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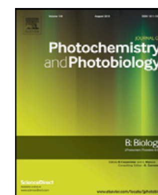

The role of acetoacetate in Amadori product formation of human serum albumin

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ABSTRACT

Amadori product is an important and stable intermediate, which is produced during glycation process. It is a marker of hyperglycemia in diabetes mellitus, and its accumulation in the body contributes to microvascular complication of diabetes including diabetic nephropathy and retinopathy. In this study, the effect of acetoacetate on the formation of Amadori products and biophysical properties of human serum albumin (HSA), after incubation with glucose, was investigated using various methods. These included circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy, and UV–visible and fluorescence spectroscopy. Our results indicated that the production of Amadori products in HSA incubated with glucose (GHSA) was increased in the presence of acetoacetate. We also detected alterations in the secondary and tertiary structure of GHSA, which was increased in the presence of acetoacetate. These changes were attributed to the formation of covalent bonds between the carbonyl group of acetoacetate and the nucleophilic groups (lysine residues) of HSA. Thus, acetoacetate can enhance the production of Amadori products through formation of covalent bonds with biomaterials.

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1. Introduction

Amadori product is a ketoamine that is produced during glycation process as a stable and important intermediate in the formation of advanced glycation end products (AGEs). Glycation is a spontaneous reaction between the free amino groups of biomacromolecules (such as proteins and lipids) and aldehyde or ketone groups of reduced sugars (such as glucose, fructose and ribose) upon covalent bond formation [1,2]. These reactions occur in three stages [3]. The first step (the early stage) is a nucleophilic attack of a carbonyl group by an electron pair of an amino group, which produces a Schiff base within hours [4]. The second step (the intermediate stage) is the rearrangement of the Schiff base to produce Amadori products within weeks [4]. The third step is the irreversible stage, which produces the AGEs through oxidation, dehydration and cyclization [5]. AGEs are yellow-brown, often fluorescent (some are non-fluorescent), intra- or inter-molecular cross-linked, and insoluble compounds [6], which contribute to pathogenesis of many diseases [7].

Amadori product is an important intermediate during AGE formation *in vivo*, and also in experimental conditions [8]. The level of Amadori products is increased in the blood and various tissues in diabetes [9], and it is a potential marker for hyperglycemia in diabetes mellitus [10]. The Amadori products of different proteins are found to be associated with diabetes and its complications [10]. Amadori products are involved in experimental hyperglycemia-induced microvascular complications [11]. They are associated with early and advanced nephropathy and retinopathy in Type 1 diabetic humans [12–14]. The increase in Amadori product concentration in diabetic animals was also linked with development and progression of diabetic retinopathy [15].

Ketone bodies are produced from the oxidation of fatty acids to provide the needed energy in the absence of available glucose in the liver [16,17]. The amount of circulating ketone bodies is from less than 5 μM after eating to more than 25 mM in acute diabetic patients [17].

Abbreviations: AA, Acetoacetate; AGEs, Advanced glycation end products; HSA, Human Serum Albumin; GHSA, glycated human serum albumin; 3BHB, 3-β-hydroxybutyrate; CD, Circular Dichroism.

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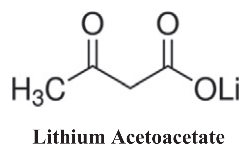
Also, the concentration of ketone bodies increases during fasting [16]. There are three types of ketone bodies in the body, namely acetoacetate (AA), 3-β- hydroxybutyrate, and acetone [18]. Acetoacetate has a linear structure with two carbonyl groups. It cans glycate the aminophospholipids in the brain and this reaction is inhibited by urea [19].



Lithium Acetoacetate

Human serum albumin (HSA) is a multifunctional protein, which acts as an antioxidant, and carrier of endocrine compounds and drugs [20]. HSA is a single polypeptide with 585 residues containing 58 lysine [21]. Lysine residues can bind covalently with carbonyl groups of other compounds, such as carbohydrates including glucose and ribose. The more reactive lysine residues of HSA are Lys 525, Lys 439, Lys 281 and Lys 199 [22], and are important target of glycation.

The formation of Amadori products and AGEs play an important role in pathogenesis of many diseases including diabetes. In addition, the concentration of AA increases during diabetes. Thus, the major objective of this study was to determine the impact of AA on Maillard reaction and its consequence on the formation of Amadori products. Here we investigated the effect of AA on formation of Amadori products and structural changes of HSA incubated with glucose.

2. Materials and Methods

2.1. Materials

Human serum albumin (96%, essentially fatty acid free), Lithium acetoacetate, nitro-blue tetrazolium (NBT), L-cysteine and 5,5′-dithiobis, 2-nitrobenzoic acid (DTNB) were purchased from the Sigma-Aldrich Company. All solutions were prepared in 50 mM sodium phosphate buffer (pH 7.4). The final concentration of HSA in each experiment was 40 mg/mL (similar to the physiological concentration in the blood), which was prepared before use. β-D-glucose and 2,4,6-trinitrobenzene sulfonic acid (TNBSA; 0.01%) were purchased from the Fluka Company. All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of AGE-HSA

The final concentration of HSA was 40 mg/mL in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA and 1 mM sodium azide. Glycation reaction of HSA was initiated by adding 16.5 mM β-D-glucose in either the presence or absence of 3 mM AA. The AA concentration was selected based on the AA concentration detected in diabetic individuals [23,24]. The incubation of HSA with all reagents was performed under sterile conditions at 37 °C, pH 7.4 and dark environment (physiological condition) for 7, 14 and 21 days. These incubation times were selected to provide sufficient time for the completion of the intermediate stage of HSA glycation and production of Amadori products [25]. Furthermore, the dark environment was chosen to mimic the physiological HSA environment in the human plasma. At the end of incubation time, all samples were dialyzed against 50 mM sodium phosphate buffer (pH 7.4) at 4 °C for 48 h, and then stored at −30 °C. Bicinchoinic acid (BCA) protein assay was used for the determination of protein concentration using a standard curve. The standard curve was generated using bovine serum albumin (BSA). Incubation of every sample was repeated three times and the results were presented in averages of three independent experiments.

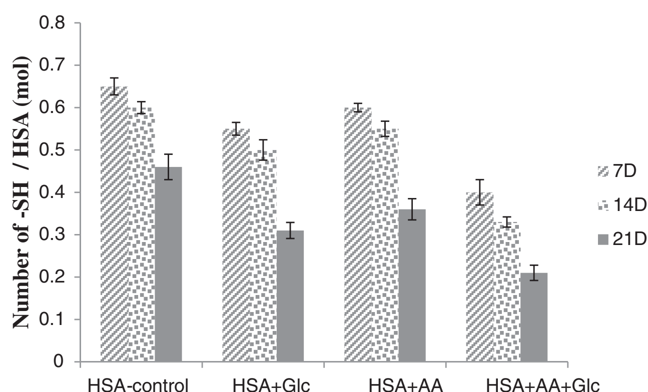


Fig. 2. Thiol group contents of HSA-control, HSA + Glc, HSA + AA and HSA + AA + Glc after 7, 14 and 21 days of incubation at 37 °C in 50 mM phosphate buffer pH 7.4.

2.3. Free Lysine Measurements

The determination of free amino groups of all HSA samples were carried out using 2,4,6-trinitrobenzene sulfonic acid (TNBSA; 0.01% (W/V)) as a sensitive reagent for quantification of primary free amino groups in available lysine residues [26]. Therefore, protein samples to be assayed were directly dissolved in buffer (0.1 M sodium bicarbonate, pH 8.5) at a concentration of 0.2 mg/mL. Then, 0.25 mL of the 0.01% (w/v) solution of TNBSA was added to 0.5 mL of each sample solution, mixed well, and incubated at 37 °C for 2 h. Following incubation, 0.25 mL of 10% SDS and 0.125 mL of 1 N HCl were added to each sample. The absorbance (335 nm) of each solution was then read against a blank prepared as

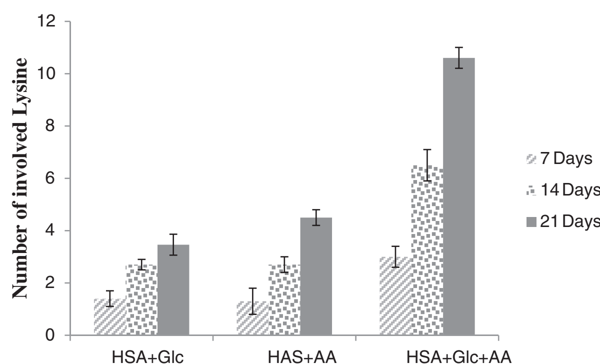


Fig. 1. Number of involved Lys residues in HSA + Glc, HSA + AA and HSA + AA + Glc after 7, 14 and 21 days of incubation at 37 °C in 50 mM phosphate buffer with pH 7.4. All data were normalized using its HSA- control.

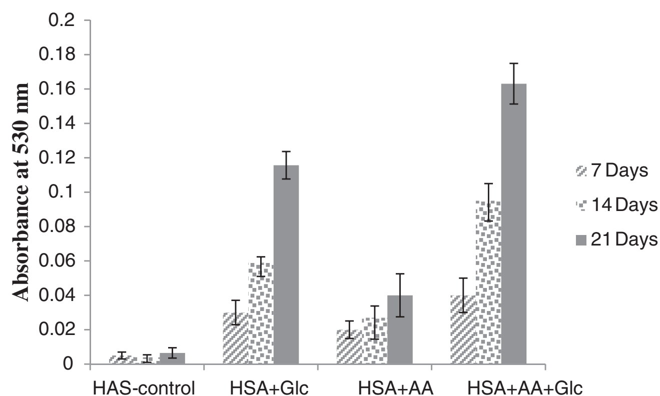


Fig. 3. Amount of Amadori products of HSA-control, HSA + Glc, HSA + AA and HSA + AA + Glc after 7, 14 and 21 days of incubation at 37 °C in 50 mM phosphate buffer pH 7.4.

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