Determination of Apparent Binding Constants for Aroma Compounds with β-Lactoglobulin by Dynamic Coupled Column Liquid Chromatography

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Apparent binding constants of aroma compounds limonene, α- and β-ionone, and terpenyl acetate, with β-lactoglobulin (BLG), were determined, using dynamic coupled column liquid chromatography, for pH values varying from 3 to 11. K_a values varied from 2.61 to 3.21 × 10^3 M^{-1} for limonene, indicating a strong interaction with BLG. Similarly, significant and close apparent binding constants were obtained for α- and β-ionone, 1.7 × 10^2 and 4.5 to 5.4 × 10^2 M^{-1}, respectively. These data indicated that a similar mechanism is involved for the binding of these two molecules. The weaker values obtained at low pH, for α-ionone relative to β-ionone, can be explained by the existence of steric hindrance. An increase of the apparent binding constant was observed, for all the compounds studied, when the pH was increased from 3 to 9. At this pH, an apparent binding constant was obtained for terpenyl acetate (1.04 × 10^2 M^{-1}), whereas this determination was not possible at pH 3 and 6. The apparent binding constant increase was in agreement with the decrease of aroma compound relative activity coefficient in the presence of BLG, previously observed at this pH. It indicated a best accessibility to the same binding site. The binding constants of all the aroma compounds studied decreased at pH 11 as a result of the important release of the BLG structure previously reported.

Keywords: *β-Lactoglobulin; aroma compounds; binding constants; dynamic coupled column liquid chromatography (DCCLC); pH effect*

INTRODUCTION

Interactions between aroma compounds and proteins, and more particularly soy and milk proteins, have been extensively studied (Langourieux and Crouzet, 1995a; O'Neill, 1996). The increase of the aroma compound retention by β-lactoglobulin (BLG) or soy proteins with chain length indicated that hydrophobic interactions were the most important mechanism involved (Damadoran and Kinsella, 1981; O'Neill and Kinsella, 1987; Jouenne and Crouzet, 2000).

Several methods were used for binding constant determination, among them, equilibrium methods: equilibrium dialysis (O'Neill and Kinsella, 1987), static headspace determination (Charles et al., 1996), fluorescence measurements (Dufour and Haertlé, 1990). However, several drawbacks were associated with the use of some of them when aroma compounds were used as ligands. Particularly, the equilibrium between aroma compounds and macromolecules reached after a long time, was generally difficult to determine, and degradations of volatile compounds during this time were evocated. Moreover, nonspecific binding and volatilization may occur.

Dynamic approaches were investigated for the study of interactions involving aroma compounds: exclusion size chromatography (Pelletier et al., 1998) and affinity chromatography using immobilized BLG (Sostmann and Guichard, 1998). Moreover, dynamic coupled column liquid chromatography (DCCLC), initially introduced for the study of polycyclic aromatic hydrocarbon solubility (May et al., 1978a,b), was successfully used for the determination of complex formation constants between hydrophobic compounds and cyclodextrins (Blyshak et al., 1989; Langourieux and Crouzet, 1995b) and aroma compounds and biopolymers (Langourieux and Crouzet, 1995c).

As indicated by Sostmann and Guichard (1998), the comparison of published data devoted to aroma compounds−protein interactions was difficult because of the wide range of external parameters used, such as salt content, protein concentration, and pH. In the case of BLG the variations of the aroma compound retention with pH, ionic strength, and urea concentration pointed up the key role played by the ternary structure of the protein in the phenomenon (Jouenne and Crouzet, 2000).

The purpose of the present work was the determination of apparent binding constants of aroma compounds with BLG, for different pH values, using DCCLC. The aroma compounds were selected in accordance with previously reported data concerning aroma compound retention and binding constant determination with BLG as receptor.

MATERIALS AND METHODS

Materials. BLG (a mixture of A and B variants, purity 90% relative to the total protein content) was obtained from Besnier Bridel Aliments (Retiers, France). Aroma compounds were from IFF (Dijon, France) and from Sigma (Saint Quentin-Fallaviers, France).

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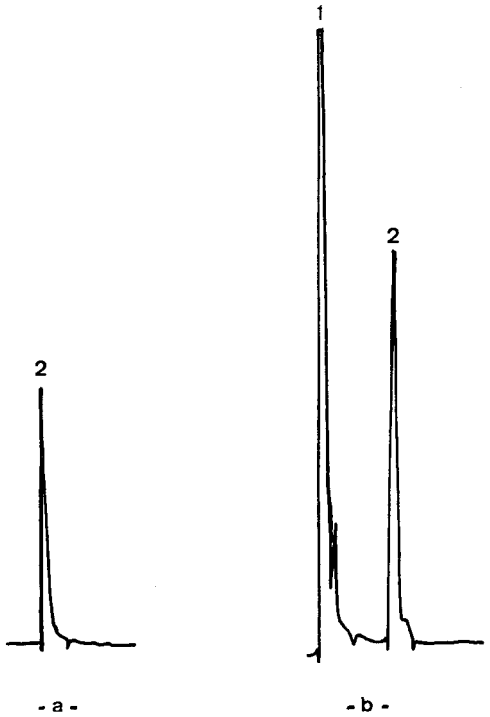

Figure 1. HPLC chromatogram of limonene eluted from the generator column at a 2 mL/min flow rate (a) with water at 25 °C, (b) with a BLG solution 4.35×10^{-4} M, pH 3, 25 °C. Spheri 5 RP18 (5 μm, 25 × 0.46 cm i.d.) column, elution solvent acetonitrile−water−trifluoracetic acid (65:35:0.1 v/v/v) at a flow rate of 1 mL/min, detection at 210 nm. Peak 1: BLG; peak 2: limonene.

DCCLC. The DCCLC equipment was similar to that previously described (May et al., 1978a,b; Langourieux and Crouzet, 1995b). The stainless steel generator column (25 × 0.46 cm) was fitted with glass beads 80−120 mesh coated with aroma compounds (1% w/w). For the determination of aroma compound solubility this column was eluted by distilled water at 25 °C at a flow rate varying from 2.0 to 4.7 mL/min using a Dosapro Milton Roy (Saint Pierre, France) minipump. The saturated aqueous solutions were introduced in the HPLC column through the sample loop (20 μL) of a 6-port switching valve (Rheodyne, Cotati, CA).

For the determination of aroma compound solubility in the presence of BLG the distilled water was replaced by an aqueous solution of this protein from 2.17 to 4.35×10^{-4} M, adjusted to the required pH by addition of sodium hydroxide or hydrochloric acid. When the aqueous solution of BLG was used instead of water for the generator column flowing, an increase in the solubility of the aroma compound (A), resulting from a complex formation, was detected by HPLC (Figure 1).

The apparent binding constant (K_a) corresponding to the equilibrium

$$\mathrm{BLG} + \mathrm{A} \rightleftarrows \mathrm{BLG} - \mathrm{A}$$

$$K_a = \frac{[\mathrm{BLG} - \mathrm{A}]}{[\mathrm{BLG}][\mathrm{A}]}$$

may be calculated (Blyshak et al., 1989), assuming that the aroma compound concentration was not depleted to an appreciable extent by complexation, according to

$$K_a = \frac{S_t - S_0}{[\mathrm{BLG}]_t\, S_0}$$

where S_0 was the solubility of the aroma compound in water, S_t its solubility in protein solution, and $[\mathrm{BLG}]_t$ the initial BLG concentration.

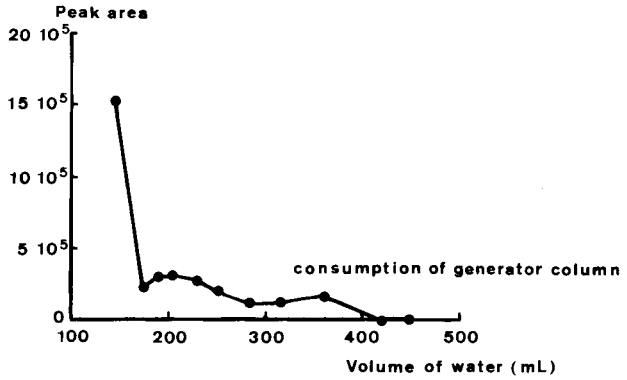

Figure 2. Variation of the chromatographic peak area of linalool eluted from the generator column at a 2 mL/min flow rate as a function of the volume (mL) of water used.

Table 1. Water Solubility at 25 °C of Aroma Compounds at 25 °C Determined by DCCLC

	solubility	
aroma compd	mg/L	mM
limonene	10.9 ± 0.8	0.08
terpenyl acetate	65.7 ± 4.3	0.33
β-ionone	105.3 ± 3.2	0.55
α-ionone	125.8 ± 8.1	0.65
L-menthol	464 ± 24.1	2.96
linalool	2.6×10^3	
acetophenone	6.9×10^3	
benzaldehyde	7.0×10^3	

HPLC. A Shimadzu (Kyoto, Japan) LC 9A pumping system fitted with a Spheri 5 RP18 (Applied Biosystem, San José, CA), 5 μm, 25 × 0.46 cm, a Varian (Walnut Creek, CA) 2550 UV detector, and a Shimadzu CR 6A integrator were used. The column was eluted with acetonitrile−water−trifluoracetic acid (65:35:0.1 v/v/v) at a flow rate of 1 mL/min. Detection was performed at 206 nm for linalool, 210 nm for limonene, 222 nm for terpenyl acetate, 240 nm for acetophenone, 242 nm for benzaldehyde, 294 nm for β-ionone, and 292 nm for α-ionone; the aroma compound concentrations were calculated from peak area using response coefficients determined for each compound from solutions of known concentration.

Hydrophobicity Estimation. The values of log *P* for the several aroma compounds used were estimated by calculation according to Rekker (1977). The log *P* value, which is defined as the logarithm of the liquid−liquid partition coefficient between water and a hydrophobic phase, generally *n*-butanol, represents the hydrophobicity of the aroma compounds.

Repeatability. The variation coefficient was determined, for each apparent binding constant, from six separate measurements.

RESULTS AND DISCUSSION

Aroma Compound Solubility. As previously reported (Pelletier et al.,1999), the use of chromatographic methods was limited by the water solubility and by the UV absorption of used ligands. The water solubility at 25 °C of several aroma compounds, in good agreement with log *P* values calculated according to Rekker (1977), were reported in Table 1. Benzaldehyde, acetophenone, and linalool were easily detected in UV, but the result of their high solubility was a rapid exhaustion of the generator column, as indicated Figure 2, for linalool.

Four aroma compounds, limonene, α- and β-ionone, and terpenyl acetate, have been finally selected on the basis of their UV absorbance, their ability for the preparation of the generator column, and the stability

Table 2. β-Ionone and Limonene−BLG Apparent Binding Constants at 25 °C, pH 3, for a Fixed BLG Concentration (2.17×10^{-4} M) and for Several Elution Volumes

	K_a (M^{-1})	
elution vol (mL)	β-ionone	limonene
20	579	
40	469	
60	457	2104
80	434	1907
100		1967
120		2418
mean	510 ± 60	2100 ± 220

Table 3. Aroma Compound−BLG Apparent Binding Constants at 25 °C, pH 3, for Several Protein Concentrations

BLG concn (M)	limonene	α-ionone	β-ionone
2.17×10^{-4}	2610 ± 580		520 ± 60
3.26×10^{-4}	3560 ± 170		450 ± 40
4.35×10^{-4}	3210 ± 210	170 ± 8	540 ± 15

of the aroma compound concentration in the generator column eluate.

Reversibility of the Phenomenon. For some aroma compounds, limonene and β-ionone, the apparent binding constant was determined at 25 °C, pH 3, for a fixed protein concentration (2.17×10^{-4} M) and for different eluent volumes. The results reported in Table 2 show that the apparent binding constant does not decrease when the volume of the injected protein solution increases from 20 to 120 mL. The constancy of K_a values is a strong argument in favor of the reversible character of the interactions between tested aroma compounds and BLG.

Determination of Apparent Binding Constants. In a first step, apparent binding constants were determined at pH 3 for different protein concentrations. This pH value was previously used for aroma compounds−BLG binding constant determination (Dufour and Haertlé, 1990; Charles et al., 1996; Pelletier et al., 1998; Sostmann and Guichard, 1998), according to the existence of a prevailing dimeric form with a very compact conformation for BLG. The values obtained for limonene and α- and β-ionone are reported in Table 3. In the conditions used it was not possible to determine terpenyl acetate apparent binding constant.

The constants obtained for limonene, 2.61×10^3 to 3.21×10^3 M^{-1}, are characteristic of strong interactions, whereas no interaction was detected by fluorescence measurements (Dufour and Haertlé, 1990). The results obtained in the present work were in apparent contradiction to those previously obtained by dynamic headspace (Jouenne and Crouzet, 1996, 2000). Using this technique, the increase of relative activity coefficient in the presence of BLG indicates a salting-out effect of the protein. However, the same parameters were not taken into account by the two methods. Determinations realized using DCCLC were not affected by the aroma compound phase change at the gas−liquid interface as in exponential dilution. Only phenomena occurring in solution were concerned, and it can be assumed that DCCLC provides a direct measure of interactions in liquid phase through the intermediary of the total solubility. A mechanical bleeding of the generator column was discarded, according to the differences observed for apparent binding constants determined at several pH (see later).

The apparent binding constants obtained for α- and β-ionone, 170 and 450 to 540 M^{-1} respectively, were much less important than those previously reported by Dufour and Haertlé (1990) for β-ionone (1.66×10^7 M^{-1}) and by Sostmann and Guichard (1998) for the two ketones (19.1×10^3 M^{-1} and 13.5×10^3 M^{-1}, respectively). The values obtained by fluorescence measurement were generally very high; as an example, Fugate and Song (1980) obtained by this method 5×10^7 M^{-1} for retinol affinity constant, whereas the constant for the retinol−β-lactoglobulin complex determined by equilibrium dialysis was only 1.5×10^4 M^{-1} (Pujol et al., 1991).

According to Dufour and Haertlé (1990), no interaction between α-ionone and BLG was detected by fluorescence measurements. They assumed that the conjugated double bonds were in the same plane for β-ionone and for retinol, and that these two compounds were bound in the hydrophobic pocket. On the contrary, the α-ionone cycle and the isoprenoid chain were in two different planes, and this more sterically hindered molecule was not able to penetrate in the pocket.

However, recent results (Le Quéré et al., 1999; Lübke et al., 2000) have shown that no spectral change in Fourier transform infrared, indicative of the absence of conformational change of BLG occurred when retinol was used as ligand at pH 2. On the contrary, α- and β-ionone induced spectral changes and gave the same difference spectra.

The results obtained in the present work for the apparent binding constants of the two ketones, as well as those reported by Sostmann and Guichard (1998), gave, independently of differences for the absolute values, the same order of magnitude and indicated very close binding mechanims.

However, the α-ionone apparent binding constant is significantly weaker than the one obtained for β-ionone, although the hydrophobicity of these two ligands was equal. So, the observed differences can be explained by conformational differences or by specificity of the sites involved in the interactions. From these results and previously reported data (Sostmann and Guichard, 1998; Le Quéré et al., 1999), it can be assumed that the binding site was the same for the two ionones, and that steric hindrance is responsible for the low value obtained for the binding of α-ionone to the compact BLG structure prevailing at pH 3. The binding site can be identified to the internal hydrophobic cluster generally considered as the retinol binding site; a direct observation of a hydrophobic ligand binding to BLG was recently reported by Wu et al. (1999) from the cocrystallization of BLG with palmitate in the central cavity. However, the presence of a second external binding site was recently suggested from ^{1}H NMR studies (Molinari et al., 1996; Ragona et al., 1997). One other possibility was the binding of these two molecule on close external binding sites, resulting from the surface exposition of residues normally hidden in the structure at low pH.

It can be assumed that the limonene binding mechanism was similar to the one of the two ionones, and that the most important value obtained for the binding constant was the result of the most important hydrophobicity of this molecule.

Effect of pH on Apparent Binding Constant Values. The apparent binding constants between BLG, limonene, α- and β-ionone, and terpenyl acetate at 2.17×10^{-4} M protein concentration in the eluate at pH 3−11

Table 4. Aroma Compound−BLG Apparent Affinity Constants for Different pH Values at 25 °C, Protein Concentration 2.17 × 10^{-4} M

aroma compd	K_a (M^{-1})			
	pH 3.0	pH 6.0	pH 9.0	pH 11.0
α-ionone	170 ± 8[a]		1000 ± 120	185 ± 50
β-ionone	520 ± 60	340 ± 30	1320 ± 70	670 ± 90
limonene	2600 ± 580	3150 ± 450	6450 ± 1700	1180 ± 210
terpenyl acetate		0	1040 ± 150	

[a] Obtained for a protein concentration 4.35 × 10^{-4} M.

are given Table 4. These results indicate, in good agreement with those obtained for aroma compound relative activity coefficients (Jouenne and Crouzet, 1996, 2000), that the apparent binding constants varied according to the pH value.

For limonene, β-ionone, and α-ionone the variation of the affinity constants with the pH is comparable with the retention variation measured by exponential dilution for all the aroma compounds. They increased from pH 3 to pH 9, then decreased when the pH value reached 11. For limonene and β-ionone the values of the apparent binding constants were three times more important at pH 9 than at pH 3; for α- ionone, the multiplicative factor was about six. Moreover, an apparent binding constant was easily determined at pH 9 for terpenyl acetate.

At pH 9, the identical values obtained for the two ketones and their increase relative to those determined at pH 3 were in favor of their binding to a same site. It can be assumed that the steric hindrance effect was minimized, resulting from the increase of the protein flexibility between pH 2 and 7 (Kella and Kinsella, 1988). The identical values obtained for the apparent binding constants for the two ionones at pH 9 can be explained by a better accessibility of these molecules to the site. Electrostatic interactions resulting from the Tanford transition occurring at pH 7.5 (Tanford et al., 1959) are possibly involved.

The increase of limonene apparent binding constant with the pH increase between 3 and 9 showed, as previously indicated, a similar behavior of this compound relative to α- and β-ionone.

The affinity constant of terpenyl acetate equal to 0 at pH 6 is equivalent to that of α-ionone at pH 9. The result obtained for this compound possessing a hydrophobicity close to that of limonene (4.64 and 4.72, respectively) can be explained by structural differences between these two compounds. The presence of an ester function induces probably a steric hindrance in the terpenyl acetate molecule and limits access to the external binding site at low pH values.

The decrease of apparent affinity constants noticed at pH 11 is the result of the alkaline denaturation of the protein involving modifications of its tertiary structure and giving, as previously reported, an important retention decrease (Jouenne and Crouzet, 2000).

Conclusion. The results obtained studying β-lactoglobulin−aroma compounds interactions by DCCLC are complementary to those obtained with one other dynamic method: exponential dilution. They indicate that the variations of the tertiary structure of the protein with pH are determinant for the intensity of aroma compounds. The measure of this intensity through the apparent binding constant is generally consistent with that obtained by relative activity coefficient.

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