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Effects of Hydration, Lipids, and Temperature on the Binding of the Volatile Aroma Terpenes by β-Lactoglobulin Powders

NIKOLAY A. MIRONOV,† VLADIMIR V. BREUS,† VALERY V. GORBATCHUK,† BORIS N. SOLOMONOV,† AND THOMAS HAERTLÉ*,§

Department of Chemistry, Kazan State University, Kremlevskaja 18, Kazan 420008, Russia, and Laboratoire d'Etude des Interactions des Molécules Alimentaires, Institut National de la Recherche Agronomique, B.P. 71627, 44316 Nantes, Cedex 3, France

The binding properties of dry proteins are relatively poorly known. Many proteins are present in emulsions and suspensions and also in dry forms. This is particularly true of dairy proteins, which are often stored and sold in powdered form. In the present work, the binding of three terpenes (α-terpinene, γ-terpinene, and terpinolene), which belong to the basic aroma components, and of decane by powdered β-lactoglobulin (BLG) was studied at different hydration levels (0.05−0.40 g of H_2O/g of protein) and temperatures (298 and 309.5 K), in the presence or absence of lipids and small concentrations of ethanol. Vapor sorption isotherms were determined for these systems by a static method of headspace gas chromatographic analysis. A cooperative effect of hydrophobic hydration was observed for the binding of aroma terpenes and decane by the solid BLG. The temperature increase from 298 to 309.5 K reduced the observed hydration threshold of BLG by 0.05−0.08 g of H_2O/g of protein. Lipids (1.2% w/w) in hydrated BLG gave at least a 2-fold increase in its binding affinity for the hydrocarbons studied, and synergic effects of the hydration and lipid on this affinity were observed.

KEYWORDS: Aroma binding; static headspace gas chromatography; vapor sorption isotherms; hydrophobic hydration; hydration threshold

INTRODUCTION

The ability of solid, dried proteins to bind different ligands in the absence of contact with liquid water (*1*−*3*) is important for the description of their behavior in solid foods or food additives. Animal and plant proteins, for example, milk proteins and flours, are often stored in dry powder form. Dairy protein powders are frequently used as ingredients in foods such as instant milks, creamers, milk chocolates, coffees with milk, and cappuccinos. Many of these preparations contain lipids and are aromatized or would have much better appeal in the aromatized form. Finally, their shelf life, even in the dried form, may be a function of their degree of hydration.

Hydration is also one of the factors defining the ligand binding of dried proteins. Protein hydration controls the kinetics of enzymatic reactions (*1*, *2*) and influences the ability of antibodies to bind antigens (*3*) in low-water conditions. Hydrophobic hydration has an attractive effect in the process of protein folding (*4*). Some studies show the presence of ordered water inside the protein binding sites (*5*), around bound organic molecules (*6*), and hydrophobic groups of amino acid residues in protein crystals (*7*). These observations imply a crucial role played by water in ligand binding by proteins (*5*). Such conclusions cannot be considered, however, as entirely proved without direct thermodynamic evidence. Most of the available thermodynamic data describing the hydration effect were obtained for binary protein−water systems (*4*, *8*). Additionally, attractive and cooperative hydration effects were observed for the binding of vapors of relatively hydrophobic compounds (benzene, dioxane, and propanols) by solid albumin (*9*).

The effect of temperature on the binding of ligand vapors by solid proteins has not previously been studied. Temperature effects were investigated for the sorption of water (*10*−*12*) and some nonpolar gases (*13*) by dried proteins.

Fatty and defatted protein powders are generally different sorbents for volatile aroma compounds because lipids in a separate phase can compete with proteins for the hydrophobic substrates. The ability of lipids to compete with other ligands for the binding sites of proteins was studied in water solutions (*14*−*16*) but not for solid dry proteins. The presence of lipids may be essential for protein stability (*17*, *18*) and thus may have an influence on the ligand-binding properties of solid proteins in foods.

This explains the interest of the present work to study the combined effects of hydration, temperature, and lipids on the binding of volatile hydrocarbons (mostly aroma terpenes obtained from citrus oils) from the vapor phase by dried and hydrated solid β-lactoglobulin (BLG). The thermodynamic study

* Author to whom correspondence should be addressed (fax +33 240 67 5244; e-mail haertle@nantes.inra.fr).
† Kazan State University.
§ Institut National de la Recherche Agronomique.

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of such systems can be more informative than the study of the aqueous solutions or suspensions of proteins in liquid organic solvents because of the independent variation of the thermodynamic activities of components in the absence of a liquid phase. Solid BLG, together with fats, is an essential component of many solid milk byproducts, end-products, and ingredients, for which the binding or release of odorants takes place at various temperatures and humidities.

MATERIALS AND METHODS

Materials and Sample Preparation. All measurements were made using bovine β-lactoglobulin (Protarmor 907 NK, Armor Protéines S.A.S.) obtained as described elsewhere (*19*). This industrial BLG preparation contained >90% protein (75% BLG and 15% other proteins, mostly glycomacropeptides), 0.6% lactose, and 1.2% fats by weight. Its ash content was 4.5%. It was used for studies in two forms: (1) without further purification and (2) after the extraction of fats with chloroform at room temperature (~23−24 °C). There were two extraction cycles. First, protein (30 g) was mixed for 1 h with 300 mL of chloroform, filtered, and then kept in the same volume of fresh chloroform for 24 h. Extraction at room temperature was preferred because extraction with organic solvents at 50−60 °C can cause a partial denaturation of protein (*20*). Both the initial un-defatted and defatted protein preparations were dried in a vacuum desiccator over P_2O_5 at 0.1 kPa for 3 weeks. Water solutions of the defatted and initial protein preparations had identical FTIR spectra in the range of amide I and amide II bands.

The terpenes studied were purchased from Aldrich (α- and γ-terpinenes, purity = 85 and 97%, respectively) and Fluka (terpinolene, purity ~ 90%). α-Terpinene and terpinolene were further purified by vacuum distillation in an argon atmosphere to the same degree of purity (97%) verified by GC analysis. Other organic compounds were of reagent grade purity (>99%) and were dried according to standard methods (*21*) before the experiment.

Equal portions of protein were placed in a series of 15-mL vials and dried in a desiccator over P_2O_5 at 1 kPa. The final weight of dried BLG in each vial was ~300 mg. The water content of the dried protein was determined from the loss of weight at room temperature and at 0.1 Pa by a micro-thermoanalyzer MGDTD-17S (Setaram). A protein sample was dried until its weight (~10 mg) did not change within 0.1% during 5−6 h. The loss of weight observed in this experiment was taken as the initial protein hydration value. The calculated masses of dry BLG in each vial for the sorption experiment were 274.5 and 276.9 mg for the defatted and un-defatted preparations, respectively.

The samples used for the vapor sorption studies were prepared by the vapor phase equilibration of dried protein powder with sorbates. To prevent direct contact between the liquid sorbate and solid protein, the liquid organic component or water plus ethanol plus hydrocarbon mixture was dispensed with a microsyringe into small open glass containers placed inside the 15-mL vials containing the dried protein preparation. For the binary protein−sorbate systems, the volume of added liquid was in the range of 1−40 μL for acetonitrile and 1−5 μL for decane. The volume of added water−organic mixture was in the range of 10−101 μL. Then the vials were sealed, without stirring, with silicone and poly(tetrafluoroethylene) (0.2 mm) linings and were kept at 298, 309.5, and 323 K for 72 h in the case of systems with acetonitrile and at 298, 309.5, and 318 K for 9−11 days in the case of systems with decane and terpenes. Estimation of sorption kinetics showed that, for decane and the terpenes studied, the equilibration time depends mostly on their rates of evaporation. Once evaporation had finished, the sorbate activity ceased to vary, within experimental error, during the first 24 h. Each studied system was equilibrated for at least 48−72 h after the end of the evaporation of all added liquid. For the determination of each sorption isotherm, a fresh portion of fatty or defatted BLG from the same lot of industrial protein was taken.

In the study of the protein hydration effect on the binding of hydrocarbons, the added liquid was a mixture of 90.1 vol % of water, 9.0 vol % of ethanol, and 0.9 vol % of hydrocarbon. In such cases, the protein hydration value was calculated as the total water content in the system including the initial protein hydration, the added water, and the water content of the vapor phase of a 15-mL vial at the water activity of 0.5. The last contribution is negligibly small and can contribute <0.0006*h* (g of H_2O/g of of protein), 0.0011*h*, and 0.0019*h* to the error of the protein hydration value at 298, 309.5, and 318 K, respectively, at the 100% error of the estimated water activity. The estimated error of the final protein hydration values is ~0.006*h*.

Determination of Vapor Sorption Isotherms. The vapor sorption isotherms were obtained by the static method of headspace gas chromatographic analysis as described earlier (*22*). This method was used to determine the sorbate uptake and thermodynamic activity in the equilibrated systems studied. In the headspace of these systems, no volatile organic impurities were detected above the protein samples. The error of the sorbate activity P/P_0 determination was in the range of 5% for $P/P_0 > 0.5$ and 10% for $P/P_0 < 0.1$. More than half of this error is systematic for the points of one sorption isotherm. It is caused by the error in the determination of peak size in the case of a saturated vapor sample of the pure liquid sorbate (2−3%) and because of the nonlinearity of the headspace dosing in the chromatographic capillary column (3−5% at $P/P_0 < 0.10$).

The sorbate uptake A (millimoles of sorbate per gram of dry protein preparation) was calculated as the difference between the total amount of sorbate in the system and its amount in the vapor phase, using the experimental value of sorbate activity and the sorbate saturated vapor pressure (*23*, *24*). The saturated vapor pressure P_0 of α- and γ-terpinene was estimated from the ratio of the heights of the chromatographic peaks measured for the headspace samples of the pure liquid terpinene and decane. In this estimation, the relative sensitivity of the flame ionization detector used for terpinenes and decane was also determined. The following values for the saturated vapor pressure P_0 were obtained: 0.22, 0.41, and 0.76 kPa at 298, 309.5, and 318 K for α-terpinene and 0.10 and 0.21 kPa at 298 and 309.5 K for γ-terpinene, respectively.

The error of the sorbate uptake A determination depends mainly on the accuracy of dosing liquid sorbate in the vials. It is true for a part of the sorption isotherms in which the sorbate fraction in the vapor phase is <10% of the total. This is the case for acetonitrile isotherms at sorbate activity $P/P_0 < 0.60$ at 298 K and $P/P_0 < 0.30$ at 309.5 K (the error of A is 3−5%), for all points of ethanol isotherms (the error is 4−6%), and for isotherms of terpenes and decane (the error is 5−7%) measured at high BLG hydration (>0.20*h*). This error is equal to 10−13% in the case of hydrocarbons studied in the systems containing low hydrated BLG (<0.10*h*), where the sorbate fraction in the vapor phase is 30−50% for defatted protein and 20% for fatty BLG.

Determination of the "Pure Liquid−Solution in Lipids" Partition Coefficients for Sorbates. The values of the coefficients of sorbate partition $A/(P/P_0)$ between its pure liquid and solution in lipids were determined for lipids extracted from the initial BLG preparation according to the procedure described under Materials and Methods. The samples of the concentrated lipid extracts in chloroform (25 mg/5 mL) were first dried in 15-mL vials at room temperature and then in a vacuum of 1 kPa until constant weight. The liquid solutes (sorbates) were equilibrated with samples of dried fats for 3 and 24 h. Then the molal concentration A (in moles per kilogram or millimoles per gram) of solute in lipids and its activity (P/P_0) were determined by headspace analysis as described above for vapor sorption isotherms. The solution of decane in lipids was studied also at unity of water activity (the presence of a liquid water phase after equilibration was checked visually) and in the presence of ethanol with activity $P/P_0 = 0.6$. No effect of water and ethanol on the $A/(P/P_0)$ value of decane was observed within experimental error (±10% for all studied solutes).

RESULTS AND DISCUSSION

Sorption Isotherms for Dried BLG. The binding properties of the dried fatty (hydration 0.05*h*, 1.2% w/w of lipids) and dried defatted BLG (0.05*h*) preparations were studied in the systems containing hydrophilic and hydrophobic sorbates at two temperatures. Vapor sorption isotherms of acetonitrile and decane were determined at 298 and 309.5 or 323 K (**Figure 1**).

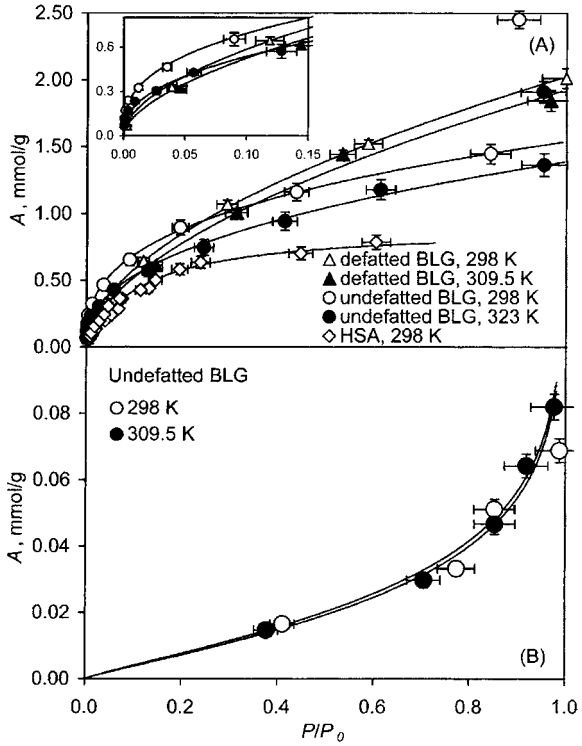

Figure 1. Sorption isotherms of acetonitrile (A) and decane (B) on the dried un-defatted (with 1.2% w/w of lipids) and defatted β-lactoglobulin preparations (0.05*h*). (Inset) Magnified initial parts of the acetonitrile isotherms. Solid lines are the approximation trends. The sorption isotherm of acetonitrile on human serum albumin (HSA) is from ref *9*.

The isotherms are the sorbate uptake A (in millimoles per gram of dry protein) functions of sorbate thermodynamic activity P/P_0. The decane sorption isotherms were determined only for the dried fatty BLG preparation because the dried defatted BLG does not bind decane, α- and γ-terpinenes, and terpinolene above 0.002 mmol/g at the sorbate activity $P/P_0 = 0.30$. This result is in agreement with the previously reported binding properties of dried albumin (*25*). Albumin with hydration 0.01*h* and 0.2% of lipids does not bind benzene or monofunctional organic compounds with a molar volume >80 mL/mol.

The Hill equation was used for calculating the acetonitrile sorption isotherms:

$$A = SC(P/P_0)^N/(1 + C(P/P_0)^N) \quad (1)$$

In eq 1 S is the binding stoichiometry, C is the sorption constant, and N is the cooperativity coefficient. The approximation parameters obtained are $N = 0.54$, $C = 0.00025$, and $S = 8.0 \times 10^3$ at 298 K and $N = 0.58$, $C = 0.048$, and $S = 42$ at 309.5 K for the dried defatted BLG and $N = 0.36$, $C = 0.060$, and $S = 27.0$ at 298 K and $N = 0.41$, $C = 0.0031$, and $S = 454$ at 323 K for the dried fatty BLG.

These isotherms have nearly the same shape as the isotherm of acetonitrile sorption by human serum albumin (*9*), where the protein hydration was 0.01*h*, lipid content = 0.2%, $T = 298$ K [approximation parameters of eq 1: $N = 1$ (fixed), $C = 9.0$, and $S = 0.90$ (**Figure 1A**)]. As distinct from the isotherms of water sorption by proteins (*10*−*12*), the isotherms of acetonitrile sorption by dried BLG reach saturation at high sorbate activities. A cooperative increase in the acetonitrile uptake A at $P/P_0 \geq 0.9$, corresponding to the formation of a separate liquid phase in the system, can be observed.

The Henderson equation (*26*) was chosen for the approximation of the decane sorption isotherms:

$$A = [-\ln[1 - (P/P_0)]/k]^{1/n} \quad (2)$$

In eq 2 k and n are the fitting parameters. This equation is one of the best among the two-parameter models tested for the approximation of the isotherm of water sorption by collagen (*27*). The approximation parameters of the decane sorption isotherms are $k = 65$ and $n = 1.15$ at 298 K and $k = 65$ and $n = 1.16$ at 309.5 K.

The absence of sorption affinity of the dried defatted BLG for decane and terpenes means that this protein preparation is not porous. The same conclusion was reached earlier in the studies of nitrogen and water sorption by dried collagen (*20*, *27*) and for the sorption of organic solvents, including acetonitrile, by dried human serum albumin (*25*). The binding selectivity of the dried defatted BLG toward the pair acetonitrile/decane is >500 at the sorbate activity $P/P_0 = 0.50$. This selectivity is 2 orders of magnitude higher than the value observed for a zeolite with a channel diameter adequate for the sorption of linear hydrocarbons (*28*).

The change of temperature from 298 K to the physiological value of 309.5 K produces a negligible decrease in acetonitrile uptake by the dried defatted BLG (**Figure 1A**). At $P/P_0 < 0.5$ the acetonitrile uptake by the dried un-defatted BLG decreases by 25% because of the temperature increase from 298 to 323 K (**Figure 1A**). This decrease exceeds the sum of the experimental errors of A values observed for two sorption isotherms (10%). A similar temperature effect on the water sorption affinity was observed for lysozyme (*10*), alcohol dehydrogenase (*11*), and hemoglobin (*12*). The sorption isotherms of decane by the dried un-defatted BLG coincide at 298 and 309.5 K (**Figure 1B**).

The effect of lipids on the binding of acetonitrile by solid BLG is rather complex. At 298 K and low sorbate activities ($P/P_0 < 0.2$), dried un-defatted BLG has a higher affinity for acetonitrile (on >34% at $P/P_0 < 0.026$) than the dried defatted BLG preparation (**Figure 1A**, inset). The other two sorption isotherms presented in **Figure 1A**, determined at 309.5 K for defatted BLG and at 323 K for un-defatted BLG, do not differ at the low activity of acetonitrile. This may be explained by a compensation of two effects: (1) lipids enhance the uptake of acetonitrile at $P/P_0 < 0.2$, and (2) the uptake of acetonitrile decreases with the rise in temperature. At the acetonitrile activity $P/P_0 > 0.4$, a significant suppression of acetonitrile uptake by lipids is observed for both sorption isotherms of the un-defatted BLG (**Figure 1A**).

To estimate the contribution of a possible separate lipid phase in the sorption of decane and acetonitrile, the partition coefficients $A/(P/P_0)$ of these compounds between the pure sorbate liquid and solution in lipids extracted from BLG were determined. In the case of decane, the value of $A/(P/P_0)$ is equal to 2.1 mmol/g at $P/P_0 = 0.18$ and $T = 298$ K. For acetonitrile, $A/(P/P_0) = 1.4$ mmol/g at $P/P_0 = 0.17$ and 0.34. Comparison of these data with the sorption isotherms obtained for dried un-defatted BLG (**Figure 1**) shows that nearly 70% of added decane with $P/P_0 = 0.18$ should be dissolved in a separate lipid phase formed by all lipids present in the BLG preparation studied. The calculated portion of acetonitrile at $P/P_0 = 0.026$ that could be dissolved in such a separate lipid phase is much lower (0.3%) and is negligible compared with the observed lipid effect at low acetonitrile activities. Therefore, only for decane can a significant portion of the lipid effect on the binding affinity of the dried un-defatted BLG be explained by the existence of a separate lipid phase.

A decrease in the acetonitrile uptake due to the presence of lipids in the dried BLG is possible if the lipids bound by the protein in water solution remain trapped in the potential acetonitrile binding sites of BLG during drying. An opposite influence of the hydrophobic compounds on the sorption of hydrophilic compounds by proteins was previously observed in low-water conditions. γ-Chymotrypsin binds much more water in the presence of hexane (*29*) and benzene (*30*). Dried albumin is able to bind a double quantity of acetonitrile in the presence of small concentrations of benzene without a separate liquid phase (*9*). Therefore, a number of competing effects of lipids on the binding of acetonitrile by a solid BLG preparation may exist.

Sorption Isotherms of Hydrated BLG Preparations. The influence of hydration and temperature on the binding affinity of β-lactoglobulin for decane and aroma hydrocarbons from citrus oils (α-terpinene, γ-terpinene, and terpinolene) was studied in the system protein + water + ethanol + hydrocarbon in the absence of a liquid phase. The binding isotherms were determined at the constant volume ratio of liquid organic components (0.9 vol % of hydrocarbon + 9.0 vol % of ethanol) and water (90.1 vol %) added to initially dried BLG samples.

The fraction of organic components in this mixture was low enough to diminish their influence on the water sorption by BLG. Besides, the concentrations of the hydrocarbons should be adequate to provide a relatively high activity for the large quantities of the added mixture ($\leq$101 μL per $\sim$300 mg of dried protein) in the absence of a liquid phase after equilibration. Ethanol was used for the dilution of hydrocarbons in order to reduce the dosing errors. The influence of ethanol on the albumin binding affinity for hydrophobic compounds does not exceed half of the hydration effect (*9*), so the ethanol contribution to the effect of the added 1:10 ethanol−water mixture is close to 5%.

The sorption isotherms obtained for terpenes and decane (**Figures 2** and **3**) are the plots of the ratio of sorbate uptake to its activity $A/(P/P_0)$ versus protein hydration, h. The typical sorption isotherms of ethanol obtained simultaneously with the sorption isotherms of hydrocarbons in the same systems are given in **Figure 4**. This presentation gives a bidimensional cross section of the multidimensional phase diagram, where both the protein hydration and the activity of organic components are variables. Full experimental data are given in **Tables 1**−**13**. The value of $A/(P/P_0)$ is the binding affinity of protein for sorbate or the partition coefficient of sorbate between its pure liquid and protein phase.

An increase in BLG hydration gives a cooperative increase in the binding affinity $A/(P/P_0)$ for decane and the terpenes studied for both the fatty and defatted BLG preparations (**Figures 2** and **3**). Below the hydration 0.1h, the binding affinity $A/(P/P_0)$ of BLG is low: 0.01−0.02 mmol/g for defatted protein and 0.04−0.20 mmol/g for the un-defatted BLG preparation. At higher hydrations, there is a stepwise increase in the binding affinity $A/(P/P_0)$ of BLG for hydrocarbons to a maximum or to saturation in most cases (**Figures 2** and **3**). Therefore, a hydration threshold exists for the binding affinity of BLG for the aroma terpenes studied and decane. Similar results were obtained for the influence of the hydration effect on the binding of hydrophobic sorbates by albumin in the absence of ethanol (*9*). The addition of ethanol to albumin in the absence of water activates the binding affinity of this protein for benzene and dioxane without reaching any threshold of ethanol content in the protein phase (*9*). Hence, the binding threshold observed in the present work should be mainly a result of BLG hydration.

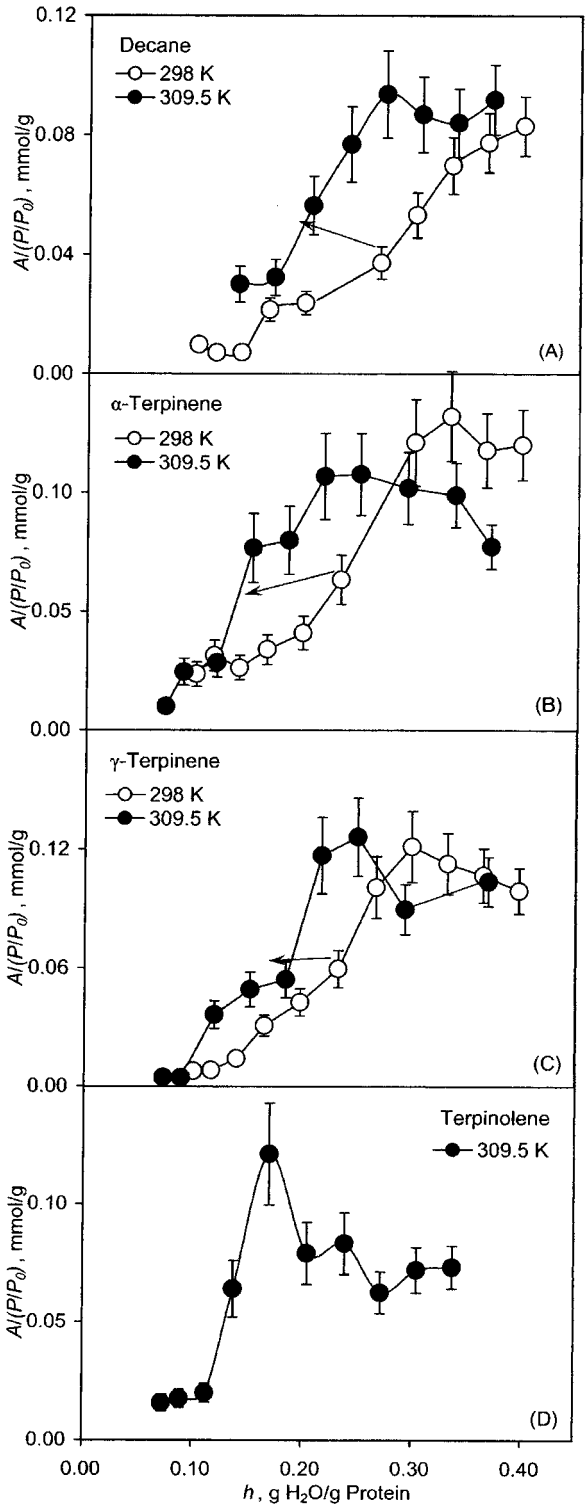

Figure 2. Hydration effect on the binding affinity $A/(P/P_0)$ of the solid defatted BLG preparation for the vapors of decane and aroma hydrocarbons in quaternary systems with water and ethanol at 298 and 309.5 K. The volume ratio of liquid hydrocarbon, water, and ethanol added to the initially dried BLG is 0.9:90.1:9.0. Full sorption data are given in **Tables 1**–**7**. Arrows show the temperature effect on the hydration threshold.

The hydration threshold h_{thr}, defined as the hydration value at the halfway point of the binding affinity increase, was calculated by the linear interpolation of the nearby isotherm points. The h_{thr} values of the defatted BLG for decane and α- and γ-terpinene are 0.26h, 0.22h, and 0.22h at 298 K and 0.19h, 0.14h, and 0.17h at 309.5 K, respectively. For terpinolene h_{thr}

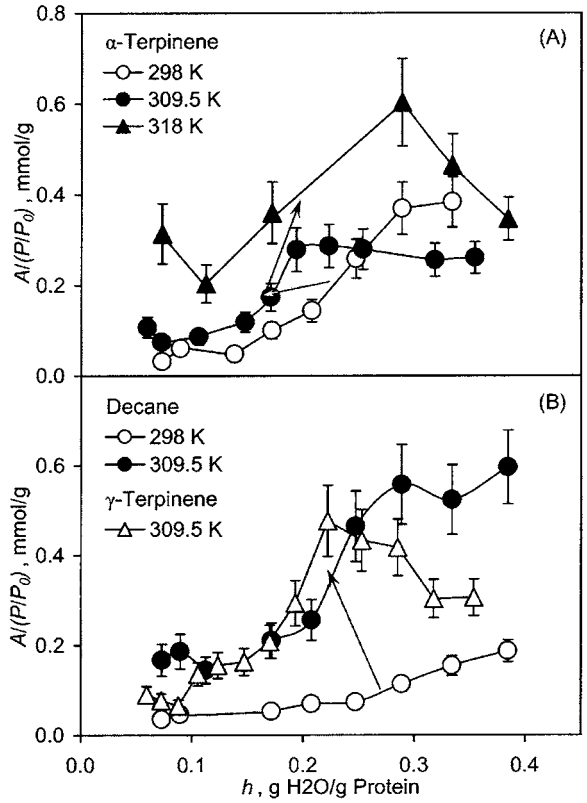

Figure 3. Effect of hydration on the binding affinity $A/(P/P_0)$ of the solid BLG preparation with 1.2% w/w of lipids for the vapors of aroma hydrocarbons and decane in quaternary systems with water and ethanol at 298, 309.5, and 318 K. The volume ratio of liquid hydrocarbon, water, and ethanol added to the initially dried BLG is 0.9:90.1:9.0. Full sorption data are given in **Tables 8**–**13**. Arrows show the temperature effect on the hydration threshold.

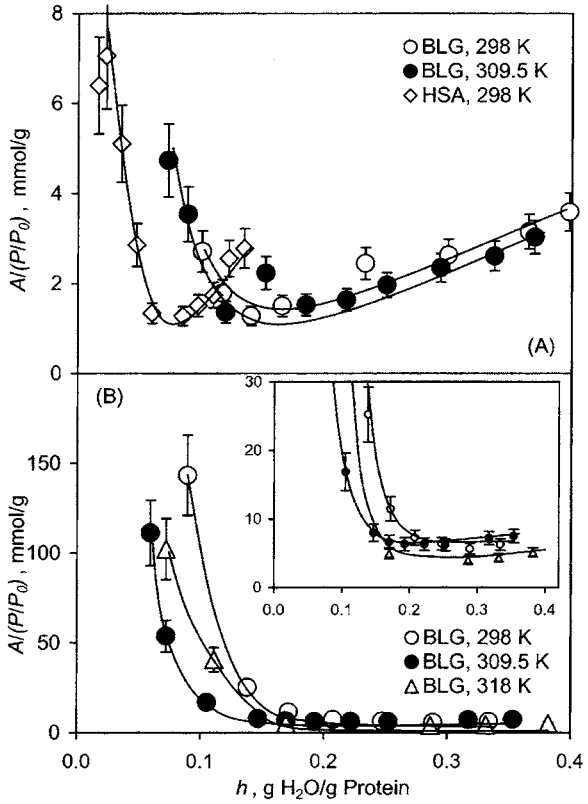

Figure 4. Effect of hydration on the binding affinity $A/(P/P_0)$ of the solid BLG preparations defatted (A) and with 1.2% w/w of lipids (B) for ethanol in quaternary systems with water and α-terpinene at 298, 309.5, and 318 K. The volume ratio of liquid hydrocarbon, water, and ethanol added to the initially dried BLG is 0.9:90.1:9.0. (Inset) Magnified initial parts of the ethanol isotherms. Full sorption data are given in **Tables 3**, **4**, and **10**–**12**. The sorption isotherm of ethanol on human serum albumin (HSA) at 298 K is from ref *9*.

$= 0.14h$ at 309.5 K. The hydration thresholds h_{thr} of the un-defatted BLG for decane and α-terpinene are $0.27h$ and $0.22h$ at 298 K and $0.22h$ and $0.16h$ at 309.5 K, respectively. For γ-terpinene $h_{thr} = 0.18h$ at 309.5 K. Hence, there is a decrease in the hydration threshold by $0.05-0.08h$ after the increase in temperature from the standard (298 K) to the physiological value (309.5 K) for both BLG preparations studied. The arrows in **Figures 2** and **3** indicate this decrease. A further increase in temperature to 318 K gives an increase in h_{thr} value to $0.20h$ for α-terpinene on the un-defatted BLG (**Figure 3A**). The presence of lipids does not influence this threshold. The value of h_{thr} is higher for decane than for the terpenes studied at the same temperatures.

The effect of hydration on the sorption of ethanol by BLG is different. The sorption isotherms in the $A/(P/P_0)$ versus h plot have a negative slope up to the hydration values of $0.15-0.20h$ (**Figure 4**). Above this hydration level, the binding affinity slightly increases for the defatted BLG and remains constant for the un-defatted BLG preparation. The isotherm of ethanol sorption by albumin from ref *9* presented in **Figure 4A** has a very similar shape despite the fact that the BLG studied is a different protein. Albumin shows the same minimal value of $A/(P/P_0)$ for ethanol but occurring at a lower level of protein hydration. The isotherms of water sorption by proteins (*10*−*12*) have nearly the same shape in the $A/(P/P_0)$ versus h plot. For BLG hydrated above $0.15-0.20h$, the obtained sorption isotherms of ethanol do not depend on the type of hydrocarbon added or on temperature (**Figure 4**; **Tables 1**−**13**).

Table 1. Sorption Isotherms of Decane and Ethanol on Defatted BLG at 298 K[a]

H_2O	decane		ethanol	
h, g/g	P/P_0	A, mmol/g	P/P_0	A, mmol/g
0.101	0.121	0.0012	0.0051	0.055
0.117	0.224	0.0016	0.0148	0.082
0.140	0.326	0.0023	0.0270	0.119
0.165	0.198	0.0043	0.113	0.147
0.198	0.243	0.0058	0.133	0.200
0.267	0.246	0.0091	0.186	0.302
0.300	0.207	0.0110	0.202	0.356
0.333	0.184	0.0128	0.218	0.41
0.365	0.187	0.0145	0.232	0.46
0.398	0.195	0.0162	0.237	0.52

[a] The volume ratio of liquid decane, ethanol, and water added to BLG with an initial hydration of $0.068h$ is 0.9:9.0:90.1.

The saturation value of $A/(P/P_0) = 0.11$ mmol/g observed for the hydrated defatted BLG (**Figure 2**) corresponds to a 2:1 (sorbate/monomer of BLG) molar ratio. The value of $A/(P/P_0) = 0.06$ mmol/g observed for terpinolene at high BLG hydration (**Figure 2D**) corresponds approximately to a 1:1 equimolar binding. Such 1:1 stoichiometry has already been observed for ligand binding by the BLG monomer in aqueous solution (*14*). The lower stoichiometry 0.5:1 was also observed for BLG (*31*) and other lipocalins (*32*, *33*) in aqueous solution. BLG is able to bind ligand (palmitate) inside its β-barrel with the stoichiometry 0.7:1 according to the X-ray data (*34*). Terpenes and

Table 2. Sorption Isotherms of Decane and Ethanol on Defatted BLG at 309.5 K[a]

H_2O	decane		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.138	0.130	0.0039	0.084	0.139
0.170	0.164	0.0053	0.121	0.182
0.205	0.130	0.0073	0.122	0.238
0.239	0.119	0.0091	0.151	0.284
0.272	0.116	0.0109	0.156	0.339
0.305	0.142	0.0123	0.174	0.389
0.337	0.164	0.0138	0.183	0.44
0.370	0.169	0.0155	0.175	0.50

[a] The volume ratio of liquid decane, ethanol, and water added to BLG with an initial hydration of 0.040*h* is 0.9:9.0:90.1.

Table 3. Sorption Isotherms of α-Terpinene and Ethanol on Defatted BLG at 298 K[a]

H_2O	α-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.101	0.070	0.0017	0.0194	0.053
0.117	0.083	0.0026	0.043	0.076
0.140	0.142	0.0037	0.084	0.108
0.165	0.155	0.0053	0.100	0.150
0.198	0.176	0.0072		
0.233	0.148	0.0094	0.107	0.261
0.300	0.113	0.0137	0.140	0.368
0.333	0.119	0.0157		
0.365	0.149	0.0175	0.152	0.48
0.398	0.162	0.0195	0.149	0.54

[a] The volume ratio of liquid α-terpinene, ethanol, and water added to BLG with an initial hydration of 0.068*h* is 0.9:9.0:90.1.

Table 4. Sorption Isotherms of α-Terpinene and Ethanol on Defatted BLG at 309.5 K[a]

H_2O	α-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.073	0.102	0.0010	0.0111	0.052
0.089	0.089	0.0022	0.0217	0.077
0.119	0.127	0.0036	0.079	0.107
0.152	0.080	0.0061	0.074	0.165
0.185	0.100	0.0080	0.132	0.201
0.217	0.094	0.0101	0.153	0.250
0.250	0.111	0.0119	0.155	0.305
0.294	0.139	0.0141	0.158	0.372
0.337	0.165	0.0163	0.167	0.44
0.370	0.234	0.0181	0.167	0.50

[a] The volume ratio of liquid α-terpinene, ethanol, and water added to BLG with an initial hydration of 0.040*h* is 0.9:9.0:90.1.

linear alkanes are good ligands for BLG in aqueous solutions (*35*). Therefore, if the solid BLG hydrated above 0.30*h* behaves as a native protein, it could bind the terpenes and decane inside the β-barrels of protein molecules. The proteins hydrated to 0.3−0.4*h* are usually regarded as native according to their enzymatic properties and specific heat capacities (*8*).

The selectivity of defatted BLG for the studied terpenes and decane above the hydration threshold does not exceed the experimental errors (**Figure 2**). This remains in agreement with the moderate binding selectivity of BLG for limonene, α-ionone, and β-ionone in aqueous solution in terms of the ligand thermodynamic activity (*36*). The temperature and the presence of lipids have no significant influence on the BLG binding selectivity (**Figures 2** and **3**).

Table 5. Sorption Isotherms of γ-Terpinene and Ethanol on Defatted BLG at 298 K[a]

H_2O	γ-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.101	0.203	0.0016	0.0022	0.056
0.117	0.295	0.0024	0.0383	0.077
0.140	0.277	0.0039	0.088	0.107
0.165	0.187	0.0057	0.102	0.149
0.198	0.183	0.0078	0.137	0.199
0.233	0.167	0.0099	0.112	0.260
0.267	0.120	0.0121	0.132	0.313
0.300	0.117	0.0142	0.140	0.368
0.333	0.143	0.0162	0.155	0.42
0.365	0.170	0.0182	0.163	0.48
0.398	0.203	0.0202	0.173	0.53

[a] The volume ratio of liquid γ-terpinene, ethanol, and water added to BLG with an initial hydration of 0.068*h* is 0.9:9.0:90.1.

Table 6. Sorption Isotherms of γ-Terpinene and Ethanol on Defatted BLG at 309.5 K[a]

H_2O	γ-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.073	0.215	0.0010	0.0150	0.051
0.089	0.325	0.0014	0.0339	0.072
0.119	0.119	0.0043	0.069	0.111
0.152	0.129	0.0064	0.093	0.158
0.185	0.153	0.0083	0.162	0.190
0.217	0.091	0.0107	0.145	0.253
0.250	0.101	0.0127	0.147	0.308
0.294	0.165	0.0148	0.156	0.373
0.370	0.190	0.0197	0.162	0.51

[a] The volume ratio of liquid γ-terpinene, ethanol, and water added to BLG with an initial hydration of 0.040*h* is 0.9:9.0:90.1.

Table 7. Sorption Isotherms of Terpinolene and Ethanol on Defatted BLG at 309.5 K[a]

H_2O	terpinolene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.073	0.096	0.0015	0.0092	0.053
0.089	0.134	0.0024	0.0159	0.079
0.112	0.178	0.0036	0.070	0.099
0.138	0.090	0.0058	0.098	0.134
0.170	0.066	0.0080	0.115	0.184
0.205	0.123	0.0098	0.133	0.234
0.239	0.141	0.0117	0.146	0.286
0.272	0.215	0.0134	0.154	0.340
0.305	0.215	0.0155	0.156	0.395
0.337	0.239	0.0175	0.166	0.45

[a] The volume ratio of liquid terpinolene, ethanol, and water added to BLG with an initial hydration of 0.040*h* is 0.9:9.0:90.1.

The binding affinity $A/(P/P_0)$ of the hydrated un-defatted BLG for terpenes, decane, and ethanol is at least twice as high as that of the defatted BLG preparation at the same hydration levels above the protein hydration threshold (**Figures 2** and **3**). To estimate the role of lipids being present in a separate phase, the partition coefficients $A/(P/P_0)$ of ethanol, decane, and γ-terpinene between their pure liquids and solutions in lipids were determined in the present work. The values of $A/(P/P_0)$ for solutions of ethanol ($P/P_0 = 0.60$), α-terpinene ($P/P_0 = 0.12$), and γ-terpinene ($P/P_0 = 0.13$) in lipids extracted from BLG are 1.5, 3.7, and 3.4 mmol/g, respectively, at 298 K. The above-mentioned value of $A/(P/P_0) = 2.1$ mmol/g for decane dissolved in lipids was not changed by the addition of ethanol with the

Table 8. Sorption Isotherms of Decane and Ethanol on Un-defatted BLG (1.2% w/w of Lipids) at 298 K[a]

H_2O	decane		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.073	0.021	0.0008	0.0003	0.028
0.089	0.033	0.0015	0.0007	0.056
0.172	0.104	0.0055	0.0154	0.193
0.208	0.105	0.0073	0.0386	0.250
0.247	0.126	0.0092	0.056	0.314
0.289	0.102	0.0115	0.070	0.384
0.334	0.090	0.0139	0.075	0.46
0.384	0.088	0.0165	0.081	0.54

[a] The volume ratio of liquid decane, ethanol, and water added to BLG with an initial hydration of 0.057*h* is 0.9:9.0:90.1.

Table 9. Sorption Isotherms of Decane and Ethanol on Un-defatted BLG (1.2% w/w of Lipids) at 309.5 K[a]

H_2O	decane		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.073	0.0048	0.0008	0.0003	0.026
0.089	0.0087	0.0016	0.0002	0.056
0.112	0.0188	0.0027	0.0009	0.095
0.172	0.0269	0.0057	0.0219	0.188
0.208	0.0293	0.0075	0.0391	0.244
0.247	0.0207	0.0096	0.059	0.305
0.289	0.0212	0.0118	0.080	0.370
0.334	0.0269	0.0141	0.083	0.45
0.384	0.0279	0.0166	0.081	0.53

[a] The volume ratio of liquid decane, ethanol, and water added to BLG with an initial hydration of 0.057*h* is 0.9:9.0:90.1.

Table 10. Sorption Isotherms of α-Terpinene and Ethanol on Un-defatted BLG (1.2% w/w of Lipids) at 298 K[a]

H_2O	α-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.089	0.0306	0.0019	0.0004	0.056
0.138	0.095	0.0045	0.0055	0.139
0.172	0.067	0.0067	0.0168	0.193
0.208	0.062	0.0090	0.0343	0.251
0.247	0.044	0.0115	0.049	0.315
0.289	0.038	0.0141	0.068	0.384
0.334	0.044	0.0169	0.073	0.46

[a] The volume ratio of liquid α-terpinene, ethanol, and water added to BLG with an initial hydration of 0.057*h* is 0.9:9.0:90.1.

activity $P/P_0 = 0.6$ and water with $P/P_0 = 1$ and at the reduced concentration of decane. The reduction of ethanol concentration in lipids provokes a decrease of its $A/(P/P_0)$ value.

Using the obtained values of $A/(P/P_0)$ for ethanol and decane dissolved in lipids, the portions of these sorbates that should be dissolved in a separate lipid phase at 298 K were calculated. For un-defatted BLG, these portions are below 17% for decane, 12% for α-terpinene, and 1% for ethanol above the protein hydration threshold. These values should be >50% to explain the observed effect of lipids on the binding affinity of BLG. Therefore, lipids activate the binding affinity of hydrated BLG for decane and terpenes in a much higher proportion than they activate the binding affinity of dried BLG, where the estimated contribution of a separate lipid phase may be up to 70% of the increase in the decane uptake. Hence, there is a certain synergy of the hydration and lipid effects on the binding properties of BLG. The absence of competition between lipids and terpenes for the binding by hydrated BLG is in agreement with the observations of independent binding of retinol and fatty acids (*15*) or of retinol and protoporphyrin (*16*) by BLG in aqueous solutions.

Table 11. Sorption Isotherms of α-Terpinene and Ethanol on Un-defatted BLG (1.2% w/w of Lipids) at 309.5 K[a]

H_2O	α-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.060	0.0173	0.0018	0.0005	0.056
0.073	0.0333	0.0025	0.0014	0.078
0.106	0.0501	0.0043	0.0078	0.131
0.147	0.0580	0.0069	0.0248	0.198
0.170	0.0484	0.0084	0.0351	0.233
0.194	0.0357	0.0099	0.042	0.269
0.223	0.041	0.0117	0.050	0.317
0.253	0.048	0.0134	0.058	0.364
0.318	0.068	0.0173	0.065	0.47
0.354	0.075	0.0194	0.071	0.53

[a] The volume ratio of liquid α-terpinene, ethanol, and water added to BLG with an initial hydration of 0.027*h* is 0.9:9.0:90.1.

Table 12. Sorption Isotherms of α-Terpinene and Ethanol on Un-defatted BLG (1.2% w/w of Lipids) at 318 K[a]

H_2O	α-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.073	0.0030	0.0010	0.0003	0.028
0.112	0.0154	0.0031	0.0023	0.094
0.172	0.0187	0.0067	0.0352	0.176
0.289	0.0231	0.0139	0.087	0.348
0.334	0.0355	0.0165	0.096	0.42
0.384	0.055	0.0192	0.098	0.50

[a] The volume ratio of liquid α-terpinene, ethanol, and water added to BLG with an initial hydration of 0.057*h* is 0.9:9.0:90.1.

Table 13. Sorption Isotherms of γ-Terpinene and Ethanol on Un-defatted BLG (1.2% w/w of Lipids) at 309.5 K[a]

H_2O	γ-terpinene		ethanol	
h, g/g	P/P_0	*A*, mmol/g	P/P_0	*A*, mmol/g
0.060	0.021	0.0019	0.0007	0.056
0.073	0.035	0.0026	0.0020	0.077
0.088	0.055	0.0036	0.0048	0.104
0.106	0.035	0.0047	0.0107	0.128
0.124	0.038	0.0059	0.0213	0.156
0.147	0.045	0.0072	0.0408	0.184
0.170	0.042	0.0087	0.0539	0.216
0.194	0.035	0.0101	0.0725	0.244
0.223	0.026	0.0120	0.0777	0.292
0.253	0.032	0.0138	0.0882	0.336
0.286	0.038	0.0157	0.0924	0.390
0.318	0.059	0.0176	0.105	0.44
0.354	0.066	0.0198	0.102	0.50

[a] The volume ratio of liquid γ-terpinene, ethanol, and water added to BLG with an initial hydration of 0.027*h* is 0.9:9.0:90.1.

Interpretation of the Hydration, Lipid, and Temperature Effects. The observed hydration threshold may be a result of the phase transition. If hydration forms a new phase of hydrated BLG for a hydrophobic sorbate, in addition to the vapor phase and the initial phase of the dried protein, there are three phases, $p = 3$, in the system with three components: protein, sorbate, and water, $c = 3$. In the $A/(P/P_0)$ versus hydration presentation of sorption isotherms, the activity of the sorbate is formally fixed and this component is excluded from the thermodynamic consideration: $c = 2$. Therefore, the number of degrees of freedom is equal to zero at the threshold hydration of BLG

according to the Gibbs phase rule: $f = c - p + 1 = 2 - 3 + 1 = 0$, which corresponds to the phase transition at $T = \text{const}$.

This phase transition may be explained by the formation of clathrates water + BLG + hydrocarbon. Such clathrates formed by ordered water molecules were observed by an X-ray method around *n*-hexane molecules bound in γ-chymotrypsin (*6*) and around hydrophobic groups of amino acid residues in collagen-like protein (*7*). The formation of ordered water structures or "hydrophobic hydration" was also found around the hydrophobic groups of proteins or protein-based polymers in water solutions (*4*). The different hydration effects on the BLG binding affinity $A/(P/P_0)$ for hydrocarbons and ethanol may be related to the "structuring" role of hydrophobic groups and/or the "structure-breaking" role of hydrophilic groups in the thermotropic transitions of protein-based polymers (*4*).

The reduction of the BLG hydration threshold during the increase in temperature is consistent with earlier data obtained for the temperature dependence of water sorption by other proteins (*10*−*12*), if the water activity rather than the water content determines the binding properties of proteins. Such an assumption has already been made to explain enzymatic activities in nonaqueous media (*37*). Hence, at higher temperatures the same water activity and ligand binding affinity can be reached at a lower protein hydration due to the negative temperature effect on water sorption by protein (*10*−*12*).

The observed effect of lipids on the binding properties of hydrated BLG may be related to the previously reported detrimental effects of delipidation on protein stability (*17*). In addition, the complexation of BLG with fatty acids enhances the stability of this protein toward urea denaturation (*18*). The elimination of fats from BLG may remove a factor stabilizing its native structure during dehydration. The defatted BLG studied is not irreversibly denatured because the amide I and amide II bands in the FTIR spectra of its water solution are identical with those of the initial un-defatted protein. The defatted BLG may be more tightly packed and thus may have a lower binding affinity for small molecules than the more native fatty preparation.

SAFETY

Hexane or acetone should be preferred instead of chloroform, which is too toxic, in food industry and laboratory studies for the elimination of fats from food proteins.

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