 5 (b) of native β-lg (*), β-lg dry-heated at 60 °C, 0.44 a_w, 4 days (○) and β-lg glycosylated with dextran at 60 °C, 0.44 a_w, 2:1 WR and 4 days (△). Results are means of two independent experiments ± standard deviation.

4. Conclusions

Glycosylation of β-lg with a high molecular weight dextran under different conditions led to the formation of high molecular weight complexes and induced polymerization of the protein by disulfide bonds. Conjugate 1, formed between β-lactoglobulin and dextran at 60 °C, 0.44 a_w and 2:1 WR, underwent less conformational changes than conjugate 2, formed at 55 °C, 0.65 a_w and 6:1 WR, as judged by fluorescence intensity measurements. As compared to the native protein, the solubility of conjugate 1 decreased at pH 4.0, but it

improved, as compared to dry-heated β-lg alone particularly around the isoelectric point of the protein. Conjugate 1 presented lower stability to heating at pH 7.0 than native β-lg but its thermal stability was higher at pH 5.0 at temperatures above 85 °C.

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