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Immobilized β-lactoglobulin on a HPLC-column: a rapid way to determine protein–flavour interactions

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To study the interaction of β-lactoglobulin (BLG) with flavour compounds a fast screening methodology was developed. BLG (variant AB, pure A and pure B) was immobilized onto a silica support, filled into a column and combined with a HPLC-system. A total of 24 different flavour compounds were injected and their retention times determined at different pHs and protein concentrations. The binding constant for each compound was calculated from the retention times and the protein concentration of the column. This simple system allows the rapid screening of many flavour compounds under a variety of external conditions like pH, salt content and flavour concentration. This procedure also permits the study of competitive effects with several flavour compounds in the solution. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Interaction of flavour compounds with proteins is known to have a strong influence on the release of flavour from foods (Franzen and Kinsella, 1974). Of all the different proteins in food, β-lactoglobulin (BLG) is one of the best known and studied (Batt *et al.*, 1994). Nevertheless, the comparison of published data concerning the interaction between BLG and other compounds (O'Neill and Kinsella, 1987; Dufour and Haertlé, 1991) is difficult because of the variety of methodologies and the wide range of external parameters (concentration, pH, salt content, temperature, presence of other compounds) influencing the results. For this reason it is desirable to have a rapid screening method to investigate the interaction of a large number of flavour compounds under various conditions to simulate different states in food. A convenient method for this purpose was found in affinity-chromatography using the immobilized protein. This technique is well known in pharmacology but has never been used in aroma research.

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MATERIALS AND METHODS

Materials

All chemicals used were reagent grade. BLG (variants AB, purity 90%) was obtained from Besnier (France), the pure variants A and B (purity 99%) from Sigma-Aldrich. Silica-Diol, LiChroprep® 25–40 μm diameter was obtained from Merck (Germany), 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) was from Sigma-Aldrich. Flavour compounds were obtained from IFF (France). Silica gel coated with immobilized BLG was filled under vacuum (water pump, 30 mbar) into an empty PEEK column (4.3 mm×5 cm) obtained from Touzart & Matignon (France). The HPLC system used was a Varian 9010 pump, a Rheodyne 9126 injector with a 50 μl loop combined with a Shimadzu SPD-6AV UV–vis spectrometric detector.

Experimental

Immobilization of the protein

The procedure used for the immobilization of BLG was that described by Nilsson and Larsson (1983). A quantity of 2.0 g silica-diol was dried under vacuum at 40°C and suspended in 5 ml acetone (dried over potassium

carbonate) and 1 ml pyridine. The suspension was cooled to 0°C (ice-bath) and 350 µl of tresyl chloride was added. After stirring for 1 h the gel was filtered, then washed with 200 ml acetone and 150 ml diethylether. The gel was then dried under vacuum. For the coupling, different amounts of BLG (70–500 mg) were dissolved in 5 ml of a 0.3 M sodium phosphate buffer, pH 7.2, containing 0.3 M sodium chloride. After centifugation (15 min, 16 000 rot min^{-1}) and filtration (0.45 µm) the protein concentration was determined spectrophotometricaly at 278 nm ($\varepsilon = 0.960\ M\ cm^{-1}$). This solution was added to a suspension of 0.7 g of the activated gel in 2 ml 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 dithioerythriol. Afterwards it was washed extensively with 0.1 M phosphate buffer, pH 7.5; 0.5 M NaCl; 1 mM dithioerithriol and 0.1 M phosphate buffer, pH 7.5; 1 mM dithioerithriol. The gel was filled into the HPLC-column. The system was equilibrated with two different eluents: (i) water, 25 mM NaCl, pH 3.0, and (ii) water, 25 mM NaCl, pH 5.5. The concentration of the injected flavour solutions ranged from 10 to 200 µg g^{-1}. Injection volume was 50 µl, detection was UV (maximum absorption for each compound, Table 1). The flow rate was 1 ml min^{-1}.

Calculation of binding constants K_B (Nilsson and Larsson, 1983) was as follows:

$$K_B = \frac{t_R - t_0}{c_P t_0}$$

where c_P = protein conentration; t_0 = void time; and t_R = retention time of the compound. The void time was determined by injection of water onto the column and used for the calculation of the column void volume (average 595 µl). The experimental protein concentrations before and after the reaction allowed, together with the void volume, the calculation of the protein concentration on the column. Protein concentrations varied from 1.69 to 5.20 mmol/l void volume.

The support material was not inert to all flavour compounds, which caused some of the compounds to be retained by the column. Retention times were determined by two methods: (i) experimentally by applying a chromatographic material treated as described above but without protein in the phosphate buffer; and (ii) by determining the linear relationship between protein content and retention time for each compound at five different protein concentrations and calculating the retention time for the protein concentration [0 mM]. Both methods gave results that were in good agreement.

Table 1. Comparison of the retention times for the experimental compounds with different BLG concentrations onto the columns at pH 3

Flavour compound	UV detection (nm)	Retention times (s) with different protein concentrations (mM)			
		0	1·69	4·48	5·20
β-Ionone	306	623	1683	3494	5400
α-Ionone	233	286	1148	2454	2910
γ-Undecalactone	212	172	820	n.d.	n.d.
β-Damascenone	234	187	580	1161	1383
2-Nonenal	226	182	470	881	1061
2-Nonanone	264	152	390	728	885
γ-Decalactone	212	144	351	689	766
1-Nonen-3-ol	200	108	243	423	521
2-Octenal	226	109	213	207	424
2-Octanone	264	95	179	246	355
Methyl benzoate	272	130	191	242	314
1-Octen-3-ol	200	82	128	157	222
2-Heptenal	226	82	120	152	198
2-Heptanone	264	65	102	125	165
γ-Octalactone	212	75	105	136	165
Benzaldehyde	254	91	113	130	155
1-Hepten-3-ol	200	63	80	89	116
δ-Octalactone	212	67	84	98	114
2-Hexenal	226	68	82	94	110
2-Hexanone	264	65	75	85	96
1-Hexen-3-ol	200	57	63	63	73
2-Pentenal	226	67	73	78	84
2-Pentanone	264	57	60	62	66
1-Penten-3-ol	200	51	53	52	57

n.d., not determined.

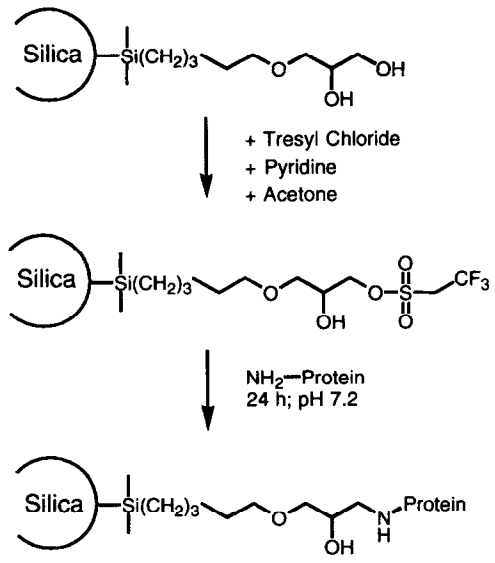

Fig. 1. Reaction scheme for the immobilization of BLG.

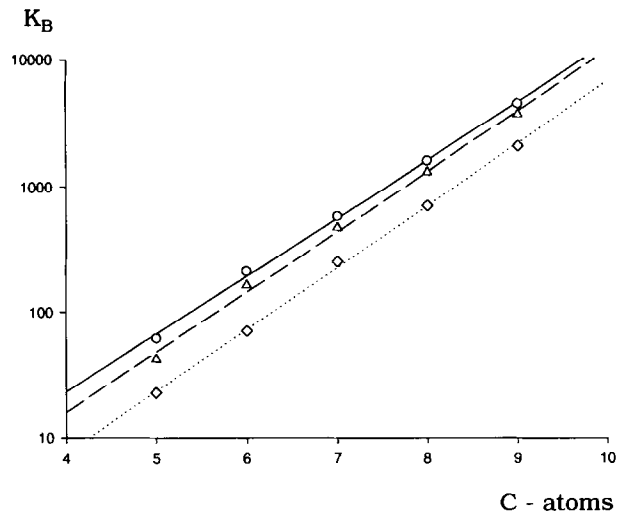

Fig. 2. dependency of the binding constant K_B on the number of carbon atoms. –○–, unsaturated aldehydes (log $y = 0{\cdot}46x - 0{\cdot}47$); –△–, ketones (log $y = 0{\cdot}48x - 0{\cdot}71$); ····◇····, unsaturated alcohols (log $y = 0{\cdot}49x - 1{\cdot}09$).

RESULTS AND DISCUSSION

Several different methods of immobilization were studied. The influence of the support material on the retention times of the flavour compounds was found to be higher for mono-alcohol-silica than for diol-silica, therefore diol-silica was the preferred material. The reaction scheme is shown in Fig. 1. To obtain a material with sufficient immobilization it was important to optimize the amount of tresyl chloride used during the activation of the silica diol. Very low amounts of this reagent (32.5 µl/1 g diol-silica), as suggested by Nilsson and Larsson (1983), were inadequate for the purpose and had to be increased. On the other hand, too large a quantity of tresyl chloride (more than 500 µl/1 g diol-silica), as described by Nakamura *et al.* (1990), gave a more reactive material. This led to inactivation of the bound protein and to lower retention times due to reduced available protein content on the column.

This technique was applied with the three samples of BLG and at three different concentrations for variant AB [1.69 mM (var.AB), 4.18 mM (var.A), 4.32 mM (var.B), 4.48 mM (var.AB) and 5.20 mM (var.AB)].

No significant difference was found between variants A and B. The retention times obtained with the three concentrations of variant AB are presented in Table 1 together with the retention times obtained on the support without protein. The measurement of the protein concentration on these columns allowed the calculation of the binding constants. The calculated binding constants for all five columns were in good agreement. In the chemical series from unsaturated alcohols (1-penten-3-ol to 1-nonen-3-ol), unsaturated aldhydes (2-propenal to 2-nonenal) and ketones (butanone to 2-nonanone) the binding constants fit an exponential function depending on the chain length of the compound. Using a logarithmic scale a linear function was obtained (Fig. 2): $\log y = a + b*x$.

For all series the parameter b was virtually the same (0.49 for unsaturated alcohols, 0.48 for ketones and 0.46 for unsaturated aldehydes), whereas parameter a varied according to the chemical class (−1.09 for unsaturated alcohols, −0.71 for ketones and −0.47 for unsaturated aldehydes). This relationship is similar to the distribution between a watery phase and oil found by McNulty and Karel (1973), suggesting a pure hydrophobic interaction without specific binding sites for these compounds. These flavour compounds were tested at two pH values (3.0 and 5.5) and no significant difference was found. This result confirms those obtained by Jouenne and Crouzet (1996) on methyl ketones.

Both reversible and irreversible binding may occur with aldehydes (Gremli, 1974; Cheftel *et al.*, 1985). As the amino groups of the BLG are bound to the support material and as short retention times are found for these

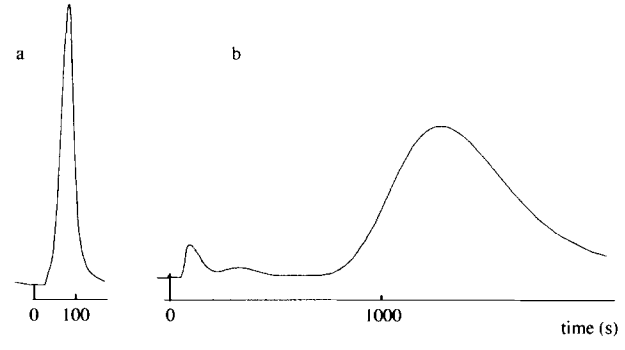

Fig. 3. Chromatographic profile obtained for two compounds with different behaviours on the column filled with 5·2 mM BLG (eluent:water, 25 mM NaCl, acidified at pH 3 with HCl): (a) 2-pentenal 100 mg litre^{-1} (UV detection: 226 nm, $t_R = 84$); and, (b) β-damascenone 40 mg litre^{-1} (UV detection: 234 nm, $t_R = 1383$).

Table 2. Binding constants of the experimental flavour compounds with BLG

Flavour compound	Binding constant K_B [M^{-1}]
β-Ionone	19143
α-Ionone	13456
γ-Undecalactone	9924
β-Damascenone	6073
2-Nonenal	4433
2-Nonanone	3629
γ-Decalactone	3230
1-Nonen-3-ol	2055
2-Octenal	1579
2-Octanone	1287
Methyl benzoate	1005
1-Octen-3-ol	699
2-Heptenal	576
2-Heptanone	465
γ-Octalactone	450
Benzaldehyde	341
1-Hepten-3-ol	250
δ-Octalactone	231
2-Hexenal	218
2-Hexanone	163
1-Hexen-3-ol	70
2-Pentenal	61
Pentanone	42
1-Penten-3-ol	23

Mean from five protein-columns with different protein content and different BLG-variants at pH 3.

compounds, only reversible interactions may occur. This was also verified by measuring the areas of the peaks which did not differ for the columns with and without protein and also with the symmetrical aspect of the peaks (Fig. 3).

Of all the compounds tested the carotenoid degradation products β-ionone, α-ionone and β-damascenone, together with γ-undecalactone, have the highest binding constants (Table 2). Nevertheless, the difference in binding constants for β- and α-ionone was 5687 M^{-1}, an effect which cannot be explained by the hydrophobicity of these two substances. More likely it indicates a specific binding of β-ionone in comparison with that of α-ionone.

Comparing the results obtained by our affinity-chromatography method with those of the published data, using other techniques, there is some agreement for substances with low binding constants (Table 3). Only β- and α-ionone differ greatly from that of the published data. A possible explanation for these differences could be the behaviour of free BLG in solution and of the immobilized protein. Immobilization of the protein could induce steric hindrance of binding sites leading to a decrease in binding constant or conformational changes leading to a better accessibility of other binding sites. In addition, the published data were also obtained using different analytical conditions. O'Neill and

Table 3. Comparison of the binding constant K_B obtained by different methods

Flavour compound	Affinity-chromatography [M^{-1}]	Reference [M^{-1}]	Method applied (authors)
2-Heptanone	465	150	Equilibrium dialysis (O'Neill and Kinsella, 1987)
2-Octanone	1287	500	Equilibrium dialysis (O'Neill and Kinsella, 1987)
2-Nonanone	3629	2440	Equilibrium dialysis (O'Neill and Kinsella, 1987)
		1250	Static headspace (own work; Charles *et al.*, 1996)
Methyl benzoate	1005	1050	Hummel and Dreyer — HPLC (own work, unpublished)
β-Ionone	19143	1670000	Fluorescence (Dufour and Haertlé, 1990)
α-Ionone	13456	0	Fluorescence (Dufour and Haertlé, 1990)

Table 4. Influence of the addition of β-ionone, methyl benzoate and γ-octalactone to the eluent on the retention time of several flavour compounds

Flavour compound	Percentage decrease of retention time when the eluent contains:		
	30 mg $litre^{-1}$ β-ionone	50 mg $litre^{-1}$ methyl benzoate	50 mg $litre^{-1}$ γ-octalactone
β-Ionone	—	1.3	6.6
α-Ionone	0.6	17.0	22.6
β-Damascenone	2.9	19.1	22.7
2-Octenal	n.d.	8.1	14.1
2-Nonenal	2.8	20.0	25.7
2-Heptanone	2.8	5.0	12.8
2-Octanone	n.d.	31.3	15.2
2-Nonanone	n.d.	n.d.	22.2
γ-Octalactone	18.4	11.6	—
δ-Octalactone	7.9	8.7	12.8
Methyl benzoate	0.6	—	13.4

n.d., not determined

Kinsella (1987) worked with a purified and lyophilized BLG in solution (1% in 20 mM phosphate buffer, pH 6.7). In these conditions, BLG is mostly dimeric (Relkin, 1996) and lyophilization could induce some changes in the protein conformation. That no binding was observed with α-ionone using the fluorescence method (Dufour and Haertlé, 1991) supports our view that the binding mechanisms for β- and α-ionone are different. In that case, binding of α-ionone cannot be measured by following the fluorescence quenching of protein tryptophans.

Using our affinity-chromatography method, it was possible to demonstrate the influence of one compound on the retention time of another. The substance with the strongest interaction, β-ionone, when dissolved in the eluent at a concentration of 30 mg litre^{-1} (Table 4) had little effect on the binding of α-ionone, β-damascenone, methyl benzoate, unsaturated aldehydes and ketones. However, β-ionone does induce a significant decrease in the retention time of γ-octalactone and to a lesser extent of δ-octalactone. Under our experimental conditions γ-deca- and γ-undecalactone, as well as the unsaturated alcohols, could not be detected due to the large UV-absorption of the added β-ionone. Furthermore, methyl benzoate at a concentration of 50 mg litre^{-1} in the eluent reduced the retention times of all the other compounds except of β-ionone and this decrease was strongest for hydrophobic substances. On the other hand, γ-octalactone, at 50 mg litre^{-1} reduced all the retention times including that of β-ionone. The reduction in retention times for most compounds was between 13 and 22%. These reductions seem to indicate two different binding mechanisms: (i) specific binding in a hydrophobic pocket for β-ionone as described before by Dufour and Haertlé (1991). From the mutual influence of β-ionone and, δ- and γ-octalactone it can be deduced that these lactones also bind to some extent into this pocket; and (ii) unspecific hydrophobic interactions for the other compounds including lactones and α-ionone. In the case of α-ionone, there is no interaction with the protein tryptophans as is the case for β-ionone. This confirms our first hypothesis that the binding mechanisms for these two compounds are different.

Affinity-chromatography has therefore been successfully applied to the investigation of flavour–protein interactions. It is now possible to rapidly screen a large number of flavour solutions: the use of the technique is only limited by the solubility of compounds in the eluent, and a sufficiently strong UV-absorption. The binding constants obtained by this method allow a quick comparison of the different chemical classes. Furthermore, our results indicate the presence of at least two different binding mechanisms in agreement with those already discussed by Dufour *et al.* (1990) for retinol and protoporphyrin IX.

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