Effect of pH on Retention of Aroma Compounds by β-Lactoglobulin

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Interactions between volatile compounds and BLG in aqueous solution were studied using static and dynamic headspace techniques (exponential dilution). The intensity of interactions between methyl ketones (C7−C9), ethyl esters (C6−C9), limonene, myrcene, and β-lactoglobulin (BLG) were estimated by determination of the relative infinite dilution activity coefficients (γ_r). For a constant pH value, the methyl ketones retention by BLG increased significantly with the hydrophobicity of the volatiles, whereas the retention reached a maximum for ethyl octanoate in the ester series, indicating a possible steric hindrance. For limonene and myrcene an unexpected increase in headspace concentration or "salting out" effect was noticed for acid pH. The variations of the retention according to the pH increase of the medium from pH 3 to pH 11 could be related to structural modifications of the BLG. The retention increase observed between pH 3 and pH 9 resulted from the flexibility modification of the protein, allowing better accessibility to the primary or the secondary hydrophobic sites, whereas the dramatic decrease observed at pH 11 was the consequence of the alkaline denaturation of BLG. Electrostatic interactions occurring at pH 7.5 could also explain the observed retention increase.

Keywords: *β-Lactoglobulin; aroma compounds; interactions; pH effect; exponential dilution*

INTRODUCTION

The interactions between aroma compounds and proteins have been known empirically for a long time; however, these interactions have only recently been scientifically investigated. A good knowledge of the physicochemical interactions occurring between aroma compounds and the main constituents of foods—lipids (Solms et al., 1973; Maier, 1975), polysaccharides (Langourieux and Crouzet, 1994), and proteins (Kinsella and Damadoran, 1980; O'Neill, 1996)—is required for the control of food flavoring and more particularly that of light foods or for understanding the phenomenona involved in the release of aroma compounds in the mouth. Such research was also prompted by the difficulties encountered in removing off-flavors from proteinaceous products such as soy or fish meals (Kinsella and Damadoran, 1980; O'Neill, 1996). Equilibrium methods, liquid−liquid partition, equilibrium dialysis, gel filtration, and ultrafiltration have been extensively used for matrix−ligand interactions; however, utilization of some of these methods involves several drawbacks when aroma compounds are used as ligands. In particular the equilibrium between aroma compounds and macromolecules is reached after a long time, generally difficult to determine, and degradations of volatile compounds may occur during this period. Moreover, nonspecific binding and volatilization may be noted, more particularly in the case of equilibrium dialysis, which is largely used for aroma compounds−protein interaction studies. Since equilibrium methods do not appear to be particularly suitable for studying interactions involving aroma compounds, headspace methods based on the volatility of these molecules have been developed for studying interactions between aroma compounds and macromolecules (Langourieux and Crouzet, 1994).

β-Lactoglobulin (BLG), the most important whey protein, possesses interesting emulsifing and foaming properties and is largely used in the food industry. BLG is soluble over a wide pH range, from pH 2 to pH 10 (Dumay, 1988). This globular protein, well characterized by X-ray crystallography (Papiz et al., 1986; Monaco et al., 1987), belongs to the superfamily of retinol binding protein (Papiz et al., 1986). Several small hydrophobic molecules, recently listed by Hambling et al. (1992) are also bound to BLG.

The monomers are associated in different ways according to the pH and temperature to give dimeric, tetrameric, and octameric forms (Hambling et al., 1992). Subtle conformational modifications of the monomer can also occur under these conditions.

In the present work, static headspace and exponential dilution were used to study interactions between several aroma compounds possessing different polarity, homologous series of methyl ketones and ethyl esters, terpenes, and BLG, at different pH values. Portions of this work have been presented elsewhere (Jouenne and Crouzet, 1996).

MATERIALS AND METHODS

Reagents. 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). Antifoaming agent polysiloxane 426 R was from Prolabo (Paris, France).

Static Headspace Measurements. Diluted solutions of volatile compounds (30 g) in water or in aqueous protein solution, adjusted to the required pH by addition of sodium hydroxide or hydrochloric acid and previously equilibrated at 25 ± 0.1 °C for 2 h, were poured into the diffusion cell (70

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mL) and maintained under magnetic stirring. An inert gas (N_2) was recirculated at 30 mL/min by means of a pump equipped with Teflon bellows. To avoid nonspecific adsorption of aroma compounds, all connections were composed of glass or Teflon tubing. The gas leaving the diffusion cell was automatically sampled every 3−6 min through a six-way electropneumatic valve positioned in the gas stream and injected into a Varian 3300 (Walnut Creek, CA) gas chromatograph fitted with a FID detector and a CB 5 silica capillary column (Chrompack, Middelburg, The Netherlands), 50 m × 0.32 mm, and isothermally operated at 150 °C. The concentration of aroma compounds increases in the gas phase to reach a constant concentration corresponding to static equilibrium. The ratio of the GC peak area obtained from a protein−water solution to the peak area from pure water, or relative area, indicates the nature and intensity of interactions; a value below 100% indicates a retention phenomenon, whereas a value above 100% indicates a "salting out" effect. The phenomenon called "salting out" occurs when a volatile compound shows an increase in headspace concentration when a salt, simple sugar, or other constituent is added to a dilute solution of this volatile compound.

Exponential Dilution Device. The exponential dilution equipment, similar to that used by Duhem and Vidal (1978), was previously described by Sadafian and Crouzet (1987). Diluted solutions of aroma compounds, 1 μL in 30 g of water or BLG solution (0−1.6% w/w), contained in the diffusion cell and maintained at 25 ± 0.1 °C, were stripped by an inert gas (N_2) at a flow rate varying from 30 to 100 mL/min according to the nature of the volatile compound. The stripping gas was dispersed into small diameter bubbles through a fritted glass disk (no. 4). The gas leaving the diffusion cell was automatically sampled every 3−6 min through a six-way electropneumatic valve thermostated at 150−175 °C and positioned in the gas stream. The gas was injected into a Varian 3300 gas chromatograph as indicated above. The foaming properties of BLG required addition of an antifoaming agent (polysiloxan), 10−25 mg in 30 g of solution.

Calculation of Activity Coefficient. Under the effect of gas bubbling and magnetic stirring a maximum in headspace concentration was reached within 1−4 min, and then an exponential decrease of the volatile compound concentration in the effluent gas was observed. This decrease is related to the infinite dilution activity coefficient γ_i^∞ by the relation (Duhem and Vidal, 1978)

$$\ln S = -\frac{D}{RT}\frac{P_{ssol}}{N}\gamma_i^\infty t + \ln S_0$$

where S = the GC peak area, t = time (min), S_0 = the GC peak extrapolated to zero time, D = the stripping gas flow rate (mL/min), N = the number of moles of solvent, R = the gas constant (mL atm mol^{-1} K^{-1}), T = the temperature (K), P_{ssol} = vapor pressure of the pure solute (atm), and γ_i^∞ = the infinite dilution activity coefficient. γ_i^∞ was calculated from the value of the slope of the straight line obtained by plotting ln S versus t.

The relative activity coefficient γ_r^∞ was defined as the ratio of γ_r^∞ in the presence of BLG to γ_r^∞ in the reference solution. A γ_i^∞ value below 100% indicated a retention phenomenon, and a value above 100% indicated a salting out effect.

Hydrophobicity Estimation. The values of log P for the several aroma compounds used were estimated by calculations 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.

Statistical Treatment. The following statistic treatment was used for the relative area and infinite dilution coefficient values. For n-sized samples (x_1, x_2, ..., x_n), the standard deviation σ has been calculated with the relation

$$\sqrt{\frac{n\sum x^2 - (\sum x)^2}{n^2}}$$

Three to six experiments were carried out for each determination.

RESULTS

1. Effect of the Antifoaming Agent. The results of the infinite dilution activity coefficient of aroma compounds used in the present work, determined in pure water in the absence and in the presence of 10 mg of antifoaming agent, are given in Table 1. A decrease of the activity coefficient of aroma compound after addition of the antifoaming agent relative to that determined in pure water was observed for all the aroma compounds. The relative values varied from 36% for myrcene to 83% for 2-heptanone. However, the values of γ_r^∞ for several antifoaming concentrations (10−25 mg in 30 g of solution) for a given aroma compound vary only weakly, 24400 ± 1250 to 20600 ± 960, for myrcene for example. Moreover, the interaction of selected aroma compounds with β-lactoglobulin expressed by the relative area obtained in static headspace experiments and by the relative infinite dilution coefficient obtained by exponential dilution in the presence of antifoaming agent (10 mg) (Table 2) indicated that the relative retention or "salting out" effects were noticeably the same in the both cases. Isoamyl acetate and 2-heptanone were not significantly bound to the protein, whereas an important "salting out" effect was observed for limonene.

2. Interaction between Aroma Compounds and BLG at Different pH. The intensity of interactions between methyl ketones, C7−C9, ethyl esters, C6−C9, limonene, myrcene, and BLG were estimated by determination of γ_r^∞ for different pH values of 3, 6, 9, and 11.

The results reported in Table 3 showed that for each methyl ketone the γ_r^∞ values decreased significantly from pH 3 to pH 9. Moreover, for a given pH value we observed also a γ_r^∞ decrease showing that the methyl ketone retention is correlated to their hydrophobicity (Table 4). In this pH range, the retention variation is the same for the three compounds. The retention decreased dramatically when the pH was 11; a slight "salting out" effect was observed for 2-heptanone at this pH.

The retention variations of ethyl hexanoate and octanoate (Table 5) were similar to those observed for methyl ketones with pH 3−9, but the behavior of ethyl nonanoate was quite different; for all the pH values its retention was lower than that of ethyl octanoate, independent of the log P values, and a "salting out" effect effect was even noted for pH 11.

For terpenes, limonene, and myrcene (Table 6) notable retention was only detected for pH 6 and 9. A "salting out" effect was noted for the other pH values notwithstanding their hydrophobic character; the calculated log P values were 4.45 and 4.75, respectively. However, the global variations of the retention were the same as those observed for methyl ketones and ethyl esters, namely, an increase from pH 3 to pH 9 and then a dramatic decrease at pH 11.

DISCUSSION

The results obtained concerning the effect of the antifoaming agent on the volatility of aroma compounds

Table 1. Infinite Dilution Activity Coefficients of Aroma Compounds in Water in the Absence (a) and in the Presence (b) of Rhodorsil 426 R, 10 mg in 30 g of Solution[a]

aroma compound	γ^{∞} (a)	γ^{∞} (b)	γ_r^{∞} (%)
limonene	47000 ± 6940	22700 ± 2500	48
myrcene	59700 ± 6300	21500 ± 1200	36
2-heptanone	4700 ± 260	3900 ± 110	83
2-octanone	11300 ± 1100	6700 ± 250	59
2-nonanone	43000 ± 4550	17000 ± 100	40
isoamyl acetate	4400 ± 190	3250 ± 180	74
ethyl hexanoate	13300 ± 1180	8370 ± 250	63

[a] ± indicates the standard deviation.

Table 2. Interaction between Aroma Compounds and BLG (2.4%, w/w, pH 3.4) in the Absence (Relative Area Determined by Static Headspace) and in the Presence (Relative Infinite Dilution Activity Coefficient Determined by Exponential Dilution) of Rhodorsil 426 R, 10 mg [a]

aroma compound	relative area (%)	γ_r^{∞} (%)
2-heptanone	90 ± 9	91 ± 2.5
isoamyl acetate	99 ± 5	95 ± 5.3
ethyl hexanoate	61 ± 6	51 ± 1.5
limonene	144 ± 14.2	102 ± 11

[a] ± indicates the standard deviation.

Table 3. Effect of pH on the Relative Infinite Activity Coefficient of Methyl Ketones in the Presence of BLG (1.6%, w/w) at 25 °C (Rhodorsil 426 R, 10 mg)

	γ_r^{∞} (%)			
aroma compound	pH 3	pH 6	pH 9	pH 11
2-heptanone	94	93	86	105
2-octanone	82	79	60	88
2-nonanone	66	47	29	86

Table 4. Physicochemical Characteristics: Infinite Activity Coefficient and Hydrophobicity of Aroma Compounds Used in the Present Work

aroma compound	γ^{∞}	log P [a]
2-heptanone	4700	1.82
2-octanone	11300	2.35
2-nonanone	43000	2.88
ethyl hexanoate	13000	3.62
ethyl octanoate	599500	4.68
ethyl nonanoate	nd	5.21
isoamyl acetate	4400	2.11
limonene	47000	4.45
myrcene	59700	4.75

[a] Rekker, 1977.

Table 5. Effect of pH on the Relative Infinite Activity Coefficient of Ethyl Esters in the Presence of BLG (1.6%, w/w) at 25 °C (Rhodorsil 426 R, 10 mg)

	γ_r^{∞} (%)			
aroma compound	pH 3	pH 6	pH 9	pH 11
ethyl hexanoate	65	54	53	107
ethyl octanoate	52	45	16	76
ethyl nonanoate	80	55	40	109

indicated that the infinite dilution coefficient was effectively decreased by addition of this agent. However, when the reference used for relative activity coefficient in the presence of BLG was water containing 10 mg of the antifoaming agent, instead of pure water, the intensity of interactions was not affected because the data obtained by exponential dilution and those obtained by static headspace were in good agreement (Table 2). It can be assumed that the antifoaming agent did not induce protein denaturation and that new interaction sites did not appear. Moreover, the constancy of the values of γ_r^{∞} for increasing content of antifoaming agent suggested that the polysiloxan molecules were not in competition with the aroma compound for the occupation of the same sites on the protein surface.

Table 6. Effect of pH on the Relative Infinite Activity Coefficient of Terpene in the Presence of BLG (1.6%, w/w) at 25 °C (Rhodorsil 426 R, 10 mg)

	γ_r^{∞} (%)			
aroma compound	pH 3	pH 6	pH 9	pH 11
limonene	102	96	74	147
myrcene	119	95	81	161

For all the pH values studied, the retention of the three methyl ketones by BLG, measured by the relative activity coefficient decrease, which increased with the length of the carbon chain, was similar to that previously obtained for these compounds by O'Neill and Kinsella (1987) using equilibrium dialysis. These results indicated the hydrophobic nature of the interactions that occurred in this case. Conversely, it can be assumed that interactions other than hydrophobic ones may be involved in the retention of ethyl esters by BLG. More particularly, the steric hindrance resulting from the length of the hydrocarbon chain and the presence of the ester group could possibly limit the affinity of ethyl nonanoate for the binding site. The intervention of a steric hindrance to the hydrophobic interaction between 5-nonanone and soy protein was proposed by Damadoran and Kinsella (1981) to explain the decrease of the binding affinity of this compound relative to that of 2-nonanone.

The fact that a significant retention decrease does not generally occur between pH 2 and 3 (Jouenne, 1997), in a pH range corresponding to the transition monomer−dimer at the temperature used (Hambling, 1992), indicates that the monomer junction area is probably not involved in the aroma compound binding (Figure 1). This point was supported by the results reported by several authors (Wishnia and Pinder, 1966; Townend et al., 1969; Robillard and Wishnia, 1972). From the retention increase observed for the aroma compounds studied in the present work when the pH was varied from 3 to 9, it can be assumed, in agreement with previously reported data (Robillard and Wishnia, 1972; O'Neill and Kinsella, 1987), that these compounds were bound to a primary binding site. This site, initially identified by several authors (Fugate and Song, 1980; Papiz et al., 1986; Dufour et al., 1990a,b, 1991; Dufour and Haertle, 1990a,b; Cho et al., 1994) at the hydrophobic core, is, more probably, the internal hydrophobic cluster recently described (Molinari et al., 1996). However, the binding of aroma compounds to a second external binding site postulated by Robillard and Wishnia (1972) and Dufour et al. (1990b) and identified by NMR studies (Molinari et al., 1996; Ragona et al., 1997) is also possible, as indicated by the determination for BLG of two sites possessing different affinity for 2-nonanone (Charles et al., 1997).

It can be assumed that interactions between aroma compounds and BLG are favored between pH 3 and 9 by the increase of the flexibility of the molecule, the surface exposure of residues previously hidden in the structure, and the unfolding of peripheric α-helix and β-sheets. According to Mills and Creamer (1975), an increase of pH from 2 to 6.5 was associated with a

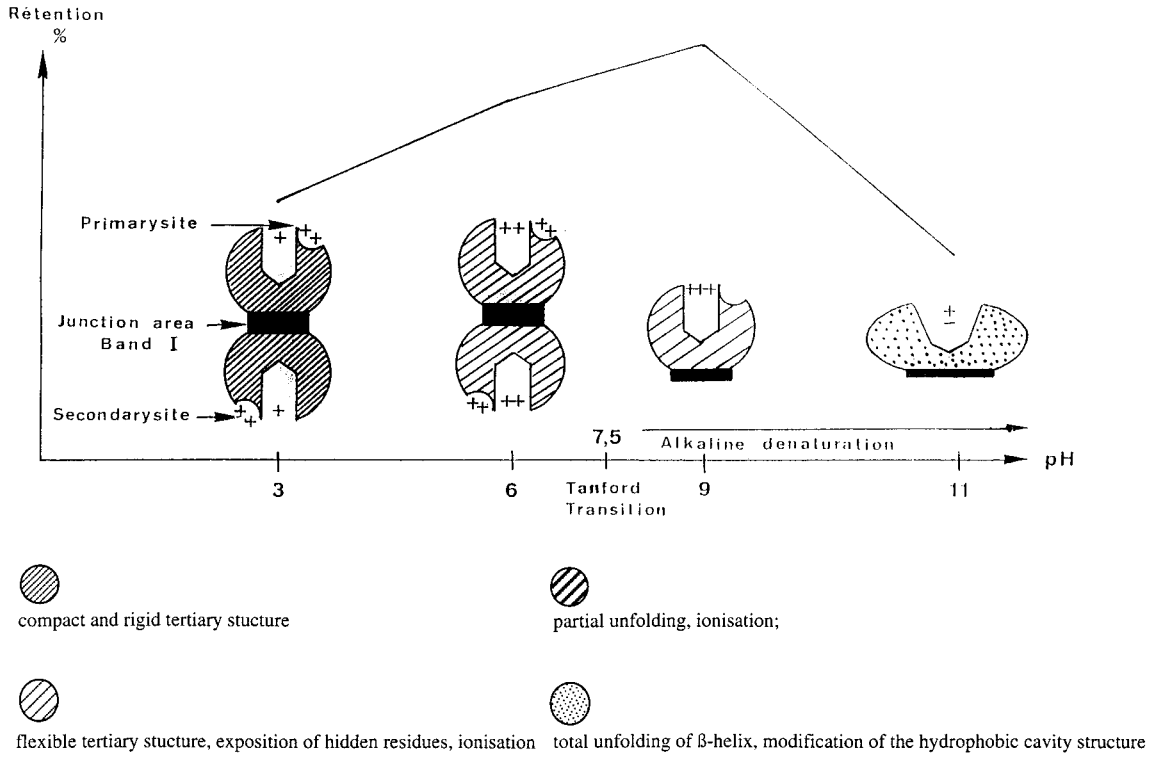

Figure 1. Modification of aroma compound retention according to structural modifications of β-lactoglobulin with pH between 3 and 11. Site accessibility: (+) medium; (++) high; (+++) very high; (±) more or less important.

conformational modification in an area near one of the tryptophan residues. The results obtained by Shimizu et al. (1985) and Kella and Kinsella (1988) showed that BLG flexibility increased between pH 1.5−2 and 7. The compacity of the protein was less important at pH 7 than at pH 2 as shown by the exposure of a residue tyrosine and a residue tryptophan to solvent (Townend et al., 1969). The increase of the affinity constant for retinol when the pH was varied between 6.5 and 7.5 was attributed to better accessibility to the cavity, resulting in conformational changes (Laligand et al., 1991). Moreover, the Tanford transition (Tanford et al., 1959) happens at pH 7.5; a carboxyle is demasked and ionized. Electrostatic interactions with polar functional groups (e.g., aldehydes, ketones, esters) similar to those occurring between free fatty acids and BLG (Spector and Flechter, 1970) can also occur.

At pH 11 the retention of all aroma compounds tested fell as a consequence of alkaline denaturation of the protein involving considerable modification of its tertiary structure.

The salting out effect detected for terpenes in the presence of β-lactoglobulin for several pH values is surprising according to the hydrophobic character of these molecules. However, the scarce results previously reported concerning this class of compounds are inconsistent. A decrease of D-limonene flavor intensity in the presence of whey protein concentrate, detected by sensory analysis, was reported by Hansen and Heinis (1992), whereas Dufour and Hartley (1990a) were not able to observe with fluorescence quenching the binding of this molecule by BLG. The "salting out" effect was not observed when other proteins were used; sodium caseinate−limonene interactions, determined by exponential dilution (Sadafian and Crouzet, 1986), showed a strong retention, probably of hydrophobic nature, as indicated by the results obtained for several aroma compounds including 2-octanone. A confirmation of limonene retention by sodium caseinate and by bovine serum albumin was provided by liquid−liquid partition studies (Sadafian and Crouzet, 1986).

CONCLUSION

Evidence for specific aroma compound−protein interactions, retention or "salting out", was obtained using headspace techniques, static headspace, and exponential dilution. In the case of BLG, the variations of retention according to the pH value of the medium were related to structural variations of the protein. However, application of these techniques is limited by the protein concentration usable, particularly due to protein foaming. Moreover, association constants cannot be obtained; in some cases they were determined by another dynamic method, namely, dynamic coupled column liquid chromatography (DCCLC) (Blishak et al., 1989).

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