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IONIC STRENGTH AND pH EFFECTS ON OPTICAL THERMOGRAPHS FOR BOVINE SERUM ALBUMIN (BSA)

EFECTOS DE LA FUERZA IÓNICA Y EL pH SOBRE LOS TERMOGRAMAS ÓPTICOS DE LA SEROALBÚMINA BOVINA (BSA)

Kongraksawech, T.1; Vázquez-Landaverde, P.1,2; Huerta-Ruelas, J.1,2; Torres, J. A.1*

1Food Process Engineering Group, Department of Food Science & Technology, Oregon State University, Corvallis, OR 97331-6602, USA.
2Centro de Investigación Avanzada y Tecnología Aplicada (CICATA), Instituto Politécnico Nacional (IPN), Querétaro, México.

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*Autor para la correspondencia/Corresponding author. E-mail: J_Antonio.Torres@OregonState.edu

Abstract
An optical method using a custom-built system was investigated to study heating effects on bovine serum albumin (BSA) as affected by pH and ionic strength (IS). Changes in the optical rotation (OR) and transmitted light (TL) of BSA in 0.01 M phosphate buffer at pH 6.1, 7, and 7.9 with IS maintained at 0.04, 0.08, and 0.16 were monitored while heating the solution at ~6 °C/min from room temperature to ~85 °C. There was no significant effect of IS on BSA's denaturation temperature (T_d) values. T_d was affected by pH with lower values observed at pH 7.9 than at pH 7 but not statistically different from values at pH 6.1. Values at pH 6.1 and 7 were not significantly different either. These observations were consistent with literature values determined by differential scanning calorimetry. Changes in the TL signal, reflecting the formation of an opaque gel, were not detected at pH 7.9, identified only at the two highest IS at pH 7, and observed always at pH 6.1, i.e., closest to the pH value for the isoelectric point (pI) of BSA reported to be around pH 4.7 to 5.2. Promoting of gel formation by IS was attributed to the screening of intra- and intermolecular electrostatic forces.

Resumen
Un método óptico basado en un sistema experimental especialmente construido para este propósito fue usado para investigar la influencia del pH y la fuerza iónica sobre los efectos térmicos en la seroalbúmina bovina (BSA). Los cambios en la rotación óptica (OR) y la luz transmitida (TL) de soluciones buffer de fosfato 0,01 M con 2,5% de seroalbúmina bovina (BSA) a pH 6.1, 7 y 7.9 y una fuerza iónica (IS) de 0,04, 0,08 ó 0,16 fueron monitoreados durante su calentamiento a ~6 °C/min desde temperatura ambiente hasta ~85 °C. No se observó un efecto estadísticamente significativo de IS sobre la temperatura de desnaturalización térmica (T_d). T_d fue afectada por el pH con valores menores observados a pH 7,9 que a pH 7 pero no estadísticamente diferentes del valor a pH 6.1. Valores a pH 6.1 y 7 tampoco fueron estadísticamente diferentes entre ellos. Estas observaciones son consistentes con valores reportados en la literatura y obtenidos por el método de Calorimetría Diferencial de Barrido (DSC). Cambios en la señal de TL, reflejando la formación de un gel opaco, no fueron detectados a pH 7,9, encontrados solo a los valores mayores de IS a pH 7, y observados consistentemente a pH 6,1, el valor de pH incluido en este estudio más cercano al punto isoeeléctrico de BSA y reportado entre pH 4,7 y 5,2. El efecto de la IS en la mayor formación de geles fue atribuido al su capacidad de bloquear fuerzas electrostáticas intra- e intermoleculares.

Keywords: Optical properties, bovine serum albumin, transmitted light
Palabras clave: Propiedades ópticas, seroalbúmina bovina, luz transmitida

INTRODUCTION

Optical techniques have been demonstrated to be powerful tools for monitoring chemical processes allowing real-time/in-situ measurements in research and process control applications (Huerta-Ruelas et al., 2007; Kongraksawech et al., 2007). Previous work reported the use of an optical system to study thermal effects on proteins. System performance and experimental procedures were evaluated by determining the thermal denaturation temperature (T_d) of bovine serum albumin (BSA) in 0.01 M phosphate buffer at pH 7 and ionic strength (IS) 0.08. The determination of T_d values was reproducible as indicated by a coefficient of variation under 3% and experimental values were in general agreement with values reported in literature (Huerta-Ruelas et al., 2007; Kongraksawech et al., 2007). BSA has a molecular weight of 66 kDa and an isoelectric point (pI) between pH 4.7
and 5.2 (de Wit, 1981; Fox, 2003; Kilara and Harwalkar, 1996; Kilara and Vaghela, 2004; Murata et al., 1993; Relkin, 1996; Walstra and Jenness, 1984) and its conformation is affected by pH and IS. The conformation transformations of BSA induced by pH changes have been described as follows (Foster, 1977; Michnik et al., 2005; Yamasaki et al., 1990):

- **F-E transition**: acidic expansion
- **N-F transition**: Native (N) conformation
- **N-B transition**: B-E transition
- **alkaline expansion**
- **N-B transition**: B-E transition

<table>
<thead>
<tr>
<th>pH</th>
<th>F-E transition</th>
<th>N-F transition</th>
<th>N-B transition</th>
<th>alkaline expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>pH 3.5</td>
<td>pH 4.5 to pH 7</td>
<td>pH 9</td>
<td>pH 11.5</td>
</tr>
</tbody>
</table>

At pH between 4.5 and 7, BSA is in its native (N) form. Lowering pH from 4.5 to 3.5 and 2, changes reversibly the N form to the fast form (F, fast migration during gel electrophoresis) and the expanded (E) form, respectively. When pH is raised from 7 to 9, the N form changes to the basic (B) form and subsequently to the E form when pH is further increased. The N form is the most compact whereas the E form is the most open structure.

Preparation of protein solutions

Solutions containing 2.5% (w/v) BSA were prepared by dispersing protein into 0.01 M phosphate buffer at pH 6.1, 7, and 7.9 and IS 0.04, 0.08, and 0.16. The solutions were stirred for 2 h to ensure complete protein dissolution and prepared separately for each experimental run. The experimental pH range was chosen based on the phosphate buffer capacity to prevent changes in the protein solution pH. A single buffer system was used to avoid a buffer system effect. Deionized water was used to prepare all solutions.

Optical system

A custom-built optical system (Huerta-Ruelas et al., 2007) and procedures previously reported for the study of thermal effects on proteins (Kongraksawech et al., 2007) were used to perform all optical measurements. A polarized laser beam obtained using a 643 nm semiconductor laser module (Model LTG650125-T, Lasermate Group Inc., Pomona, CA) and a Glan Taylor polarizer (Model MGTYB20, Karl Lambrecht Corp., Chicago, IL) was modulated at 50 KHz using a photoelastic modulator (Model PEM-80, Hinds International, Inc., Hillsboro, OR) (Kemp, 1969). The modulated laser beam passed through the sample contained in a controlled-heating cell and then through a Rochon polarizer acting as analyzer (Model RPPC-0912, Lambda Research Optics, Inc., Costa Mesa, CA). A silicon detector (Model UV-005, OSI Optoelectronics, Inc., Hawthorne, CA) with a Low Noise Single JFET Op-amp TL-071 (OSI Optoelectronics, Inc.) with gain defined according to system transmitted signal to operate in the linear response range (i.e., 2 to 7 V output) was used to observe changes in optical rotation (OR) and transmitted light (TL) changes of the laser beam. The electric signal generated was filtered using a lock-in amplifier (Model 5207, EG&G Princeton Applied Research, Princeton, NJ) operated with a constant time of 1 s and 12 dB/octave filter. The lock-in amplifier sensitivity was adjusted for each protein concentration so as to maximize the signal to noise ratio. Sample temperature and optical signals were collected in real-time by a computer data acquisition card (DAQ 6036E, National Instrument Inc., Austin, TX) controlled using LabView™ (Version 7 Express, National Instrument Inc.).

Sample handling and measurement procedures previously reported (Kongraksawech et al., 2007) were implemented as follows. Characteristic points, Td and TL1, in the OR and TL signal profiles plotted against temperature were identified using OriginLab® (Version 6.0, OriginLab Corporation Inc., Northampton, MA). First, a significant slope change point in the OR or TL profile was visually identified and marked with Origin’s data marker tool. Next, two additional data points at 15 °C below and 10 °C above the slope change point were marked. Origin’s Linear Fit tool was then used for linear fits between the data points below/above the slope change point and the slope change.

MATERIALS AND METHODS

BSA (A7906, batch 112K0589) was obtained from Sigma Chemical Co. (St. Louis, MO). Dibasic sodium phosphate (Na₂HPO₄) and monobasic sodium phosphate (NaH₂PO₄) were purchased from EM Industries, Inc. (Gibbstown, NJ) and EMD Biosciences, Inc. (San Diego, CA), respectively. Analytical grade NaCl was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI).
point. The intersection points from the fitted lines in the OR and TL profiles were designated as the Td, indicating conformational changes of BSA, and TL1, revealing protein gel formation, respectively. If the difference between estimated and calculated values was more than 0.5 °C, an additional iteration was performed. In cases when the OR or TL signal showed a sudden rather than a gradual change in slope, Td and TL1 values were determined as the last temperature point before the sudden change. When BSA formed turbid gels or aggregated significantly, the TL signal reached values lower than 2 V, the lowest level, defined by its signal to noise ratio, that the detector can use for an acceptable linear response to changes in the OR signal. The temperature at this point was defined as TL2. Finally, each protein solution was tested in triplicate and values were reported as the average.

Statistical analysis

Experimental data were analyzed using SPSS (Version 13, SPSS Inc., Chicago, IL). The analysis of variance (ANOVA) was used to determine statistical differences among Td values. Tukey-HSD (95%) was used to determine significant pH and IS effects on Td values.

RESULTS AND DISCUSSION

Unheated BSA measurements

OR values for unheated 2.5 % BSA in 0.01 M phosphate buffer at all pH and IS levels tested were measured for 5 min. The average value and standard deviation of the OR signals collected showed statistical differences among some conditions but a fine scale graph resolution was required to show them visually (Figure 1). The extremely small standard deviations indicated that the optical system provided stable signals. Moreover, the small differences among the average OR values for unheated samples are not practically significant when compared to the much larger changes observed during the heating of protein solutions (Figures 2-3). A probable source for these small average OR differences between samples is the always present uncertainty in weighing components and measuring volumes during sample preparation. Furthermore, these small differences showed no trend in OR values for solutions at pH 6.1, 7, and 7.9 and IS 0.04, 0.08, and 0.16 suggesting that the pH and IS levels used in this study had no significant effect on the conformation of unheated BSA. Also, the amounts of NaCl added to the protein solution to adjust IS values may have reduced intramolecular repulsion forces in BSA molecules further increasing the stability of the N form at the pH near neutral tested in this study. This is consistent with published work by Foster (1977), Yamasaki et al. (1990) and Michnik et al. (2005) who reported that BSA occurs in the N form at pH between 4.5 and 7.

Effect of pH on the thermal stability of BSA

The determination of BSA’s Td values on the basis of optical thermographs had good reproducibility with an average coefficient of variation under 2 % (Table 1). At pH 7.9, Td values were lower than at pH 7 but they were not statistically different from those at pH 6.1. Values at pH 6.1 and 7 were not significantly different either (Table 1). The effect of pH on Td values reflected the conformational stability of BSA as affected by the buffer pH. Foster (1977), Michnik et al. (2005) and Yamasaki et al. (1990) reported that BSA has the same conformational structure (N form) at pH between 4.5 and 7. Barone et al.
example, Barone et al. (1992) examined the heat denaturation of defatted serum albumin (10^4 M) in 0.2 M tris-HCl buffer. DSC determinations (0.5 °C/min) of T_d at pH 6.3, 7, and 8 gave values of 63, 64.5, and 61 °C, respectively. Yamasaki et al. (1990) investigated the effect of pH on the thermal denaturation of defatted BSA (2 %, 0.1 M NaCl) in water with pH adjusted by NaOH or HCl finding that at pH 7, BSA had the maximum T_d value, 64.3 °C, followed by values of 63.9 and 60.9 °C at pH 5.61 and 8.01, respectively. Finally, Giancola et al. (1997) reported the T_d values of defatted BSA (20 mg/mL) in 0.01 M phosphate buffer with 0.15 M NaCl at pH 6, 7, and 8 were 66.8, 64.05, and 59.6 °C, respectively.

(1995) also concluded that the maximum structural stability of BSA was in the 6-7 pH range. These conclusions appear consistent with the observation that BSA in buffer at pH 6.1 and 7 had no significant difference in T_d values and that the T_d value at pH 7 was higher than that of pH 7.9 (Table 1).

Although, T_d values obtained from optical thermographs (Table 1) were slightly higher than reported values obtained from DSC measurements, the effect of pH followed the behavior reported by several authors. For example, Barone et al. (1992) examined the heat denaturation of defatted serum albumin (10^4 M) in 0.2 M
Table 1. Effect of pH and IS on the thermal denaturation (T_d, °C) and transmitted light change temperature (TL_1, TL_2, °C) for 2.5% BSA in 0.01 M phosphate buffer. 1 & 2 = indicate homogenous subsets of mean T_d for pH. a = indicate homogenous subsets of mean T_d for IS. * indicates no change in TL observed during heating to 90 °C. ** indicates that the TL signal remained always above 2V.

<table>
<thead>
<tr>
<th>pH</th>
<th>IS</th>
<th>T_d</th>
<th>% CV</th>
<th>TL_1</th>
<th>TL_2</th>
</tr>
</thead>
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<tr>
<td>6.1</td>
<td>0.04</td>
<td>68.8</td>
<td>2.6</td>
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<td>-</td>
</tr>
<tr>
<td>6.1</td>
<td>0.08</td>
<td>68.1</td>
<td>2.6</td>
<td>71.6</td>
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</tr>
<tr>
<td>6.1</td>
<td>0.16</td>
<td>68.7</td>
<td>2.6</td>
<td>72.0</td>
<td>77.8</td>
</tr>
<tr>
<td>7.0</td>
<td>0.08</td>
<td>70.4</td>
<td>2.6</td>
<td>72.1</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>0.16</td>
<td>69.5</td>
<td>2.6</td>
<td>72.4</td>
<td>-</td>
</tr>
<tr>
<td>7.9</td>
<td>0.08</td>
<td>67.9</td>
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<tr>
<td>7.9</td>
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<td>68.1</td>
<td>0.6</td>
<td>72.0</td>
<td>77.8</td>
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</table>

It is possible that the lower T_d values reported in the literature as compared to the ones obtained in this study reflect differences in the BSA sample used. Defatted BSA has lower thermal stability than the nondefatted BSA used in this study (Gumpen et al., 1979; Michnik, 2003). Also, DSC determinations of T_d values published in the literature are based on energy peaks in the thermogram assumed to indicate the protein conformational change point. In the approach used in this study, the OR slope change point in the optical thermogram is assumed to coincide with the conformational change of the protein. Therefore, a comparison of experimental and reported T_d values must consider the difference of the principle used between the DSC method and the experimental technique used in this study (Kongraksawech et al., 2007).

Examples of optical profiles for 2.5 % BSA in 0.01 M phosphate buffer adjusted to pH 0.04 and pH 6.1, 7, and 7.9 during heating from room temperature to ~85 °C are shown in Figure 2. At this IS level and pH 6.1, BSA formed an opaque gel as evidenced by the decrease in TL with heating beginning at TL_1 whereas, BSA in buffer at pH 7 and 7.9 formed transparent gels. At higher IS levels (optical profiles not shown), turbid gels were formed also at pH 7 (Table 1). Intermolecular repulsion forces may be responsible for the formation of turbid BSA gels in the 4.8 6 pH range and clear ones below pH 4.8 or above pH 6 (Jensen et al., 1950; Donato et al., 2005). At pH approaching the BSA isoelectric point, pH 4.7 to 5.2 (de Wit, 1981; Fox, 2003; Kilara and Harwalkar, 1996; Kilara and Vaghela, 2004; Murata et al., 1993; Relkin, 1996; Walstra and Jenness, 1984), intermolecular repulsion forces are minimal, leading to rapid formation of gels during heating with less interstitial spaces for binding water. If the pH of the BSA solution is far from the pl, higher intermolecular repulsion forces lead to a more gradual gel formation with more interstitial spaces for binding water (Jensen et al., 1950; Donato et al., 2005).

A second significant slope change in the OR signal for BSA solutions prepared at pH 6.1 and IS 0.04 (Figure 2a) appeared at temperature ~77 °C. This behavior was observed consistently in every replication of this pH and IS test condition (data not shown). This may be interpreted as caused by optically active protein groups becoming hidden during slow protein aggregation causing the slope change and the continuous increase in the OR signal. At the same pH but higher IS levels, BSA aggregated rapidly, therefore a sudden increase in the OR signal was observed.

Effect of IS

The statistical analysis of experimental T_d values showed no significant effect of IS. Previous studies (Gumpen et al., 1979; Kilara and Harwalkar, 1996; Yamasaki et al., 1990; Yamasaki et al., 1991) have shown that salts have a significant effect on electrostatic forces in proteins. Intramolecular electrostatic repulsion forces are shielded by the presence of salt at low concentration increasing the conformational stability of proteins. As observed in this study, the differences between T_d values of BSA at IS 0.04, 0.08, and 0.16 (0.01-0.15 M NaCl) were not statistically significant (Table 1). Yamasaki et al. (1991) found that NaSCN stabilized BSA when added at concentrations in the 0.02 M range and had the opposite effect at higher concentrations while NaCl up to 2 M increased continuously the T_d value.

Optical profiles during heating of 2.5 % BSA in 0.01 M phosphate buffer adjusted to pH 7 and IS 0.04, 0.08, and 0.16 are shown in Figure 3. Changes in transmitted light characterized by TL_1 values were observed at IS 0.08 and 0.16 but not at IS 0.04 indicating that gel turbidity was promoted by IS. In addition, BSA at IS 0.16 formed a more turbid gel than at IS 0.08. Similar behavior was also observed in other optical profiles (data not shown). The gel turbidity increase with salt concentration can be explained by the ability of salt to reduce electrostatic forces between BSA molecules (Yamasaki et al., 1991; Murata et al., 1993) facilitating intermolecular interactions.

CONCLUSIONS

The analysis of optical thermographs confirmed that thermal effects on BSA are both pH and IS dependent. The effect of pH on the thermal stability of BSA correlated well with conformational transformations reported in the
literature and obtained from DSC studies. BSA gel formation was influenced by pH as well. At pH approaching its pI value, BSA formed opaque gels. In the presence of low NaCl concentrations, thermal denaturation of BSA was not affected but protein gel formation was promoted. These results showed $T_d$ and TL values obtained using a custom-built optical system used can be applied to study thermal effects on proteins.

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