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Investigation of Binding Behavior of α- and β-Ionones to β-Lactoglobulin at Different pH Values Using a Diffusion-Based NOE Pumping Technique

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Diffusion-based NMR techniques were employed to study effects of pH on β-lactoglobulin (BLG) conformation and binding affinity to α- and β-ionone. In the first part of the study, the influence of pH on the diffusion coefficient of BLG in D_2O solution was investigated using a stimulated-echo NMR experiment. The diffusion coefficient of BLG decreased with increasing pH values. A significant decrease in the diffusion coefficient observed at pH 11 may be due to total unfolding (denaturation) of the protein, resulting in hydrophobically driven self-aggregation. A diffusion-based NOE pumping technique was then applied to determine the relative binding affinities between α- and β-ionones and BLG at pH values varying from 3 to 11. An increase in signal intensities for β-ionone with increasing molar concentration ratios between β-ionone and BLG was observed at all pH ranges studied. The increased signal intensities reflect increased relative binding affinity. The greatest binding affinity occurred at pH 9 and the lowest at pH 11. α-Ionone showed binding evidence only at pH 9, and the binding was significantly weaker than that obtained for β-ionone at the same pH. The high affinity observed for both aroma compounds at pH 9 may be due to a flexible conformation of BLG at this pH so that the flavor ligand accessibility increases. Conversely, alkaline denaturation occurring at pH 11 gives rise to relatively lower binding affinity compared to that observed at the other pH values.

KEYWORDS: β-Lactoglobulin (BLG); α-ionone; β-ionone; pH; PFG-NMR spectroscopy; diffusion coefficient; relative binding affinity; diffusion-based NOE pumping

INTRODUCTION

Whey protein is a major ingredient in a variety of foods because of its various functionalities, including emulsifying and foaming properties. β-Lactoglobulin (BLG) is the major protein in the whey fraction, and its conformation and physical properties are well characterized (*1*−*3*). BLG is also known to have a high affinity for a wide range of flavor compounds, such as esters, alcohols, ionones, ketones, and aldehydes (*3*−*11*). The ability of BLG to either carry off-flavors or selectively bind flavor components from a flavor blend can cause an imbalance in perceived product flavor and directly influence product acceptability.

There have been many studies on the influence of volatile−protein interactions on flavor release and perception (*12*). These studies indicate that volatile−protein interaction mechanisms differ depending on the nature of the aroma compounds, the conformational states of proteins, temperature, pH, and the presence of other ingredients, such as, salt, alcohol, and fat (*13*). In an attempt to obtain a better understanding with regard to the interactions, many spectroscopic and chromatographic methodologies have been introduced. Concomitantly, however, some inconsistent results have been obtained by the different methods. Among the discrepancies observed, the binding affinity of ionone isomers (α and β) to BLG is still contradictory.

Diffusion-based NMR techniques have been recently employed in the study of volatile−macromolecule interactions (*14*). Diffusion is an important molecular characteristic that reflects molecular weight and conformational states of compounds relative to changes in chemical surroundings. In particular, diffusion measurement combined with a nuclear Overhauser effect (NOE) technique (called diffusion-based NOE pumping) is a powerful monitoring tool to identify small ligands having binding affinity to a macromolecule.

In this study, a diffusion-based NOE pumping technique was used to investigate the interactions between BLG and α- and β-ionones and relative binding affinities between individual pairs were determined for different pH values. Differences in binding behavior were related to changes in the diffusion coefficient of BLG, which were in turn related to changes in the potential conformational states at the various pH values studied.

MATERIALS AND METHODS

Materials. BLG (mixture of A and B variants, purity = 90%) was purchased from Sigma (St. Louis, MO) and used without further

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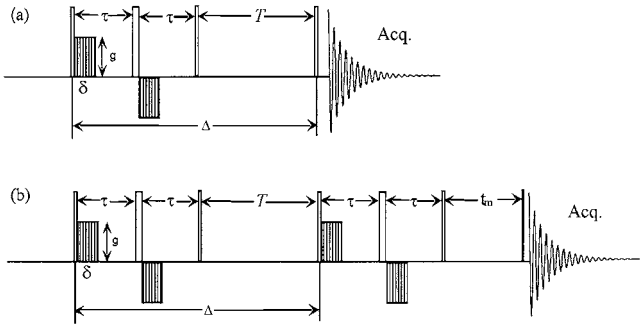

Figure 1. Pulse sequences of (a) stimulated-echo pulsed field gradient (STE-PFG) and (b) diffusion NOE pumping. The narrow and wide bars represent 90° and 180°, respectively. τ and T are interpulse delays between 90° and 180° and between two 90°s, respectively. t_m on sequence b is the mixing time for NOE buildup. g and δ, respectively, indicate the amplitude and duration of the gradient pulse, and Δ is the diffusion time. (Reprinted with permission from ref *14*. Copyright 2002 American Chemical Society.)

purification. The flavor compounds used for this study, α-ionone (90%) and β-ionone (95%), were also purchased from Sigma Chemical Co. Deuterium oxide (D_2O, 99.9%) and sodium deuterioxide (NaOD, 99.5% of D, 30% w/w in D_2O) were obtained from Cambridge Isotope laboratories, Inc. (Andover, MA).

Sample Preparation. BLG solution (0.97 mM, ~1.7%, w/v) was prepared in D_2O and adjusted to different pH levels (pH 3, 6, 9, and 11) with 1 N HCl solution (in D_2O) or 30% NaOD solution. All pH measurements were performed using a microelectrode (Wilmad, Buena, NJ) connected to a pH meter (Analytical Technologies Inc., Boston, MA) calibrated with aqueous pH buffers. The pH values reported here were based on the pH meter readings without correction for isotope effects.

Each flavor compound was directly added to the pH-adjusted BLG solution to give final concentrations ranging from 0.5 to 10 mM. All of the solutions were equilibrated at room temperature for at least 12 h before further analysis.

Instrumentation. All NMR experiments were carried out at 500 MHz using a Bruker DRX-500 instrument equipped with a BGU-2 field gradient accessory capable of delivering *z*-field up to 590 mT/m. In all experiments, the probe temperature was maintained at 298 ± 1 K, and standard 5 mm NMR tubes were used.

Measurement of Diffusion Coefficients. Diffusion coefficients of BLG in solutions varying from pH 3 to 11 were measured by pulsed field gradient NMR (PFG-NMR) spectroscopy using a stimulated-echo (STE) pulse sequence (*15*; **Figure 1a**).

The PFG-NMR spectroscopy is based on the attenuation of individual proton resonances under the influence of linear field gradients (*16*). The relationship between the amplitude (I) of the signal and the diffusion coefficient (D) is shown in the following equation:

$$I = I_0 \exp[-D(\Delta - \delta/3)\gamma^2 g^2 \delta^2] \quad (1)$$

I and I_0 are, respectively, the intensity of the NMR signal in the presence and absence of external gradient pulses ($g\delta$); Δ is the diffusion time; γ is the nuclear magnetogyric ratio; and g and δ are, respectively, the amplitude and duration of the gradient pulse (*16*). In our experiments, gradient strength, g, in the z direction was calibrated by $D_{H_2O} = 2.3 \times 10^{-9}$ m^2 s^{-1} at 298 K and then linearly attenuated from 0.68 to 32.4 g/cm during the experiments. The gradient duration time (δ) and diffusion time (Δ) were optimized to 5 and 200 ms, respectively, and kept constant for all of the spectra. The spectra were processed on a Silicon Graphics Indigo workstation. The calculation of the diffusion coefficient at a given pH was determined by integration of a series of peaks (at ~0.85 ppm) with increasing gradients, following a peak fitting procedure. Because the resonances used for integration were broad and overlapped, the intensities of the signals could not be determined directly by integration or peak height measurement. The peak fitting procedure involves specifying the initial value of peak number, center, width, and height, and then the peak is fit to a Lorentzian shape by varying the variables until the difference between the real and simulated spectra reaches a minimum. The resonance intensities were determined by integration of the simulated peaks.

The diffusion coefficient was calculated from the slope obtained by plotting the natural logarithm of resonance intensity versus the square of the gradient amplitude. The experimental data were successfully fit to a linear equation producing a single diffusion coefficient for a set of peaks.

Diffusion-Based NOE Pumping Technique. A diffusion-based NOE pumping technique (**Figure 1b**, refer to ref *14*) was applied to determine the relative binding affinities between the two ionones and BLG at different pH values (3, 6, 9, and 11). The gradient length (δ) and NOE mixing time were selected to be 3 ms and 400 ms, respectively. The proton resonance at ~1 ppm was selected for both aroma compounds (obtained from the difference spectra, **Figures 2c** and **3c**) because of the strong signal intensity. The signal intensity was integrated and normalized relative to that obtained for the lowest concentration ratio to yield the relative binding affinity:

$$\text{relative binding affinity} = \frac{\text{signal intensity obtained with a given molar concentration ratio}}{\text{signal intensity obtained with the lowest molar concentration ratio}}$$

RESULTS AND DISCUSSION

Effect of pH on the Diffusion Coefficient of BLG. The diffusion coefficient is an important molecular property and is closely related to molecular weight and conformational features (e.g., polymerization and molecular shape). The conformation of BLG is largely influenced by pH changes (*17*); therefore, the resulting changes in the diffusion coefficient can be readily related to the structural modifications occurring in the protein at the pH values studied. These structural modifications can also then be related to binding behavior with small ligands.

The diffusion coefficient of BLG was measured in D_2O solutions varying in pH (pH 3, 6, 9, and 11) using an STE experiment (**Figure 1a**). The diffusion coefficient of BLG was greatest at pH 3 [$(13.33 \pm 0.05) \times 10^{-11}$ m^2 s^{-1}] and lowest at pH 11 [$(6.49 \pm 0.04) \times 10^{-11}$ m^2 s^{-1}], with intermediate values at pH 6 and 9 [(9.46 ± 0.04) and $(9.31 \pm 0.03) \times 10^{-11}$ m^2 s^{-1}, respectively]. Results indicated that BLG at pH 3 diffuses twice as fast in solution compared to that at pH 11. The significant increase in diffusion coefficient of BLG observed at pH 3 may be attributed to the specific conformational state where a very compact and spherical monomer is in rapid equilibrium with its dimer under the acidic condition (*9*, *18*, *19*). Dimer formation prevails as the pH increases to pH 6.0 because of the configuration changes around the isoelectric point (pI) of the protein (pI = 5.4) (*20*). The result may account for the slow diffusion coefficient observed at pH 6 compared to pH 3. The observation that a decrease in diffusion coefficient of BLG occurs with continuously increasing pH (above pH 9) may suggest the conformational transitions of BLG such as an increase in reactivity of a buried carboxyl group, a free thiol group, and a change in the environment of some amino acid residues (*21*, *22*). These structural changes may lead to an enhanced surface hydrophobicity (*20*), so hydrophobically driven self-aggregation may be favored. Several time-dependent changes involving conformational and new aggregation processes occurring above pH 8 are related to irreversible denaturation of the protein (*21*); thus, a possible formation of higher order aggregates under the high pH conditions is expected. However, at higher pH values the self-aggregation behavior becomes more complicated and the association properties of the protein are affected by many other factors such as temperature, protein concentration, and ionic strength as well as pH. In addition, monomer−oligomer equilibrium or self-aggregation as a func-

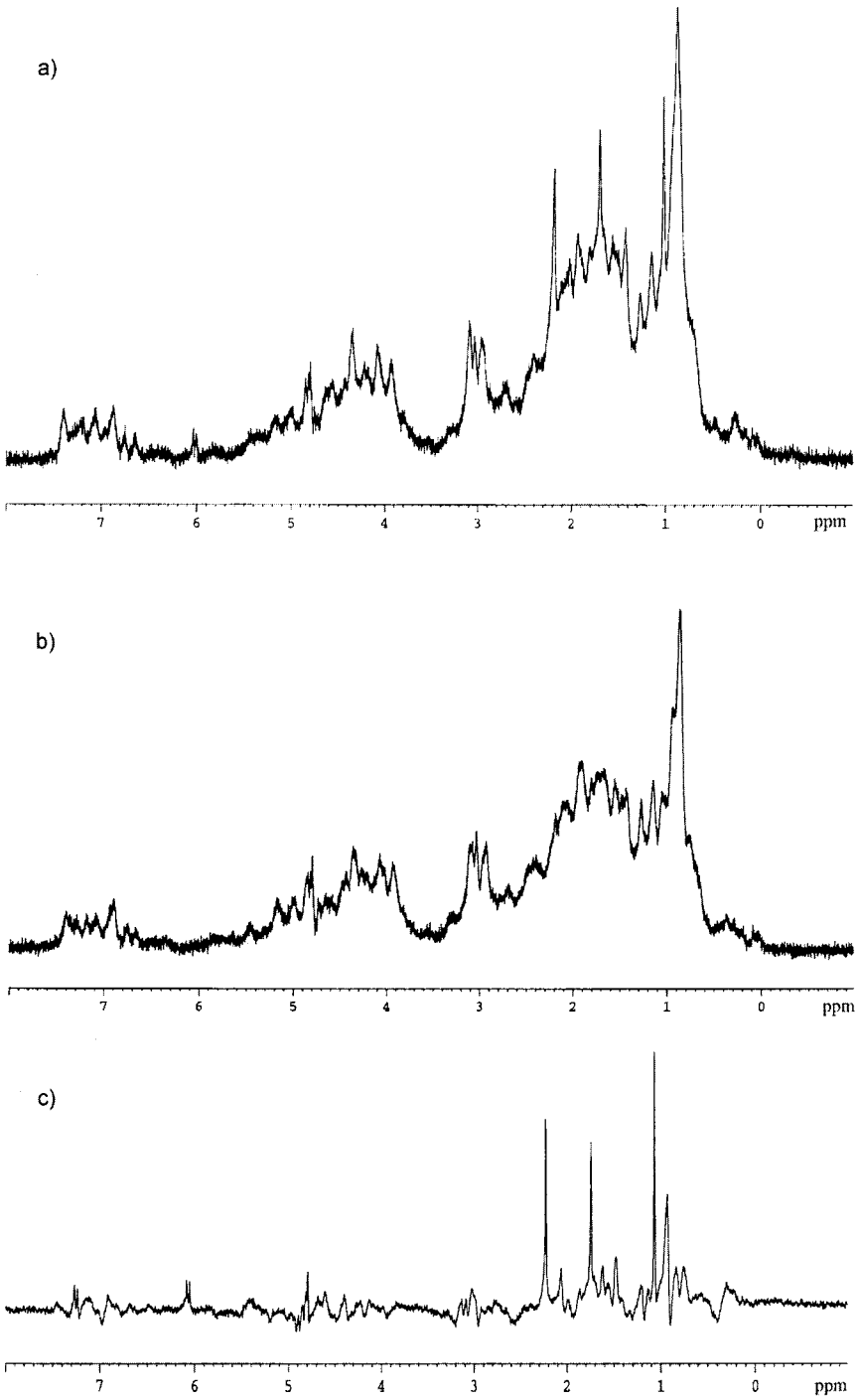

Figure 2. ^{1}H NMR spectra obtained by diffusion-based NOE pumping technique: (a) diffusion NOE pumping technique for α-ionone (0.5 mM) in the presence of BLG (0.97 mM); (b) diffusion NOE pumping technique for BLG (0.97 mM) alone; (c) difference spectrum for (a) subtracted from (b).

tion of surrounding pH changes is controlled by different mechanisms (*19*), so it is difficult to fully relate the self-aggregation of the protein and diffusion constants to pH effects alone. Calculation of the hydrodynamic radii of the BLG aggregates (according to the Stokes−Einstein equation or by dynamic light scattering) at variable pH values is necessary to fully explain the pH effect on the protein conformation. In addition, the activity coefficient and the volume fraction of the solute should be considered, particularly for more concentrated solutions (*23*).

Determination of Relative Binding Affinities between α- and *β*-Ionones and BLG at Different pH Values. The diffusion-based NOE pumping technique has shown a novel ability to screen small drug compounds with binding affinity to BSA (*24*). Recently, the technique was successfully applied to the study of binding between several selected aroma compounds and BSA or cacao bean tannin extract (*14*). However, the technique was used to provide only qualitative information on the binding phenomena. In the current study, we have modified the diffusion-based NOE pumping technique to provide more quantitative information on binding affinities between two ionone isomers and BLG at different pH values. By calculating changes in signal intensity for the individual aroma compounds at various ligand/protein ratios, we are able to calculate relative binding affinity.

BLG is known to bind a variety of small hydrophobic molecules (*17*). Among them *β*-ionone is reported to possess a binding affinity to BLG (*4*, *11*, *25*). However, the binding

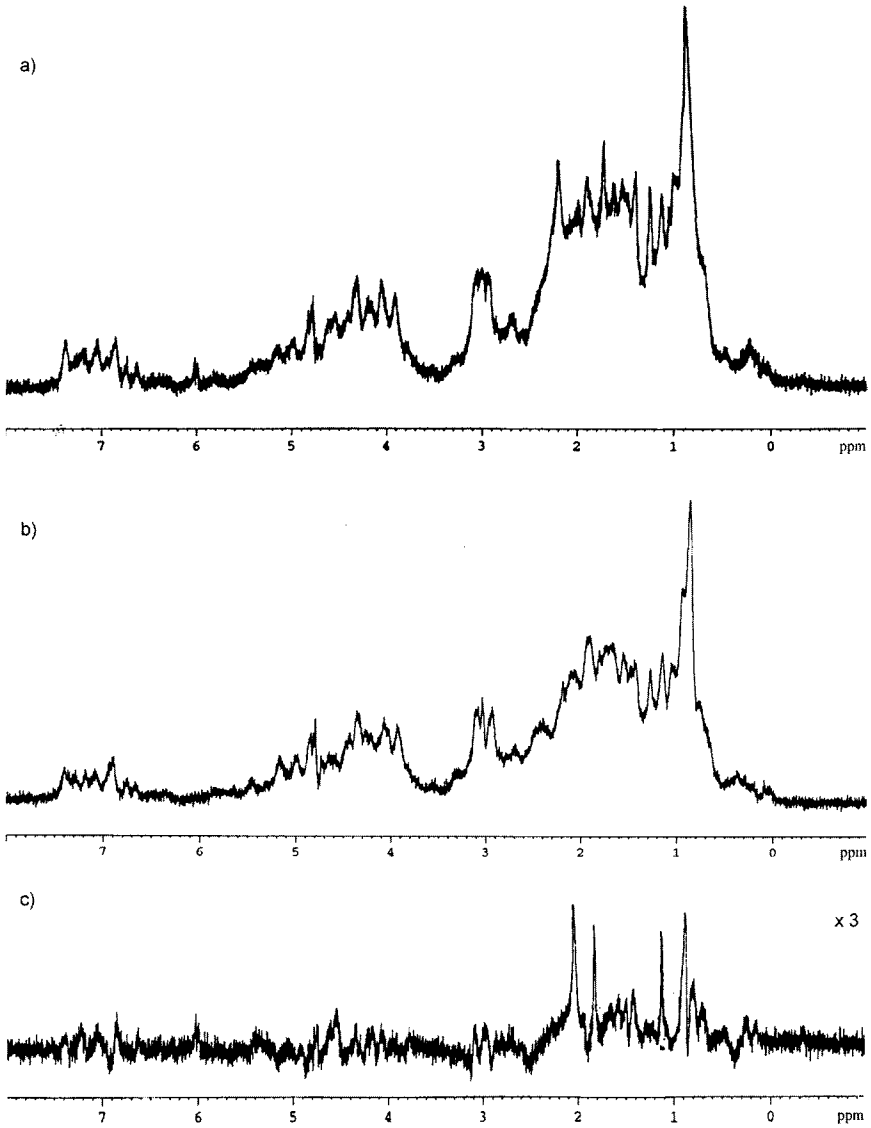

Figure 3. ^{1}H NMR spectra obtained by diffusion-based NOE pumping technique: (a) diffusion NOE pumping technique for β-ionone (0.5 mM) in the presence of BLG (0.97 mM); (b) diffusion NOE pumping technique for BLG (0.97 mM) alone; (c) difference spectrum for (a) subtracted from (b); three times expanded.

affinity of α-ionone to BLG is still ambiguous. Liquid chromatography techniques (*6*, *10*) have provided some evidence that α-ionone could bind to BLG with relatively weaker binding affinity than β-ionone; infrared spectroscopic analyses (*26*) indicated that both ionone compounds bind to BLG in a similar binding pattern illustrated by the same difference spectra. On the other hand, Dufour and Haertlé (*4*) concluded that α-ionone did not bind to BLG because it did not induce any fluorescence quenching of the protein and suggested that the steric hindrance of α-ionone may interfere with accessibility to specific binding sites.

Therefore, we determined the relative binding affinities between α- and β-ionones and BLG using the diffusion-based NOE technique at different pH values (pH 3, 6, 9, and 11). For individual pH values, solutions having different concentration ratios (0.25−5) of the aroma compounds and BLG were prepared. Because of the limited sensitivity of the NMR instrument for the nonpolar aroma compounds in aqueous solutions, the minimum concentration of α- and β-ionone added was 0.5 mM with a maximum concentration of 10 mM. With an excess of 10 mM, the BLG spectrum was significantly changed, which may be due to a conformational change induced by increasing ligand concentration (*27*). To exclude possible competition between the ligands for the same binding site, individual flavored solutions containing BLG were prepared and tested for this study.

Figures 2c and **3c** are the difference spectra for α- and β-ionones, respectively, obtained at pH 9 (molar concentration ratio of 5 between the aroma compounds and BLG) by means of a diffusion-based NOE pumping technique. The individual spectra were obtained by subtraction of a BLG spectrum (b) from that of an aroma/BLG mixture (a). After subtraction, proton signals appear only in the region between 0.5 and 3 ppm, which corresponds to four methyl groups of the ionones (*28*). The difference spectra clearly indicate that both α- and β-ionones bind to BLG at this pH.

As shown in **Figure 4**, the change in the signal intensity was obtained as a function of the molar concentration ratios between the flavors and the protein, reflecting relative binding affinities. For β-ionone, the largest increase in the signal intensity was observed at pH 9 and the smallest increase at pH 11. The increase in signal intensity for α-ionone was noticeable only at pH 9, which shows an increase similar to that observed for β-ionone at pH 3.

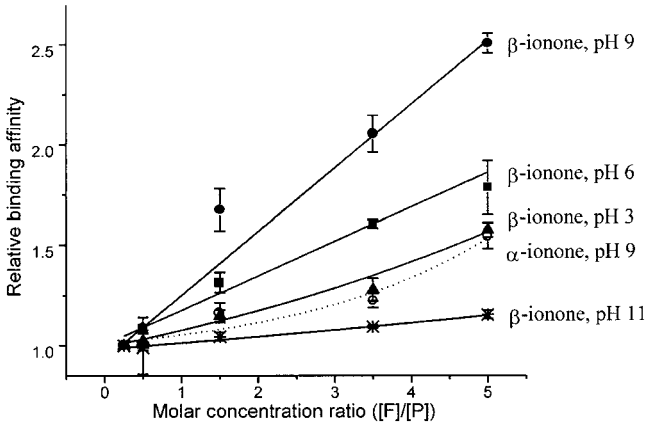

Figure 4. Binding affinity relative to the lowest molar concentration ratio (0.25) between α- and *β*-ionone (F) and *β*-lactoglobulin (P; 0.97 mM) in D_2O solutions (25 °C) at different pH values. *β*-Ionone at pH 9 (●), pH 6 (■), pH 3 (▲), and pH 11 (∗) and α-ionone at pH 9 (○). F and P, respectively, represent initial concentration of ionones and *β*-lactoglobulin added to the mixtures.

The variations of the relative binding affinity of BLG as a function of pH changes are consistent with Jouenne and Crouzet's findings (*9*). Retention (expressed as relative infinite dilution activity coefficients) of methyl ketones (C7−C9) and ethyl esters (C6−C9) in BLG containing aqueous solution was greatest at pH 6 and lowest at pH 11. An increase of binding affinity from pH 3 to pH 9, observed for *β*-ionone, can be explained by increasing flexibility of the protein conformation with an increase in pH. Elevated susceptibility of BLG to surface denaturation at pH above 9 compared to that at pH 3 indicated a more open flexible molecular structure at alkaline pH values (*18*). Unfolding of BLG conformation progressively increased with increasing pH values from pH 1.5 to pH 7 as determined by UV difference spectroscopy (*19*). The conformational flexibility may result in increased accessibility of the aroma compounds to the binding site of the protein. The relatively low binding affinity at pH 11 is most likely a consequence of the alkaline denaturation of the protein, which may be explained by the significant decrease in diffusion coefficient of BLG [$(13.33 \pm 0.05) \times 10^{-11}$ m^2 s^{-1} and $(6.49 \pm 0.04) \times 10^{-11}$ m^2 s^{-1} at pH 3 and 11 respectively]. Alkaline denaturation can induce partial or complete unfolding of the protein conformation and subsequently lead to exposure of hidden hydrophobic residues, resulting in molecular aggregation to minimize the physical contact with surrounding water molecules.

Many previous findings show that BLG adopts various oligomeric states according to pH changes. This event would be more greatly facilitated with relatively high concentrations of the protein and ligands such as used in this study. However, the association and aggregation exhibited by BLG are complex, and many factors are attributed to the association behavior of the protein. On the basis of our results and other previous findings, we may assume that the self-aggregation of BLG could be promoted above pH 9, accompanied by increased surface hydrophobicity. These irreversible conformational changes associated with aggregation may not be transmitted to the binding sites, and the structures at the binding joints are probably not involved in the ligand binding (*9*, *29*). With these assumptions, the binding affinity of ligands for the protein would be increased with increasing pH to pH 9. However, under severe alkaline conditions (such as pH 11 in the study), BLG experiences total unfolding of the tertiary and quaternary structures including several modifications of binding sites, resulting in decreased binding affinity.

The relative binding behavior of the ionones to BLG observed in this study are also in reasonable agreement with the previous findings observed by liquid chromatographic techniques (*6*, *10*). Jouenne and Crouzet (*10*) concluded that α-ionone has significantly weaker binding affinity than *β*-ionone, although their hydrophobicities are equal. The different binding affinities between these two compounds may then be attributed to the conformational differences in relation to the specificity of the BLG binding site.

To facilitate the instrumental analysis, the experiments were conducted in highly concentrated flavor solutions, which may not occur in natural foods and beverages. In addition, the conformation of the protein could be changed as a result of the increasing flavor concentrations as well as by the pH changes (*30*). Thus, the relative binding affinities obtained at high ligand (aroma) to protein concentration ratios cannot be fully compared with those obtained at lower ratios or in more complex food systems.

In this study we describe the application of diffusion-based NOE pumping techniques for monitoring binding affinity of small flavor molecules to macromolecules as a function of variable solution conditions. We showed that the relative binding affinities for both α- and *β*-ionones to BLG were greatest at pH 9, with *β*-ionone having a greater binding affinity than α-ionone. This result may be explained by increased flexibility of BLG with increasing pH, so that the ligands can easily approach the binding sites. However, the severe alkaline conditions above pH 9 should result in total unfolding of the protein structure, including complete modification of the binding sites, leading to a reduced binding affinity to ligands. The diffusion-based NOE pumping techniques proved to be useful tools for relating changes in macromolecule conformation to changes in macromolecule diffusion coefficient and binding behaviors. However, these studies were done in simple model systems and may not completely explain processes occurring in more complex systems. Techniques to enhance NMR sensitivity at low ligand concentrations may be necessary to fully understand interactions that occur in real food matrices.

LITERATURE CITED

(1) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of *β*-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383−385.

(2) Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. Crystal structure of the trigonal form of bovine *β*-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *J. Mol. Biol.* **1987**, *197*, 695−706.

(3) O'Neil, T. E.; Kinsella, J. E. Binding of alkanone flavors to *β*-lactoglobulin: effects of conformational and chemical modification. *J. Agric. Food Chem.* **1987**, *35*, 770−774.

(4) Dufour, E.; Haertlé,ds T. Binding affinities of *β*-ionone and related flavor compounds to *β*-lactoglobulin: effects of chemical modifications. *J. Agric. Food Chem.* **1990**, *38*, 1691−1695.

(5) Pelletier, E.; Sostmann, K.; Guichard, E. Measurement of interactions between *β*-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. *J. Agric. Food Chem.* **1998**, *46*, 1506−1509.

(6) Sostmann, K.; Guichard, E. Immobilized *β*-lactoglobulin on a HPLC-column: a rapid way to determine protein-flavour interactions. *Food Chem.* **1998**, *62*, 509−513.

(7) Andriot, I.; Harrison, M.; Fournier, N.; Guichard, E. Interactions between methyl ketones and β-lactoglobulin: sensory analysis, headspace analysis, and mathematical modeling. *J. Agric. Food Chem.* **2000**, *48*, 4246−4251.
(8) Guichard, E.; Langourieux, S. Interactions between β-lactoglobulin and flavour compounds. *Food Chem.* **2000**, *71*, 301−308.
(9) Jouenne, E.; Crouzet, J. Effect of pH on retention of aroma compounds by β-lactoglobulin. *J. Agric. Food Chem.* **2000**, *48*, 1273−1277.
(10) Jouenne, E.; Crouzet, J. Determination of apparent binding constants for aroma compounds with β-lactoglobulin by dynamic coupled column liquid chromatography. *J. Agric. Food Chem.* **2000**, *48*, 5396−5400.
(11) Muresan, S.; van der Bent, A.; de Wolf, F. A. Interaction of β-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: nonlinear quenching effects related to protein−protein association. *J. Agric. Food Chem*. **2001**, *49*, 2609−2618.
(12) Kinsella, J. E. Flavor perception and binding to food components. In *Flavor Chemistry of Lipid Foods*; Min, D. B., Smouse, T. H., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; pp 376−403.
(13) Lubbers, S.; Landy, P.; Voilley, A. Retention and release of aroma compounds in foods containing proteins. *Food Technol*. **1998**, *52*, 68−74, 208−214.
(14) Jung, D.-M.; de Ropp, J. S.; Ebeler, S. E. Application of pulsed field gradient NMR techniques for investigating binding of flavor compounds to macromolecules. *J. Agric. Food Chem.* **2002**, *50*, 4262−4269.
(15) Tanner, J. E. Use of the stimulated echo in NMR diffusion studies. *J. Chem. Phys*. **1970**, *52*, 2523−2526.
(16) Stilbs, P. Fourier transform pulsed-gradient spin−echo studies of molecular diffusion. *Prog. NMR Spectrosc.* **1987**, *19*, 1−45.
(17) Hambling, S. G.; McAlpine, A. S.; Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry—1: Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 141−190.
(18) Shimizu, M.; Saito, M.; Yamauchi, K. Emulsifying and structural properties of β-lactoglobulin at different pHs. *Agric. Biol. Chem.* **1985**, *49*, 189−194.
(19) Kella, N. K. D.; Kinsella, J. E. Enhanced thermodynamic stability of β-lactoglobulin at low pH. *Biochem. J.* **1988**, *255*, 113−118.
(20) Mckenzie, H. A. In *Milk Proteins: Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic Press: New York, 1970; Vol. 2, pp 257−329.
(21) Townend, R.; Herskovits, T. T.; Timasheff, S. N. The state of amino acid residues in β-lactoglobulin. *Arch. Biochem. Biophys.* **1969**, *129*, 567−580.
(22) Tanford, C.; Bunville, L. G.; Nozaki, Y. The reversible transformation of β-lactoglobulin at pH 7.5. *J. Am. Chem. Soc*. **1959**, *81*, 4032−4036.
(23) Everhart, C.; Johnson, C. S. The determination of tracer diffusion coefficients for proteins by means of pulsed field gradient NMR with applications to hemoglobin. *J. Magn. Reson.* **1982**, *48*, 466−474.
(24) Chen, A.; Shapiro, M. J. NOE pumping: a novel NMR technique for identification of compounds with binding affinity to macromolecules. *J. Am. Chem. Soc.* **1998**, *120*, 10258−10259.
(25) Lübke, M.; Guichard, E.; Tromelin, A.; Le Quéré, J. L. Nuclear magnetic resonance spectroscopic study of β-lactoglobulin interactions with two flavor compounds, γ-decalactone and β-ionone. *J. Agric. Food Chem.* **2002**, *50*, 7094−7099.
(26) Lübke, M.; Guichard, E.; Le Quéré, J. L. Infrared spectroscopic study of β-ionone interactions with flavour compounds. In *Flavor Release: Linking Experiments, Theory and Reality*; Roberts, D. D., Taylor, A, J., Eds.; American Chemical Society: Washington, DC, 2000; pp 142−152.
(27) Damodaran, S.; Kinsella, J. E. Flavor protein interactions. Binding of carbonyls to bovine serum albumin: thermodynamic and conformational effects. *J. Agric. Food Chem*. **1980**, *28*, 567−571.
(28) *The Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra*; Pouchert, C. J., Behnke, J., Eds.; Aldrich Chemical Co.: Milwaukee, WI, 1993; Vol. 1, p 643.
(29) Wishnia, A.; Pinder, T. W. Hydrophobic interactions in proteins. The alkane binding site of β-lactoglobulins A and B. *Biochemistry* **1966**, *5*, 1534−1542.
(30) Dufour, E.; Roger, P.; Haertlé, T. Binding of benzo(α)pyrene, ellipticine, and cis-parinaric acid to β-lactoglobulin: influence of protein modifications. *J. Protein Chem.* **1992**, *11*, 645−652.

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