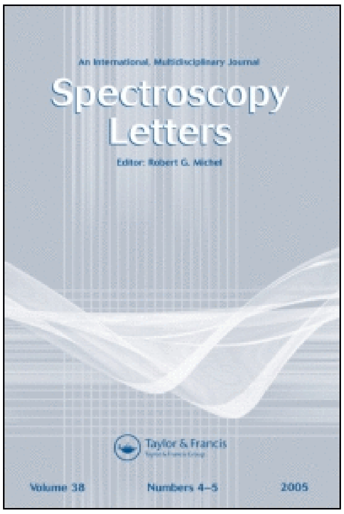

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FTIR-ATR Study of pH Effects on Egg Albumin Secondary Structure

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Abstract: The pH effects on the secondary structures of egg albumin were investigated using Fourier transform infrared–attenuated total reflection (FTIR-ATR) technique with a single-bounce diamond crystal. The albumin was first denatured in a series of solutions with pH ranging from 1 to 12. The albumin film was then cast on the ATR crystal from the albumin solution for the IR spectrum collection. Significant secondary structure spectral differences were observed for these films. The findings are presented in terms of the shape and position of the albumin amide I band between 1600 and 1700 cm^{-1}.

Keywords: Acid/base denaturation, albumin secondary structure, FTIR-ATR

INTRODUCTION

Albumin is a class of simple, water-soluble proteins that are found in egg white, blood serum, milk, and many other animal and plant fluids and tissues. Albumin has been the subject of many investigations because of its important roles in maintaining normal biochemical functions. Albumin is known to have a secondary structure that includes alpha-helices, parallel beta sheets, antiparallel beta sheets, and random coils. The secondary

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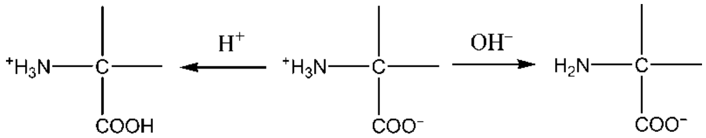
structure of albumin is sensitive to environmental changes, including changes in pH. Addition of an acid or a base to an albumin solution changes the number and the distribution of the charges on the protein:



$$\underset{COOH}{{}^{+}H_3N-\overset{|}{\underset{|}{C}}-} \xleftarrow{H^+} \underset{COO^-}{{}^{+}H_3N-\overset{|}{\underset{|}{C}}-} \xrightarrow{OH^-} \underset{COO^-}{H_2N-\overset{|}{\underset{|}{C}}-}$$

At low pH values, the net positive charge on albumin will increase, as carboxyl and amino groups are protonated, whereas at high pH values, the net negative charge will increase. These changes in the ionic interactions can destabilize albumin structure causing denaturation by:

- The development of like charges that cause internal repulsion
- The loss of opposite charges that may have been involved in attractive forces
- The loss of surface charges that may have kept individual albumin molecules apart.

However, quantitative evaluation of secondary structure in albumin under varied conditions remains difficult and challenging.[1,2]

Application of IR spectroscopy to the analysis of protein secondary structure is based on the sensitivity of peptide group absorptions to the polypeptide chain conformation. In particular, the absorptions of amide I band (between 1600 and 1700 cm^{-1}), mainly associated with the C=O stretching vibration and directly related to the protein backbone conformation, have been extensively used for the study of secondary structures (Table 1).[3–5]

In this study, we report our results of the pH effects on the secondary structure spectral features of egg albumin using Fourier transform infrared–attenuated total reflection (FTIR-ATR) technique. Commonly available household chemicals are used to produce solutions with pH values ranging from 1 to 12. The conformations of albumin under varied conditions, including extreme conditions, may provide insights into its denatured states under physiologic conditions. Our findings are presented in terms of the

Table 1. The approximation of protein secondary structure based on amide I band

	Amide I band
Alpha-helix	1652–1654 cm^{-1}[4–6]
Random coils	1642–1648 cm^{-1}[4–6]
Parallel beta sheets	1631–1637 cm^{-1}[4–6]
Antiparallel beta sheets	1624–1693 cm^{-1}[5,6]

shape and position of the albumin amide I band. Deconvolution procedure will be applied to these peaks in a later report.

MATERIALS AND METHODS

Solution Preparation

Basic Solutions

Solutions of base were prepared using 1.0 g of each base (sodium hydrogen carbonate, borax, sodium carbonate, and sodium hydroxide) in 100 mL of deionized water.

Acidic Solutions

Boric acid, vinegar (5% acidic acid solution), and 0.10 M hydrochloric acid were used to provide the acid solutions with varied pH values. The boric acid solution was prepared by dissolving 1.0 g boric acid in 100 mL deionized water. The 0.10 M hydrochloric acid was diluted from the certified 1 N hydrochloric acid (Fisher Scientific, Hanover Park, IL, USA).

Albumin Solutions

Four grams of albumin was added to each of the above solutions. The mixture was then stirred for 5–10 min to remove the gel-like substance that was formed initially. All solutions stood for 18 hr before testing.

pH Measurements

A pH electrode and amplifier (PASCO Scientific Model CI-6507) connected to a PASCO Science Workshop Computer Interface System (PASCO Scientific, Roseville, CA, USA) was used to measure the pH of the deionized (DI) water as well as that of each of the acid and base solutions. The electrode was dual point standardized using the buffers of $pH = 7$ and $pH = 4$ or 10. Because the electrode cannot measure the pH of the DI water directly due to DI water's low ionic strength, 1.0 g of sodium chloride (p.a.) was added to 100 mL of DI water for the pH measurement.

FTIR-ATR Spectroscopy

Spectra were acquired using a single-reflection ATR apparatus with a diamond internal reflection crystal (Courtesy of Thermo Scientific, Waltham, MA, USA). The ATR unit was installed in a Mattson Genesis II FTIR spectrometer (SpecTech). A drop of albumin solution was placed onto the surface of the ATR diamond crystal and allowed to air-dry. Spectra were recorded at 32 scans and 4 cm^{-1} resolution for both the background and sample collections.

Relative Polypeptide Chain Lengths of Albumin

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a BioRad MiniProtein Cell System (with a 4–20% BioRad Ready Gel) (Hercules, CA, USA) to determine the polypeptide length of the albumin treated with a strongly basic solution (pH ≥ 8.9) relative to that of the control albumin (albumin dissolved in DI water). The above albumin solution was diluted with DI water to a concentration of 2 mg albumin/mL DI water. The running buffer was prepared by diluting 10 mL of PAGEgel Running Buffer (20x) to 200 mL with DI water. The running sample was prepared by mixing 20 μL of the diluted albumin solution, 45 μL of DI water, 25 μL of PAGEgel LDS Sample Buffer 4x, and 10 μL of reducing agent (2-mercaptoethanol). The running sample (20 μL) was loaded to the sample well of the gel, which was submerged in the running buffer. A voltage of 150 V was applied across the gel for 2 hr. The staining solution was prepared by dissolving 0.125 g of Coomassie Brilliant Blue in a mixture of 50 mL of methanol, 10 mL glacial acetic acid, and 40 mL DI water. The relative polypeptide length of the treated albumin to that of the control albumin was measured using the R_f value, calculated as the ratio of the distance migrated by the treated albumin to that migrated by the control albumin.

RESULTS

The FTIR-ATR Spectrum of Albumin Film Formed from DI Water

The IR amide I band of albumin film cast from DI water (pH = 6.4) is shown in Fig. 1. The band is found to center around 1633 cm^{-1}, indicative of the presence of a large amount of parallel beta sheets (an estimate based on the peak heights at 1633 cm^{-1}, 1642 cm^{-1}, and 1652 cm^{-1} indicates approximately 45% beta structure and 35% alpha-helices in the sample). Because the major component of egg albumin is ovalbumin,[6] the result is in agreement with those reported by Kurdryashova et al.[5] In their IR-ATR study of protein secondary structure, Kurdryashova et al. found about 47% beta structure and about 35% alpha-helices in the native ovalbumin.

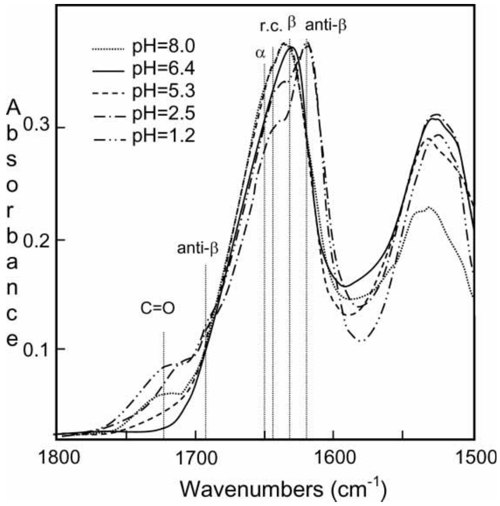


Figure 1. The amide I region in the FTIR-ATR spectra of egg albumin films. The films were formed onto the ATR diamond crystal surface from the albumin solutions of pH values of 1.2, 2.5, 5.3, 6.4, and 8.0.

The Acid Effects on Spectral Features

Figure 1 also shows the amide I peaks of the egg albumin films formed from acid solutions of different pH values. For the albumin treated with the boric acid solution (pH = 5.3), the amide I band is shifted to 1642 cm^{-1} from 1633 cm^{-1}, suggesting an increased amount in the irregular structures and a decreased amount in the beta-sheet structures. This observation may indicate a partial opening of the ordered structure into random turns and bends. Because the alpha-helical and random structures show absorptions fairly close together, without applying the deconvolution procedure, it is difficult to say if any significant increase in the alpha-helices has taken place.

For the albumin samples treated with more acidic solutions (5% acetic acid, pH = 2.5; 0.1 M HCl, pH = 1.2), two new peaks, 1624 cm^{-1} and 1693 cm^{-1}, clearly appear in their IR-ATR spectra. These peaks are assigned to the antiparallel beta sheets, typical for aggregated protein.[5,7] The absorptions due to the random coils at 1642 cm^{-1}, however, are reduced for both samples, although the film treated with the hydrochloric acid solution contains more disordered structures, as indicated by the higher absorption. The formation of this newly ordered conformation might result from the more loosened and unfolded form of albumin in the strongly acidic solution. As the albumin acquires more net positive change in the more acidic solution, turns and bends would repel each other further, resulting in the more random structures. This disorder at a larger scale might allow the chains to pack into the new arrangement, the antiparallel beta structure, upon forming the films on the ATR crystal.

It might also be possible that the antiparallel beta sheets are already formed in the strongly acidic solution prior to the film formation.

Also observed in the IR spectra of the albumin films treated with the acidic solutions is the new shoulder formed at 1728 cm^{-1}. The shoulder increases in intensity as the solution pH decreases. This shoulder, attributed to the C=O stretching of the carboxyl groups, might result from a redistribution of these carboxyl groups in albumin due to the secondary structural change.

The Base Effects on Spectral Features

The amide I band of the egg albumin film formed from sodium hydrogen carbonate solution (pH = 8.0) is also shown in Fig. 1. It can be seen that this amide I band is similar to that of the film formed from the boric acid solution (pH = 5.3): the main peak also occurs at 1642 cm^{-1}, indicating an increased randomness in the structure compared with the film treated with deionized water. This weak base albumin structure may be quite similar to the weak acid albumin structure. However, spectral differences do exist for these samples. For instance, the shoulder at 1728 cm^{-1} due to the C=O stretching is more prominent for the film obtained from the pH = 8.0 solution compared with that from the pH = 5.3 solution. Then again, these differences might come from the presence of the hydrogen carbonate ions in the pH = 8.0 solution. Additional work will be done in the future to see if denaturing albumin in different buffer systems would generate the same spectral changes for these films.

Figure 2 shows the FTIR-ATR spectra of the albumin films formed from pH = 8.9 (borax), 10.7 (sodium carbonate), and 12.0 (sodium hydroxide) solutions, respectively. The continued base denaturation of albumin causes the spectral change in a fundamentally different way compared with those associated with the acid or weak base denatured albumin:

1. The amide I peak height decreases dramatically as the solution pH increases. On the other hand, the peaks in the range of 1425 to 1335 cm^{-1}, likely due to the CH deformation vibrations, become progressively prominent in the IR spectra. Because amide I is primarily due to the C=O stretching of the backbone, its decrease implies a lost of C=O groups due to a backbone cleavage. The dominant absorption features at 1425–1335 cm^{-1} may be reasonably attributed to various cleavage products that are rich in CH groups. This suggestion is supported by the SDS-PAGE results. The R_f values for the albumin samples treated with borax, sodium carbonate, and sodium hydroxide solutions are 1.3, 1.5, and 2.9, respectively, indicating that the polypeptide chain length of the control albumin (albumin treated with DI water) is the longest and that of the albumin treated with sodium hydroxide solution the shortest.
2. In addition to its decreased height, the amide I peak gradually returns back to its initial frequency of 1633 cm^{-1} (beta sheets) from 1642 cm^{-1}

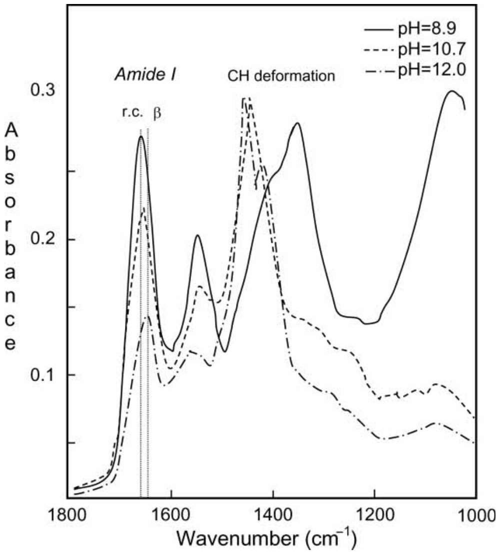


Figure 2. The FTIR-ATR spectra of egg albumin films in the region 1000–1800 cm^{-1}. The films were formed onto the ATR diamond crystal surface from the albumin solutions of pH values of 8.9, 10.7, and 12.0.

(random structures). The faster decrease in the amount of the random structures may suggest that the disordered chains are the weak points at which backbone cleavages likely take place. A significant amount of beta sheets appears to remain even in the sample treated with the sodium hydroxide solution (pH = 12.0).

CONCLUSIONS

We have shown that FTIR-ATR using a single-bounce diamond crystal can be effective at probing the pH-induced changes in the secondary structures of egg albumin films. Based on the amide I band position, the albumin film formed from deionized water appears to have a high content in the beta-sheet structure, which absorbs at 1633 cm^{-1}. The amide I bands show similar profiles for the albumin films formed from mild acid (pH = 5.3) and mild base (pH = 8.0) solutions, indicative of a similar denaturing process under these mild conditions, although spectral differences do exist. For the films formed from strong acids (pH < 3), newly ordered structures or the antiparallel beta sheets may be present as indicated by the appearance of the new peaks at 1624 cm^{-1} and 1693 cm^{-1}, and the amounts of random structures, signified at 1642 cm^{-1}, are not high. Our results also suggest that denaturing egg albumin in strongly basic solutions (pH $\geq$ 8.9) may result in backbone cleavage. This suggestion is supported by the SDS-PAGE measurements. The cleavage product film appears to retain the originally ordered structures and is rich in CH groups. Our future effort will include investigating how different buffer systems used to denature albumin will affect the IR-ATR spectra of the albumin films. We will also apply the deconvolution software such as GRAMS/Al to quantify the amounts of different secondary structures in various denatured states of egg albumin and other proteins.

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