Measurement of Interactions between β-Lactoglobulin and Flavor Compounds (Esters, Acids, and Pyrazines) by Affinity and Exclusion Size Chromatography

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Interactions between flavor compounds and β-lactoglobulin have been studied by two high-performance liquid chromatography methods. Affinity chromatography using immobilized β-lactoglobulin is a rapid method to calculate the global affinity between different flavor molecules and a protein. The binding constant for each compound was calculated from its retention time on the column with immobilized protein on a silica support. Hydrophobic interactions were found with esters (the binding constant increases by increasing the hydrophobic chain length), and no interaction was found with volatile acids and pyrazines studied. This method gives information only on the global affinity. The number of binding sites and binding constants can be calculated using the exclusion size chromatography for compounds with high water solubility and high UV absorption.

Keywords: *β-Lactoglobulin; flavor compounds; interactions; affinity chromatography; binding constants*

INTRODUCTION

Flavor perception in food is highly influenced by interactions between volatile aroma compounds with a variety of nonvolatile food matrix components. Understanding the processes that influence the release and binding behavior of flavor volatiles from the food matrix is of major significance for improving flavor quality. The binding of volatiles to proteins has been extensively studied (O'Neil, 1996) and particularly with soy proteins or β-lactoglobulin (BLG), the most abundant milk protein in the whey. Moreover, BLG is often used as an emulsifier in salad dressing. This protein is relatively well characterized (Batt et al., 1994). It has been postulated that this protein belongs to the superfamily of proteins (Papiz et al., 1986) involved in the strong interactions with small hydrophobic molecules. Futterman and Heller (1972) demonstrated that 1 mol of retinol was bound per monomer of BLG. The site of fixation could be located in the hydrophobic pocket of the protein (Pervaiz and Brew, 1985). Dufour and Haertlé (1990), using fluorescence quenching, showed that β-ionone, due to its structure analogy with retinol, was also bound in the hydrophobic pocket of BLG. Only a few other volatile compounds such as methyl ketones, alcohols, and aldehydes were studied (O'Neil and Kinsella, 1987; Jasinski and Kilara, 1985). Hydrophobic interactions were found for ketones and alcohols, and covalent or hydrogen binding was found with aldehydes (Damodaran and Kinsella, 1980; O'Keefe et al., 1991). However, the obtained data often differ among the authors, due to different experimental conditions and methodologies used. In the present work, we decided to analyze compounds belonging to other chemical classes, such as esters, acids, and pyrazines. Charles et al. (1996) found different mechanisms of interaction between BLG and, respectively, isoamyl acetate and ethyl hexanoate. We decided thus to study a wide range of esters to determine the mechanism of interaction. In a previous study, Sostmann and Guichard (1998) demonstrated with methyl ketones that affinity chromatography could be a good method to rapidly screen flavor compounds susceptible to interact with BLG. As affinity chromatography gives indication only of global affinity, when possible we used exclusion size chromatography to determine the number of binding sites and affinity constants (Hummel and Dreyer, 1962).

MATERIALS AND METHODS

Reagents. BLG (variants AB, purity 90%) was obtained from Besnier (France). Silica-diol, LiChroprep (25−40 μm diameter), was obtained from Merck (Darmstadt, Germany) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) from Sigma-Aldrich. Flavor compounds were from IFF (France).

Affinity Chromatography. *Immobilization of the Protein.* The procedure used for the immobilization of BLG was that described by Sostmann and Guichard (1998). A quantity of 2.0 g of silica-diol was dried under vacuum at 40 °C and suspended in 5 mL of acetone (dried over potassium carbonate) and 1 mL of pyridine. The suspension was cooled to 0 °C (ice bath), and 350 μL of tresyl chloride was added. After 1 h of stirring, the gel was filtered and then washed with 200 mL of acetone and 150 mL of diethyl ether. The gel was then dried under vacuum. This tresyl-activated support is mechanically and chemically stable and ready to be coupled with proteins (Nakamura et al., 1990). For coupling, 500 mg of BLG was dissolved in 5 mL of a 0.3 M sodium phosphate buffer, pH 7.2, containing 0.3 M sodium chloride. After centrifugation (15 min, 16 000 rpm/min) and filtration (0.45 μm), the protein concentration was determined at 278 nm ($\epsilon = 0.960$ M/cm). This solution was added to a suspension of 0.7 g of the activated gel in 2 mL of phosphate buffer. After 24 h of continual stirring at room temperature, the material was filtered. Protein concentration was determined in the filtrate to calculate the amount of bound protein. The residue was treated for 1 h with 0.2 M Tris-HCl buffer, pH 8.0, containing 1 mM dithioerythritol. Afterward, it was washed extensively

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with 0.1 M phosphate buffer, pH 7.5/0.5 M NaCl/1 mM dithioerythritol. The gel was filled under vacuum (water pump, 30 mbar) into an empty polyether ether ketone (PEEK) column (4.3 mm × 5 cm) (Touzart & Matignon, France). HPLC system used was a Varian 9010 pump, a Rheodyne 9126 injector with a 50 μL loop, and a Shimadzu SPD-6AV UV−vis spectrometric detector. The system was equilibrated with (25 mM NaCl, pH 3.0) eluent at a flow rate of 1 mL/min. Flavor compounds were injected in water solution (100 and 200 μL/L) and detected at their maximum absorption (200 nm for esters and acids and 270 nm for pyrazines).

Calculation of Binding Constant K_b (Nilsson and Larsson, 1983):

$$K_b = (t_R - t)/c_P t_0$$

where c_P is the protein concentration, t_0 is the void time, t_R is the retention time of the compound on the column with protein, and t is the retention time of the compound on the column without protein.

The void time (t_0) was determined by the injection of water onto the column and used for the calculation of the column void volume (on average 595 μL). Protein concentration on the column was determined from the concentrations measured before and after the reaction.

The repeatability of the retention times on the columns realized without protein (t) was determined with 12 esters injected on two different columns. Variation coefficients were lower than 12%. Flavor compounds were injected on two columns realized with different protein concentrations (8.61 and 20.8 mM).

Hummel and Dreyer Method. A Waters 6000 pump was equipped with a 20 μL Rheodyne injector, a Lichrosorb 100 Diol column (250 mm × 4.6 mm i.d., 10 μm particle size, Merck), and a UV spectrophotometric detector (Waters). A multichannel chromatography work station was used for quantification. The composition of eluent was pure water with NaCl (50 mM) adjusted at pH 3 with HCl, filtered at 0.45 μm, and containing different concentrations of flavor compound. The flow rate was 1 mL/min.

Calculation of Thermodynamic Constants. The concentration of free ligand in the eluent was between 5 and 80 mg/L. The concentration of bound ligand was determined by an internal calibration method. The binding of flavor compound with BLG causes a negative peak at the retention time of the flavor compound (Figure 1b). When increasing flavor compound concentrations were injected with the BLG, for a constant flavor concentration in the eluent, the trough area decreased and a positive peak appeared (Figure 1d). Concentration of the bound flavor compound (C_0) was calculated from the regression equation ($A = 7.4C - 445$ for ethyl benzoate, with $R^2 = 0.9988$), when the peak area (A) was equal to zero.

For a protein having a number of equivalent and independent binding sites (n), the reversible interactions between flavor compounds and protein can be represented thermodynamically by the Scatchard equation (eq 1) or its double-reciprocal form (eq 2):

$$\nu/L = K_b n - K_b \nu \quad (1)$$

$$1/\nu = (1/n) + (1/nK_b L) \quad (2)$$

where ν is the number of moles of flavor compound bound per mole of protein, K_b is the intrinsic affinity constant, and L is the concentration of free compound (Scatchard, 1949).

RESULTS AND DISCUSSION

Affinity Chromatography. No interaction was found for acetic, propionic, and butyric acids, 1*H*-pyrazole, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and tetramethylpyrazine. Most of the esters studied interact with BLG, except methyl acetate, methyl propionate, and ethyl acetate.

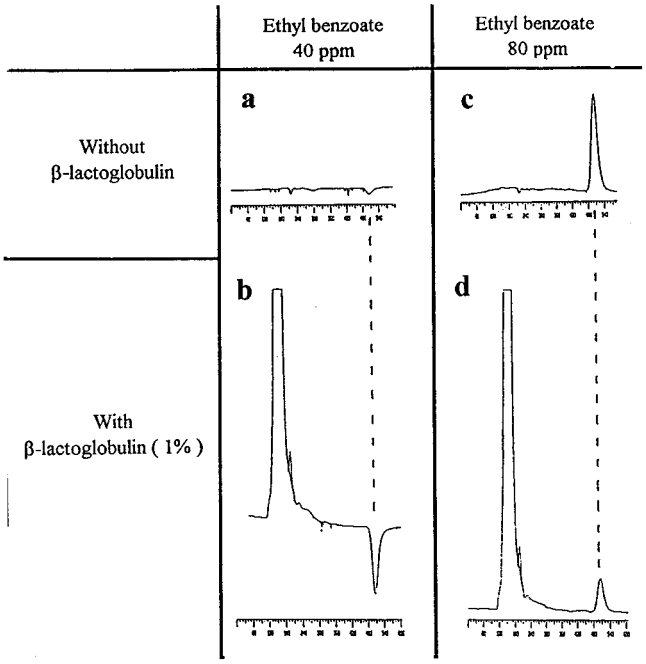

Figure 1. Hummel and Dreyer type chromatograms obtained for a 40 ppm ethyl benzoate concentration in the eluent (flow rate, 1 mL/min; pressure, 31 bar; support, Lichrosorb Diol, 10 μm): injection of ethyl benzoate 40 ppm without BLG (a) or with BLG (b); injection of ethyl benzoate 80 ppm without BLG (c) or with BLG (d).

The differences of the retention times between the columns with and without protein were used to calculate the global affinity between BLG and esters (Table 1). The repeatability was good (variation coefficient < 15%), except for methyl butyrate, ethyl propionate, and propyl acetate. For these compounds, no difference in the retention times was found with the column realized with the lower concentration in protein and only a small difference with the other column. The global affinity calculated for these compounds ($K_b < 15$) is too low to be associated with any significant interaction. Considering the series of methylic, ethylic, propylic, and butyric esters, the global affinity increases by increasing the acid chain length.

An exponential function was found for these series following the equation:

$$\log K_b = an + b$$

where n is the number of carbon atoms. Parameter a increases and b decreases from the methylic to butyric series (Figure 2).

The binding constant also increases for the same acid chain length and increasing alcohol chain length. Considering a total number of carbon atoms of 7 (Figure 3), the global affinity is minimal for propyl butyrate in which three carbon atoms are present on each side of the C=O function. The same observation can be done with a total number of carbon atoms of 8. When ramified chains are present, global affinity is lower than for the corresponding linear chain (for example, K_b = 153 M^{-1} for isoamyl acetate and 214 M^{-1} for pentyl acetate). Two phenyl esters were also studied with a higher affinity for ethyl benzoate than for methyl benzoate. All of these observations lead to the conclusion that hydrophobic interactions occur because the global affinity increases when the length of one of the two hydrophobic chains increases. Voilley et al. (1991)

Table 1. Retention Times and Binding Constants for the Experimental Compounds with Different BLG Concentrations onto the Columns at pH 3

	retention time (s) with protein concentration of			binding	variation
flavor compound	0 mM	8.61 mM	20.08 mM	constant K_b[a] (M^{-1})	coefficient (%)
methyl acetate	55		53	nd[b]	
methyl propionate	59	57	62	nd	
methyl butyrate	65	66	78	14	98
methyl hexanoate	84	141	221	244	0
methyl heptanoate	118	283	478	676	7
ethyl acetate	59	55	59	nd	
ethyl propionate	62	64	73	14	47
ethyl butyrate	71	84	103	55	3
ethyl pentanoate	76	124	179	196	9
ethyl hexanoate	100	232	392	543	6
ethyl heptanoate	143	511	863	1434	15
propyl acetate	63	64	75	13	84
propyl propionate	68	84	107	70	1
propyl butyrate	75	117	175	180	1
propyl pentanoate	102	206	355	450	1
propyl hexanoate	143		831	1225	
butyl formate	65	77	97	53	10
butyl acetate	68		113	81	
butyl propionate	80	121	192	189	8
butyl butyrate	101	206	371	466	4
butyl pentanoate	154	403		534	
pentyl acetate	70	125	180	214	13
hexyl acetate	85		381	569	
hexyl propionate	127		715	1128	
isopropyl acetate	56	59	66	17	15
isobutyl acetate	65	79	103	63	12
isopentyl acetate	78	109	172	153	15
methyl benzoate	123		290	297	
ethyl benzoate	171		551	677	

[a] Mean from the two protein columns. [b] nd, not determined.

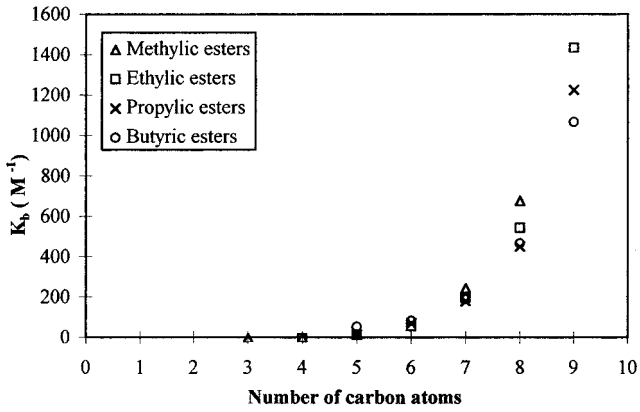

Figure 2. Dependency of the binding constants (K_b) on the number of carbon atoms (n): methylic esters (log $K_b = 1.31n - 1.68$; $R^2 = 0.994$); ethylic esters (log $K_b = 1.15n - 1.29$; $R^2 = 0.994$); propionic esters (log $K_b = 1.09n - 1.13$; $R^2 = 0.983$); butyric esters (log $K_b = 0.78n - 0.04$; $R^2 = 0.987$).

also found hydrophobic interactions between caseins and esters. Affinity chromatography gives lower values than those obtained by static headspace analysis by Charles et al. (1996) for ethyl hexanoate ($nK_b = 863$) and isoamyl acetate ($nK_b = 627$). Some binding sites could be hindered due to the chemical binding of the protein onto the chromatographic support, or the protein could be denatured. This phenomenon seems to be more important for isoamyl acetate (factor of 4) than for ethyl hexanoate (factor of 1.6). A study of competition between these two esters and 2-nonanone was conducted in our laboratory by static headspace analysis (Bernal, 1996). The fixation of ethyl hexanoate was reduced in the presence of 2-nonanone, whereas isoamyl

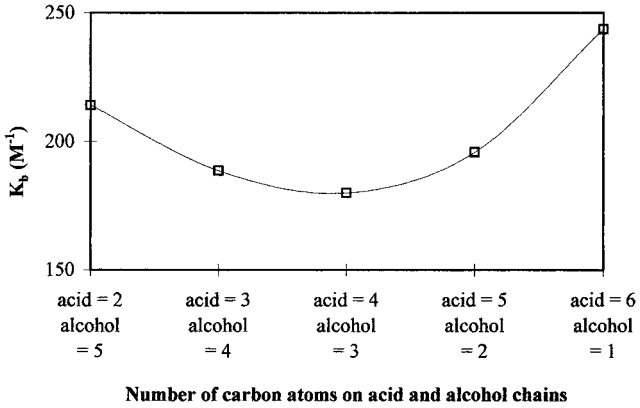

Figure 3. Dependency of the binding constant K_b on the acid and alcohol chain length for a total number of carbon atoms of 7.

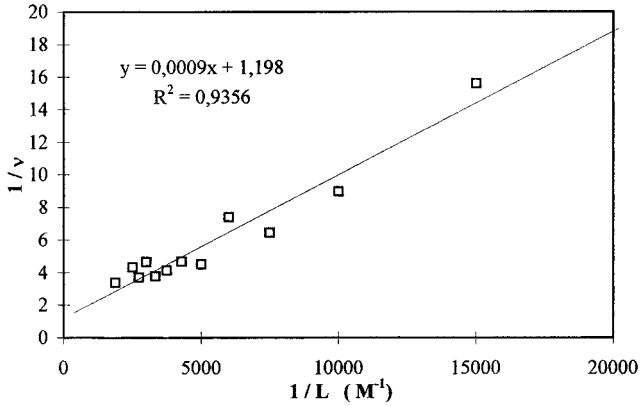

Figure 4. Interaction between BLG and ethyl benzoate (double-reciprocal form of Scatchard equation).

acetate was not influenced. We can thus deduce that 2-nonanone and ethyl hexanoate are bound in the hydrophobic pocket and that isoamyl acetate is bound on a less specific binding site. The existence of two different types of binding sites on BLG has already been demonstrated by Cho et al. (1994). We suppose that the hydrophobic pocket is less affected by the immobilization of the protein than other sites at the surface of the protein.

Hummel and Dreyer Method. As affinity chromatography gives no information on the number of binding sites, the Hummel and Dreyer method was used to determine thermodynamic constants. Due to their highest water solubility, good UV absorption at a specific wavelength ($\lambda = 270$ nm), and good separation from the peak of BLG, only 2-acetylpyrazine and methyl and ethyl benzoate could be analyzed by using this technique.

No interaction was found with 2-acetylpyrazine, confirming the results obtained by affinity chromatography with other pyrazines.

Injection of BLG with the esters led to the observation of a negative peak corresponding to the bound ester (Figure 1b). The exact amount of bound ester was determined by internal calibration. This calculation was repeated for different concentrations of ester in the eluent (free ester) to develop a Klotz plot or double-reciprocal form of Scatchard plot (Figure 4). The thermodynamic parameters obtained for the two esters are listed in Table 2. As for affinity chromatography, the binding constant increases from methyl benzoate to ethyl benzoate, by increasing the aliphatic chain

Table 2. Interaction Parameters of Two Benzoic Esters Obtained by the Hummel and Dreyer Method

	interaction parameter		
flavor compound	K_b (M^{-1})	n	nK_b (M^{-1})
methyl benzoate	280	0.9	250
ethyl benzoate	1339	0.8	1110

length, suggesting hydrophobic interactions. The number of binding sites is lower than unity (0.9 and 0.8). At a pH of 3.0, the protein could be in a monomeric conformation as postulated by Cheftel et al. (1982) or a mixture of monomers and dimers (Casal et al., 1988). Our results are more in favor of a monomeric conformation, with eventually a small proportion of dimers. Concerning ethyl benzoate, the global affinity calculated with the Hummel and Dreyer method (1110 M^{-1}) is higher than the value obtained by affinity chromatography (676 M^{-1}). As for the other esters, we can conclude that these differences are due to differences in protein conformation in solution and on the chromatographic support, resulting in the hindrance of binding sites.

Conclusion. Affinity chromatography using immobilized BLG allows a rapid measurement of global affinity between the protein and different flavor molecules. This method could be very useful to determine the flavor compounds that can interact with proteins in different food products (dairy products, emulsions with protein as emulsifiers). The obtained data are repeatable but lower than data obtained with different methods. Hydrophobic interactions were found with esters. However, this method gives information only on the global affinity. The number of binding sites and affinity constants can be calculated using the Hummel and Dreyer method, when possible. This last method is limited to the study of flavor compounds with high water solubility and high UV absorption and is time-consuming, because a wide range of concentrations must be tested to obtain the saturation curve.

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