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# Interaction properties of β-lactoglobulin and benzaldehyde and effect on foaming properties of β-lactoglobulin

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**Abstract**

Quenching of tryptophan fluorescence intensity and absence of covalently bound fluorescent products in β-lactoglobulin solutions containing benzaldehyde at various molar ratios, indicated that β-lactoglobulin monomer and benzaldehyde might associate through a non covalent binding mechanism. The affinity constant determined from perturbation of tryptophan spectrofluorescence spectra was close to that previously observed by other authors for retinol binding to β-lactoglobulin. In parallel foaming properties of solutions of β-lactoglobulin alone or in mixture with benzaldehyde were investigated through a conductimetric method. It was observed that addition of benzaldehyde to β-lactoglobulin led to enhanced foaming properties in comparison with β-lactoglobulin alone. These results were discussed in terms of formation of non covalently bound complexes with a specific surface activy. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* β-Lactoglobulin; Benzaldehyde; Heat denaturation; Ligand binding; Foams; Aroma compound

## 1. Introduction

Since many years, β-lg the major bovine whey protein has been studied extensively (Futterman & Heller, 1972; Hegg, 1980; Jones & Wilkinson, 1976; Robillard & Wishnia, 1972; Wishnia & Pinder, 1966) for its interaction properties with a large variety of small hydrophobic ligands, and more recently tryptophan spectrofluorimetry has been used to determine the constant affinity and the total number of binding sites (Dufour & Haertlé, 1990a, b, 1991; Frapin, Dufour & Haertlé, 1993; Fugate & Song, 1980; Laligant, Dumay, Casas Valencia, Cuq & Cheftel, 1991; Wang, Allen & Swaisgood, 1997). From its core-structural pattern, as revealed by X-ray crystallography (Monaco, Zanotti, Spadon, Bolognesi, Sawyer & Eliopoulos, 1987; Papiz et al., 1986), it has been suggested that hydrophobic ligands may bind inside the central calix, and from recent studies (Brownlow et al., 1997; Qin, Creamer, Baker & Jameson, 1998; Wu, Perez, Puyol & Sawyer, 1999), there is evidence that it binds fatty acids inside the central calyx, as for retinol. Aliphatic aldehydes have been shown to react with protein amino groups, causing formation of covalently bound fluorescent products (Stapelfeldt & Skibsted, 1994). However, Wang et al. (1997) have observed that binding properties of β-lactoglobulin (prepared in non denaturing conditions) to all-trans-retinal are very similar to those obtained with retinol and retinoids, and Andriot, Marin, Ferron, Relkin and Guichard (1999) have recently observed by high performance liquid chromatography with a radioametric detection that labelled benzaldehyde binds strongly to β-lg, but no covalent linkages have been detected either by Raman or infra-red spectroscopy (E. Guichard, personal communication) or by electro-spray mass-spectrometry Relkin, Molle & Marin, 2000).

On the other hand interfacial properties of globular proteins are known to be influenced by their conformational state in solution (Dickinson & Matsumura, 1994; Graham & Phillips, 1976; Zhu & Damodaran, 1994) and it has been shown that β-lactoglobulin may compete for interfaces with model lipids or surfactants (Atkinson, Dickinson, Horne & Richardson, 1995; Horne, Atkinson, Dickinson, Pinfield & Richardson, 1998; Magdassi,

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Vinetsky & Relkin, 1996; Miller, Fainerman, Makieski, Grigoriev, Wilde & Krägel, 1999; Wilde & Clark 1996) and that ethanol has effect on foaming properties of β-lg solutions (Ahmed & Dickinson, 1991).

In the field of protein-volatile compounds interactions and their effects on protein foaming properties which is important in food technology, we have studied as model systems mixtures of β-lactoglobulin (β-lg) and aroma compounds such as isoamyl acetate, benzaldehyde and 2-5 dimethyl pyrazine (Marin, 1998). In this manuscript results obtained with a β-lg concentrate prepared by ultra-diafiltration and benzaldehyde (BZA) are presented by paying attention to possible formation of Maillard reaction products.

## 2. Materials and methods

### 2.1. Protein solutions

The β-lactoglobulin (β-lg), variants A and B, was obtained by micro-diafiltration of the soluble phase of skimmed milk. It was kindly supplied by Besnier Bridel (Retiers, France). The whey protein concentrate contained 93.5% protein of which more than 95% β-lg, less than 5% α-lactalbumin, approx 2% lactose, 0.9% salt and traces of fat (J.J. Maugas, personal communication). It was dispersed in Milli-Q water and dialysed against distilled water 50 mM NaCl during 24 h at 4°C. After centrifugation (15,000 *g* for 15 min), the ionic strength was adjusted by addition of NaCl (50 mM) and pH was adjusted to pH 6 (2 M NaOH). The protein concentration was determined either by the biuret method with bovin serum albumin as a standard, or by spectrophotometry (Cary 100, USA) at 280 nm ($OD_{1\%} = 9.6$). The purity of the protein sample and quantification of β-lg in monomeric form was checked by non reducing SDS-PAGE stained with Coomassie blue. We used a Phastsystem apparatus (Pharmacia, France) following the instructions of the manufacturer. Quantification of individual peaks of the electrophoregraph pattern of the protein solution was made by the use of an image-treatment software (NIH image 1.61). This analysis indicated that β-lg molecules are mainly in the monomeric form (Marin, 1998). The lactose content in the supernatant which was obtained after dialysis and centrifugation was determined (less than 0.5 wt.%) by using the Boerhinger lactose/D-galactose kit. Benzaldehyde (BZA), kindly supplied by International Flavour and Fragrance (Longvic, France), was 96% pure and its solubility in water was equal to 2.6 g $l^{-1}$ (Espinosa-Diaz, Seuvre & Voilley, 1998). It was dissolved in 50 mM NaCl at pH 6 and added to the corresponding protein solutions to achieve mixtures at different molar ratios, using 18,400 Da and 106 Da for the monomer molecular weight of β-lg and BZA, respectively.

### 2.2. Differential scanning calorimetry (DSC) measurements

Heat-induced conformational changes of β-lg (~1 wt.% concentration) alone or in mixture with BZA were monitored by μ-DSC (Setaram-France) at 1.2°C $min^{-1}$ scan rate. $T_p$ is defined as the peak temperature, $\Delta H_{app}$ as the apparent enthalpy change involved in the overall heat-induced reactions within the protein molecules. $\Delta H_{app}$ was determined from the area between the curve and a straight line drawn from the beginning to the end of the transition. The experimental results were the average of three independent thermogramms obtained after substraction of the thermogramm obtained in a the second heating run.

### 2.3. Spectrofluorimetric measurements

We used a spectrofluorimeter Aminco-Bowman SLM series 2 in the ratio mode with both excitation and emission slit-widths equal to 4 nm. The fluorescence spectra of unheated of pre-heated β-lg solutions in the absence or presence of benzaldehyde were measured at 20°C in a thermostatted quatz cells (1 × 1 cm path). The following procedure was used : 1 ml of β-lg from the stock solution (6.5% w/w) and 150 μl of aqueous solution of BZA at various concentrations were mixed in order to get BZA/β-lg molar ratios ranging between 0 and 2. After dilution to 10 μM β-lg, such mixtures were used for spectrofluorimetric measurements.

Intrinsic tryptophan fluorescence emission spectra of β-lg (290 and 330 nm for the wavelength of excitation and emission, respectively) were recorded between 300 and 400 nm. To eliminate the possibility of non-specific interactions with tryptophan indole groups in β-lg (Cogan, Koppelmann, Mokady & Shinitsky, 1976), titration of *N*-acetyl-L-tryptophanamide by benzaldehyde were made but any fluorescence quenching was observed. To check if β-lg amino groups react with the carbonyl group of benzaldehyde we used the spectrofluorimetric procedure applied previously by Stapelfeldt and Skibsted (1994) to study the interaction properties of β-lg to aliphatic aldehydes. That procedure is based on the hypothesis that reaction between protein lysine groups and aldehyde, causes formation of new fluorescent products at 340–370 nm excitation maximum wavelength and 400–450 nm emission maximum wavelength. In our experimental conditions (pH 6, absence of alcohol, low protein concentration, benzaldehyde at concentration lower than its limit of solubity in water), even after a heat-treatment at 37°C for two-weeks we did not observed a fluorescent signal. However, when a mixture of β-lg solution (0.06 wt.%) in phosphate buffer (pH 6.8) and excess of benzaldehyde (1 mol $l^{-1}$) dissolved in 1-octonol, as previously performed by Stapelfeldt and Skibsted, was heat-treated at 85°C overnight, a fluorescent

signal with maximum intensity at 420 nm (excitation maximum at 370 nm) was observed (Fig. 1). From this spectrofluorimetric analysis and within the hypothesis that base Schiff reaction is favoured at neutal pH, low water activity and is enhanced by temperature and time of heat-treatment (Fox, Loncin & Weiss, 1983), we concluded that our physicochemical conditions (absence of 1-octanol and concentration of benzaldehyde lower than 5 mM) are not favourable for the formation of an enamine by reaction between protein amine groups and benzaldehyde.

Therefore, the binding properties of benzaldehyde to β-lg were determined through tryptophan fluorescence quenching characteristics and the apparent dissociation constant ($K_d$) and number of benzaldehyde bound to one β-lg monomer were determined according to the method of Cogan et al., 1976, by the use of the following equation:

$$P_0 a = -K_d/n + [\mathrm{BZA}]a/n(1-a) \quad (1)$$

where $P_0$ is the total protein concentration (mol $l^{-1}$), [BZA] is the total concentration of benzaldehyde (mol $l^{-1}$), $a$ is the fraction of free binding sites on the protein molecule, and $K_d$ ($= 1/K_a$) is the apparent dissociation constant. The fraction of the free binding sites is deduced from the relative variation of the maximum fluorescence intensity, following the relationship:

$$a = (F - F_{min})/F_0 - F_{min} \quad (2)$$

where $F_0$ is the fluorescence intensity observed in the absence of BZA, $F$ is the fluorescence intensity observed in the presence of BZA at a concentration [BZA] and $F_{min}$ its plateau value.

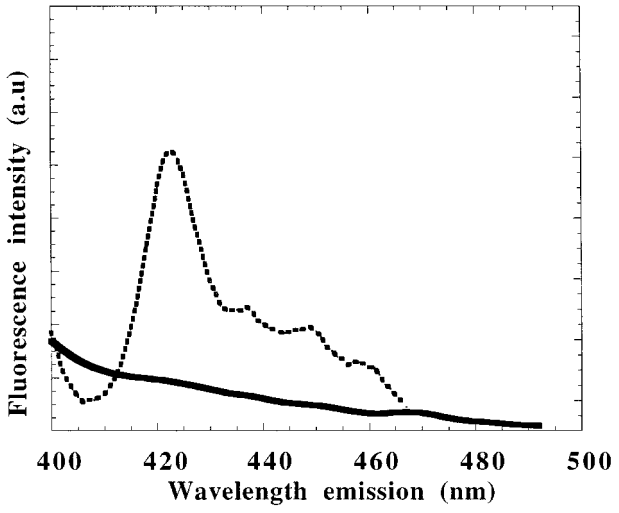


Fig. 1. Spectrofluorimetric spectra of BZA/β-lg mixtures in water and in 1-octanol (350 nm excitation wavelength). Bold curve: BZA/β-lg mixture in distilled water after heat-treatment at 76°C for 30 min. Dotted line: BZA/β-lg mixture in 1-octanol after heat-treatment at 85°C overnight.

### 2.4. Foaming properties

Foaming properties of pure protein solutions were compared to those of protein mixtures with BZA at molar ratios, $R = [\mathrm{BZA}]/[\beta\text{-lg}]$ equal to 1 or 2. Foaming properties were characterised through their foam formation and stability. We used a foam analyser (PM 930, Grosseron, Saint Herblain-France) based on conductimetric measurements of the liquid under the foam (Loisel, Popineau & Guéguen, 1993). The foam was generated in a cylinder glass colonne by sparging air (15 ml $min^{-1}$) into an initial volume (12 ml) of protein solutions (0.5% in 50 mM NaCl). The foaming ability was evaluated by the sparging time needed to reach a certain total of foam volume (50 ml) that was detected by an on-line camera, while the volume of liquid under the foam was measured by conductivity for 20 min, including the step of foam formation and liquid drainage after air sparging. Conductivity measurements of the liquid, between two large electrodes which are located at the bottom of the cylinder glass colonne, were made as a function of time ($C_t$) and with reference to the conductivity of the buffered test solution. The volume of liquid entrained in the foam ($V_L$) was calculated from the change in conductivity of the liquid at the base of the foam, according to Loisel et al. (1993):

$$V_L = V_{init}[1 - (C_t/C_{init})], \quad (3)$$

where $V_{init}$ is the volume and $C_{init}$ is the conductivity of sample solution introduced into the apparatus. The volume of liquid entrained in the foam ($V_L$) was then used as an indication of foamability (foam forming ability) and long-term foam stability. The foam density was calculated as the volume of liquid in the foam ($V_L$) at the specified time divided by the volume of air incorporated in constant volume of foam (50 ml), and foamability was defined as the initial foam density, immediately after sparging ($D_0$). Long-term stability of the foam was evaluated by the half drainage time, $t_{1/2}$, which was defined as the time need to drain half the liquid volume [1/2 ($V_{max} + V_{min}$)] retained in the foam after air sparging. $V_{max}$ is the volume of liquid incorporated during the air sparging and $V_{min}$ is the volume which remained in the foam at the end of the experiment.

## 3. Results

### 3.1. Heat-induced conformational changes of β-lactoglobulin

The heat-induced conformational changes of β-lg alone or in mixture with BZA were investigated by high-sensitive DSC. Examples of thermograms are shown in Fig. 2. Comparison with pure protein solution

indicated an increase in $T_p$ ($\Delta T_p \sim 1.4°C$) but no change in $\Delta H_{app}$ (19 J $g^{-1}$), when BZA was added at molar ratio equal to 1. However, for BZA/β-lg molar ratio equal to 2, $T_p$ value (78. 2°C) was equal to that of pure β-lg solution but $\Delta H_{app}$ was lower (14 J $g^{-1}$). Addition of surfactants has been shown to cause a large increase in $T_p$ and a large decrease in $\Delta H_{app}$ (Hegg, 1980; Magdassi et al., 1996; Waninge, Paulsson, Nylander, Ninham & Selers, 1998), but addition of isoamyl acetate, a small molecular weight hydophobic molecule did not lead to significant changes in both $T_p$ and $\Delta H_{app}$ (Marin & Relkin, 1999). The results obtained in the present study indicated that when BZA is added to β-lg solutions, there is a slight increase in $T_p$ for $R = 1$. This could be explained by a specific effect of BZA binding to β-lg monomers, leading to stabilisation of the folded state of β-lg (Privalov & Potekhin, 1986; Relkin, 1996).

### 3.2. Spectrofluorimetric properties of benzaldehyde and β-lactoglobulin mixtures

Examples of fluorescence spectra obtained with BZA/β-lg mixtures are shown in Fig. 3a. The corresponding relative variation in maximum fluorescence intensity, obtained after subtraction of benzaldehyde spectra and correction from the inner filter effect (Lakowicz, 1986), indicated a decrease toward a plateau value for $R \sim 1$, similar to previous results reported by other authors (Dufour & Haertlé, 1990a, b, 1991; Frapin et al., 1993; Fugate & Song, 1980; Laligant et al., 1991; Wang et al., 1997) who have used a similar methodology. Interaction of β-lg molecule and aldehyde compounds might give rise to Schiff base formation between protein exposed Lys groups and benzaldehyde. Such a reaction which is favoured by a low water activity and basic pH could be reflected too by fluorescence quenching of Trp fluorescence (Stapelfeldt & Skibsted, 1994). However, in light of our spectrofluorimetric experiments in the 400–500 nm emission wavelength range and 350 nm excitation wavelength (see Fig. 1) and in agreement with the results of Wang et al., for binding properties of all-trans-retinal to native β-lg, we assume that the observed fluorescence quenching in our experimental conditions might be caused by radiationless energy transfer upon a close approach of benzaldehyde to one of the two tryptophanyl groups of β-lg, by analogy to the retinol binding mechanism (Fugate & Song). Analysis of the experimental data, according to the Eq. (1), gave a linear plot (Fig. 3b) from which we deduced the dissociation constant ($K_d = 1.6\ 10^{-7}$ M) and the number of binding sites per monomer ($n \sim 1$). These affinity parameters of BZA for β-lg are very close to those observed by spectrofluorimetric measurements for a large kind of hydrophobic ligands including fatty acids (Frapin et al.), retinol and retinoids (Laligant et al.; Wang et al.); β-ionone and protorporphyrin (Dufour et al., 1990a, b). However, they are very much lower than those obtained by other methodologies such as equilibrium dialysis (Puyol, Perez, Ena & Calvo, 1991) or chromatography

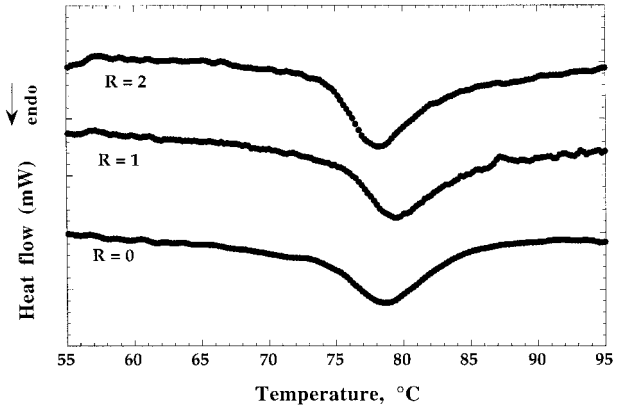


Fig. 2. Examples of thermograms obtained with pure β-lactoglobulin solution ($R = 0$) and mixtures of benzaldehyde and β-lactoglobulin at BZA/β-lg molar ratios $R = 1$ and $R = 2$.

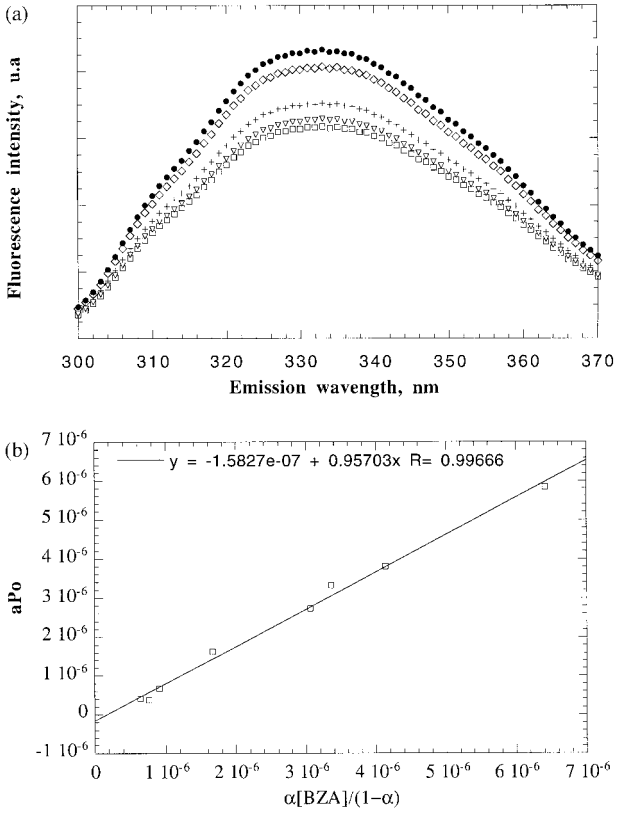


Fig. 3. Tryptophan spectrofluorimetric properties of β-lactoglobulin solutions in the absence of benzaldehyde and in the presence of benzaldehyde at various BZA/β-lg molar ratios, $R$ (10 μM protein concentration, 290 and 333 nm emission excitation wavelengths). (a) Examples of tryptophan spectrofluorimetric spectra obtained with protein solutions are $R = 0$; 0.2; 0.6; 1 and 2 (from top to bottom). (b) Cogan representation of tryptophan spectrofluorescence intensity obtained at various molar ratios.

(Pelletier, Sostmann & Guichard, 1998). Further investigations are needed to clarify this difference.

### 3.3. Foaming properties

The formation of β-lg-based foams and their stability against liquid drainage were monitored by conductimetric measurements during 20 min, including the first step of air sparging (until the foam volume in the collonne reached 50 ml) and the step of foam destabilisation. In those experimental conditions, $D_0$ the initial foam density increased by approx 15 and 35% for the mixture of β-lg and BZA at the molar ratios $R=1$ and $R=2$, in comparison with pure protein solution (Fig. 4a). As foam density in bubbling tests is inversely correlated to foam expansion in whipping tests (Loisel et al., 1993), our results indicated that the presence of the aroma compound enhanced the foamability of β-lg. In parallel, Fig. 4b shows that the addition of BZA at $R=1$ and $R=2$ caused an increase in the index of foam stability against liquid drainage, $t_{1/2}$ (see Section 2), by approx 28%. In a similar recent study performed on mixtures of β-lg and isoamyl acetate we have shown that addition of isoamyl acetate caused increase in $D_0$ by ~36% ($R=1$) and 60% ($R=2$) and in $t_{1/2}$ by ~74% ($R=1$ and $R=2$), while the spectrofluorimetric titration experiments have shown that the fluorescence quenching plateau was observed at $R \geqslant 2$ (Marin & Relkin, 1999). Therefore, it seems that isoamyl acetate which binds specifically to β-lg with a higher affinity constant ($K_d = 1.4\ 10^{-8}$ M) enhanced more the foaming properties of β-lg than did BZA ($K = 1.6\ 10^{-7}$ M).

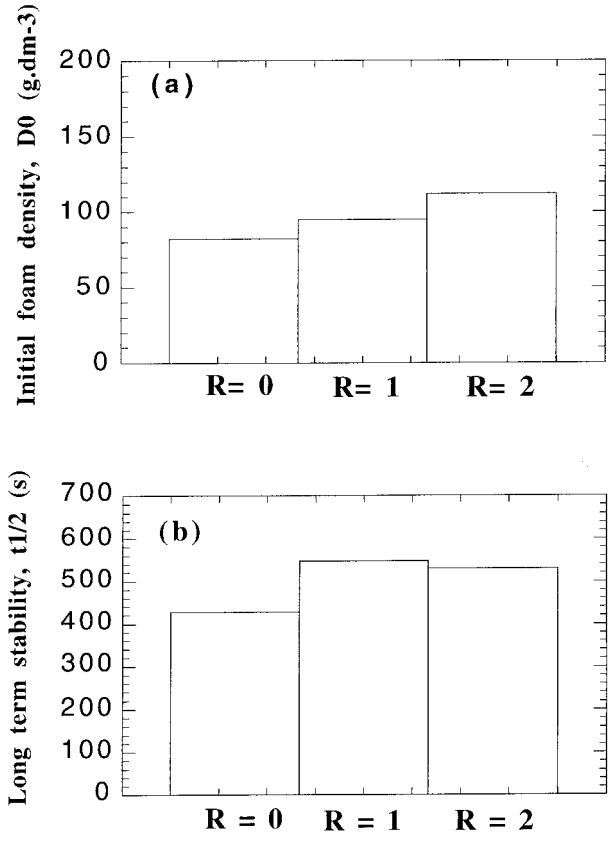


Fig. 4. Foam properties of β-lactoglobulin solutions (0.5 wt.%) in the absence ($R=0$) and in the presence of benzaldehyde at BZA/β-lg molar ratios equal to $R=1$ and $R=2$ (see text for methodology). (a) Variation of initial foam density, $D_0$. (b) Variation of long-term stability, $t_{1/2}$ against liquid drainage.

## 4. Conclusion

The results obtained in the present study, indicated that BZA and β-lg can form non covalent bound complexes with a specific surface activity that could explain the enhancement of foaming properties of BZA/β-lg mixtures, similarly to isoamyl acetate/β-lg ones.

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