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# Thermal Glycation of Proteins by D-Glucose and D-Fructose

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**Abstract.** The dry thermal glycation method was used to conjugate D-glucose and D-fructose with bovine serum albumin. Reactions were conducted at 50–104°C for 30 min. Depending on temperature, different levels of substitution were achieved. In the case of D-glucose, average substitution levels of up to 53 mol glucose/mol bovine serum albumin were obtained. D-fructose turned out to be less reactive than D-glucose. The pH of the reaction mixture was also found to affect the efficiency of the glycation reaction. The levels of substitution were estimated using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and SDS-polyacrylamide gel electrophoresis. Glycated molecules, and a process of glycation itself are implicated in diabetes complications. Hypothetically bovine spongiform encephelopathy (BSE) was also derived from glycation.

**Key words:** glycation; bovine serum albumin; D-glucose; D-fructose; conjugate.

## Introduction

Protein glycation is initiated by the condensation reaction between the carbonyl group of the reducing sugar and the amino group of the protein. The resulting Schiff base is subsequently stabilized by Amadori rearrangement. The Schiff base and the Amadori product are referred to as the early glycation products. Then a complex series of reactions follows leading to the formation of many different, highly reactive intermediates (e.g. glyoxal[21, 26], methylglyoxal[1, 21], 3-deoxyglucoson[12]) and, in the later stages of the Maillard reaction, to highly reactive species called advanced glycation end-products (AGEs)[16]. AGEs are a heterogenous group of high-molecular aggregates stabilized by non-reducible cross-linking components with characteristic fluorescence spectra[19]. Their structures are generally unknown; only a small number of AGE structures has been elucidated (e.g. pentosidine[11, 18], N-(carboxyalkyl) lysines: $N^{\varepsilon}$-(carboxymethyl)lysine[2] and $N^{\varepsilon}$-(carboxyethyl)lysine[1]).

Increased levels of AGEs are found in the tissues and sera of diabetic patients and in aged individuals[4, 22]. Accumulation of AGEs leads to tissue damage via alteration of the structures and functions of tissue proteins[15], stimulation of cellular responses[25], through receptors specific for AGE-proteins[10, 17], and via generation of reactive oxygen intermediates[27]. AGEs also react with DNA, thus causing mutations and DNA transposition[8]. Thermal processing of proteins and carbohydrates brings major changes in allergenicity. AGEs are antigenic and represent many of the important neoantigens found in cooked or stored foods[9].

While the role of AGEs has been thoroughly inves-

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tigated, only a few reports on the significance of the early glycation products have been published so far. Amadori products, like AGEs, were found to inhibit nitric oxide synthase activity[24] and generate superoxide anions that interfere with nitric oxide-mediated vasodilatory responses[23]. It has also been shown that free radicals generated by both Schiff base and Amadori products may contribute to oxidative modifications of vascular wall lipids[14]. Taking into consideration that Amadori products are the predominant form of glycated proteins, their role in diabetic vascular complications deserves thorough study.

A simple method for the synthesis of Amadori products is the previously described dry thermal protein glycation[5, 7]. The procedure enables direct attachment of reducing sugars to proteins without prior modifications. It suffices to mix the carbohydrate with a protein, lyophilize them together and then briefly heat. The conjugates obtained by this procedure are stable over a wide pH range and in the presence of dissociating substances. The conditions of the reaction do not affect the biological integrity of the coupled carbohydrates or the biological activity of the proteins. In the present report we show the results of the dry thermal glycation of bovine serum albumin (BSA) by D-glucose and D-fructose. Depending on temperature, the method provides different levels of substitution. Neoglycoproteins prepared by dry thermal glycation might be a useful tool when investigating the pathophysiological role of Amadori products.

## Materials and Methods

All reagents were of analytical grade. BSA, D-glucose and D-fructose were purchased from Sigma.

BSA (20 mg/ml) was mixed with the same volume (150 µl) of sugar solution (40 mg/ml). To examine the influence of pH on the yield of glycation, 300 µl of the initial aqueous solutions were treated with 150 µl of 0.1 M phosphate buffers of different pH value. The samples were frozen at –60ºC and lyophilized. The lyophilizates were heated at 50–104ºC for 30 min, dissolved in 300 µl of water and dialyzed to remove unbound sugar and phosphates.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 8% gel under reducing conditions according to LAEMMLI[13].

Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry (MS) was run on a Kratos Kompact SEQ time of flight (TOF) instrument (Schimadzu Kratos Analytical, UK), using sinapinic acid (10 mg/ml in 0.1% trifluoroacetic acid in 50% acetonitrile/50% water solution) as the matrix. The spectrometer was operated at 20 kV accelerating voltage with pulsed extraction. The average degree of sugar incorporation in BSA was estimated by the MALDI-TOF MS using the centre of the distribution of the singly-charged molecular ions.

## Results

### *Influence of temperature*

Reactions of BSA with D-glucose and D-fructose were conducted at temperatures varying from 50 to 104ºC for 30 min. Reaction temperature had a substantial effect on the efficiency of glycation in both BSA/D--glucose and BSA/D-fructose systems. The data of sample analysis obtained by MALDI-TOF MS (Fig. 1) and SDS-PAGE (Fig. 2) are listed in Table 1. Analysis of the conjugates by MALDI-TOF MS revealed that in the case of the glucation reaction (glycation by glucose), the average level of substitution obtained at 50ºC was 4.7 mol glucose/mol BSA; this increased with temperature. At 104ºC, we obtained a level of substitution which was as high as 53 mol glucose/mol BSA. The average substitutions of the BSA-D-glucose conjugates were higher than those found in the corresponding BSA-D-fructose samples in the whole analyzed temperature range. When the BSA/D-fructose samples

**Table 1.** Effect of reaction temperature on BSA substitution with D-glucose and D-fructose

| Temperature (ºC) | Mol glucose/mol BSA | | Mol fructose/mol BSA | |
|---|---|---|---|---|
| | MALDI-TOF MS | SDS-PAGE | MALDI-TOF MS | SDS-PAGE |
| 104 | 53.0 | 57.1 | 26.6 | 75.1 |
| 95 | 46.1 | 47.6 | 22.2 | 51.8 |
| 90 | 41.0 | 41.5 | 18.2 | 40.5 |
| 82 | 32.7 | 37.2 | 9.2 | 18.9 |
| 72 | 20.4 | 19.5 | 3.7 | 16.7 |
| 67 | 11.7 | 12.6 | 2.6 | 8.1 |
| 50 | 4.7 | 1.4 | 0.4 | – |

3 mg BSA and 6 mg D-glucose or D-fructose in 300 µl water were lyophilized and heated for 30 min at different temperatures.

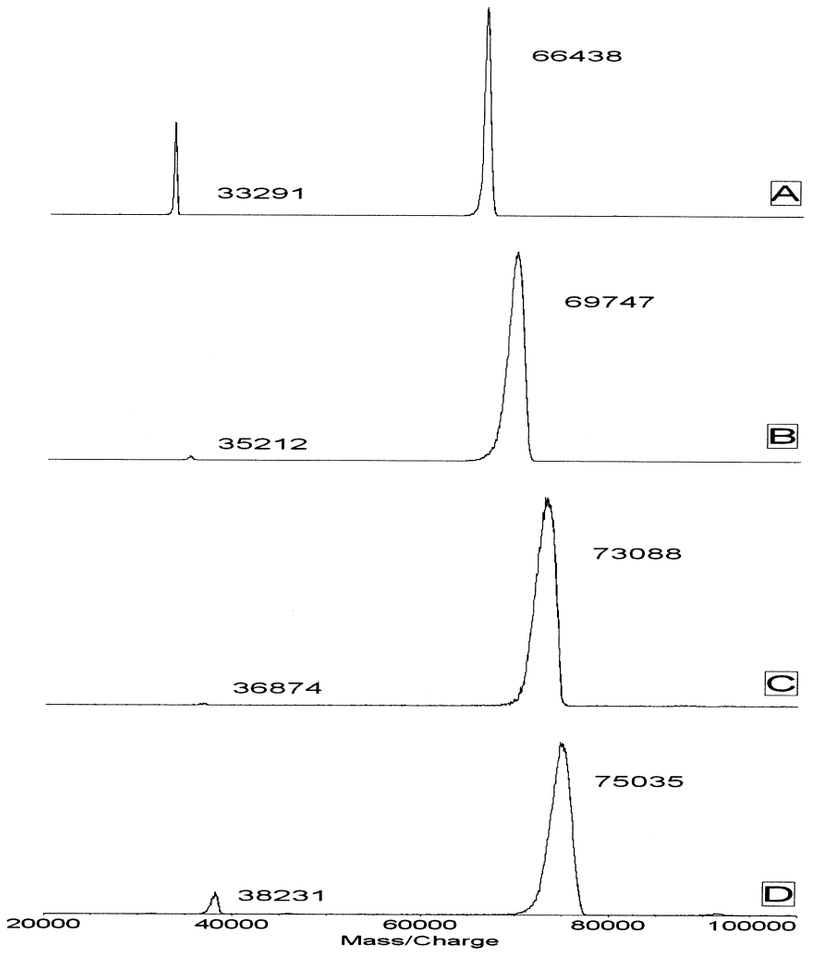


**Fig. 1.** MALDI-TOF mass spectra of: **A** – BSA; **B** – BSA-D-glucose conjugate after reaction at 72ºC; **C** – BSA-D-glucose conjugate, reaction at 90ºC; **D** – BSA-D-glucose conjugate, reaction at 104ºC

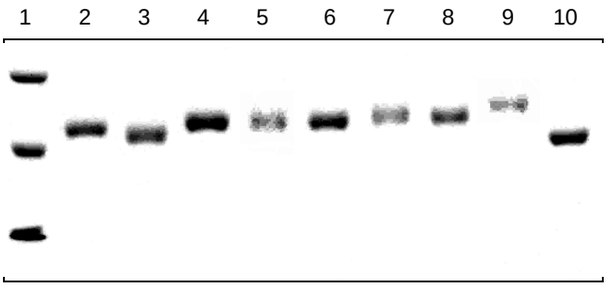


**Fig. 2.** Temperature dependence of conjugation. SDS-PAGE in 8% gel in the presence of 1% 2-mercaptoethanol[13]. Lane: **1** – standard of molecular weight (94, 67, 44 kDa); **2** – BSA-D-glucose conjugate, reaction at 82ºC; **3** – BSA-D-fructose conjugate, reaction at 82ºC; **4** – BSA-D-glucose conjugate, reaction at 90ºC; **5** – BSA-D--fructose conjugate, reaction at 90ºC; **6** – BSA-D-glucose conjugate, reaction at 95ºC; **7** – BSA-D-fructose conjugate, reaction at 95ºC; **8** – BSA-D-glucose conjugate, reaction at 104ºC; **9** – BSA--D-fructose conjugate, reaction at 104ºC; **10** – BSA

were heated at 104ºC, substitution averaged 26.6 mol fructose/mol BSA. This finding indicates that D-glucose is more reactive than D-fructose. The results are consistent with others reported for glycation experiments conducted under dry-heating conditions[28]. We noticed, however, that heating of the BSA/D-fructose sample at 104ºC produced aggregated material of brown color. The sample of BSA/D-fructose heated at 95ºC turned yellow. In the corresponding BSA/D-glucose sample we did not notice any insoluble complexes or visible yellowing. Fig. 1 depicts the MALDI-TOF mass spectra of BSA and relevant conjugates. The BSA spectrum contains a sharp peak for singly-charged protein (m/z 66 438) and doubly-charged protein at m/z 33 291. Panels B-D show the MALDI-TOF mass spectra of the conjugates. The peaks are broader than those for BSA, since every conjugate sample contains a range of

protein molecules with a different number of coupled sugar residues. Making use of the average molecular weights of the conjugates obtained from the spectra, the average number of sugar residues bound to BSA was calculated.

*Influence of pH*

The pH-dependence of conjugation in both BSA/D--glucose and BSA/D-fructose systems was investigated using phosphate buffers. In the case of BSA/D-fructose samples heated at 81°C, the degree of substitution was the highest at alkaline pH (pH 8.1 and 8.9; Table 2, Fig. 3). The lowest substitution levels were obtained at acidic pH (5.5 and 4.5) and in the sample which contained no buffer. At neutral pH (with buffer added) the reaction went with much better efficiency than at acidic pH, and with worse efficiency than at alkaline pH.

In the case of BSA/D-glucose samples, there was only a slight difference in the efficiency of the reaction between pH 4.5 and 5.5, or in pure water (Table 2, Fig. 4). Higher levels of substitution were obtained at alkaline

**Table 2.** Effect of pH on glycation efficiency

| pH | Mol glucose/ /mol BSA | Mol fructose/ /mol BSA |
|---|---|---|
| 4.5 | 45.4 | 10.2 |
| 5.5 | 50.8 | 8.9 |
| 6.8 | 56.5 | 19.8 |
| 7.0 | 61.2 | 25.5 |
| 8.1 | 56.2 | 32.0 |
| 8.9 | 68.6 | 32.7 |
| $H_2O$ | 48.6 | 10.0 |

3 mg BSA and 6 mg D-glucose or D-fructose in 450 µl 0.033 M phosphate buffer or water were lyophilized and heated for 30 min at 81°C. The levels of substitutions were calculated from the average molecular weights of the conjugates obtained from MALDI-TOF mass spectra.

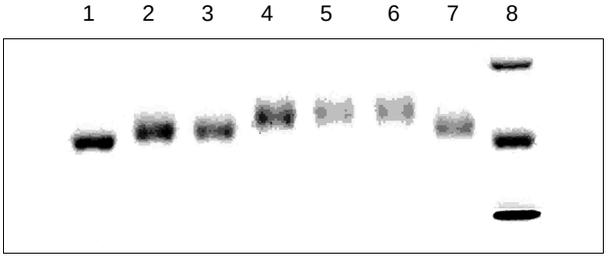


**Fig. 3.** Effect of pH on high temperature conjugation of BSA and D-fructose. SDS-PAGE in 8% gel in the presence of 1% 2-mercaptoethanol[13]. Lane: **1** – BSA; **2** – reaction at pH 4.5; **3** – at pH 5.5; **4** – at pH 7.0; **5** – at pH 8.1; **6** – at pH 8.9; **7** – no buffer added; **8** – standard of molecular weight (94, 67, 44 kDa)

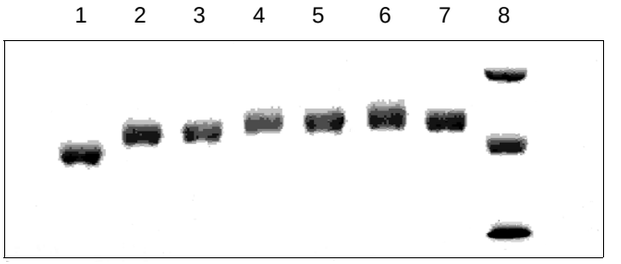


**Fig. 4.** Effect of pH on high temperature conjugation of BSA and D-glucose. Explanations see under Fig. 3

(8.1 and 8.9) and neutral pH. The effect of pH on conjugation is also associated with the salt effect. The salt effect was previously described[8] and can be responsible for the single deviation of substitution level at pH 8.1.

## Discussion

Dry thermal protein glycation is a simple method for the preparation of BSA-fructose and BSA-glucose conjugates. Selecting the appropriate temperature, we can achieve the desirable degree of substitution. However, if Amadori compounds are to be the products of the reactions, high heating temperatures should be avoided. Insoluble complexes are formed for the reactions of BSA with D-glucose at temperatures higher than 104°C, and there is a visible yellowing of the sample, which indicates the occurrence of advanced glycation reactions. In the case of the reaction of BSA with D-fructose, soluble, non-yellowed products are obtained at temperatures lower than 95°C. The pH of the reaction mixture also contributes (to some extent) to the level of substitution. The highest substitution was achieved with alkaline pH. However, we do not recommend the use of an alkaline reaction environment of the reaction. The influence of pH on the advanced Maillard reaction rate is far more distinct. Low pH inhibits the advanced Maillard reaction, whereas alkaline pH enhances the rate of AGE production[3].

We noticed that the dilution of the sample prior to lyophilization and heating also exerted a certain influence on the efficiency of the glycation reaction. Thus, the lower the concentration of BSA and sugars, the higher the level of substitution which could be achieved. We suppose that at higher concentration the exterior layer of the protein acts as an insulator. As a result, we achieved a lower level of substitution (32.7 mol D-glucose/mol BSA) at a protein concentration of 3 mg/300 µl than at the concentration of 3 mg/450 µl (48.6 mol D-glucose/mol BSA) at a similar temperature (compare Tables 1 and 2).

Because there had been conflicting reports on the reactivities of glucose and fructose, we tried to compare these two sugars. Analysis of the BSA-D-glucose and BSA-D-fructose conjugates by MALDI-TOF MS (Table 1) revealed that D-glucose was more reactive than D-fructose in the whole temperature range investigated. The substitution levels of BSA-D-glucose conjugates were higher than those of BSA-D-fructose conjugates at corresponding temperatures. Other researchers, who had compared the reactivities of D-glucose and D-fructose under dry-heating conditions, reported similar results[28]. SDS-PAGE electrophoresis, however, revealed that BSA-D-fructose conjugates heated at 95 and 104°C had higher molecular weights than BSA-D-glucose conjugates incubated at corresponding temperatures (Fig. 2). BSA-glucose conjugates migrated as sharp bands, whereas BSA-D-fructose conjugates produced at 95–104°C migrated in the form of broad bands. BSA-D-glucose and BSA-D-fructose conjugates produced at 90°C had similar molecular weights. In the lower temperature range (50–82°C), electrophoresis pattern and MS indicated that D-glucose was more reactive than D-fructose. Generally, in the case of BSA-D-fructose conjugates, the molecular weight values calculated from electrophoresis were higher than those obtained from MALDI-TOF MS. We do not know what exactly contributes to the discrepancies in molecular weight values. In the case of BSA-D--glucose conjugates there were no essential differences in the molecular weight values between the samples analyzed by MALDI-TOF MS and those examined by SDS-PAGE electrophoresis. We suppose that in the case of BSA/D-fructose mixtures heated at higher temperatures, intramolecular cross-links were formed, thus lowering mobility during electrophoresis and yielding artificially higher molecular weights. This might indicate the occurrence of advanced glycation reactions, which become even more probable, since we noticed that fructose-containing samples heated at higher temperatures (95 and 104°C) turned yellow, while those containing glucose remained colorless. Moreover, in the case of fructose, some other structures retarding migration of BSA-D-fructose conjugates in the gel are likely to have formed, e.g. double sugar adducts[20].

We recommend MALDI-TOF MS as a convenient method for the assessment of molecular weights of neoglycoconjugates. With this technique it is possible not only to determine the average incorporation value, but also to obtain information on the range of distribution.

Glycated macromolecules and their receptors play an important role in diabetes complications and neurodegenerative diseases. Reducing carbohydrates react with proteins during the high-temperature processing of feeding stuff. Cattle, in comparison to other mammals, have low glucose levels that minimize spontaneous glycation of their own proteins. It therefore can not be precluded that feeding cattle with high temperature glycated products induce bovine spongiform encephalopathy (BSE)[6].

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