JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Conjugation of Bovine Serum Albumin and Glucose under Combined High Pressure and Heat

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ABSTRACT: The effect of combined heat and pressure on the Maillard reaction between bovine serum albumin (BSA) and glucose was investigated. The effects in the range of 60−132 °C and at 0.1−600 MPa on the lysine availability of BSA were investigated at isothermal/isobaric conditions. The kinetic results showed that the protein−sugar conjugation rate increased with increasing temperature, whereas it decreased with increasing pressure. The reaction followed 1.4th order kinetics at most conditions investigated. A mathematical model describing BSA−glucose conjugation kinetics as a function of pressure and temperature is proposed. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were used to verify BSA−glucose conjugation and to identify the glucosylated sites. These indicated that the application of combined high pressure and high temperature resulted in significant differences in the progression of the Maillard reaction as compared to heat treatments at atmospheric pressure.

KEYWORDS: high pressure, bovine serum albumin, glucose, glycation kinetics, Maillard reaction, MALDI-TOF-MS, SDS-PAGE

INTRODUCTION

The utilization of high pressure, an emerging technology in the area of food processing, has constantly increased over the last two decades.[1] In the food industry, the main task of high pressure processing (HPP) is to increase the shelf life of foods by inducing structural changes to the cell membrane of micro-organisms and inactivating important enzyme systems, which disrupt the micro-organisms' metabolism. HPP is considered an attractive alternative to traditional heat pasteurization and sterilization processes since it often only minimally alters the taste, texture, appearance, or nutritional value of the exposed food system. Apart from its food preservation capabilities, HPP has also shown potential to manipulate biochemical transformation reactions and the functionality of food ingredients such as fats, proteins, and carbohydrates.[1]

Bovine serum albumin (BSA) is a protein component of whey and blood. Its physiochemical and structural properties have been well-characterized,[2] making it a desirable model protein for food systems. Limited research investigating the effects of HPP on BSA shows that the solution properties of the protein can be affected by pressure. For example, partial unfolding of BSA has been observed at pressures as low as 200 MPa.[3] However, the protein is relatively stable up to 400 MPa due to its rigid and compact, globular structure stabilized by 17 disulfide bonds.[4] Nevertheless, similarly to other proteins, the changes to BSA structure largely depend on the magnitude and duration of the pressure applied.

As with thermal treatment, the pressure-induced unfolding of BSA can rapidly lead to aggregation. BSA tends to aggregate and form oligomers at pressures above 400 MPa, depending on concentration and treatment time.[5] The aggregation of BSA after pressure treatment is considered to be mainly due to the formation of intermolecular disulfide bonds via −SH/−SS interchange,[5] although hydrophobic-induced oligomerization of the protein due to the exposure of hydrophobic residues cannot be ruled out. High pressures have a substantial effect on the secondary structure of BSA. These structural changes mainly consist of a decrease in α-helical structure accompanied by a slight increase in β-sheet structure.[6] Although these changes to BSA might occur at low pressure (e.g., 400 MPa), most studies report that pressures between 800 and 1000 MPa (these pressures are higher than those usually used in food processing applications, e.g., up to 600−700 MPa) are required to bring upon alterations to the secondary structure of the protein.[7] For example, Hayakawa et al.[7] reported a decrease in BSA α-helix content of up to 50% at 1000 MPa and 20−40 °C after 10 min. In comparison to heat treatment, HPP of BSA up to 600 MPa results in negligible modifications to its secondary structure (3% as compared to 40% at 80 °C).[4] Structural modifications are mostly reversible over time because the disruption of hydrogen bonds and Van der Waals forces is confined to the course of pressurization. However, irreversible structural modifications can occur, depending on the pH, protein concentration, and other experimental conditions.[7,8] The presence of polysaccharides reportedly increases the pressure and heat stability of BSA and enhances the reformation of its secondary structure after pressure treatment.[9] This is largely related to complex formation between the protein and the polysaccharide inhibiting protein−protein interactions.[8,10] BSA glycated with sugar is also known to possess increased heat stability.[11]

The Maillard reaction, a nonenzymatic browning reaction, involves the interaction between carbonyls and amines resulting in the formation of a wide range of products, including color and odor compounds. The reaction can have both beneficial and detrimental outcomes, depending on the food system.[12]

Received: November 9, 2010
Accepted: February 17, 2011
Revised: February 15, 2011
Published: March 11, 2011

Generally, it is suggested that Maillard reactions are hampered by high pressure.[13,14] On the other hand, it has been reported that Maillard reactions are enhanced at pressures as high as 600 MPa, under certain pH conditions.[15,16]

The objective of this study was to investigate the extent of Maillard-induced interaction between the BSA and the reducing sugar, α-D-glucose (Glc), in a model system subjected to different pressure−temperature conditions. The reaction was carried out at 1:10 w/w BSA:Glc and pH 9.0 using bicine buffer, which is known to be pressure stable.[17] It has to be stressed that buffers, particularly phosphate buffers, are known to influence the Maillard reaction by accelerating sugar−amine condensation, sugar fission, and reductones production.[18−20] Unfortunately, there are no published reports on the conformational changes of BSA and its aggregation due to the use of bicine buffer at alkaline pH. At pH 9.0, BSA isomerizes reversibly to the basic B form. If stored for 3−4 days at low temperature (3 °C) and ionic strength at the same pH, it changes to the aged A form, which is known to consist of less α-helices.[21,22] In this study, the effect of the combined heat and high pressure treatments in the range of 60−132 °C and at 0.1−600 MPa isothermal/isobaric conditions, on the extent of conjugation, were investigated using the *o*-phthaldialdehyde (OPA) method. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed for further evidence that conjugation of BSA and Glc had occurred, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was carried out to elucidate the glucosylated sites.

EXPERIMENTAL SECTION

Materials. BSA was purchased in a dried state from AusGenX (#PBSA010701, Arundel, QLD, Australia). The chemical composition (by weight %) provided by the manufacturer was as follows: 96% protein (100% purity), 2% moisture, 1.1% ash, and 0.024% fatty acid. Anhydrous Glc (#MKBB8469) was acquired from Sigma Aldrich (St. Louis, MO).

The BSA−Glc mixtures were prepared by dissolving 2.5 mg/mL BSA and 25 mg/mL Glc in 0.05 M bicine buffer (#039K5437, Sigma) adjusted to pH 9.0. The high ratio of Glc to BSA was chosen to ensure a good degree of irreversible glucosylation.[23] This is because sugars tend to react faster than amino compounds in the Maillard reaction, and they might caramelize, isomerize, fragment, or form diglucosylamines, whereas amino compounds might even be regenerated from the initial condensation product.[12,24] From the published literature, it was found that a common ratio for BSA:Glc mixtures is 1:5, which translates to a ratio of 12:1 for glucose per lysine residue. A ratio twice as high was used in the present study, as it was shown that a large excess of glucose results in increasing attachment of glucose per BSA molecule.[9]

Thermal and High Pressure Treatments. BSA−Glc solutions were treated under isothermal/isobaric conditions at temperatures ranging from 70 to 132 °C and a pressure of 0.1 to 600 MPa. Samples were pipetted into 2.0 mL crimp vials (#03390540, Agilent Technologies Inc., Santa Clara, CA) and were sealed with an 11 mm seal PTFE/rubber cup (#43605304AS, Agilent Technologies). Heat-only treatments at 70−90 °C were performed in a temperature-controlled water bath (#WB20, Ratek, Boronia, VIC, Australia), whereas treatments at 100−132 °C were performed in a shaking water bath (#SWB20, Ratek, Boronia, VIC, Australia) filled with glycerol (#38199, Merck, Darmstadt, Germany). Treatment times at isothermal temperatures ranged from 0 to 120 min, with shorter treatment times at higher temperatures. Following treatment, the samples were immediately cooled in iced water, and chemical analysis was performed within 4 h. The initial free (i.e., chemically available) amino group concentration (C_0) was defined as the concentration measured after heating the glass vials to the target temperature (approximately 1 min) followed by immediate cooling of the sample in iced water. All experiments were performed at least in duplicate.

Pressurization experiments under isothermal conditions were performed using a multivessel high pressure unit (#U111, Unipress, Warsaw, Poland) as described previously.[25] The target temperature was varied between 70 and 120 °C, and the pressure ranged from 200 to 600 MPa. The BSA−Glc solution was filled with no airspace into cryogenic vials (#5000−1012, Nalgene, Rochester, NY). The cap of each vial was pierced with a needle to insert the thermocouple. Samples were sealed and stored at approximately 4 °C before they were placed into the pressure vessels and preheated to the target temperatures. Pressurization was started when the sample reached a temperature level, which would result in the target temperature after compression heating. The compression rate was set to 21 MPa/s to achieve an operational pressure of 600 MPa in less than 30 s. Note that preheating times are very short but vary slightly with the pressure−temperature conditions applied. Typically, preheating times are in the range of 30−75 s (e.g., approximately 60 s for treatment at 110 °C and 600 MPa).[25] Treatment time was started as soon as isobaric and isothermal conditions were reached. A data acquisition system (2700 Integra multimeter, Keithley Instruments, Cleveland, OH) connected to the high pressure multivessel apparatus U111 software for data acquisition (Version 2.1c, Unipress, Warsaw, Poland) was used to monitor the pressure and temperature history of each sample. Samples were immediately stored on ice after treatment for further analysis. The initial free amino group concentration (C_0) was defined as the concentration found after pressure build-up followed by immediate pressure release and storage on ice for up to 4 h. All experiments were performed at least in duplicate.

SDS-PAGE. Monodimensional SDS-PAGE under reducing conditions was used. In brief, 50 mL of NuPAGE MES SDS running buffer (20×) (#685344, Invitrogen, Carlsbad, CA) was mixed with 950 mL of Milli-Q water. The gel running tank (XCell SureLock Novex mini cell, Invitrogen) was set up with a precast NuPAGE 4−12% Bis-Tris gel 1.0 mm × 17 well (#9111174, Invitrogen). Two hundred milliliters of the diluted running buffer and 500 μL of NuPAGE antioxidant (#690288, Invitrogen) were placed into the inner chamber of the tank, and 800 mL of the diluted running buffer was added into the outer chamber.

BSA−Glc samples were diluted 1:4 with running buffer solution, and 25 μL of this solution was injected into 1.7 mL plastic vials (#070101-229, Axygen Scientific Inc., Union City, CA). Twenty microliters of NuPAGE LDS sample buffer (4×) (#700243, Invitrogen) and 5 μL of NuPAGE sample reducing agent (10×) (#658227, Invitrogen) were added, and the vials were stirred and heated for 10 min at 70 °C to reduce the disulfide bonds. Before the gel was loaded with 10 μL of each sample, the samples were thoroughly stirred. SDS-PAGE was run for 35 min at 200 V.

An 0.1% naphthol blue-black (#27697HJ, Sigma Aldrich, St. Louis, MO) in 7% (v/v) acetic acid (#k29637017132, BDH Laboratory Supplies, Poole, United Kingdom) solution was prepared and filtered through a #4 filter paper (#46123, Whatman, Maidstone, United Kingdom) to remove particles. The gel was washed overnight in 7% acetic acid solution (pH 2.38) after being stained for 2 h in the naphthol blue-black solution. It was then transferred to a G Box transilluminator and gel imaging system (SynGene, Cambridge, United Kingdom) for photography.

OPA Assay. Measurement of the free amino groups of the BSA−Glc solution was performed using a modified OPA method[26] suitable for microplates and fluorescence readers as described by Lochmann et al.[27] Aqueous 0.1 M boric acid buffer (#33082, Merck, Darmstadt, Germany) was prepared and adjusted to pH 9.5. A 0.5 M OPA solution was prepared by dissolving OPA (#1388274, Sigma Aldrich) in ethanol. The OPA solution was stored at 4 °C in the dark to increase its shelf life.

N-Acetyl-L-cysteine (NAC) (#038K0711, Sigma Aldrich) was dissolved in boric acid buffer at 0.05 M NAC and was also stored at 4 °C. The OPA−NAC reagent for protein analysis was prepared daily by gentle mixing of 0.75 mL of OPA, 0.75 mL of NAC, and 6.0 mL of boric acid buffer. Background fluorescence could be decreased by storing the reagent for at least 2 h at 4 °C.[27]

Heat- and/or high pressure-treated samples were diluted 1:10 to be within the linear range of the assay, and 100 μL was pipetted into the wells of a black microplate optiplate 96F (#810635, Perkin-Elmer, Waltham, MA). A Flash microplate reader (4.00.25, Thermo Fisher Scientific) with SkanIt software (v2.4.3.37) was used to perform the fluorometric measurements. After a short incubation period at 25 °C, 200 μL of OPA reagent was automatically dispensed at medium speed into each well. Five minutes of shaking at 360 spm was followed by a 5 min settle delay for the reaction to occur. Fluorometric measurements at an excitation wavelength of 340 nm and an emission wavelength of 455 nm were measured using an excitation bandwidth of 12 nm for 100 ms.

Kinetic Data Analysis. The amount of free amino groups (C/C_0) was determined by dividing the number of free amino groups after treatment (C) by the initial number of free amino groups in the protein (C_0). The literature showed that most kinetics relating to the Maillard reaction have been described using an *n*-th order reaction model (eq 1).[20] Integration of eq 1 yields eq 2 introducing the specific inactivation rate constant k', which is dependent on the initial number of free amino groups C_0 (eq 3).[28]

$$\frac{\mathrm{d}C}{\mathrm{d}t} = -kC^n \tag{1}$$

$$\frac{C}{C_0} = [1 + k' \cdot t \cdot (n-1)]^{1/(1-n)} \tag{2}$$

$$k' = \left[k \cdot C_0^{(n-1)}\right] \tag{3}$$

where t is the time (min), k is the rate constant (min^{-1}), and n is the reaction order.

The reaction order n of eq 2 was determined by minimizing the cumulative sum of standard error of fit (ΣSD) over a wide range of reaction orders (1.0−2.0 in 0.1 increments) using Table Curve 2D (v.5.01, Systat Software Inc., Chicago, IL). After the reaction order was fixed to the lowest ΣSD, the k' values were obtained by regressively fitting eq 2 to the kinetic data set of all isothermal/isobaric conditions tested. C_0 was assumed to be constant in all experiments. This was done to obtain comparable values of the inactivation rate, which can then be used for secondary modeling.[29]

Modeling BSA Glucosylation at Isothermal/Isobaric Conditions. The effect of pressure and temperature on the k' value was expressed using a thermodynamically based model (eq 4), which has been successfully used to describe biochemical reactions as a function of pressure (p) and temperature (T):[30,31]

$$\ln(k') = \ln(k_0') - \frac{\Delta V_0}{RT}(p - p_0) + \frac{\Delta S_0}{RT}(T - T_0) - \frac{\Delta\beta}{2RT}(p - p_0)^2 + \frac{\Delta C_p}{RT}\frac{(T - T_0)^2}{2T_0} - \frac{2\Delta\alpha}{RT}(p - p_0)(T - T_0) \tag{4}$$

The equation couples the volume difference ΔV_0 and the change of the entropy ΔS_0, the compressibility factor β, the heat capacity C_p, and the thermal expansion factor α. Δ denotes the change of the corresponding parameter as the pressure or temperature is changed. These thermophysical constants were estimated by nonlinear regression analysis of the estimated k' values at different pressure−temperature combinations using a statistical program (Table Curve 3D v4.0 Statistical Package, Systat Software Inc., Richmond, CA).

The performance of the secondary model (eq 4) was assessed by calculating the coefficient of determination (R^2) (eq 5) and the accuracy factor (Af) (eq 6), which is a simple multiplicative factor indicating the spread of results around the prediction.[32]

$$R^2 = 1 - \frac{\sum_i[\ln(k_{obs}') - \ln(k_{pred}')]^2}{\sum_i[\ln(k_{obs}') - \ln(\bar{k}_{obs}')]^2} \tag{5}$$

where k_{pred}' is the predicted specific rate constant, k_{obs}' is the observed specific rate constant at a given pressure−temperature combination, and $\bar{k}_{obs}'$ is the mean of the observed specific rate constants.

$$Af = \exp\left\{\frac{1}{N}\sum_{i=1}^{N}\left[\log\left(\frac{k'_{pred}}{k'_{obs}}\right)\right]^2\right\}^{(\ln 10)/2} \tag{6}$$

Here, N is the number of rate constants used for the model. The smaller the Af value, the more accurate are the absolute errors in the model. A value of 1 indicates perfect agreement of the model to the fitted data.

MALDI-TOF-MS. Selected heat- and/or high pressure-treated samples were investigated with MS to determine the sites of Glc attachment to the BSA macromolecule. Five microliters of trypsin (sequencing grade trypsin−porcine) was added to 50 μL of the sample, which was subsequently digested at 37 °C for 16 h. Digested samples were cospotted onto a MALDI target plate with a matrix solution of 10 mg/mL of α-cyano-4-hydroxycinnamic acid (Laser BioLabs, Sophia-Antipolis, France) in 50% acetonitrile/0.1% trifluoroacetic acid. The samples were analyzed on an 4700 Proteomics Analyzer with MALDI-TOF/TOF (Applied Biosystems, Foster City, CA) in reflectron mode with a mass range of 800−3500 Da and a focus mass of 1400 Da at 1500 shots per spectra. The 4700 Series Explorer software automatically selected the 15 most intense peptides as precursor masses for tandem mass spectrometry (MS/MS) analysis and acquired them in the order of decreasing intensity. MS/MS analysis was carried out in reflector mode with a relative precursor mass window of 50 resolutions with metastable ion suppression on and spectra summed up to 2500 shots/spectrum.

Peptide mass fingerprinting (PMF) and MS/MS data were compiled by the GPS explorer software version 3 (Applied Biosystems) and searched against the National Center for Biotechnology Information (NCBI), nonredundant and Swiss-Prot databases using the MASCOT search engine (version 1.9, Matrix Science Inc., London, United Kingdom) with all taxonomy selected.

RESULTS AND DISCUSSION

Gel Electrophoresis (SDS-PAGE). Gel electrophoresis has been used to analyze Maillard reaction products[33] and, therefore, could be used to investigate BSA−Glc samples following heat and high pressure treatment. Figure 1 shows the SDS-PAGE profile of BSA−Glc solutions treated at different pressure−temperature conditions. Control BSA (lane A) and BSA−Glc (lane B) solutions showed no significant differences in their SDS-PAGE profile, indicating that no change occurred to BSA at room temperature when Glc was added (Figure 1). This is in line with results from Ajandouz et al.,[12] who obtained one band at ∼66 kDa and another at ∼200 kDa for unheated BSA in the presence of Glc.

The SDS-PAGE profiles of all pressure−temperature treated samples show an increase in the amount of high molecular weight species (Figure 1). Lanes E and F, which correspond to samples treated at atmospheric pressure (0.1 MPa) at 110 °C for 10 and 30 min, respectively, showed four distinct bands, whereas high pressure-treated samples (lanes C and D) resulted in more

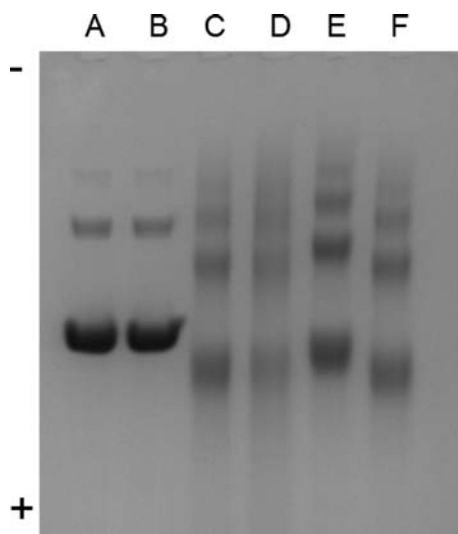

Figure 1. SDS-PAGE for BSA−Glc solutions. Lanes: A, BSA; B, BSA−Glc solution; C, BSA−Glc treated at 200 MPa, 110 °C, 30 min; D, 600 MPa, 110 °C, 30 min; E, 0.1 MPa, 110 °C, 10 min; and F, 0.1 MPa, 110 °C, 30 min.

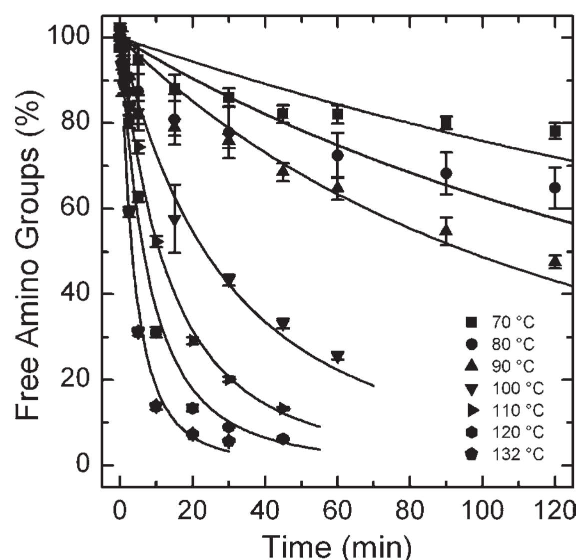

Figure 2. Isothermal glucosylation kinetics of BSA−Glc in bicine buffer (0.05 M, pH 9.0) at atmospheric pressure and 70−132 °C. Solid lines are obtained by fitting the data to eq 2 with n = 1.4 (see the text for further details).

diffuse and less intense bands, despite the same amount of protein loaded into each well (Figure 1). The decrease in band intensity indicates the formation of high molecular weight material that was too large to enter the gel. All of the treated samples show an increase in the formation of protein cross-linked products having high molecular weight.[34] Such cross-linking has been observed for thermal-treated[35] as well as high pressure-treated BSA samples.[8] Ledesma-Osuna et al.[36] reported that BSA−Glc bands migrate slower and broader in an SDS-PAGE gel, which can indicate glycation. Smearing of bands also points toward different states of protein association and possibly conjugation as glycated samples are heterogeneous protein species with a different number of sugar moieties attached.[34] Nevertheless, pressure−temperature treatment also caused a decrease in molar mass as compared to the untreated BSA−Glc samples. This is especially obvious for longer treatment times (Figure 1; lanes C, D, and F), whereas lane E with only 10 min treatment did not show many low molecular weight species, indicating a time dependence of the molecular degradation. Literature states that apart from high molecular weight polymers the formation of low molecular weight compounds is expected under some circumstances due to protein hydrolysis and fragmentation reactions.[19,37] Indeed, side reactions take place throughout the course of the Maillard reaction, which produce fission products of low molecular weight. On the other hand, a downward shift of the protein bands can also be due to protein aggregation and changes in the shape of the BSA molecules after pressure−temperature treatment, which can impede the diffusion of the remaining material into the gel.

Impact of Heat and High Pressure on Protein−Sugar Conjugation. Isothermal heating of the BSA−Glc solution at atmospheric pressure was carried out between 70 and 132 °C for up to 120 min and resulted in a decrease of free amino groups over time as measured by the OPA method (Figure 2). This is likely caused by the glucosylation of the ε-amino group of the lysine residues of BSA at high temperatures.[35] For example, heating at 80, 90, 100, and 110 °C for 30 min yielded a decrease in free amino groups of 22, 24, 57, and 80%, respectively. Glucosylation kinetics also indicated a pronounced increase in the reaction rate at temperatures higher than 90 °C (Figure 2). This might be explained by the complex reaction pathway of the Maillard reaction, which consists of several simultaneous and interrelated reactions, each possibly with different temperature sensitivity.[20] Often, the applied temperature determines which path of the reaction route prevails, complicating the comparison of results obtained at different temperatures.[20] High temperatures, especially when applied to a system at high pH, have been found to be the most important factor enhancing the Maillard reaction.[12,23,24] The kinetics shown in Figure 2 also indicate that the rate of BSA glucosylation decreases over time, causing a "tailing" of the kinetic curve. Martins and Van Boekel[38] attributed this to the decrease in pH over time, due to the formation of acidic products, which consequently slows the Maillard reaction. The BSA−glucose interaction might also be slowed after longer incubation times due to alkaline degradation of glucose.[39] Another important aspect to consider is that steric factors might inhibit the progression of amino group occupation. Lysine groups that are easily accessible may conjugate rapidly with Glc, whereas the remaining lysine groups become increasingly inaccessible. Prolonged incubation also decreases the number of remaining reactive groups, thereby decreasing the reaction rate.[40]

Melanoidins can be formed over the course of the Maillard reaction and simultaneous caramelization of sugars resulting in insoluble colored complexes.[41] Indeed, visible browning of the BSA−Glc solutions was observed in the current study after 5 min of heating at 120 °C (Figure 3), whereas no color formation was detectable at 90 °C within 90 min (data not shown).

Figure 4 shows the decrease in free amino groups of BSA heated in the presence of Glc in bicine buffer (0.05 M, pH 9.0) at 110 °C and 0.1, 200, 400, and 600 MPa, respectively. It is evident that the reaction rate is decreased with higher pressures. This trend occurred at all temperature and treatment times investigated, with higher pressures resulting in more BSA-free amino groups remaining unoccupied after defined treatment times. For instance, after 30 min of heating at 110 °C and atmospheric

Figure 3. Color formation of BSA−Glc conjugates in bicine buffer (0.05 M, pH 9.0) after 0−45 min heating at 120 °C.

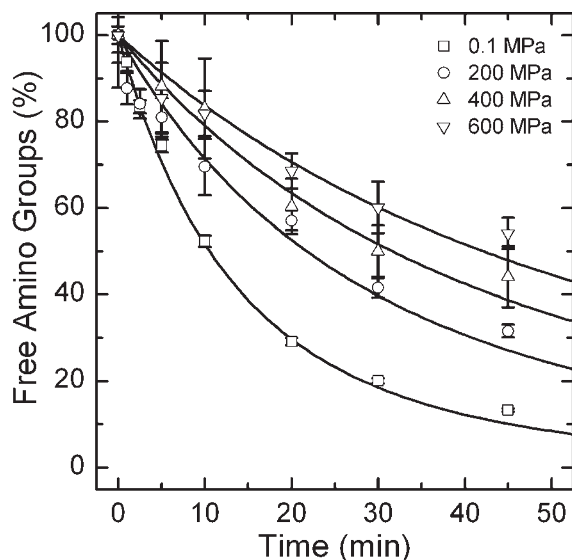

Figure 4. Glucosylation kinetics of BSA−Glc in bicine buffer (0.05 M, pH 9.0) at 110 °C and 0.1, 200, 400, and 600 MPa. Solid lines are obtained by fitting the data to eq 2 with a 1.4th order reaction (see the text for further details).

pressure, only 20% free amino groups of BSA remained available, whereas after 30 min at 110 °C and 200, 400, and 600 MPa, the amount of free amino groups was approximately 42, 50, and 60%, respectively (Figure 4). Notably, the residual free amino groups after 30 min of heating at 110 °C and 200 MPa was twice as high as after 30 min of heating at 110 °C and atmospheric pressure.

The effects of pressure and temperature on reaction kinetics are antagonistic in molecular terms as an increase in pressure at constant temperature leads to a decrease in the entropy of the system.[42] The deceleration of the Maillard reaction under pressure is generally associated with a decrease in the reaction rate due to a volume increase of the reaction products relative to the volume of the reactants.[43] The complexity of the Maillard reaction, however, being an intricate network of reactions, exacerbates the determination of one activation volume $\Delta V^{\#}$. The literature states that several pathways of the Maillard reaction have a positive activation volume $\Delta V^{\#}$, the most important of them being the decomposition of the Amadori rearrangement product.[44] Because the conducted experiments monitor the Maillard reaction as a whole without considering any intermediates, it can only be assumed that the glucosylation reaction as a whole has a positive activation volume $\Delta V^{\#}$ and is, therefore, suppressed by pressure. Nevertheless, other possible factors, which include pressure-induced changes to hydrophobic and electrostatic interactions, the ion equilibriums, and/or pH that affect the stability of proteins and colloidal structures, cannot be overruled.[43,45]

It can be assumed that in the high pressure, high temperature environment under investigation, BSA denaturation occurs readily, transforming the protein into a molten globule, which ultimately results in a random coil polymer. Upon unfolding, BSA possibly exposes more lysine groups over time, further changing the kinetics of the Maillard reaction. To investigate the accessibility of the free amino groups on unfolding of the protein, several solutions of BSA were exposed to different heat and high pressure treatments. Results showed that high pressure treatment for up to 45 min at 600 MPa and 70 °C does not alter the availability of free amino groups, indicating minimal unfolding of BSA under these conditions. On the other hand, high temperature treatment at 120 °C and atmospheric pressure for 1 min lead to an increase of approximately 10% in the availability of lysine ε-amino groups (Figure 2). Considine et al.[43] also reported that BSA is relatively resistant to pressure denaturation, whereas it is easily denatured by heat. However, combined heat and HPP yielded a faster unfolding of the protein, exposing possibly all available lysine groups after 30−45 min depending on the pressure temperature regime applied.[43] Thus, it is likely that pressure has a synergistic effect on the heat denaturation of BSA.

Modeling of BSA−Glucose Conjugation. Close inspection of the decrease of free amino groups under isobaric/isothermal conditions indicated some deviations from simple first-order kinetics. Thus, the complete experimental data set was fitted to an *n*-th order reaction model (eq 2) with reaction orders ranging from 1.0 to 2.0. Determination of the ∑SD of all 24 tested pressure−temperature combinations yielded the lowest value for a 1.4th-order model. The lines in Figures 2 and 4 interpolating the experimental data points show the fit using the 1.4th-order reaction model. Upon fixing the reaction order, the k' constant remained as the only parameter in eq 2 and was estimated in a global approach using linear regression analysis. The resulting k' values of the different pressure−temperature combinations investigated are shown in Table 1.

In the literature, the Maillard reaction is mostly considered to be of either first- or second-order, depending on which reaction is measured.[38] Thus, a reaction order of 1.4 is a realistic representation of the amino group occupation in a BSA−Glc model system. Nevertheless, the use of *n*-th order kinetics is a simplification, which does not give an insight into the molecular mechanisms of the Maillard reaction. However, for engineering and modeling purposes, this approach is deemed appropriate.[20]

The kinetic data were also used to develop a mathematical model that describes the pressure and temperature dependence of k'. A polynomial model, which is based on the change in Gibbs free energy (eq 4), provided a good functional relationship of the obtained k' values with pressure and temperature. Table 2 shows the estimated model parameters obtained by nonlinear regression fitting of eq 4 to the experimental data set using a reference pressure and temperature of p_0 = 200 MPa and T_0 = 373.15 K. Although different reference pressures and temperatures have been used, the values of ΔG_0, ΔV_0, ΔS_0, $\Delta\beta$, ΔC_p, and $\Delta\alpha$ are in the same order of magnitude as reported for other food biopolymers.[31] The parity plot (Figure 5) showing the natural logarithm of the observed versus the predicted k' values indicates no significant heteroskedasticity problems as the deviations from the bisector are small, and thus, the associated accuracy is high (R^2 = 0.984). The high accuracy of the model was also confirmed by the accuracy factor (*Af*) (eq 6), which indicated a low prediction error of 2.4%. Thus, statistical data suggest that the

Table 1. Specific Rate Constants k' (10^{-3} min^{-1}) for Combined Pressure (p in MPa)−Temperature (T in °C) Glucosylation of BSA in 0.05 M Bicine Buffer (pH 9.0) Using a Reaction Order n = 1.4[a]

p/T	70	80	90	100	109	110	120	123	132
0.1	2.941 ± 0.430[b]	5.121 ± 0.886	8.325 ± 0.947	34.180 ± 1.892	58.482 ± 3.052	73.207 ± 2.057	123.952 ± 8.317	143.691 ± 9.431	243.435 ± 23.354
200	ND	2.736 ± 0.613	3.225 ± 0.618	10.116 ± 0.937	ND	35.470 ± 3.020	ND	ND	ND
400	ND	1.181 ± 0.083	2.650 ± 0.396	9.321 ± 0.326	ND	24.389 ± 1.427	51.508 ± 2.631	ND	ND
600	0.286 ± 0.030	0.941 ± 0.300	1.3428 ± 0.138	6.0234 ± 0.562	ND	18.308 ± 1.413	34.145 ± 1.713	ND	ND

[a] ND, not determined. [b] Standard error of regression.

Table 2. Estimated Model Parameter Values for Glucosylation of BSA in 0.05 M Bicine Buffer (pH 9.0) Using eq 4 and a Reference Pressure and Temperature of p_0 = 200 MPa and T_0 = 373.15 K

parameter	estimate
k_0' (10^{-2} min^{-1})	2.378 ± 0.066[a]
ΔV_0 (10^{-5} cm^3 mol^{-1})	1.210 ± 0.246
ΔS_0 (10^2 J mol^{-1} K^{-1})	2.371 ± 0.123
$\Delta\beta$ (10^{-14} cm^6 J^{-1} mol^{-1})	−1.610 ± 0.868
ΔC_p (10^2 J mol^{-1} K^{-1})	4.794 ± 4.427
$\Delta\alpha$ (10^{-8} cm^3 mol^{-1} K^{-1})	−4.730 ± 1.880
R^{2b}	0.984
Af	1.024

[a] Standard error of regression. [b] Coefficient of determination of the model.

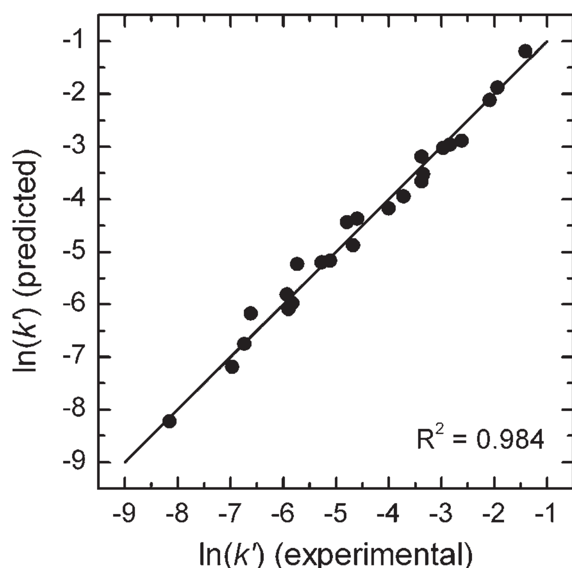

Figure 5. Correlation between the experimental k' values of BSA−Glc conjugation determined at isothermal/isobaric conditions and the predicted k' values using eq 4 and model parameter values of Table 3.

secondary model performs well in the description of the performed experiments.

Substituting k' of eq 2 by the pressure−temperature relations of eq 4 allows the calculation of pressure−temperature combinations that result in the same glucosylation rate of BSA. Using the pressure as an independent variable and constant glucosylation ratio ($C \times C_0^{-1}$) and treatment time, the equation can be solved for the temperature. Figure 6 shows lines of pressure−

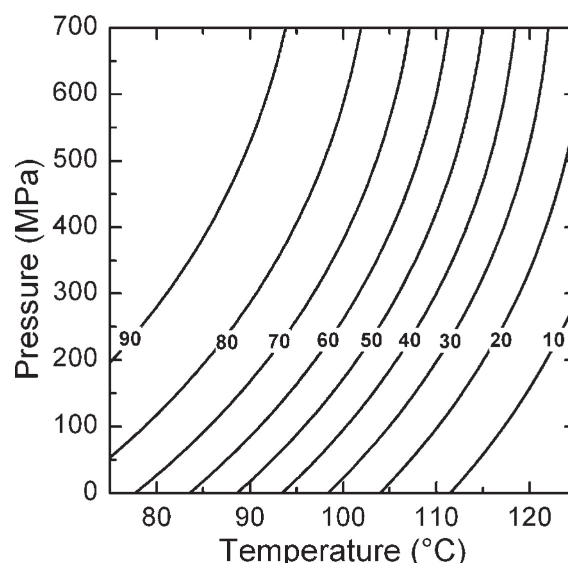

Figure 6. Pressure−temperature isorate diagram for BSA−Glc conjugation in bicine buffer (0.05 M, pH 9.0) with lines for 10−90% free amino groups after 30 min of isothermal/isobaric treatment.

temperature combinations that lead to different levels of free amino groups of BSA after 30 min. For example, at atmospheric pressure, a temperature of approximately 84 °C is required to leave 60% of BSA's free amino groups unglucosylated after 30 min, whereas at 600 MPa, a temperature of approximately 110 °C is required to give the same extent of glucosylation. In contrast, at atmospheric pressure, this temperature results in a decrease of the free amino groups to approximately 12%, showing that pressure hampers the glucosylation reaction of BSA.

MALDI-TOF-MS. To identify the modified lysine residues in the BSA molecule, MALDI-TOF-MS analysis was carried out. This method was previously used to study the glycation of BSA[46] but has not yet been used for the investigation of proteins glycated under high pressure. In addition to BSA alone (control), the following samples were selected for the MALDI-TOF-MS measurements: Sample 1 heated at 110 °C at atmospheric pressure for 10 min, sample 2 heated at 110 °C at atmospheric pressure for 30 min, sample 3 heated at 110 °C at and 200 MPa for 30 min, and sample 4 heated at 110 °C at and 600 MPa for 30 min. These samples were chosen as they allow investigation of the effect of heating time (samples 1 and 2) and pressure (samples 3 and 4). In addition, MALDI-TOF-MS measurements of samples 1 and 3 allow comparison between samples treated using different pressure and time conditions but showing similar OPA results (please see Figure 4 for comparison).

Table 3. Determination of Modified Lysine Groups of Glucosylated BSA with MALDI-TOF-MS

treatment conditions				
pressure (MPa)	temperature (°C)	time (min)	peptide sequence	modified lysine group
0.1	110	10	^{66}LVNELTEFAK*^{75}	K75
			^{157}FWGK*YLYEIAR^{167}	K160
			^{198}GACLLPK*IETMR^{209}	K204
			^{286}YICDNQDTISSK$^{\#}$LK$^{\#299}$	K297 or K299
0.1	110	30	^{66}LVNELTEFAK*^{75}	K75
			^{198}GACLLPK*IETMR^{209}	K204
			^{286}YICDNQDTISSK$^{\#}$LK$^{\#299}$	K297 or K299
200	110	30	^{66}LVNELTEFAK*^{75}	K75
			^{198}GACLLPK*IETMR^{209}	K204
			^{286}YICDNQDTISSK$^{\#}$LK$^{\#299}$	K297 or K299
			^{548}K*QTALVELLK^{557}	K548
			^{548}KQTALVELLK*^{557}	K557
600	110	30	^{66}LVNELTEFAK*^{75}	K75
			^{157}FWGK*YLYEIAR^{167}	K160
			^{198}GACLLPK*IETMR^{209}	K204

Table 3 gives a summary of the modified lysine groups detected with MALDI-TOF-MS. At room temperature, BSA in 0.05 M bicine buffer (pH 9.0) did not show any glycation in the presence of Glc (data not shown). Upon heat treatment at 110 °C, lysine residues K75, K160, K204, and K297/K299 were glucosylated after 10 min as well as after 30 min of heating at different pressures (200 and 600 MPa). Interestingly, K160 was not always detected as modified after 30 min of treatment, which might be an indication of BSA degradation or fragmentation after prolonged heating times at high temperatures. Other authors also report a modification of K75, K160, K297, and K299 of BSA, indicating that these easily accessible amino groups are commonly involved in the Maillard reaction.[47−49]

In this study, the modified sites, as determined by MALDI-TOF-MS, were mostly limited to these four lysine residues in the BSA polypeptide. Under these conditions, the extent of glycation, as determined by OPA, was approximately 50%. Thus, it is likely that more lysine sites are involved in the glycation reaction. Furthermore, fragmentation reactions of carbohydrates coupled to peptides throughout the course of the Maillard reaction are known to interfere with the identification of glycopeptides.[36] In addition, it is possible that the ions in the glucosylated peptides, relative to the nonglucosylated peptides, which coelute together, were not sufficiently abundant to be detected.[36] Despite these experimental limitations, five modified lysine residues were identified for 30 min of treatment at 200 MPa and 110 °C, that is, lysine residues K75, K204, K297/299, K548, and K557 (Table 3). Glycation of K548 was also identified by Frolov et al.,[48] but modification of K557 has not been reported in the literature to our knowledge. Site specificity has been linked to local acid base catalysis, making lysine more reactive when in a Lys-Lys, Lys-His-Lys environment or when close to a disulfide bridge.[50] However, it is also a consequence of BSA's three-dimensional structure influencing accessibility to the amino group.[36] Approximately 48% of the 59 lysine residues of BSA are very accessible, 28% exhibit an intermediate level of exposition, and 24% are poorly accessible.[35] K557 might be one of the lysine groups that is rather inaccessible but becomes available for glucosylation upon heat and HPP. On the other hand, the detection of K548 and K557 can also be explained by the slow rates of reaction, including degradation, under combined pressure−heat conditions as compared to the relatively quick degradation or rearrangement of lysine residues at heat-only conditions. Thus, glucosylation products may exist long enough to be detected under pressure, whereas without pressure they degrade or rearrange quickly after formation and cannot be detected.

It is worth noting that OPA results for treatments at 0.1 MPa, 110 °C, 10 min and 200 MPa, 110 °C, 30 min are relatively similar with 52 and 50% available amino groups, respectively, whereas MALDI-TOF-MS detected differences in three modified lysine residues. Thus, even though the progression of glycation is similar as shown by OPA, the modified groups of conjugation are different, reflecting the structural heterogeneity of the BSA glycoforms with respect to the modified sites.

Further studies of biopolymer systems with different proteins, amino acids, sugars, and carbohydrates are required to generalize the effects of combined high pressure/temperature treatments on the Maillard reaction, since the underlying mechanisms of action responsible for protein-reducing sugar conjugation under high pressure is still not fully understood. The knowledge generated from the present study might be useful to tailor the extent and nature of the modified residues for BSA-reducing sugar conjugates formed under high pressure. Enhancement and control of the functional properties of food systems such as increased stability of emulsions or modification of textural and solubility properties by applying specific high pressure−temperature−time conditions might allow for the production of value-added foods.[51] Ultimately, the investigation of the progression of the Maillard reaction in the high pressure/temperature domain might permit the specific control of protein glycation and potentially the generation of Maillard-generated flavor compounds. This might be of interest if chemical changes and associated off-flavors as well as the loss of nutritional value are an unwanted side reaction during heat treatments, for example, in the dairy or fruit juice industries. Because the application of heat on systems rich in protein and sugars commonly results in undesired nonenzymatic browning and formation of toxic

Maillard products, the use of high pressure can also be used for sterilization of nonfood products relatively high in pH such as pharmaceutical solutions.[52]

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Funding Sources

We gratefully acknowledge the Victorian State Government for the Science and Technology Infrastructure grant.

■ ACKNOWLEDGMENT

We thank Christine Oliver for fruitful discussions on the Maillard reaction.

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