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# Chicken albumin exhibits natural resistance to glycation

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ABSTRACT

Glycation of proteins and subsequent production of advanced glycation end products (AGEs) is a major contributor to the pathophysiology of diabetes. The objective of the present study was to compare the glycation of avian and human serum albumin to elucidate the mechanisms by which protein glycation in birds is prevented in the presence of naturally high plasma glucose concentrations. Solutions of purified chicken and human serum albumin (CSA and HSA) were prepared with four different glucose concentrations (0, 5.56, 11.1, and 22.2 mM) and incubated at three temperatures (37.0, 39.8, and 41.4 °C) for seven days. The solutions were sampled on Days 0, 3, and 7 and analyzed by liquid chromatography-electrospray ionization-mass spectrometry for the presence of glycated albumin. Four-way repeated measures ANOVA ($p = 0.032$) indicate that all independent variables (albumin type, glucose concentration, temperature and time) interacted to affect the degree of glycation. With increasing glucose concentration, the glycation of both HSA and CSA increased with time at all temperatures. In addition, HSA was glycated to a greater extent than CSA at the two higher glucose concentrations for all temperature conditions. Glycation was elevated with increasing temperatures for HSA but not CSA. The results suggest an inherent difference between human and chicken albumin that contributes to the observed differences in glycation. Further research is needed to characterize this inherent difference in an effort to elucidate mechanisms by which avian plasma protein is glycated to a lesser degree than that of mammals (humans).


## 1. Introduction

As reported by the Centers for Disease Control and Prevention, diabetes remains the seventh leading cause of death in the United States (Xu et al., 2016). Hyperglycemia, the hallmark of diabetes, promotes the production of advanced glycation end products (AGEs), which originate from the nonenzymatic reaction of a reducing sugar, such as glucose, with proteins. This initial reaction results in the production of a Schiff base that can rearrange to form an Amadori product, such as glycated hemoglobin. Under euglycemic or hypoglycemic conditions, glucose can detach from Schiff bases and Amadori products whereas hyperglycemic conditions promote the irreversible rearrangement of Amadori products to AGEs (Brownlee et al., 1984; Peppa et al., 2003; Ulrich and Cerami, 2001). Once formed, AGEs tend to accumulate and are considered to be the primary cause of the majority of complications associated with diabetes including vascular diseases, neuropathy, and nephropathy (Brownlee et al., 1984; Cade, 2008; Peppa et al., 2003; Singh et al., 2014). Atherosclerosis risk in particular is strongly correlated with AGE concentrations (Chang et al., 2011) as AGEs promote collagen cross-linking and increased susceptibility of low density lipoproteins to oxidation, critical steps in the development of atheromas (Basta et al., 2004). Albumin, hemoglobin, and collagen are just a few examples of proteins that are subject to glycation in mammals. Damage induced by AGEs is typically initiated by their interaction with receptors for AGEs, i.e. RAGE, resulting in increased oxidative stress (Ramasamy et al., 2011; Singh et al., 2014; Szwergold and Miller, 2014).

Birds are a unique model to examine potential strategies for preventing the development of hyperglycemia-mediated pathologies. The normal plasma glucose concentration of many avian species is 1.5–2 times those of mammals of similar body mass (Braun and Sweazea, 2008). Moreover, birds are resistant to the glucose-lowering effects of insulin, which may maintain the relative hyperglycemia compared to mammals (Braun and Sweazea, 2008; Sweazea and Braun, 2005; Sweazea et al., 2006). In addition, of the few species that have been examined, protein glycation is quite low (Holmes et al., 2001). For example, a study examining hemoglobin found glycation to be in the range of 0.5–1.0% in ducks, chickens, and turkeys. In contrast, for mammals the range was 1.7–5.8% glycation (Beuchat and Chong, 1998). Hummingbirds, which have the highest fasting plasma glucose

*Abbreviations:* AGEs, advanced glycation end-products; CSA, chicken serum albumin; HSA, human serum albumin; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry.

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concentrations of all vertebrates measured (300–756 mg/dL in the fasted and fed states, respectively), were found to have glycated hemoglobin levels of 3.7–4.6% (Beuchat and Chong, 1998). Although higher than other avian species, the percent glycation is lower than what the World Health Organization has established as a guideline for diagnosing diabetes in humans, 6.5% (WHO, 2011).

Iqbal et al. (1999) concluded that protein glycation in chickens is not likely the main cause of glucose-derived crosslinks and that the accumulation of glycated products is less in birds than mammals. In fact, recent studies have shown that the receptor for AGEs (i.e. RAGE) first appeared in mammals and is not present in the avian genome (Sessa et al., 2014). The lack of RAGE in birds could also explain why high blood glucose concentrations do not cause the same complications as observed in humans with hyperglycemia. However, this does not explain the protective mechanisms that exist in birds to reduce glycation of plasma proteins. Considering these remarkable characteristics of birds, it is perhaps not surprising that they outlive mammals of comparable body size (Braun and Sweazea, 2008; Holmes et al., 2001; Speakman, 2005). Moreover, these data suggest that the production of glycated proteins as well as hyperglycemia-mediated inflammation and oxidative stress, as opposed to elevated plasma glucose concentrations per se, are what promotes pathologies.

The goal of the present study was to identify factors that contribute to lower levels of protein glycation in avian species, despite their naturally high glucose concentrations. As a prior study described high protein turnover in birds (Jaensch, 2013), the present study utilized isolated albumin to eliminate this factor. Moreover, other data have described a lower concentration of plasma proteins in birds, which would result in less overall glycation (Bartholomew and Dawson, 1954). Thus, we examined protein glycation in HSA and CSA samples matched for protein concentration. The reversible exothermic nature of the protein glycation reaction suggests that temperature may impact the reaction. Therefore, all experiments were carried out at temperatures relevant for humans and chickens. Normal body temperature of birds is approximately 39–42 °C (Whittow, 1999), slightly higher than the normal body temperature of humans (Baumann and Baumann, 1977). Urbanowski et al. (1982) incubated purified human serum albumin in vitro for 24 h at temperatures ranging from 5 to 55 °C in the presence of constant concentrations of either D-galactose or D-glucose. The authors observed a positive correlation between incubation temperature and protein glycation with both sugars (Urbanowski et al., 1982). Considering the naturally low levels of protein glycation in birds, we hypothesized that chicken serum albumin would be more resistant to glycation compared to human serum albumin at any temperature, time or glucose concentration.

Albumin was chosen as it is the most abundant circulating protein (Kim and Lee, 2012; Singh et al., 2014) and it exhibits a relatively high degree of glycation when exposed to glycemic conditions over time (Austin et al., 1987). In addition, previous work has shown that the susceptibility of albumin to glycation does not differ greatly across mammalian species as it does for hemoglobin (Rendell et al., 1985). Albumin has a half-life of 12 to 21 days in humans (Rondeau and Bourdon, 2011). This is significantly shorter than the half-life of hemoglobin (Kim and Lee, 2012). Data show a strong correlation between the glycation of albumin over a 3-week period and that of hemoglobin over a 3-month period (Kim and Lee, 2012). Therefore, the use of albumin as a marker of protein glycation allows an assessment of blood glucose control over a shorter-period of time (Kim and Lee, 2012; Rondeau and Bourdon, 2011). The use of liquid chromatography (LC) and mass spectrometry (MS), known as LC-MS, to analyze protein glycation (Borges et al., 2011 & 2014) allows for higher throughput and greater sensitivity than prior analyses (Wuhrer et al., 2005) and has been widely implemented to measure percent glycated albumin (Borges et al., 2011; Brede et al., 2016; Dong et al., 2014; Rabbani et al., 2016). Another advantage to this technique is the ability to pinpoint and quantify individual albumin molecules with two (i.e. doubly-glycated), rather than just one, glucose molecules attached. This level of precision in measurement allows for a more thorough and detailed examination of the extent of protein glycation in a sample that is not available using other methods (Wuhrer et al., 2005).

## 2. Material and methods

### 2.1. Materials

Purified albumins were purchased from commercial suppliers. Chicken serum albumin (CSA) from Equitech-Bio, Inc., Kerrville, TX (Cat No. CSA62 Lot #CSA62-1254) and human serum albumin (HSA) from Sigma Aldrich, St. Louis, MO (Cat No. A3782 Lot #SLBD7204V). The reported purity was ≥96% for CSA and >99.9% for HSA. HEPES-buffered saline solution prepared in deionized water was used as vehicle (134.3 mM NaCl (Cat. No. S9888, Sigma-Aldrich, St. Louis, MO), 6 mM KCl (Cat. No. P4504, Sigma, St. Louis, MO), 1.0 mM $MgCl_2$ (Cat. No. M2670, Sigma-Aldrich, St. Louis, MO), 1.8 mM $CaCl_2$ (Cat. No. C3881, Sigma-Aldrich, St. Louis, MO), and 9.14 mM NaHEPES (Cat. No. H3784, Sigma, St. Louis, MO)). A stock solution of 27.8 mM (0.5 mg/μL) D-glucose (Cat. No. G8270, Sigma-Aldrich, St. Louis, MO) was also prepared.

### 2.2. In vitro incubations

Following the preparation of 8.4 mg/mL HSA or CSA in HEPES-buffered saline, the pH was adjusted to 7.4 (VWR Symphony pH meter, Ross Electrode, Radnor, PA) and the solutions were sterilized using a syringe filter. Glucose stock (0.5 mg/μL) was then added to the albumin-HEPES solutions to achieve the following final concentrations of glucose (in mM): 0, 5.56 (normal human), 11.11 (normal avian), and 22.22. Aliquots (100 μL) of each solution were then transferred to PCR tubes and incubated in triplicate at 37.0 °C (normal human), 39.8 °C (normal avian, high human), and 41.4 °C (high avian) using a thermal cycler (MyCyler; BioRad, Hercules, CA) set to hold each temperature indefinitely. Aliquots (2 μL) were extracted from all samples after vortexing at baseline (Day 0, prior to incubation), Day 3, and Day 7 and were stored at −80 °C until analyses.

### 2.3. Measurement of percent glycated albumin by LC-ESI-MS

All aliquots were diluted 30:1 with 0.1% trifluoroacetic acid (TFA) and analyzed intact by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) on a Dionex Ultimate 3000 HPLC (Thermo Scientific, Sunnyvale, CA) equipped with a 1:100 flow splitter connected to a Bruker Maxis 4G quadrupole-time-of-flight (Q-TOF) mass spectrometer (Billerica, MA). A trap-and-elute form of LC-MS was carried out in which 15 μL samples were loaded at 10 μL/min in 80/20 water/acetonitrile containing 0.1% formic acid (loading solvent) onto a Bruker-Michrom protein captrap configured for bi-directional flow on a 6-port diverter valve. The flow over the captrap was then switched to the micropump, set at 2 μL/min, and ramped over 5 min from 80% water containing 0.1% formic acid (Solvent A) / 20% acetonitrile (Solvent B) to 90% acetonitrile and held for 3 min. The captrap eluent was directed to the mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the *m/z* range of 300 to 3000 with a nominal resolving power of ~60,000 m/Δm FWHM. ESI settings for the Agilent G1385A capillary microflow nebulizer ion source were as follows: End plate offset −500 V, capillary −3500 V, nebulizer nitrogen 2 bar, dry gas nitrogen 3.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 4 GHz. Spectra rate control was by summation at 1 Hz.

HSA eluted over a period of about 1 min; under the above conditions, HSA ranged in charge state from +32 to +71. Raw mass spectra were averaged across this timeframe, smoothed 0.0482 Da, baseline subtracted 0.85, charge deconvoluted and baseline subtracted 0.85