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pH-induced changes in adsorbed β-lactoglobulin molecules measured using atomic force microscopy†

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Received 8th July 2008, Accepted 21st August 2008
First published as an Advance Article on the web 9th October 2008
DOI: 10.1039/b811609a

We have used atomic force microscopy (AFM) imaging and force spectroscopy to study β-lactoglobulin (β-LG) food protein molecules adsorbed onto a mica surface. In particular, we have studied the effect of in situ changes in pH on several different properties: the topographical morphology of the adsorbed β-LG molecules, adhesion of the β-LG molecules to the underlying mica substrate, and the mechanical unfolding of the β-LG molecules. In AFM images, the structure of the adsorbed protein layer was observed to change dramatically with changes in pH. This result was consistent with the mechanical unfolding of single protein molecules within the adsorbed protein layer at different pH values performed using AFM. The short rupture length (~50 nm) measured for the fully unfolded protein at an acidic pH value of 2.5 is in good agreement with the dominant single molecule population measured previously for this pH value. Unfolding β-LG molecules from the same protein layer at a neutral value of pH = 6.8 resulted primarily in longer rupture lengths, corresponding to dimers of β-LG. AFM force–distance curves collected at pH = 9 were dominated by a large repulsion between the AFM tip and the adsorbed protein layer, which is likely due to the extended nature of the molecules because of irreversible denaturation for pH values greater than 9. This work provides a novel insight into the mechanisms of protein adsorption onto surfaces and shows that AFM force spectroscopy is a promising tool for probing in situ conformational changes in single molecules under various conditions.

Introduction

Proteins are large complex amphipathic molecules containing a combination of ionic, polar and non-polar regions. Because of this, they interact strongly with a wide range of surfaces, from lipid membranes in living organisms to metal pipes in food processing plants. In particular, the adsorption of proteins at liquid–solid interfaces is of increasing interest because of its implications for safety in the biomedical, biotechnology, advanced materials and food industries. For example, adsorption of proteins onto food processing equipment surfaces is a severe problem which can lead to change dramatically with changes in pH. This result was consistent with the mechanical unfolding of single protein molecules within the adsorbed protein layer at different pH values performed using AFM. The short rupture length (~50 nm) measured for the fully unfolded protein at an acidic pH value of 2.5 is in good agreement with the dominant single molecule population measured previously for this pH value. Unfolding β-LG molecules from the same protein layer at a neutral value of pH = 6.8 resulted primarily in longer rupture lengths, corresponding to dimers of β-LG. AFM force–distance curves collected at pH = 9 were dominated by a large repulsion between the AFM tip and the adsorbed protein layer, which is likely due to the extended nature of the molecules because of irreversible denaturation for pH values greater than 9. This work provides a novel insight into the mechanisms of protein adsorption onto surfaces and shows that AFM force spectroscopy is a promising tool for probing in situ conformational changes in single molecules under various conditions.

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† Submitted as a contribution to the Soft Matter web theme on Food Science.
changes and biomolecular processes in real time under native conditions, AFM also allows the manipulation of molecules and the measurement of the strength of molecular interactions with piconewton sensitivity. It is relatively straightforward to attach biomolecules to the AFM tip so that the interaction between these molecules and the sample, which could be a protein, nucleic acid, or cell surface, can be measured.\(^{19,20}\) Perhaps one of the most exciting demonstrations of the potential of the AFM to measure biomolecular interactions has been the recent observations of the mechanical unfolding of single proteins. Several groups have shown that a protein, held between the tip and a solid support, can be mechanically unfolded by pulling on the molecule with the AFM tip.\(^{21,22}\) The external mechanical force plays the role of the denaturant and leads to sequential unfolding of the three-dimensional structure of individual proteins.

In the present work we first describe our use of AFM imaging together with AFM scratching to investigate structural changes of adsorbed \(\beta\)-LG layers on an underlying hard mica surface in response to \textit{in situ} changes in pH. Then we correlate these measurements with changes in protein conformation due to \textit{in situ} changes in pH probed by AFM-based force spectroscopy.

**Experimental**

**Sample preparation**

AFM studies of proteins require the protein molecules to be immobilized on a substrate that is flat and rigid. A wide variety of biological molecules have been adsorbed onto the basal surface of muscovite mica, and these studies have shown that the mica surface is an excellent substrate for biological molecules since the molecules can be easily distinguished from the underlying mica surface and, furthermore, were found to retain their native-like structure. For these reasons, mica was chosen as the substrate in the present study.

\(\beta\)-LG was purified from whey protein isolate (New Zealand Dairy Products) by preparative ion chromatography on Q Sepharose (GE Healthcare) with a procedure described by Andrew et al.\(^{23}\) The \(\beta\)-LG solution was dissolved in a 20 mM imidazole buffer at room temperature and then the pH was adjusted to a value of 6.8 by adding HCl. This solution was then filtered through a 0.8 \(\mu\)m filter (Millex-HV, Millipore Co., Billerica, MA). 60 \(\mu\)l of the \(\beta\)-LG solution (0.5 mg ml\(^{-1}\)) was incubated with a freshly cleaved muscovite mica surface for 30 min. Longer periods of incubation produced similar results. After incubation, the surface was rinsed with 200 \(\mu\)l of the same imidazole buffer with pH = 6.8 to remove non-adsorbed proteins, to prevent any further adsorption and to prevent drying of the protein layer.

**Atomic force microscopy (AFM) measurements**

AFM images and force–distance measurements were performed in contact mode at room temperature using a Multimode AFM with a Nanoscope IV Controller (Digital Instruments, Veeco, Santa Barbara, CA). V-shaped Si\(_3\)N\(_4\) cantilevers with oxide-sharpened tips (Olympus, spring constant of 0.047 N m\(^{-1}\)) were used. The spring constants of the cantilevers were determined using the thermal noise technique provided with the instrument software.

AFM images were recorded in both height and deflection modes. The height images provided quantitative information on sample surface topography. A higher contrast of morphological details was typically observed in the deflection images. Force–distance curves were measured using a \(z\) velocity of 0.5 \(\mu\)m s\(^{-1}\), both on approach and retraction, with an interaction time of \(\sim\)1 s between the sample and the AFM tip. For all measurements the maximum loading force was 1 nN. In the force–distance curves shown below, the deflection of the cantilever was subtracted from the measured piezo displacement values. For each sample, several images and hundreds of force–distance curves were collected and they were analyzed using the Nanoscope IV software (Version 6.13r1, Digital Instruments, Veeco).

All AFM measurements were performed under imidazole buffered solutions. The pH of the fluid above the adsorbed protein layers was changed \textit{in situ} to achieve an acidic value of 2.5 and an alkaline value of 9.0, as measured using pH indicator paper, by adding small amounts (typically \(~\sim\)30 \(\mu\)l) of hydrochloric acid (0.1 M HCl) or sodium hydroxide (0.1 M NaOH), respectively. This procedure allowed the same region of the protein layer to be investigated using the same AFM tip for different pH values.\(^{24}\)

**Results**

**AFM imaging of \(\beta\)-lactoglobulin at different pH values**

In Fig. 1 are shown representative AFM height and deflection images recorded in contact mode for \(\beta\)-LG molecules adsorbed onto a freshly cleaved mica surface prepared in an imidazole buffer (pH = 6.8) and then imaged at different pH values. The AFM images were recorded on the same sample using the same AFM tip and tip surface forces between 0.1 and 0.3 nN. As can be seen in Fig. 1A, at neutral pH (pH = 6.8) the morphology of the adsorbed protein layer was homogeneous, corresponding to uniform coverage of the mica surface with \(\beta\)-LG molecules and no evidence for the existence of large protein aggregates, and the protein layer was stable upon repeated AFM scanning. Line scans of the AFM height images shown in Fig. 1A revealed that the height of the \(\beta\)-LG molecules ranged from 1 to 2.5 nm at this pH value (pH = 6.8). After changing the pH in the liquid above the same protein layer to an alkaline value (pH = 9) by introducing a small volume of NaOH, the surface morphology was significantly different from that measured at neutral pH. The protein layer was weakly adsorbed on the mica surface, as revealed by the displacement of the proteins by the AFM tip during scanning using low forces (\(~\sim\)0.1 nN) (see the AFM height image in Fig. 1B). In addition, the AFM deflection image shown in Fig. 1B indicates that the mica surface is not fully covered with \(\beta\)-LG molecules with large areas, such as that indicated by the white box, containing very few protein molecules. The line scan shown in Fig. 1B revealed globular molecules which ranged from 0.5 to 1.5 nm, which is smaller than the size of the \(\beta\)-LG monomers (\(~\sim\)3.6 nm). The surface morphology also changed dramatically when the pH of the solution above the same protein layer was changed from a neutral value to an acidic value (pH = 2.5). As can be seen in Fig. 1C, the mica surface was fully covered and protein

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*Soft Matter, 2009, 5, 220–227 | 221*
aggregates with different heights and widths were present over the entire surface. However, some small areas, such as that highlighted by the white box, were similar to the overall morphology seen at neutral pH. Line scans performed on the AFM height image showed a height distribution that ranged from 0.5 to 10 nm.

During AFM imaging using contact mode in buffer solutions, the forces between the tip and the sample are sufficiently small such that there is just a small amount of damage to the soft protein layers (few proteins could be displaced during the scanning), but we observed that continuous scanning with a large value of the applied force could deform the soft surface. Therefore, AFM tip “scratching” of the surface can be used to investigate the adhesion between the protein molecules and the underlying mica surface. In this study, we have used the AFM tip to scratch small areas (1 \times 1 \mu m^2) of the adsorbed protein layer, potentially exposing the underlying mica surface, at different pH values using a relatively large value of the applied force of about 10 nN. As shown in Fig. 2A, which was collected at neutral pH (pH = 6.8), there was evidence of scratching of the protein layer from the observation of the accumulation of material at the left and right ends of the horizontal scratches. The scratched area was still uniformly covered by \( \beta \)-LG molecules with vertical and lateral extents that were the same as those in the sample outside the scratched area. The height of the scratched protein layer was estimated from line scans of the height image to be about 2 nm. It was not possible to estimate the thickness of the unscratched protein layer since there were no bare regions on the mica substrate.

On the same sample, we changed the pH value of the solution above the protein layer to an alkaline value (pH = 9) and we

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**Fig. 1** AFM height (left) and deflection (right) images of \( \beta \)-LG molecules adsorbed onto a mica surface under imidazole buffer and at different pH values. The height profiles correspond to cross-sections of the AFM images along the white lines. (A)Neutral condition: pH = 6.8; (B) alkaline condition: pH = 9; and (C) acidic condition: pH = 2.5. All of the images correspond to a sample area of 2 \times 2 \mu m.

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performed scratching of the surface using the same value of the tip surface force (10 nN). This force was large enough to remove the adsorbed protein layer, exposing the flat underlying mica surface, as indicated by the flatness of the scratched area and the corresponding height profile shown in Fig. 2B. From line scans of the AFM height images, we estimated the thickness of the adsorbed protein layer to be 0.5 nm.

The pH value of the solution above the protein layers was also changed from a neutral to an acidic value (pH = 2.5). After scratching the protein layer using the same value of the loading force (10 nN), the morphology of the scratched area was significantly different from that observed for pH = 6.8 and pH = 9. As can be seen in Fig. 2C, the scratched area was much rougher than the unscratched areas, with protein aggregates that were about 2 nm in height.

**AFM force spectroscopy at different pH values**

AFM force spectroscopy is a well-established technique for investigating individual molecules or molecular complexes on surfaces. In the present study, we have used AFM force spectroscopy to investigate the mechanical stability of β-LG molecules at different pH values. In these measurements, the AFM tip was pushed onto the protein layer adsorbed onto the mica surface under a buffer solution, applying forces of ~0.5–1 nN for ~1 s. Within this time period, the β-LG molecules adsorbed onto

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**Fig. 2** AFM height (left) and deflection (right) images showing the effect of AFM tip scratching on the adsorbed β-LG layer at different pH values. To scratch the protein layer, small areas (1 × 1 μm²) were first scanned using a large applied force (10 nN), and then 3 × 3 μm² images were collected of the same areas using smaller forces (maximum of 1 nN). The height profiles correspond to cross-sections of the AFM images along the white lines. (A) Neutral condition: pH = 6.8; (B) alkaline condition: pH = 9; and (C) acidic condition: pH = 2.5.
the AFM tip, and then the AFM tip was pulled away from the adsorbed layer while recording the cantilever deflection (force) as a function of the displacement of the AFM cantilever. Upon retraction, we measured the force exerted on the AFM cantilever due to the β-LG molecule as a function of the displacement of the AFM cantilever. An adhesive force between the AFM tip and the β-LG molecule corresponds to a negative deflection. At neutral pH (pH = 6.8), almost all of the force–distance curves collected on different spots on the same sample indicated adhesion between the AFM tip and the β-LG layer. A large fraction (about 85%) of the retraction curves displayed a sawtooth pattern with two or more peaks (see Fig. 3A), with the distance between each peak corresponding to the length of the peptide regions which represent a barrier against unfolding. The remainder of the measurements showed a single unbinding peak in the retraction curves. Multiple force–distance curves collected at the same spot on the protein layer yielded reproducible force–distance curves. For each experimental condition, several hundred force–distance curves were collected. Statistical analysis of these curves allowed us to reliably identify the force peaks characteristic of the mechanical unfolding of the β-LG molecules. In Fig. 3B and C are shown histograms of the measured rupture force and the corresponding rupture length measured on the same sample using the same AFM tip. The rupture force histogram was asymmetric with a mean rupture force value of 180 pN (Fig. 3B). The rupture length histogram revealed that most unbinding events were accompanied by nonlinear elongation forces with rupture lengths ranging from 15 to 220 nm (mean value ≈ 100 nm). We suggest that these elongation forces and large rupture lengths are due to the mechanical unfolding of the adsorbed β-LG molecules. Similar data were obtained using five different AFM tips and five samples. Force spectra performed on the same sample, using the same AFM tip, at an alkaline value of pH = 9 were substantially different from those measured at neutral pH = 6.8. As shown in Fig. 4, all of the force–distance curves showed a significant repulsion between the AFM tip and the protein layer for both the approach and the retraction parts of the force–distance curves. The average repulsion distance, defined as the distance at which the force deviated significantly from zero due to the repulsive interaction, was about 100 nm. For the acidic value of pH = 2.5, force spectroscopy measurements were performed on the same sample using the same AFM tip as for the neutral pH value. As can be seen in Fig. 5, the measured force–distance curves were dramatically different from those measured for pH = 9, with a significant adhesion observed between the AFM tip and the β-LG layer, and the adhesion forces were much larger with shorter rupture lengths than those measured at neutral pH (cf. Fig. 3). From the histogram of the unbinding forces we obtain a mean value of 260 pN (Fig. 5B) which is significantly larger than that measured at neutral pH. The rupture lengths for the unbinding events varied from 10 nm to 120 nm with a mean value of ~50 nm (Fig. 5C) that was significantly smaller than that measured at neutral pH.

We note that the significant changes observed in the interaction between the AFM tip and the protein layer with changes in pH, e.g. rupture force and rupture distance histograms, were...
reversible for changes in pH between neutral and acidic values, but not for changes in pH between the neutral and alkaline values.

**Discussion**

**AFM imaging**

Protein adsorption is a complicated process which shows a significant dependence on the solution conditions (temperature, pH and ionic strength), the nature of the solid surface and other factors, such as the concentration of the protein solution and the incubation time with the solid surface. In the present study, deposition of β-LG molecules at neutral pH (pH = 6.8) resulted in multilayer coverage of the mica surface, as determined by scratching of the protein layer with the AFM tip, but there was no indication for the existence of large aggregates (cf. Fig. 1A). Although both the mica surface and β-LG molecules have a net negative charge at pH = 6.8,[25,26] the AFM tip scratching experiment revealed strong protein adsorption to the mica surface since little evidence of the scratching of the protein layer was observed. Protein adsorption onto surfaces at a given pH value is determined by the conformation of the protein molecules as determined by protein–buffer interactions, and the interplay of attractive and/or repulsive interactions between the protein molecules, and between the protein molecules and the surface.[1,2,27] We suggest that the ionic composition of the buffer (20 mM imidazole) might influence the adsorption of β-LG molecules to the mica surface. Since we could not reach the underlying mica surface by scratching the adsorbed protein layer with a reasonably large value of the applied force (~10 nN), indicating strong adsorption of the β-LG molecules to the mica, it is not surprising that the measured heights (~2 nm) of the globular molecules were much smaller than the known size of the protein (~3.6 nm) at neutral pH.[28] β-LG is known to adsorb strongly even at low bulk concentrations to both hydrophilic and hydrophobic surfaces including the air–water interface, and conformational changes may take place upon protein surface adsorption.[29,30] In addition, native globular proteins form more condensed and more tightly packed layers than random-coil denatured proteins.[31]

When the pH value of the solution above the adsorbed protein layer was changed to an alkaline value (pH = 9), the interaction between the β-LG molecules and the mica surface was dramatically affected such that it was possible to scratch and remove the adsorbed protein layer to expose the underlying mica surface (Fig. 1B). Because the maximum height (~1.5 nm) measured for the adsorbed layer was less than half the size of the protein in its native conformation (~3.6 nm), it is clear that the protein was denatured and partially unfolded at this pH value but not tightly bound to the mica substrate. Thus, the net charge of the β-LG molecules, which is ~2 at this value of pH,[32] affected its surface-active properties. In addition we have found that when the pH of the same adsorbed protein layer was changed to a neutral value of pH = 6.8 after being held at pH = 9 for about 1 h, the morphology of the film was indistinguishable from that observed at pH = 9 (Fig. 1B). This finding confirms that the β-LG molecules undergo an irreversible denaturation at alkaline pH values, as has been shown previously using various techniques.[28,33] Furthermore, it was demonstrated in several studies that the irreversible alkaline denaturation of β-LG occurs in two stages. In the first stage, the α-helical content decreases significantly and only certain β-sheets unfold. Then, in the second stage of the alkaline denaturation, the remaining β-sheets unfold though not completely. This two stage denaturation process is consistent with the molecular size distribution given by line scans of the AFM height images in the present study (ranging from 0.5 to 1.5 nm, as shown in Fig. 1B).

The primary cause of the aggregation of the β-LG molecules with changes in pH is thought to involve electrostatic interactions between molecules.[14] In some cases, protein aggregation proceeds by random sticking of the molecules, leading to unstructured aggregates; in other cases the proteins form specific contacts, leading to aggregates of well-defined structures such as amyloid fibres. In our case, at pH = 2.5, the AFM results indicate that the aggregates that are formed have a thickness of two or three protein monomers with no clearly defined structure (Fig. 1C). In particular, the structure of the aggregates observed

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**Fig. 5** (A) Representative curves of the retraction portion of AFM force–distance curves, recorded between the AFM tip and the adsorbed β-LG layer under imidazole buffer at pH = 2.5, showing a sawtooth pattern corresponding to the mechanical unfolding of single β-LG molecules. (B) Histogram of rupture forces measured from hundreds of force–distance curves showing a mean rupture force of 260 pN. (C) Histogram of rupture length showing a mean rupture length of 50 nm.
in our study differs from those formed after heat-induced denaturation at pH = 2 under salt (NaCl) conditions.\(^3\)\(^5\) Also, our AFM images revealed that β-LG molecules at pH = 2.5 adhere strongly to the mica surface and the high force (10 nN) used to scratch the protein layer results only in the disruption of some of the protein–protein structures. In addition, the structural changes of the protein layer induced by changing the pH from pH = 6.8 to pH = 2.5 were reversible: by subsequently increasing the pH value back to 6.8, AFM imaging revealed a similar morphology to that observed originally at pH = 6.8 (Fig. 1A).

**AFM force spectroscopy**

The stability of the native protein structure is sensitive to the degree of protonation of various side-chain groups. Thus, pH-dependent conformational changes can reflect differences in interchain interactions due to changes in the ionization state of individual amino acid side-chains. Changes in the force–distance curves between the AFM tip and a protein molecule picked randomly from the surface measured as a function of pH can be attributed to pH-induced conformational changes of the protein molecule. The application of a stretching force in AFM force spectroscopy unravels a protein along a specific direction, and reveals what the secondary structure of the protein was before the molecule was stretched. In this study, at neutral pH, stretching β-LG molecules results in force–distance curves which have characteristic sawtooth patterns, with each force peak due to a disruption of intra- and intermolecular interactions which represents a barrier against unfolding. When the externally applied force overcomes the strength of these molecular interactions in a given domain, all amino acids in that domain unfold spontaneously, with the measured force dropping abruptly to zero. Although most force–distance curves show common force peaks at the same value of the separation distance between the tip and the sample surface, there are variations in the magnitude of the maximum force measured for the individual peaks (see Fig. 3A and 5A). The retraction portion of the force–distance curves measured at neutral pH displays two populations of unfolding events with different rupture lengths (see Fig. 3). Since each β-LG molecule consists of 162 amino acid residues which form a thread that is 58.32 nm long (assuming a contour length per residue of 0.36 nm) when fully unfolded, the population characterized by the short rupture length that is ~60 nm long and consists of essentially two individual force peaks can be attributed to the unfolding of a single β-LG molecule. The second population of unfolding events with a longer rupture length of ~120 nm are likely to correspond to the unfolding of β-LG dimers. Other oligomerization states (trimer, tetramer) may also be present with rupture lengths ranging up to 220 nm as suggested by other studies.\(^3\)\(^6\) In addition, the average unfolding force of ~180 pN measured for a pulling speed of 0.5 μm s\(^{-1}\) is in qualitative agreement with the values measured in other AFM protein unfolding studies.\(^2\)\(^1\)\(^2\)^\(\text{21}\)\(^2\)^\(\text{22}\)

At pH = 2.5, a peak with a relatively large maximum force is typically present in the retraction portion of the force–distance curves at a small value of separation distance (<20 nm) (see Fig. 5A). This peak indicates a substantial adhesion between the AFM tip and the protein molecules (due to the large value of the maximum force), as well as a substantial adhesion of the protein molecules to the underlying mica substrate and between the protein molecules themselves (due to the rupture of the bond between the AFM tip and the protein molecule at a relatively small distance). The adhesion between the AFM tip and the protein layer is likely to occur because the protein molecules are positively charged while the AFM tip is negatively charged at this value of pH. The charge on each individual molecule varies from zero at the isoelectric point of pH = 5.1 to around +20 at pH = 2, as reported previously.\(^3\)\(^5\) Since the β-LG molecules strongly interact with each other and with the mica surface (negatively charged) under acidic conditions, the β-LG molecules show a significant resistance to AFM mechanical unfolding. As for the neutral pH condition, the mechanical unfolding of the β-LG molecules occurs in multiple steps, but with significantly larger forces (~260 pN for the average unfolding force, see Fig. 5). The short rupture length (average ~50 nm) is very close to the length of a single, fully unfolded β-LG molecule. However, a significant fraction (~20%) of the force–distance curves show a longer rupture length with a maximum at ~120 nm. This result is consistent with earlier work which shows that, for pH<3, the dimers dissociate into monomers while preserving their native conformations.\(^2\)\(^8\)\(^3\)\(^3\)\(^3\) We conclude that β-LG shows a remarkable stability to low values of pH, resisting denaturation even at pH = 2.5. A fundamental understanding of the driving forces and mechanisms involved in protein aggregation, however, is still outstanding.

It should be noted that all transitions in β-LG that occur at pH values between pH = 2 and pH = 9 do not cause any appreciable changes in the native-like β-barrel conformation of β-LG. In contrast, for pH>9, β-LG molecules undergo an irreversible unfolding transition with global disruption of both secondary and tertiary structures.\(^2\)\(^8\)\(^2\)\(^3\)\(^3\)\(^3\) The observation of a large repulsion between the AFM tip and the adsorbed protein layer in AFM force–distance curves collected at pH = 9 is likely because of the net negative charge on the β-LG molecules and the extended nature of the molecules due to the irreversible unfolding transition at large values of pH. We propose that in our measurements performed under alkaline conditions, the pH of the protein layer may be slightly higher than 9 since the β-LG denaturation was irreversible and the very large electrostatic repulsion between the tip and the protein layer persisted after changing the pH to a neutral value. This irreversible denaturation for pH values greater than 9 has been attributed to thiol/disulfide exchange caused by the interaction between the free cysteine group and the broken disulfide bond.\(^3\)\(^8\)

**Conclusion**

The β-LG molecule has been widely studied, in large part because of its importance to the food industry. The aim of the present work was to shed new light on the properties of β-LG molecules adsorbed onto hard surfaces by using AFM imaging, scratching, and mechanical unfolding of the adsorbed molecules under aqueous conditions with in situ changes in pH. The surface morphology of the adsorbed protein layer changes substantially with changes in pH from neutral to acidic or alkaline conditions. While β-LG forms small aggregates under acidic conditions, it undergoes an irreversible denaturation at pH = 9. The significant advance of the present study of β-LG molecules is in the use of an
AFM tip to stretch and unfold adsorbed molecules at different pH values, for in situ changes in pH. Using this methodology we demonstrated that the adsorbed β-LG molecules maintain their known conformations, dimers and monomers, under neutral and acidic conditions respectively. The large electrostatic repulsion between the AFM tip and the adsorbed protein layer at pH = 9 was consistent with the denaturated state of the β-LG molecules. Further detailed AFM imaging and force spectroscopy analysis are needed to achieve a better understanding of the pH-dependent behavior of β-LG which may provide valuable insight into its biological role.

Acknowledgements

We thank Milena Corredig for providing the purified protein. The authors gratefully acknowledge financial support from the Advanced Foods and Materials Network (AFMnet), the Canadian Foundation for Innovation and the Natural Sciences and Engineering Research Council of Canada. JRD acknowledges support from the Canada Research Chairs (CRC) program.

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