Chemistry of Natural Compounds, Vol. 41, No. 3, 2005

GLYCOSYLATION OF BOVINE SERUM ALBUMIN WITH D-[^{14}C]-GLUCOSE

R. I. Kublashvili[1] and D. Sh. Ugrekhelidze[2]

UDC 547.917

Certain parameters of the Maillard reaction between bovine serum albumin (BSA) and D-[^{14}C]-glucose were investigated.

Key words: glycosylation, bovine serum albumin, D-[^{14}C]-glucose.

In continuation of our studies of the melanoidin reaction [1], we investigated certain parameters of the Maillard reaction between bovine serum albumin (BSA) and D-[^{14}C]-glucose.

Figure 1 shows the effects of temperature and BSA:glucose mole ratio on the reaction at pH 7.00. It has been found earlier that BSA undergoes the Maillard reaction mainly with the free ε-amines of lysine and terminal amines under mild conditions (temperature <50°C, slight excess of glucose) [8, 9]. The molecular weight of the studied BSA was 67,000. The number of free amines, according to our analysis, was 58 (amine N ~1.2%). Therefore, the equivalent mass of BSA in this reaction is 1155 g. Labeled C of glucose is not incorporated at all into BSA at 25°C with a 1:1 BSA:glucose equivalent ratio during the first 500 h of incubation of the reaction mixture (Fig. 1, 1). Increasing the glucose concentration (1:5 BSA:glucose equivalent ratio) increases the irreversible incorporation (Fig. 1, 2). However, the degree of incorporation in both instances is exceedingly low. Increasing the reaction temperature to 45°C increases significantly the irreversible incorporation of the label (Fig. 1, 3 and 4). The extent of the process increases sharply at 65°C even with a 1:1 equivalent ratio of reagents (Fig. 1, 5). Therefore, the rate of the Maillard reaction depends more on temperature and less on the molar ratio of the reagents.

Increasing the basicity of the medium increases the extent of the Maillard reaction between BSA and glucose (Fig. 2). Increasing the glucose concentration in the reaction medium also increases markedly the degree of incorporation of label into BSA (Fig. 2, 4). We investigated comparatively low glucose concentrations. Under these conditions, a strong color developed only at 65°C in neutral and basic media. A certain trend was observed: after 192 h of reaction, according to radioactivity measurements (Fig. 2, 2-4), no further incorporation of ^{14}C into BSA was observed. However, according to spectrophotometric measurements, the strength of the solution coloration increased signficantly during the period between 192 and 240 h.

A definite induction period was observed for the reaction of BSA and glucose. This decreased with increasing temperature (Figs. 1 and 2). The induction period was followed by a rather long period during which the radioactivity of the BSA glucosylation product increased almost linearly with time (Fig. 1). The spectrophotometric studies also indicated that BSA glucosylation has an induction period [2]. Apparently, this induction period corresponds to the formation of an N-glucoside. It is known that N-glucosides are the most unstable of all glucosides and undergo hydrolysis even in neutral media. Therefore, the initial step in the reaction of glucose and BSA is reversible. The N-glucoside bond is hydrolyzed during dialysis. As a result, the glucose separates from BSA. However, the Amadori rearrangement occurs simultaneously with N-glucoside hydrolysis to form first aminoketoses and then various products, usually called "final glycosylation products" [3]. Just this last reaction step correlates with irreversible incorporation of glucose into BSA.

Saturation ensues after a certain time under these glucosylation conditions. BSA no longer binds glucose (Fig. 2). Table 1 shows that this saturation ensues regardless of the fact that BSA still contains enough free amines to bind glucose.

1) I. Dzhavakhishvili Tbilisi State University, Tbilisi, 0128, Georgia; 2) Institute of Biochemistry and Biotechnology, Academy of Sciences of Georgia, e-mail: devi_ugrekhelidze@hotmail.com. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 275-277, May-June, 2005. Original article submitted September 15, 2004.

 0009-3130/05/4103-0340

TABLE 1. Amount of Bound (1-6)-[^{14}C]-D-Glucose and Glucosylated Primary Amines of BSA

Glycosylated BSA preparation conditions				Glucose bound to BSA, mg/g	Glucosylated primary amines in BSA, % (by bound ^{14}C)
BSA:glucose equivalent ratio	pH	mp, °C	time, h		
1:1	7.0	25	672	1.0	0.6
1:5	7.0	25	672	1.3	0.8
1:1	7.0	45	480	7.3	4.7
1:5	7.0	45	480	11.3	7.2
1:1	5.0	65	240	94.0	60.3
1:1	7.0	65	240	103.6	66.4
1:1	8.0	65	240	106.6	68.3
1:5	8.0	65	240	113.3	72.6

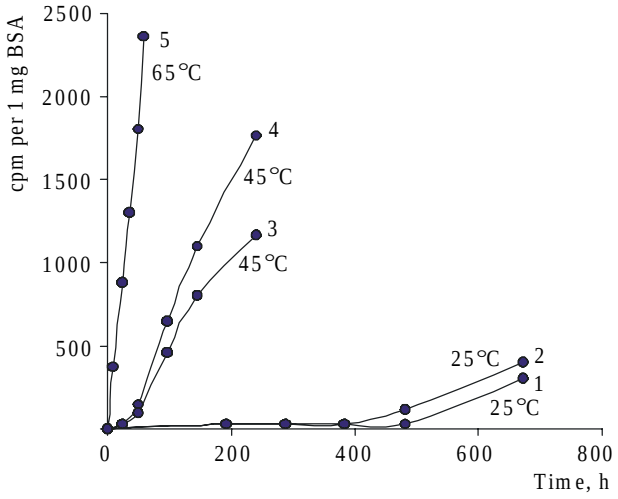

Fig. 1

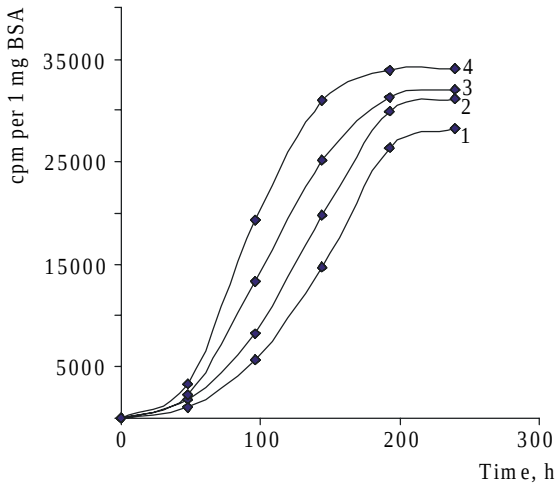

Fig. 2

Fig. 1. Effect of temperature and equivalent ratio of reagents on rate of irreversible incorporation of ^{14}C into BSA (0.06 M phosphate buffer, pH 7.00). BSA:glucose equivalent ratio: 1:1 (1, 3, 5) and 1:5 (2, 4).
Fig. 2. Effect of pH and equivalent ratio of reagents on rate of irreversible incorporation of ^{14}C into BSA (0.06 M phosphate buffer, 65°C). BSA:glucose equivalent ratio 1:1 at pH: 5.0 (1), 7.0 (2), 8.0 (3), and 8.0 (4).

The minimal degree of glucosylation (0.6% glucosylated primary BSA amines) in our experiments was found for a 1:1 BSA:glucose equivalent ratio at 25°C, pH 7.0, and reaction time 672 h. The maximal degree of glucosylation was seen for a 1:5 BSA:glucose equivalent ratio at 65°C, pH 8.0, and reaction time 240 h (Table 1). It should be noted that incubation of glucose with BSA (phosphate buffer, pH 7.4, 37°C, 40 d) produced BSA in which the degree of lysine ε-amine glucosylation was 75.7% of its total amount [4].

Blocking the free amines causes the protein to lose the ability to bind glucose. We acetylated BSA and studied the ability of the resulting acetylated albumin to bind glucose. The acetylated product contained 55.5 moles of bound acetyls per 67,000 g of BSA. Thus, 95% of the free amines of the protein were blocked. Glucosylation of acetylated BSA was investigated in neutral medium at 45 and 65°C to avoid any possibility of hydrolytic cleavage of acetyls. As it turned out, glucose C atoms were not irreversibly incorporated into BSA under these reaction conditions.

Acid hydrolysis of the BSA glucosylation products indicated that they contained a certain amount of hydrolytically cleavable glucose. However, most of the labeled glucose C was incorporated into the BSA as various transformation products (Table 2).

TABLE 2. Glucose Formation by Hydrolysis of Glucosylated BSA (for BSA:Glucose Equivalent Ratio 1:5, 65°C, pH 8.0, Reaction Time 240 h)

Temperature, °C	Time, h	Glucose, %
	0.1 N HCl	
25	24	0
25	48	Tr.
100	9	0.5
	1N HCl	
100	12	2.8
100	24	4.3
	6 N HCl	
100	24	0.7

It should be kept in mind that glucose usually undergoes drastic transformations on heating in strongly acidic media. Apparently, this was the reason for the low glucose content in the hydrolysate resulting from the use of 6 N HCl.

EXPERIMENTAL

Reagents. Bovine serum albumin (BSA, SERVA Electrophoresis GmbH) and (1-6)-[^{14}C]-D-glucose (B/O "Isotope," Russia) were used. (1-6)-[^{14}C]-D-Glucose was purified by 2D paper chromatography (Whatmann 3, BuOH:AcOH:H_2O, 4:1:5, and AcOEt:AcOH:H_2O, 3:1:3) and diluted with nonradioactive D-glucose. The specific radioactivity of the final (1-6)-[^{14}C]-D-glucose was 909.1 Bq/mmol. The radioisotopic purity was 100% (according to autoradiography). Other reagents were chemically pure and analytically pure.

N-Glucosylation of BSA. N-Glucosylation of BSA was studied in vitro by incubating a mixture of BSA and (1-6)-[^{14}C]-glucose (9.36 or 46.8 mg glucose per 60 mg BSA) in phosphate buffer (30 mL, 0.06 M, pH 5.0, 7.0, and 8.0). Toluene (1 mL) was added to the reaction mixtures as an antiseptic. The reaction mixtures were incubated in hermetically sealed flasks in the dark at 25, 45, and 65°C for several days. Samples (5 mL) were collected from the reaction mixtures after certain time intervals and dialyzed against distilled water (water exchanged four times daily) at 15-18°C in SERVAPOR® dialysis sacs (retain proteins with MW >12,000). The optimal dialysis time was established in a separate experiment by measuring the radioactivity of a sample every day during the dialysis. The ^{14}C activity usually equilibrated after 2-3 d. The volumes of the dialyzed solutions were adjusted to 10 mL with distilled water. The formation of N-glucosylation products was monitored by determining the radioactivity of dialyzed solution (1 mL). Each N-glucosylation variant was carried out in duplicate. The radioactivity in each parallel experiment was determined three times. The results were processed using the Excel computer program. The absorption (λ = 470 nm) of the melanoidin complex was measured and the free primary amines were determined for BSA glucosylation using the same experiments but with BSA and nonradioactive D-glucose.

Acid hydrolysis of N-glucosylated BSA was performed as follows: N-glucosylated BSA (20 mg) was heated at 100°C with HCl (2 mL) of various molarities for 9, 12, 24, or 48 h and neutralized. (1-6)-[^{14}C]-Glucose in the hydrolysate was determine by autoradiography.

Preparation of Aminoacetyl-BSA. Aminoacetyl-BSA was prepared by the literature method [5]. BSA (1 g) was dissolved in sodium acetate solution (20 mL), cooled with ice, stirred slowly, and treated over a period of an hour with acetic anhydride (1.2 mL). When the smell of acetic anhydride disappeared the resulting acetylated BSA was separated quantitatively by dialysis. The resulting product contained 55.6 moles of bound acetyls per 67,000 g of BSA.

Analytical Methods. Primary amines in BSA and aminoacetyl-BSA were determined by Van Slyke measurement of the volume of nitrogen released by reaction with nitrous acid [6]. The amount of acetyls in aminoacetyl-BSA was calculated by titration of the acetic acid liberated by hydrolysis of aminoacetyl-BSA with *p*-toluenesulfonic acid [7]. Spectrophotometric measurements were made on a SPECORD UV VIS recording spectrophotometer. Radioactivty was determined using an LKB

1215 RACKBETA II liquid scintillation counter in Lumac LSC Aqualuma® (LUMAC, B. V. Schaesberg, Netherlands) high-efficiency scintillant. The quenching curve was determined and inserted into the counter program in order to calculate automatically from the actual data (counts per minute) the radioactivity in units of cpm [8].

REFERENCES

1. R. I. Kublashvili and D. Sh. Ugrekhelidze, *Khim. Prir. Soedin.*, 336 (2005).
2. A. Mohammad, H. Fraenkel-Conrat, and H. S. Olcott, *Arch. Biochem.*, **24**, 157 (1949).
3. F. K. Yeboah, I. Ali, and V. A. Yaylayan, *J. Agric. Food Chem.*, **47**, 3164 (1999).
4. A. Kuniyasua, N. Ohgamia, S. Hayashia, A. Miyazakib, S. Horiuchib, and H. Nakayama, *FEBS Lett.*, **537**, 85 (2003).
5. H. S. Olcott and H. Fraenkel-Conrat, *Chem. Rev.*, **41**, 151 (1947).
6. *Analytical Methods in Protein Chemistry* [in Russian], Izdatinlit, Moscow (1963), p. 600.
7. J. F. Riordan and B. L. Vallee, *Methods in Enzymology*, C. H. W. Hirs, ed., Vol. XI, Academic Press, New York and London (1967), p. 565.
8. LUMAC, *Safety and Saving in Liquid Scintillation*, Schaesberg, Netherlands (1982/1983), p. 26.