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Original article

Adsorption kinetics of β-lactoglobulin at the air/solution interface: impact of pre-heating and addition of isoamyl acetate

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Abstract — We compared the kinetics of adsorption at the air/solution interface of β-lactoglobulin alone or in mixture with isoamyl acetate at a concentration where it remains water-soluble. Addition of isoamyl acetate to β-lactoglobulin, at molar ratios equal to 1 or 2, led to a decrease in the surface tension in the short-time region, but the steady-state value of surface tension was close to that of pre-heated solution of β-lactoglobulin alone, only for molar ratio equal to 2. Pre-heating mixtures at this molar ratio increased the adsorption kinetics more significantly in comparison with non-heated mixtures and pre-heated pure solutions of β-lactoglobuline. In parallel, tryptophan spectrofluorimetric spectra obtained from non-heated or pre-heated solutions indicated in both cases that addition of isoamyl acetate to β-lactoglobulin were accompanied by a quenching of fluorescence intensity, while a slight red-shift in wavelength of maximum fluorescence intensity occured only for pre-heated mixtures. The results were discussed in terms of formation of β-lg/IAA complexes with a specific activity at the air/solution interface. © Inra/Elsevier, Paris.

β-lactoglobulin / aroma compound / protein-ligand complex / surface activity / air-water interface

Résumé — Cinétiques d'adsorption de la β-lactoglobuline à l'interface air/liquide : influence du préchauffage et de l'addition d'acétate d'isoamyl. Nous avons comparé les cinétiques d'adsorption à l'interface air/liquide de la β-lactoglobuline en l'absence ou en présence d'acétate d'isoamyl, à des concentrations où le composé d'arôme est encore soluble dans l'eau. Nous avons observé que pour des rapports molaires d'acétate d'isoamyl/β-lactoglobuline égaux à 1 ou 2, les valeurs de la tension de surface aux temps courts sont inférieures à celle de β-lactoglobuline pure, mais la valeur aux temps longs n'est proche de celle de la β-lactoglobuline préchauffée qu'au rapport molaire égal

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à 2. La cinétique d'adsorption à l'interface air/liquide, de la β-lactoglobuline est nettement accélérée lorsque le mélange au rapport molaire égal à 2 a été préchauffé. Parallèlement, les spectres de fluorescence du tryptophane, obtenus à partir des différentes solutions étudiées, ont montré une diminution de l'intensité de fluorescence pour tous les mélanges β-lg/AIA, alors qu'un léger déplacement vers le rouge de la longueur d'onde d'intensité maximale n'a été observé que dans le cas des mélanges préchauffés. Les différences de cinétique d'adsorption de la β-lactoglobuline, observées dans les différentes conditions étudiées, sont discutées en termes de formation de complexe entre la β-lg et le composé d'arôme, pouvant avoir une activité de surface spécifique. © Inra/Elsevier, Paris.

β-lactoglobuline / composé d'arôme / complexe protéine-ligand / activité de surface / interface eau–air

1. INTRODUCTION

The interfacial properties of globular proteins are influenced by their conformational state in solution [5, 6,13, 14] and heat-treatment which is an important step in food processing can affect the rate and extent of protein adsorption at the air/fluid interfaces [13, 14, 30, 34]. On the other hand, there is competitivity for interfaces between lipids or surfactants and β-lactoglobulin [3, 16, 23, 38], and ethanol [1], with consequences on the film properties of β-lg solutions at the interfaces.

Many years ago [11, 12, 15, 17, 29, 31, 35, 39], β-lg the major bovine whey protein was studied extensively for its interaction properties with a large variety of small hydrophobic ligands, and more recently tryptophan spectrofluorimetry has been used to determine the affinity constant and the total number of binding sites [7, 8, 10, 19, 37]. From its core-structural pattern, as revealed by X-ray crystallography [25, 28], it has been suggested that hydrophobic ligands may bind inside the central calyx, and from recent studies [4, 32, 40], there is evidence that it binds fatty acids inside the central calyx, as for retinol.

The aim of this study was to compare the adsorption kinetics of β-lg at the air/solution interface, when pre-heated in 50 mmol·L^{-1} NaCl, pH 6 or when in mixture with isoamyl acetate, a small molecular-weight aroma compound at concentrations where it remains water-soluble.

2. MATERIALS AND METHODS

β-lactoglobulin (β-lg) in powdered form 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 % was β-lg), less than 5 % α-lactalbumin, approx 2 % lactose, 0.9 % salt and traces of fat (Maugas, personal communication). It was dispersed in Milli-Q water and dialysed against Milli-Q water for 24 h at 4 °C, to remove lactose and salts. After centrifugation (15 000 *g* for 15 min), the pH of proteins in the supernatant was adjusted to pH 6 (2 mol·L^{-1} NaOH) and its ionic strength to 50 mmol L^{-1} NaCl. The protein concentration was determined either by the biuret method with bovine serum albumin as a standard or by spectrophotometry at 280 nm with OD (1 %) = 9.6. Electrophoretic analysis [21] indicated that more than 80 % of proteins in the supernatant are β-lg monomers. Isoamyl acetate (IAA), was kindly supplied by International Flavour and Fragrance (Longvic, France). Its degree of purity and its solubility in water were 87 % and 2.4 g·L^{-1} [9]. The aroma compound was dissolved in 50 mmol·L^{-1} NaCl at pH 6 and added to protein solutions (5.4 µmol·L^{-1} for spectrofluorimetry or 0.27 mmol·L^{-1} for tensiometry) to achieve mixtures at different R = [IAA]/[β-lg] molar ratios, by using 18 400 g·mol^{-1} and 130 g·mol^{-1} for the monomer molecular mass of β-lg and IAA, respectively.

2.1. Tensiometry

Protein solutions (0.27 mmol·L^{-1}) were pre-heated from 20 °C to 80 °C at 2 °C·min^{-1} and then cooled in an ice bath before surface tension measurements were taken. Under this condition

of pre-heating, the protein solubility did not significantly change. Kinetics of surface tension decay were measured continuously using a processor tensiometer (Kruss K12) equipped with a platinium Wilhelmy-plate. The new interface was created by pouring out the solution, then allowed to equilibrate for 2 min before measuring up to 4 h. Each value of surface tension was automatically determined and independent experiments were done at least twice. In this study, the value of the solvent (50 mmol·L^{-1} NaCl, pH 6) surface tension, γ_0 was equal to 72.5 mN ± 0.25 mN·m^{-1} and it did not significantly change when acetate isoamyl was added up to a concentration of 1 mmol·L^{-1}, which corresponded to that of IAA when mixed with β-lg at a IAA/protein molar ratio ~ 4.

The repeatability of tensiometric measurements, evaluated from independent measurements at 20 ± 0.5 °C, was less than 2 %, and determination of protein concentration after tensiometric measurements indicated no significant change, relative to concentration of stock solutions.

2.2. Spectrofluorimetric measurements

Absorbance and fluorescence measurements were made with a Carry 100 and SLM AB_2 Aminco Bowman spectrophotometer and spectrofluorimeter, respectively. 2 mL of β-lg solutions were put in a thermostatically controlled quartz-cell (1 cm pathlength). For β-lg alone or in mixtures with IAA at IAA/β-lg molar ratios up to 4, the stock solutions were diluted to 5.4 µmol·L^{-1} by 50 mmol·L^{-1} NaCl, pH 6 and tryptophan emission spectra were recorded between 300 and 400 nm with excitation at 290 nm and 2 nm excitation and emission slit widths. ANS-dye binding properties of β-lg alone from non-heated and pre-heated solutions (0.27 mmol·L^{-1}, 50 mmol·L^{-1}NaCl and pH 6) from 20 °C to 80 °C at 2 °C·min^{-1} were monitored at 380 nm (excitation) and 475 nm (emission) wavelengths. Interactions with ANS-dye in excess were quantified from the initial slope of the maximal fluorescence intensity versus protein concentration plots. This procedure [2, 33] was used to determine the index of surface hydrophobicity of protein molecules from unheated and pre-heated stock solutions. The variation in the intensity photon flux as a function of wavelength was minimized by using a ratio amplifier mode, as specified by the manufacturer. The fluorescence measurements were corrected from absorbance effects following the procedure of Lackowicz [18]. All spectra were made at 20 °C and in duplicate, and NaCl buffer spectra containing IAA or ANS-dye were substracted from those of protein solutions in mixture.

3. RESULTS AND DISCUSSION

3.1. Surface adsorption at the air/solution interface

The ability of IAA and β-lg molecules to adsorb at the air/water interface was evaluated through surface tension kinetics, $\gamma = f(t)$. The surface tension value (72.5 ± 0.25 mN·m^{-1}), observed with 50 mmol·L^{-1} NaCl solutions in the absence or presence of IAA at various concentrations up to 1 mmol·L^{-1}, was independent of time and in agreement with published data [36]. However, solutions containing β-lg alone or in mixture with IAA at various molar ratios showed different kinetics of surface tension. From *figure 1a*, it is seen that the steady-state value of surface tension observed with solution of β-lg alone is 46.3 mN·m^{-1}, in agreement with that of β-lactoglobulin found by other authors [23] under similar conditions (0.1 mmol·L^{-1} in sodium phosphate buffer at pH 7), and with that of lysozyme (another globular protein), but higher (by approx. 2 mN·m^{-1}) than that of β-casein, a random-coil protein [36]. The curves in *figure 1a* indicate that addition of IAA to non-heated protein solutions leads to increased rates of adsorption in the short-time region. In the long-time region $\gamma_\sim$ the steady-state value of γ (45.7 mN·m^{-1}) is slightly lower than for β-lg alone, only for mixture at R = 2. For this molar ratio of IAA/β-lg, $\gamma_\sim$ is similar to that of pre-heated β-lg alone (*figure 1b*). The curves in *figure 1b* show kinetics of adsorption obtained with solutions of β-lg alone or in mixture with IAA, which have been heated/cooled prior to tensiometric measurements. Comparison between non-heated solution of β-lg alone and pre-heated solution, indicate that pre-heating causes a more rapid decrease in the short-time values

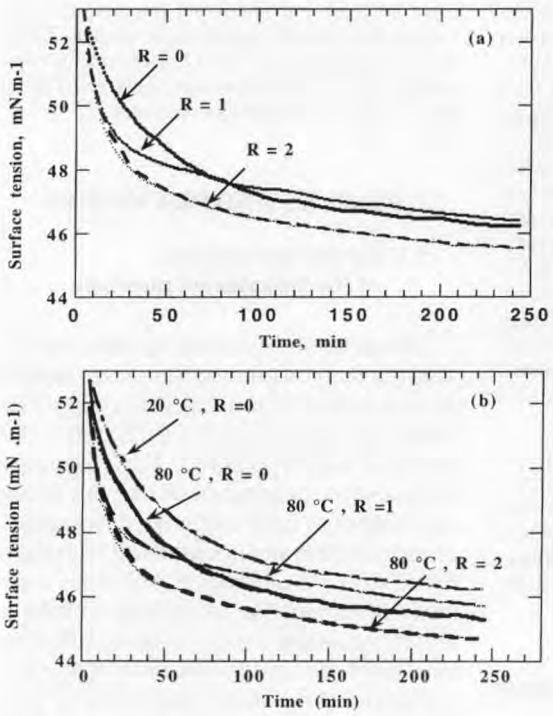

Figure 1. Kinetics of adsorption at the air/solution interface for β-lactoglobulin solutions (0.27 mmol·L^{-1} concentration in 50 mmol·L^{-1} NaCl, pH 6) in the absence of isoamyl acetate (R = 0) and in the presence of isoamyl acetate, at IAA/β-lg molar ratios, equal to 1 (R = 1) and 2 (R = 2). (**a**), non-heated solutions. (**b**), pre-heated solutions from 20 to 80 °C, at 2 °C·min^{-1}.

Figure 1. Cinétique d'adsorption à l'interface air/solution de β-lactoglobuline (0,27 mmol·L^{-1} dans 50 mmol·L^{-1} NaCl, pH 6), en l'absence d'acétate d'isoamyl (R = 0) et en présence d'acétate d'isoamyl aux rapports molaires AIA/β-lg égaux à 1 (R = 1) et 2 (R = 2). (**a**), solutions non chauffées. (**b**), solutions préchauffées de 20 à 80 °C à 2 °C·min^{-1}.

of γ(t) for IAA/β-lg mixtures than for β-lg alone. However, the value of $\gamma_\sim$ (44.7 mN·m^{-1}) decreases more significantly, only for mixtures at R = 2. These results indicated that the ability of β-lg molecules to adsorb at the air/water interface seems to be enhanced in the presence of IAA, an hydrophobic small-molecular-weight molecule, at concentrations where it remains water-soluble. Decreases in surface tension values of β-lg in the presence of sodium dodecyl sulfate (SDS) or dodecyltrimethylammonium bromide (DTAB) have been previously observed [20]. In this last study, the surface tension values have been observed to reach a minimum for surfactant/β-lg molar ratio close to 1, where formation of complexes between β-lg and surfactant molecules have been shown. On the other hand, it has been observed that the addition of small molecule surfactants to β-lg at a low surfactant/β-lg molar ratio has consequences not only on surface tension kinetics, but also on composition, thickness and rheological properties of the surface layers [1, 16, 23]. Furthermore, addition of a non-ionic surfactant (hexaoxyethylene dodecylether, $C_{12}E_6$) to β-lg at molar ratios less than 0.2, have indicated little protein displacement from the air-water interface [16]. And, addition of cetyltrimethylammonium bromide (CTAB) to β-lg at a molar ratio equal to 2, has been observed [23] to cause a decrease in surface tension of about 1 mN·m^{-1}, in the range of that observed in the present study for pre-heated IAA/β-lg mixture.

3.2. Spectrofluorimetric properties

Local protein conformational changes of β-lg from a solution at 0.27 mmol·L^{-1} which has been pre-heated from 20 °C to 80 °C, at 2 °C·min^{-1} were monitored through ANS-dye binding properties to β-lg at various

concentrations and by tryptophan spectrofluorimetry, after dilution to ~ 5.4 µmol·L^{-1}.

The evolution of ANS-fluorescence intensity, as a function of protein concentrations, obtained by dilution of the stock solutions (0.27 mmol·L^{-1}) before or after the heat treatment is shown in *figure 2*. The index of the protein surface hydrophobicity, deduced from the initial slope of these curves, increased by approx. 55 % (insert in *figure 2*) on pre-heating, indicating a significant increase in hydrophobicity. This result is in fair agreement with our previous result [33], found with β-lg solutions (2.4 mmol·L^{-1}) in 100 mmol·L^{-1} NaCl and pH 7, which had been pre-heated at 80 °C. As in the previous study, performed with stock solutions at ten-fold protein concentration, the present result can be correlated with formation of new, more solvent accessible hydrophobic parts of the protein, when heated up to 80 °C and cooled to 20 °C. This could explain the increased adsorption properties of pure β-lg, after heating/cooling.

The effects of IAA on the tryptophan fluorescence properties of un-heated and pre-heated β-lg are shown in *figure 3*. Under our experimental conditions, we observed no change in the wavelength of maximum fluorescence intensity for non-heated mixtures, relative to non-heated β-lg alone (*figure 3a*). As expected from earlier studies [24], tryptophan spectrofluorimetric spectra (*figure 3b*) showed that pre-heating the pure protein solution from 20 °C to 80 °C, led to increased value of maximum fluorescence intensity (+15 %) and to a Stokes' shift (3 to 4 nm), in compararison with unheated proteins, where the maximum wavelength of fluorescence intensity was observed at 333 nm. The increased value in maximum fluorescence intensity and the red-shift in its wavelength indicates more exposure of initially buried hydrophobic residues of β-lg molecules to the aqueous medium, after the heating/cooling. However when the mixtures have been heated/cooled, the curves in *figure 3b* show

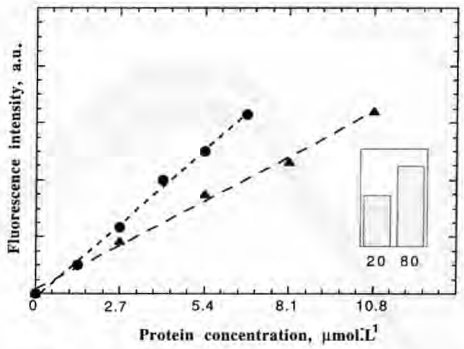

Figure 2. Variation of ANS-fluorescence intensity as function of β-lactoglobulin concentrations from stock solutions (0.27 mmol·L^{-1} in 50 mmol·L^{-1}NaCl, pH 6), without pre-heating (triangles) or with pre-heating from 20 to 80 °C, at 2 °C·min^{-1} (circles), the figure in the insert shows comparison between the surface hydrophobicity index (see text) for non-heated and pre-heated solutions.

Figure 2. Variation de l'intensité de fluorescence de l'ANS de différentes solutions de β-lactoglobuline, issues de la solution-mère (0,27 mmol·L^{-1} dans 50 mmol·L^{-1} NaCl, pH 6), sans traitement thermique (triangles) ou avec prétraitement thermique de 20 à 80 °C, à 2 °C·min^{-1} (cercles), la figure insérée indique la comparaison entre les valeurs de l'indice d'hydrophobicité de surface (voir texte) pour les solutions non chauffée et préchauffée.

a slight blue-shift (~2 nm) in the wavelength of maximum fluorescence for pre-heated mixtures, relative to β-lg alone, or a red-shift (2 to 3 nm), relative to non-heated solutions. The small blue-shift emission maximum observed from heat-treated mixtures relative to heat-treated β-lg alone, might suggest a change in tryptophan local environment only when β-lg + IAA have been pre-heated. However, for both un-heated or pre-heated mixtures the relative fluorescence intensity decreased toward a plateau value at R > 2. The intrinsic fluorescence of tryptophan residues in proteins is known to be sensitive to protonation or deprotonation of neighbouring residues, but in our experimental conditions, addition of IAA did not affect the solution pH. Therefore,

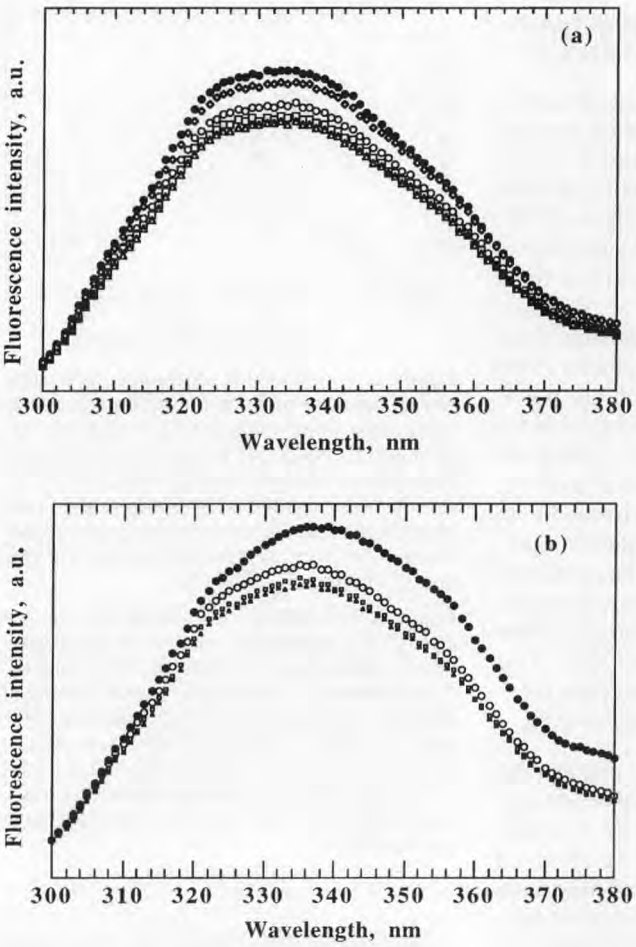

Figure 3. Changes in tryptophan spectrofluorimetric spectra of β-lactoglobulin solutions containing isoamyl acetate at IAA/β-lg at various molar ratios (open symbols), relative to β-lg alone (closed symbols). The protein solutions (0.27 mmol·L^{-1} concentration in 50 mmol·L^{-1} NaCl, pH 6) have been diluted to 5.4 µmol·L^{-1}. Increasing IAA/β-lg molar ratios, R, are from top to bottom. (**a**), non-heated solutions, R = 0, 1, 2, 3 and 4. (**b**), pre-heated solutions from 20 to 80 °C, at 2 °C·min^{-1}, R = 0, 2, 3 and 4.

Figure 3. Modification des spectres de fluorescence du tryptophane observée à partir de solutions de β-lactoglobuline contenant de l'acétate d'isoamyl à différents rapports molaires (symboles vides), en comparaison avec la β-lg pure (symboles pleins). Les solutions protéiques (0,27 mmol·L^{-1} dans 50 mmol·L^{-1} NaCl, pH 6) ont été diluées à 5,4 µmol.L^{-1}. (**a**), solutions non chauffées, R = 0, 1, 2, 3 et 4. (**b**), solutions préchauffées de 20 à 80 °C, à 2 °C·min^{-1}, R = 0, 2, 3 et 4.

we can assume, according to several previous studies on interaction properties of β-lg and hydrophobic molecules [7, 8, 10, 19, 26, 27], that the perturbation in fluorescence when IAA was added could reflect binding properties of the aroma compound to β-lg. The results obtained in the present study could be explained on the basis of a specific surface activity of β-lg + IAA complexes. This property may be related to the functional properties of proteins in biotechnology, such as enhancement of foaming properties of β-lg in presence of IAA, as shown recently [22].

4. CONCLUSION

In this work we showed that addition of IAA to β-lg, at concentrations used in food applications, led to an increase in β-lg surface activity. This hypothesis was based on the observed decrease in surface tension values. With regard to the quenching of fluorescence intensity observed when β-lg was in mixture with IAA, at increasing IAA/β-lg molar ratios, it might be suggested that when IAA bound to β-lg this led to the formation of complexes with a specific surface activity. Further experiments are needed to deter-

mine the values of a constant for binding IAA to β-lg.

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