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Food Chemistry 71 (2000) 301–308

Food Chemistry

www.elsevier.com/locate/foodchem

Interactions between β-lactoglobulin and flavour compounds

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Received 4 January 2000; received in revised form 11 April 2000; accepted 20 April 2000

Abstract

The aim of this study was to report on the interactions between a well characterised macromolecule, β-lactoglobulin, and a wide range of flavour compounds, in different media. Flavour compounds interacting with the protein were screened using different methods, and thermodynamic constants were calculated. Within the same chemical class, the affinity constant increased with increasing chain length, suggesting hydrophobic interactions. The influence of ethanol, NaCl, pH and temperature were discussed as a function of the protein conformation. The influence of β-lactoglobulin at the oil–water interface, on the release of different flavour compounds, was investigated in model oil/water emulsions. Even if the addition of fat induces a greater change in flavour release than the addition of protein in water solution, β-lactoglobulin at the oil/water interface limits the transfer of hydrophobic compounds from oil to water and thus induces a lower flavour perception.

1. Introduction

Flavour perception in foods is highly influenced by interactions between flavour compounds and a variety of non-flavour matrix components. Understanding the mechanisms which influence flavour binding or release behaviour of flavour compounds from the food matrix is of major significance for improving flavour quality. The strength and the nature of the interactions will affect the release of the aroma during food processing or consumption.

The aim of this study was to determine the interactions between a well characterised macromolecule, and a wide range of flavour compounds, in different media. In order to apply the results to real food systems, such as dairy products, a milk protein, β-lactoglobulin (BLG) was chosen since it is the most extensively characterised and best described whey protein (Batt, Brady & Sawyer, 1994) and it has good emulsifying properties. This globular protein belongs to the superfamily of retinol binding proteins (Papiz et al., 1986). The existence of two independent binding sites was proposed by Narayan and Berliner (1997). The main binding site for fatty acids is located in the central cavity, as demonstrated by Wu, Perez, Puyols and Sawyer (1999) after co-crystallisation of BLG with palmitate. This protein is known to interact with many flavour compounds, such as aldehydes and ketones (O'Neil & Kinsella, 1987), ionones (Dufour & Haertlé, 1990) and hydrocarbons (Wishnia & Pinder, 1966), however, no information is available on the mechanisms involved in the binding.

This paper reports the most relevant results obtained by the different participants to the French DGAL programme and COST action 96, who worked on the same batch of BLG.

2. Materials and methods

2.1. Materials

All chemicals used were reagent grade. BLG (variants AB, purity 90%) from the same batch was provided by Besnier-Bridel (France). Flavour compounds from different chemical classes were obtained from IFF. Some homologous series were studied in order to find out rules governing retention.

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0308-8146/00/$ - see front matter
PII: S0308-8146(00)00181-3

2.2. Experimental

2.2.1. Affinity chromatography

Immobilisation of the protein on the Silica-Diol support and calculation of binding constants Kb were already described by Sostmann and Guichard (1998). Silica gel coated with immobilised BLG was filled under vacuum (water pump, 30 mbar) into an empty polyether ether ketone (PEEK) column (4.3 mm×5 cm) obtained from Touzart and 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.

2.2.2. Headspace analysis

2.2.2.1. Aqueous solutions. Analyses were done in triplicate in amber flasks (40 ml) closed with mininert valves (Supelco). Analysed solutions (10 ml), with or without protein (3%), were stirred at 30°C until equilibrium. The vapour phase (1 ml) was injected on a Carlo Erba 8000 gas chromatograph equipped with a DB-Wax column (J&W Sci., i.d. 0.32 mm, 30 m, film thickness 0.5 μm). Temperatures of injector and detector were respectively 250 and 260°C. The H_2 carrier gas velocity was 1.9 ml min^{-1}.

2.2.2.2. Emulsions. Three protein concentrates were tested as emulsifier: α-lactalbumin (PSDI 4200, MD-Food), β-lactoglobulin (Besnier Bridel) and a mixture of α-lactalbumin and β-lactoglobulin (73:27, w:w). Proteins were solubilised in a citric acid / sodium citrate buffer (0.1 M; NaCl 25 mM, pH 3) at a concentration determined in order to have 0.5% of protein in the final emulsions (30% oil). Flavour compounds (40 mg), protein solution (31.5 g) and sunflower oil (13.5 g) were equilibrated (30°C, 1 h) before homogenization with an Ultra-Turax at 8000 rpm for 3 min at 5°C.

Table 1
% Binding of flavour compounds by β-lactoglobulin (BLG) measured by headspace methods at pH 3[a]

	Exponential dilution	Static headspace
Acetophenone	10 (c)	11 (a)
Benzaldehyde	16 (c)	23 (a), 20 (b)
Linalool	17 (c)	7 (a), 15 (b)
2-Heptanone	7 (c)	20 (a)
2-Octanone	18 (c)	42 (a)
2-Nonanone	41 (c)	60 (a), 56 (b), 44 (d)
Ethyl hexanoate	34 (c)	49 (a), 39 (c)
Ethyl octanoate	49 (c)	46 (c)
Ethyl nonanoate	21 (c)	40 (c)
Isoamyl acetate	1 (c)	16 (a), 14 (b), 7 (d)
Limonene	−6(c)	−44 (c)

[a] 3% BLG : (a) Charles, Bernal and Guichard, 1996; (b) Espinoza-Diaz, 1999; 2% BLG : (c) Jouenne and Crouzet, 1996; (d) Roozen and Legger, 1998.

Headspace analysis was done on aliquots of 10 ml emulsions, at equilibrium (30°C) without stirring.

2.2.3. Sensory analysis

A trained panel composed of 16 assessors performed sensory evaluations in a tasting room equipped with booths. Computerized data acquisition and treatment were done with the Fizz software (Biosystemes, Dijon, France). Training consisted of ranking of reference aroma compounds at increasing concentrations (eight different concentrations for each aroma compound). Assessors were then asked to evaluate the odour intensity of the aroma without or with protein (1%), one compound per session, on a linear scale according to the eight reference samples.

3. Results and discussion

3.1. Comparison of the percentage of binding of aroma compounds by BLG obtained by headspace methods

The percentage of flavour compounds bound to BLG (Table 1) measured at pH 3 by a headspace method, static or dynamic, are in good agreement. The differences observed are mainly due to the difference in the percentage of BLG used (2 or 3%). An increase in the percentage of BLG always induces an increase in the percentage of binding, except for linalool. For limonene, a salting out effect was observed in the presence of protein. However using dynamic coupled column liquid chromatography (DCCLC) method, retention was observed. In fact, the methods do not measure the same parameters. DCCLC does not consider the transfer from the dilute solution to the gas phase and it can be assumed that this method provides a direct measurement of molecular interactions (Jouenne & Crouzet, 1996, 1998; Langourieux & Crouzet, 1995).

Binding of the studied flavour compounds could be of a different nature. For example, an increasing percentage of retention on increasing chain length for the series of two-alkanones and ethyl esters suggests hydrophobic binding, whereas benzaldehyde could be partially covalently bound (Sostmann, Bernal, Andriot & Guichard, 1997). Therefore, headspace measurements only give some information on the mechanism involved for the binding and other additional methods have to be used to determine the nature of the binding.

3.2. Comparison of binding constants of aroma compounds with BLG obtained by different methods

Table 2 presents the thermodynamic parameters calculated by exclusion chromatography, equilibrium dialysis, static headspace, fluorimetry, dynamic coupled column liquid chromatography and affinity chromato-

Table 2
Comparison of binding constants obtained with different methods on the same batch of BLG (pH = 3)[a]

	Exclusion chromatography (a)			Equilibrium dialysis (b)			Static headspace (c)			Fluorimetry (d)			Dynamic coupled column liquid chromatography (e)	Affinity chromatography (f)
	n	Ka	nKa	n	Ka	nKa	n	Ka	nKa	n	Ka	nKa	nKa	nKa
Benzaldehyde	0.3	1800	540 (h)				0.57	533	304 (h)	0.8	2.5×10^7	2×10^7 (g)		341
2-Nonanone				0.5	1756	969 (i)	0.5	1667	833					
Methyl benzoate	0.9	280	250											
Ethyl benzoate	0.8	1339	1110											297
Ethyl hexanoate							0.85	1018	863					677
Isoamyl acetate							0.08	7510	627					543
Limonene													2610	153
α-Ionone													170	13 000
β-Ionone				0.9	11 700	10 550				0.8	1.9×10^6	1.5×10^6	520	19 000
γ-Undecalactone				1.0	5900	5900				1.0	8900	8900		10 000

[a] (a) Pelletier, Sostmann and Guichard, 1998; (b) Muresan et al., 1999 (pH 7.1); (c) Charles et al., 1996; (d) Muresan and Leguijt, 1998 (pH 7.1); (e) Jouenne and Crouzet, 1998; (f) Sostmann and Guichard, 1998; (g) Marin and Relkin, 1998 (pH 6); (h) Guichard, non published data; (i) Espinoza-Diaz, 1999.

graphy. As the protein is under a dimeric form at pH 3, the number of binding sites calculated for 2-nonanone (0.5 mol per mol of BLG) means that there is 1 mol of ketone bound per dimer. Except for isoamyl acetate, the number of binding sites calculated for the esters is close to 1, which means 1 mol bound per monomer. Therefore, the mechanism of binding should be different from that found for 2-nonanone. Even if affinity chromatography only gives a value for global affinity (nKa), this value is comparable with those obtained with the other methods, when available. However, these values are often lower which may indicate that some binding sites could be less accessible due to the immobilisation of the protein onto the chromatographic support.

The large discrepancy observed between the binding constants obtained through a fluorimetric method and the other mentioned methods cannot be only attributed to the difference in pH of the solution. It appears that the constant estimated by means of fluorescence spectroscopy is overestimated for some ligands such as β-ionone. This is due to excessive quenching that occurs even if the ligand is not in direct contact with the BLG fluorophores (Muresan, de Wolf, van der Bent & Leguijt, 1999). This is not the case for γ-undecalactone. In addition, there is also a mismatch between the values of the binding constant obtained with affinity chromatography and DCCLC. The bad fit was not expected and has probably something to do with principles of the two methods. There is some evidence that α-ionone could bind to BLG. This is not in agreement with the previous work of Dufour and Haertlé (1990). These authors assumed that α-ionone did not bind to BLG since it did not induce any quenching of the protein fluorescence. It seems rather that there is not enough vicinity between the tryptophan (TRP) residue and α-ionone so that fluorimetry is unable to detect the interaction between BLG and α-ionone. The difference in the binding constants between β-ionone and α-ionone is not related to hydrophobicity since it is similar for both compounds. There is a structural feature which might account for a specific binding of β-ionone in comparison with that of α-ionone. The conjugation of the cyclohexenyl ring double bond with the double bond of the isoprenoid tail could bring some structural constraints which would explain this difference (Dufour & Haertlé, 1991). Jouenne and Crouzet (1998) also gave some evidence in favour of a specific binding of β-ionone. They showed that the binding constant of β-ionone was higher at pH 11 than at pH 3. At pH 11, the secondary structure of the peripheric zone of the β-barrel is destroyed. The β-barrel itself is also highly affected and hydrophobic interactions are no longer possible. This means that the β-ionone ring links with the TRP 19 residue in a manner involving a stronger binding. This assumption is supported by the work of Fugate and Song (1980) who showed that the binding of retinol was not affected by the presence of 8 M urea or sodium dodecyl sulfate.

3.3. Relation between the binding properties and the hydrophobicity of aroma compounds

A good linear correlation was found between the logarythm of the binding constant measured by affinity chromatography and hydrophobicity of the molecule, for series of ketones, aldehydes, alcohols and lactones (Fig. 1) and for series of methylic, ethylic, propylic and butyric esters (Fig. 2). These compounds may bind into the hydrophobic pocket of the protein, by hydrophobic interactions. However, for terpene alcohols and phenolic compounds, such a linear relationship could not be obtained (Reiners, Nicklaus & Guichard, 2000),

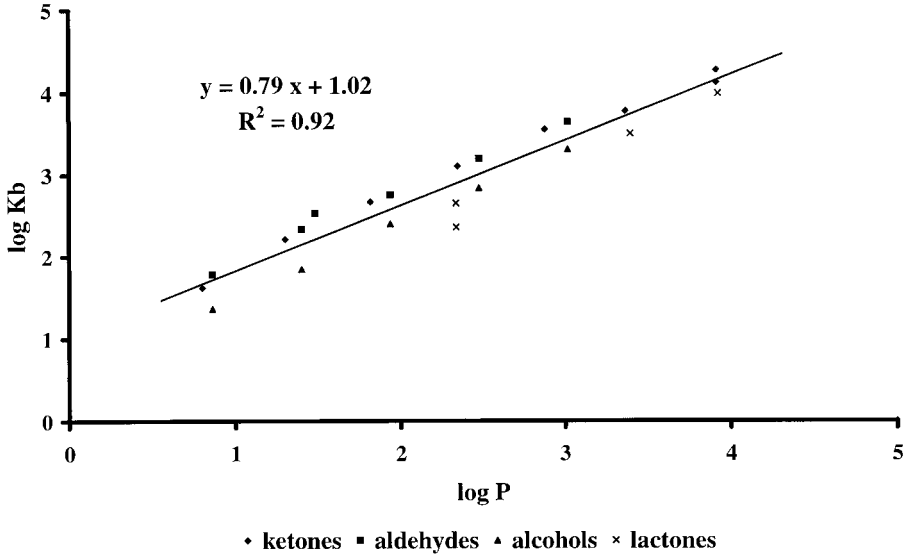

Fig. 1. Interactions between β-lactoglobulin and ketones, alcools, aldehydes, lactones. relation between the logarithme of the binding constant (log *Kb*) obtained by affinity chromatography (Sostmann & Guichard, 1998) and the hydrophobicity (log *P*) of the flavour compound.

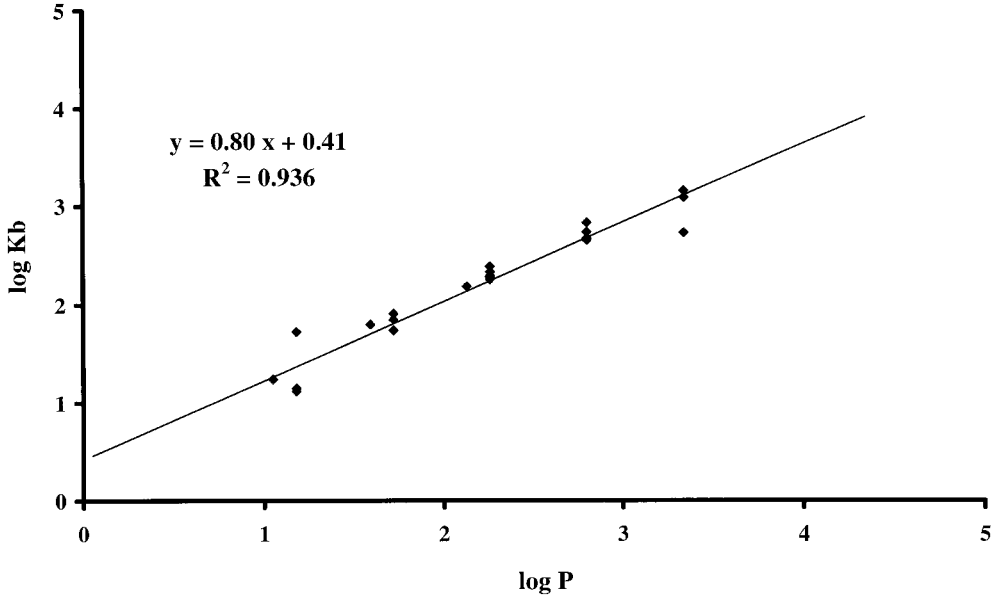

Fig. 2. Interactions between β-lactoglobulin and esters, relation between the logarithme of the binding constant (log *Kb*) obtained by affinity chromatography (Pelletier et al., 1998) and the hydrophobicity (log *P*) of the flavour compounds.

showing that the stereochemistry of the molecule influences the binding, as already discussed for β-ionone and α-ionone.

3.4. Competitions between ligands: different binding sites?

Different studies on the competition between ligands were undertaken. The binding of 2-nonanone induced a decrease of the binding of ethyl hexanoate and vice-versa (Jouenne, Chalier & Crouzet, 2000). These two ligands are in competition for their binding into the hydrophobic pocket of the protein. Muresan and Leguijt (1998) found competitions between γ-undecalactone, β-ionone and retinol, which was in agreement with the competitions between γ-octalactone and β-ionone found by Sostmann and Guichard (1998). Moreover, Sostmann and Guichard found no competition between β-ionone and α-ionone. The fixation of the two ionones did not induce the same conformational changes on the protein, as shown by Lübke, Guichard and Le Quéré (in press), using infrared spectroscopy. This is another confirmation that the binding mechanism should be different for these two compounds.

3.5. Influence of the medium on the retention

The influence of pH on the retention of aroma compounds by BLG has been extensively studied by Jouenne and Crouzet (1996, 2000). The retention of methyl ketones increased from pH 3 to 9, due to the flexibility modification of the protein (Shimizu, Saito & Yamauchi, 1985), allowing a better accessibility to the primary or secondary binding sites. The decrease in retention observed at pH 11 was a consequence of the alkaline denaturation of the protein.

Fig. 3 points out the effect of salt content, ethanol and temperature on the percentage of bound benzaldehyde to BLG (Andriot, Marin, Feron, Relkin & Guichard, 1999). The decrease of the retention observed in the presence of salt could mainly be explained by a salting out effect. Indeed, without protein the volatility of benzaldehyde in the presence of salt was higher than in water. Another explanation could be that the change in the polarity at the protein surface in the presence of salt may influence the retention of benzaldehyde. No effect of ethanol was observed in the presence of salt. In fact ethanol increases the solubility of benzaldehyde, which decreases its volatility, in contrast to the salting-out effect of NaCl. No effect of heat treatment was observed in water. However, when the heat treatment was realised in the presence of salt or ethanol, the percentage of retention was divided by two. In this case a denaturation of the protein may occur, leading to a change in the viscosity and cloudiness of the protein solution.

3.6. Incidence of the binding properties on aroma perception

Fig. 4 shows the results obtained for five aroma compounds presenting different binding properties. No significant effect was found for vanillin, the compound with the lowest affinity for the protein. The same observation was made by Dolezal and Velisek (1999) who found no direct correlation between the perception of vanillin in water solutions with different amounts of BLG, and the concentration of BLG. On the other hand, the odour of eugenol, a compound which has a higher affinity for BLG, is significantly reduced by addition of BLG. In the case of methyl ketones, we were expecting the most significant effect for 2-nonanone, compound with the highest affinity for BLG, but it was not the case. In fact BLG solutions are odorant and the panelists found that it was difficult to perceive the differences between the odour of 2-nonanone and that of BLG, in contrast to the other ketones. Another explanation could be that due to an extensive binding of 2-nonanone to BLG, other compounds could be released, which might be responsible of the odour of BLG. However, this experiment showed a significant decrease in the odour perception of aroma compounds which present a high affinity for BLG.

Differences in aroma perception were also noticed when BLG was handled as a gel. The influence of different parameters (i.e. pH, salt concentration and heating rate) on gel microstructure and banana flavour release have been investigated (Langton, Ramard & Leufvén, 1998; Pålsgård Jones & Dijksterhuis, 1998). These authors showed that gels with an open and irregular structure were perceived as having a stronger banana flavour and aftertaste than gels with an homogenous and dense microstructure. However, in the case of gels, it is difficult to confirm whether flavour entrapment occurs due to diffusion control in a macromolecular network rather than to binding, or whether both mechanisms occur. If denatured BLG is still able

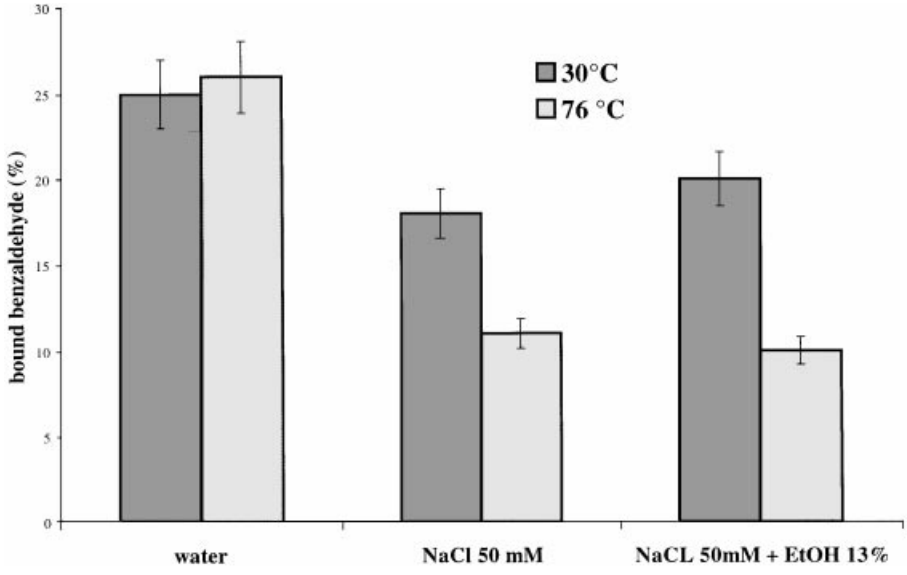

Fig. 3. Interactions between β-lactoglobulin and benzaldehyde, incidence of the medium on the percentage of bound flavour (Andriot et al., 1999).

to bind aroma compounds, there could be an intra- and inter-molecular binding. There is some evidence that heat-denatured BLG is also able to bind aroma compound when no gel is formed (Fig. 3). Marin and Relkin (1998) also showed, using a fluorimetric method, that the amount of bound benzaldehyde to BLG increased after heat treatment of BLG at 76°C for 10 min.

3.7. Incidence of BLG at the oil–water interface

It was important to point out the influence of interactions between aromas and BLG in a more complicated system, including oil. Most of the flavour compounds are hydrophobic and thus more soluble in oil than in water. Moreover, lipids interact with proteins

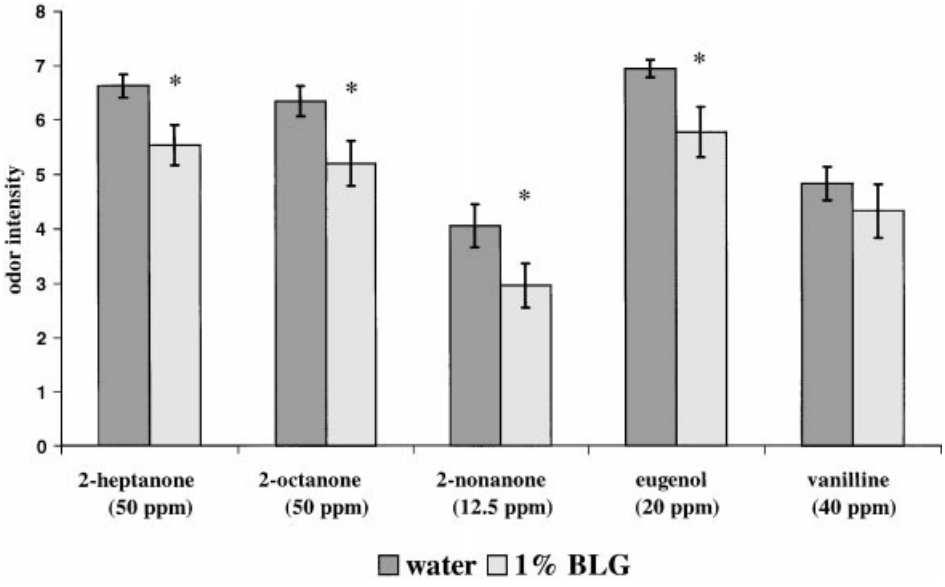

Fig. 4. Influence of the presence of β-lactoglobulin on the odour perception of different flavour compounds (Reiners et al., 2000; Andriot, Harrison

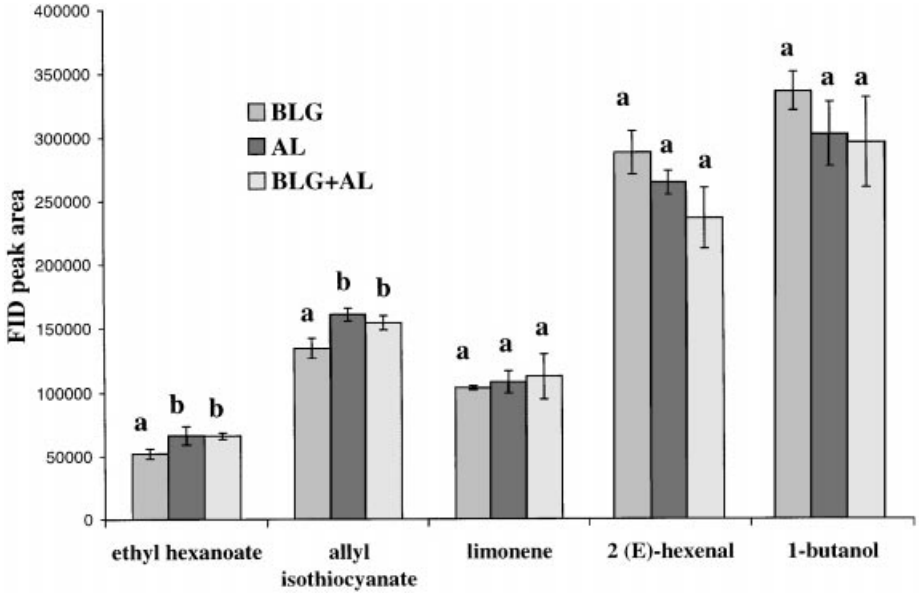

Fig. 5. Static headspace analysis of flavour compounds. Effect of the nature of the protein used as emulsifier on flavour release from an oil-in-water (30/70) emulsion (0.5% protein in citric acid/sodium citrate buffer 0.1 M, NaCl 25 mM, pH 3). BLG = β-lactoglobulin; AL = α-lactalbumin; BLG + AL = mixture of BLG and AL (27:73, w:w). Peak areas with the same letters are not significantly different at the 5% level (Charles, Lambert et al., in press).

and should be in competition with flavour compounds for the same binding sites. Different studies were carried out to show the influence of BLG, in binary oil/water systems, or in emulsions.

Rogacheva, Espinoza-Diaz and Voilley (1999) showed that the presence of BLG at the oil–water interface increases the resistance to the transfer of aroma compounds, in the function of the hydrophobicity of the aroma, the diffusion and partition coefficients. In the case of benzaldehyde, the overall resistance was mainly due to the transfer through the oil–water interface, which could be explained by strong binding (Andriot et al., 1999). Castelain and Dumont (personal communication) showed that the rate of transfer of benzaldehyde from water-to-oil and oil-to-water decreased in the presence of BLG. For 2-nonanone there was also an increase in the interfacial resistance between oil and water, explained by a lower oil–water partition coefficient in the presence of protein.

When whey proteins were used as emulsifiers (Charles, Lambert, Brondeur, Couthauden & Guichard, in press), the nature of the protein had an effect on flavour release from the emulsion (Fig. 5). Flavour release of ethyl hexanoate and allyl isothiocyanate was significantly lower from emulsions with β-lactoglobulin than from emulsions with α-lactalbumin or a mixture of both ($P < 0.05$). For the other flavour compounds (which present a lower affinity for BLG), no significant differences were observed. The decrease in flavour release could partly be explained by a limitation of the transfer from oil to water at the protein interface and then by interactions of these compounds with the protein in the bulk phase. These results obtained on model systems contributed to the explanation of the differences in flavour perception found in real salad dressings (Charles, Rosselin, Sauvageot, Beck & Guichard, in press).

4. Conclusion

The presence of β-lactoglobulin in aqueous solutions induces a decrease in the volatility of the most hydrophobic flavour compounds, mainly due to hydrophobic interactions into the central cavity of the protein. This retention induces a significant decrease in the odour perception.

In emulsions, the presence of β-lactoglobulin at the oil/water interface increases the resistance to mass transfer for the most hydrophobic compounds, which may induce some persistence of the flavour notes during eating.

Acknowledgements

This programme was a collaborative study with ENSBANA-Dijon (A. Voilley), ENSIA-Massy (P. Relkin), GBSA-Montpellier (J. Crouzet) and INRA-Nantes (J.P. Dumont), partly financed by the French Ministery of Agriculture and Fisheries, and a collaboration within COST action 96 "Interactions between food matrix and small ligands influencing flavour and texture", ETH-Zürich (S. Langourieux and F. Escher), Wageningen Agricultural University (J. Roozen), ATO-DLO-Wageningen (T. Leguijt), University-Prague (J. Velisek). The author thank all the scientists and their collaborators for their participation. The authors thank D. Aravantinos (IFF-Dijon) for providing the flavour compounds and Besnier-Bridel for the BLG samples.

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