Non-covalent binding of benzaldehyde to β-lactoglobulin: characterisation by spectrofluorimetry and electrospray ionisation mass-spectrometry

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β-Lactoglobulin (β-lg), which is found in the milk of several mammal species, is the most abundant protein in bovine whey (McKenzie, 1971). The interaction properties of β-lg with a large variety of small hydrophobic ligands have been extensively studied (Sawyer *et al.* 1998). β-Lg belongs to the super-family of hydrophobic molecule transporters called the lipocalins, which characteristically bind hydrophobic ligands inside a central calyx (Godovac-Zimmermann, 1988; Perez *et al.* 1989; Brownlow *et al.* 1997; Wu *et al.* 1997; Qin *et al.* 1998).

At present, whey protein concentrates containing a high percentage of β-lg are commercially available in a very large quantity, but most of the processes used in milk technology involve heat-treatments that are known to affect the initial conformational state of β-lg. Such conformational changes have consequences for both the physicochemical and functional properties in food systems, including a decrease in the availability of lysine, due to Maillard reactions (Léonil *et al.* 1997) and a decrease in the affinity constant for binding to retinol (Laligant *et al.* 1991) and to flavour compounds (O'Neil & Kinsella, 1988). In the field of protein-volatile compound interactions, we have recently reported a significant enhancement of foaming properties of β-lg solutions (50 mM-NaCl, pH 6) in the presence of aroma compounds such as isoamyl acetate (Marin & Relkin, 1999), benzaldehyde (BZA; Marin & Relkin, 2000) and vanillin (Relkin & Vermersh, 2000). The observed increase in foaming properties of β-lg was postulated to be due to formation of surface active complexes between β-lg and aroma compounds.

In the present work we have investigated the interaction between a β-lg concentrate (prepared by ultra-diafiltration on an industrial scale) and BZA; particularly, evidence was sought for the presence of covalently bound monomers, dimers and lactolated monomers.

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materials and methods

Preparation of protein and benzaldehyde solutions

β-Lg variants A and B were obtained by micro-diafiltration of the soluble phase of skimmed milk. It was kindly supplied by Besnier Bridel (F-35240 Retiers, France). The whey protein concentrate contained 93·5 g protein/100 g (of which more than 95 % was β-lg and less than 5 % was α-lactalbumin); approximately 2 g lactose, 0·9 g salt/100 g and traces of fat were also present (J. J. Maugas, personal communication). It was dispersed in Milli-Q water and extensively dialysed against water for 24 h at 4 °C to remove free residual salts and lactose. After centrifugation (15000 ***g*** for 15 min), the concentration of residual lactose was less than 0·5 % (dry matter basis), as determined by the use of the Boehringer kit (cat. no. 176 303, Mannheim, Germany). The protein solution was adjusted to pH 6 (2 M-NaOH). Protein concentration was determined by either the Biuret method with bovine serum albumin as a standard or spectrophotometrically at 280 nm using $OD_{1\%} = 9{\cdot}6$.

BZA, which was kindly supplied by International Flavour and Fragrance (Longvic, 21 600-France), was 96 % pure. It was dissolved in Milli-Q water at pH 6 and added to the corresponding protein solutions to achieve mixtures at different molar ratios, using 18400 and 106 as the monomer molecular masses of β-lg and BZA, respectively.

Characterization of the β-lactoglobulin sample

The protein composition of the β-lg sample and the presence of covalently bound monomers were checked by SDS-PAGE (Phastsystem; Pharmacia F-91898 Orsay, France) in reducing and non-reducing conditions, respectively. The electrophoretographs were stained with Coomassie blue (Marin, 1999) and scanned (SNAPSCAN 12–12 AGFA). Individual protein peaks were quantified using image-treatment software (NIH image 1.61).

Aldehyde can react with primary amines via condensation reactions to form Schiff's bases accompanied by an increase in mass of β-lg. Formation of irreversible covalently bound molecular species between BZA and β-lg was monitored by the use of on-line combined liquid chromatography–mass spectrometry, as described previously (Mollé *et al.* 1998).

Spectrofluorimetric measurements

Spectrofluorimetric measurements were performed by using an Aminco-Bowman SLM series 2 spectrofluorimeter, as described elsewhere (Marin & Relkin, 2000). When a ligand binds non-covalently to a protein, the interaction parameters may be derived from Scatchard analysis (Cantor & Schimmel, 1979) of modification to the intrinsic tryptophan fluorescence signals, as a function of ligand/protein molar ratio. The fraction of the binding sites (ν) was deduced from the relative variation of the maximum fluorescence intensity, following the relationship:

$$\nu = \frac{F_0 - F}{F_0 - F_{sat}}, \tag{1}$$

where F_0 is the fluorescence intensity observed in the absence of BZA, F is the fluorescence intensity observed in the presence of BZA, and F_{sat} the plateau value corresponding to saturation of the β-lg binding sites. L_{free}, the concentration of free BZA was calculated from the relation:

$$L_{free} = L_0 - \nu P_0, \tag{2}$$

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where L_0 and P_0 are the total concentration of BZA and β-lg, respectively. In the most simple case, where β-lg binds ligand at equivalent binding sites, K_d the dissociation constant, may be derived from the double reciprocal plot (Cantor & Schimmel, 1979):

$$\frac{1}{\nu} = \frac{1}{n} + \frac{K_d}{(nL_{free})}, \tag{3}$$

where n is the total number of protein binding sites.

RESULTS

Tryptophan fluorescence spectra (280 nm excitation wavelength) observed for pure β-lg solution and mixtures of β-lg and BZA at various molar ratios (R) indicated a fluorescence quenching towards a plateau value for R $\sim$ 1, without a change in the wavelength of maximum fluorescence intensity, in comparison with pure protein solutions. The fluorescence isotherm corresponding to the relative decrease in fluorescence intensity, $(F_0 - F)/F_0$, as a function of R is shown in Fig. 1(*a*). This isotherm presents a break in its trend which can be located at R $\sim$ 1. For analysis of the tryptophan spectrofluorimetric data (eqns 1–3), the fluorescence plateau value (F_{sat}) was considered to be equal to the lowest value observed, within experimental uncertainties. Application of that procedure gave a linear double reciprocal plot (Fig. 1*b*), from which the interaction parameters are $K_d = 0{\cdot}6\ \mu$M and $n \sim 1$ ($r = 0{\cdot}96$). Fitting the experimental points with $n = 1$, led to $K_d = 0{\cdot}63 \pm 0{\cdot}05\ \mu$M.

The SDS-PAGE patterns (not shown) obtained in reducing conditions confirmed the presence of more than 95% β-lg and less than 5% α-lactalbumin. Under non-reducing conditions, patterns indicated the presence of approximately 10% disulphide-bound β-lg dimers and 90% monomers. The chromatograms obtained with solutions of β-lg mixed with BZA (R = 1, 2 or 3) were very similar to that obtained with β-lg alone (Léonil *et al.* 1997). Analysis by reversed-phase HPLC on-line with ESI-MS and the reconstructed spectra indicated the presence of two main peaks with relative molecular masses equal to 18279 and 18365 (corresponding to monomers of β-lg variants A and B), and two further weak peaks with relative molecular masses equal to 18604 and 18689. The difference of 324 between relative molecular masses of the main peaks and the weaker peaks indicated that the protein sample used for the present study contained a relatively high proportion of β-lg molecules (82·4% and 84·8% for the variants B and A, respectively), a small fraction of mono-lactolated β-lg (17·6% and 15·2% for the variant B and A, respectively), but the absence of irreversible covalently bound molecular species formed between β-lg and BZA.

DISCUSSION

Both SDS-PAGE and ESI-MS experiments indicated that the β-lg sample contained approximately 90% monomers, slightly more than 15% was mono-lactolated. Under the experimental conditions (absence of salts, pH 6), the protein would naturally form dimers linked by hydrophobic interactions.

Tryptophan fluorescence is an indirect method to study the interaction properties of proteins with small molecular weight molecules. The fluorescence quenching observed in mixtures of BZA and β-lg for R $\sim$ 1 and above could be due to binding of BZA at one of the three potential specific binding sites of β-lg (see Sawyer *et al.*

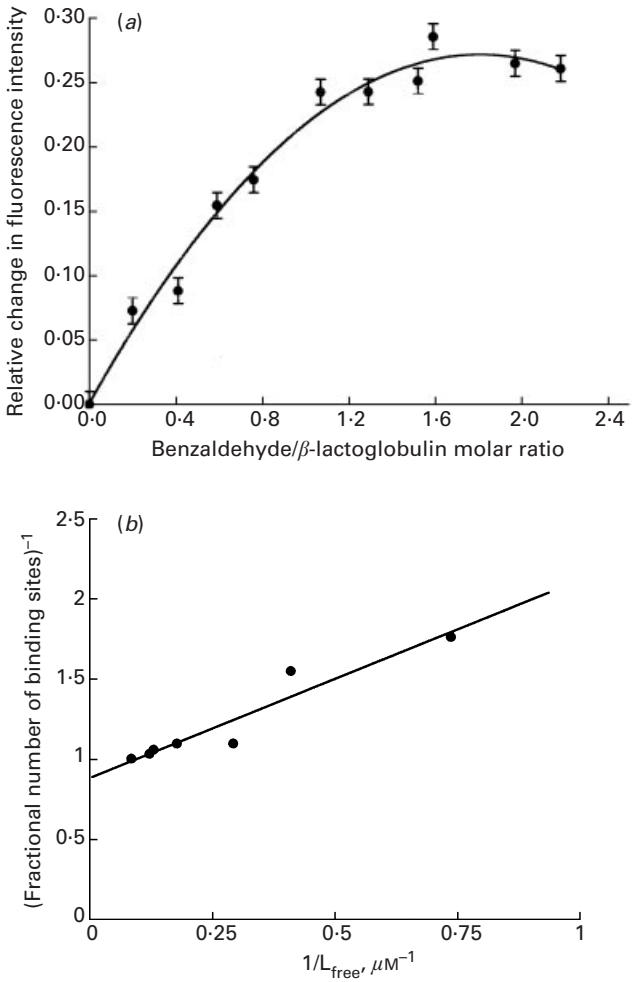

Fig. 1. (*a*) Quenching of tryptophan fluorescence obtained from mixtures at various benzaldehyde β-lactoglobulin molar ratios, R. (β-Lactoglobulin concentration 7·8 μM, pH 6; excitation and emission wavelengths 280 and 330 nm, respectively.) (*b*) Analysis of fluorescence intensity following the double reciprocal plot (see text).

1998 for a recent review). Similar observations have been reported for several other hydrophobic molecules (Fugate & Song, 1980; Dufour & Haertlé, 1990; Wang *et al.* 1997). The apparent K_d value ($0{\cdot}63 \pm 0{\cdot}05$ μM) derived from our fluorimetric data was higher than the values (0·02 to 0·08 μM) determined by Fugate & Song (1980) for retinol (at both pH 2 and pH 8) and by Wang *et al.* (1997) for all-trans-retinal (at $5 < \text{pH} < 7$), for a β-lg sample prepared in non-denaturing conditions. However, it was close to the value reported by Dufour & Haertlé (1990) for β-ionone and for palmitate and stearate (Frapin *et al.* 1993). This difference in the dissociation constant values could be due either to a difference in ligand structure and/or to perturbation in the initial tertiary structure of β-lg. Previous studies on the effect of protein conformational and chemical modifications on binding of retinol (Laligant *et al.* 1990) or alkanone flavours (O'Neil & Kinsella, 1988) indicated that protein conformational changes resulted in an increase in K_d and also in the total number of binding sites. In the present study, where β-lg was shown to contain approximately 10% covalently bound dimers and approximately 15% mono-lactolated monomers,

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we can postulate that the observed modification of tryptophan spectrofluorimetry might be explained by complex formation between β-lg monomers (or non covalently bound dimers) and BZA with a stoichiometry equal to $n = 1$ (or $n = 2$). Further experiments are needed to investigate the possibility of non-reversible modification of lysine groups by condensation reaction with BZA, and to explain the molecular interaction mechanism between BZA and β-lg.

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