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Journal of Chromatography B, 660 (1994) 265–270

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Quantitation of the glycation intermediate 3-deoxyglucosone by oxidation with rabbit liver oxoaldehyde dehydrogenase to 2-keto-3-deoxygluconic acid followed by high-performance liquid chromatography

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First received 6 April 1994; revised manuscript received 4 July 1994

Abstract

A simple and sensitive method for the detection of 3-deoxyglucosone was developed using oxidation with crude oxoaldehyde dehydrogenase to 2-keto-3-deoxygluconic acid followed by high-performance liquid chromatography (HPLC). Oxoaldehyde dehydrogenase was prepared from rabbit liver and partially characterized. 2-Keto-3-deoxygluconic acid produced from 3-deoxyglucosone by oxoaldehyde dehydrogenase was derivatized with 1,2-diamino-4,5-methylenedioxybenzene, and the fluorescent products were detected and quantitated by HPLC using a solvent containing borate. In the presence of borate, 2-keto-3-deoxygluconic acid was completely separated from N-acetylneuraminic acid. The detection limit of 3-deoxyglucosone was 2.5 pmol/injection (10 μl) at a signal-to-noise ratio of 3. This method was used to confirm the inhibitory effect of aminoguanidine on glycation.

1. Introduction

Posttranslational glycosylation of proteins by attachment of O- and N-linked oligosaccharides is mediated by regulated mechanisms involving complex enzyme systems [1]. There are also many enzymes that remove sugar chains from proteins [2,3]. In contrast, non-enzymatic glycosylation of proteins by reducing sugars occurs accidentally and appears not to be regulated [4]. Polymerization and browning of proteins in the presence of glucose or other reducing sugars are now well known phenomena [4–6]. The chemical basis for this process is the initial production of Schiff's base by the reaction between the amino groups of the protein and the reducing end of the sugar, followed by Amadori rearrangement [4]. The subsequent production of 3-deoxyglucosone (3-DG) from this Amadori adduct is important in the formation of advanced glycosylation end products (AGEs) which accounts for the changes occurring in proteins during aging and in diabetes mellitus [6,7]. It is well established that aminoguanidine-HCl is capable of trapping 3-DG and Amadori products and thus can prevent the production of AGEs [8]. Because sugars are used as a primary energy

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SSDI 0378-4347(94)00313-0

source by living organisms, this unexpected and uncontrolled glycosylation reaction is inevitable.

Presently, the glycation intermediate 3-DG is detected by mass spectrometric analysis [7]. Although this method is highly sensitive it is tedious and requires expensive equipment. In this report, we present a simple method for the detection of 3-DG by first converting it to 2-keto-3-deoxygluconic acid (3-DGA) by treatment with oxoaldehyde dehydrogenase (OAD) followed by HPLC of a fluorescent derivative of the α-keto acid. We have used this new technique to confirm the inhibitory effect of aminoguanidine on glycation.

2. Experimental

2.1. Materials

The following compounds and materials were obtained commercially: D(−)-fructose was from Merck (Darmstadt, Germany); 2,4-dinitrophenylhydrazine, dextrose and 1,2-diamino-4,5-methylenedioxybenzene (DMB) from Wako Pure Chemical Industries (Osaka, Japan); β-nicotinamide adenine dinucleotide (NAD, sodium salt) and β-nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) from Sigma (St. Louis, MO, USA); and aminoguanidine-HCl from Tokyo Kasei Industries (Tokyo, Japan). All other reagents were of the highest grade available and were used without further purification.

2.2. Sample preparation

3-Deoxyglucosone

3-DG was prepared as reported previously [9]. Briefly, a 2.0 *M* solution of fructose in 2.0 *M* sodium acetate, pH 5.5, was heated at 100°C for 2 h and the 3-DG formed was purified by descending paper chromatography using butanol–acetic acid–water (4:1:1, v/v) as solvent. The purity of the 3-DG preparations was checked by thin-layer chromatography (TLC) according to reported methods [10]. TLC was carried out on silica-gel plates (Kieselgel 60 from Merck) using two different solvent systems: chloroform–methanol–water (7:3:0.3, v/v), and ethyl acetate–methanol–water (4:1:1, v/v). Detection of 3-DG on TLC plates and paper was performed by spraying with a saturated solution of 2,4-dinitrophenylhydrazine in 2 *M* HCl. Quantitation of 3-DG was performed by the reported method for the dicarbonyl compound [10].

Crude enzyme

Livers dissected from rabbits and other species were homogenized in 4 volumes of 50 m*M* phosphate buffer, pH 7.0, containing 1 m*M* β-mercaptoethanol using a Polytron homogenizer. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was dialyzed against the same buffer to remove low-molecular-mass materials and stored at −80°C until used as the crude enzyme source for OAD.

2.3. Procedure

A 25-μl volume of sample ($>$200 pmol 3-DG) was mixed with 25 μl each of 0.1 *M* Tris-HCl buffer, pH 8.6, 50 m*M* β-NAD and the crude enzyme preparation, and the mixture was incubated at 37°C for 3 h. The reaction mixture (12.5 μl) was diluted with 12.5 μl of distilled water and then acidified by addition of 25 μl of 50 m*M* sulfuric acid. Finally, 50 μl of DMB solution, prepared as reported by Hara et al. [11], was added and the mixture was heated at 50°C for 2.5 h.

2.4. High-performance liquid chromatography

The DMB derivative of 3-DGA was analyzed by HPLC on a reversed-phase column (250 × 4.6 mm I.D., Inertsil ODS-2, 5 μm; Gasukuro Kogyo, Tokyo, Japan) using acetonitrile–methanol–water or 10 m*M* sodium borate buffer pH 7.0 (9:7:84 v/v) as mobile phase at a flow-rate of 0.75 ml/min. The DMB derivative was detected by spectrofluorimetry (Shimadzu RF-530) using an excitation wavelength of 373 nm and an emission wavelength of 448 nm as previously reported [11,12].

2.5. Aminoguanidine treatment

Bovine serum albumin (BSA) was dissolved at a concentration of 20 mg/ml in 0.2 *M* phosphate buffer (pH 7.4) containing 0.5 *M* glucose and 0.02% sodium azide (to prevent bacterial growth). Aminoguanidine was added at final concentrations of 0–50 m*M* (0, 0.5, 1.0, 5.0, 10.0 and 50.0 m*M*) and the mixtures were incubated at 37°C for up to 41 days. Aliquots of the reaction mixture were collected at various time intervals, and the concentration of 3-DG measured as described above. Another batch of BSA solutions containing a larger number of different concentrations of aminoguanidine was prepared to learn more about the concentration dependency of aminoguanidine. The BSA solution were prepared as described above except for the concentrations of aminoguanidine. Aminoguanidine was added at final concentrations of 0, 0.5, 0.7, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 20.0, 30.0, 40.0 and 50.0 m*M*. The 3-DG concentrations in the reaction mixtures were measured after 41 days.

3. Results

3.1. Optimization of assay conditions

The DMB derivatives of 3-DGA and N-acetylneuraminic acid (NANA) could not be separated by HPLC using previously reported conditions (Fig. 1A) [11]. In order to separate these two sugar derivatives, the water in the mobile phase was substituted with 10 m*M* sodium borate buffer, pH 7.0. This caused a shift in the elution position of the DMB derivative of NANA such that it separated completely from the derivative of 3-DGA (Fig. 1B). Furthermore, the fluorescence intensity of the DMB derivative of 3-DGA was observed to be higher in the borate-containing solvent. The production of 3-DGA by rabbit liver OAD is illustrated in Fig. 2. The results show that the 3-DGA produced was not further metabolized during prolonged incubation. Identical quantitation curves were obtained using buffers with a pH of 8.6 or 11 for the enzyme incubation. A linear relationship was observed up to a concentration of 1.0 m*M* 3-DG. The detection limit of the DMB derivative of 3-DGA was approximately 2.5 pmol/injection (10 μl) at a signal-to-noise ratio of 3. For lower concentrations, noise mainly from OAD was

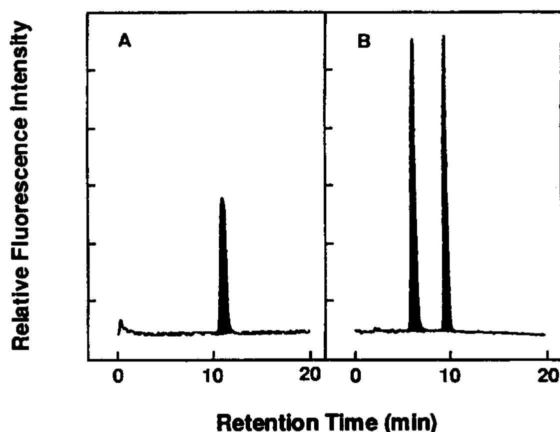

Fig. 1. HPLC profile of mixed DMB derivatives of N-acetylneuraminic acid and 2-keto-3-deoxygluconic acid. (A) Mobile phase: acetonitrile–methanol–water (9:7:84, v/v). (B) Mobile phase: acetonitrile–methanol–10 m*M* borate buffer, pH 7.0 (9:7:84, v/v). DMB derivative of 2-keto-3-deoxygluconic acid (slower peak) was completely separated from N-acetylneuraminic acid (faster peak).

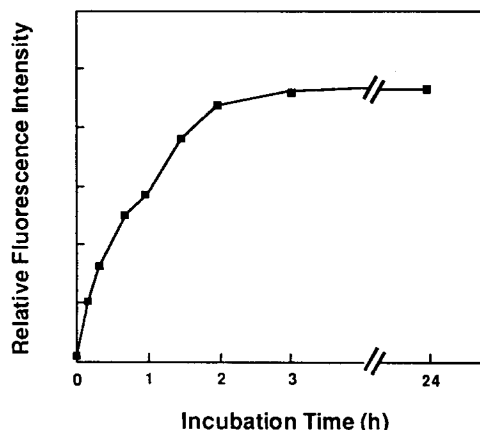

Fig. 2. Time course of conversion of 3-DG to 3-DGA by rabbit liver oxoaldehyde dehydrogenase. Purified 3-DG (1 m*M*) was treated with rabbit liver oxoaldehyde dehydrogenase and at various time intervals the DMB derivative of the product was quantitated by HPLC as described in the text.

observed and a linear relationship could not be obtained.

3.2. Characterization of oxoaldehyde dehydrogenase

The liver is the most commonly used source of oxoaldehyde dehydrogenase [13–16]. We compared the activity of the enzyme from livers of different animals toward 3-DG and methylglyoxal. Detection of the activity toward 3-DG was carried out using the method described above; the activity toward methylglyoxal was estimated by spectrophotometric measurement at 340 nm of the NADH produced from NAD [13]. In agreement with the report by Hata et al. [14], the rabbit liver enzyme was found to be more active against 3-DG than the enzymes isolated from livers of other animals (Fig. 3). The pH optimum of OAD for the 3-DG substrate was broad, and OAD exhibited a high activity even at pH 11. When NADP was used as coenzyme instead of NAD, less activity was observed at all pH values measured (Fig. 4).

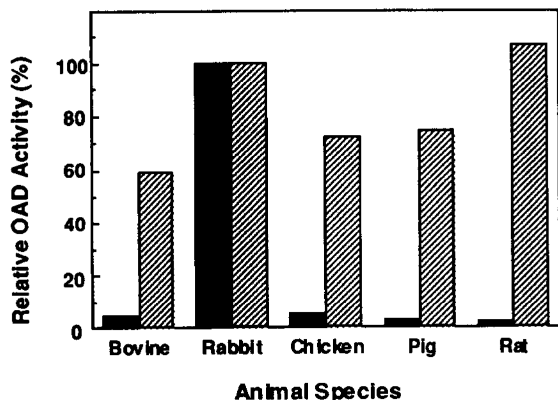

Fig. 3. Differences between liver oxoaldehyde dehydrogenase activities form several species toward the substrates methylglyoxal and 3-DG. Detection of activity toward 3-DG (black area) was carried out by quantitation of the DMB derivative of 3-DGA. Activity toward methylglyoxal (shaded area) was estimated by the spectrophotometric measurement of NADH derived from NAD.

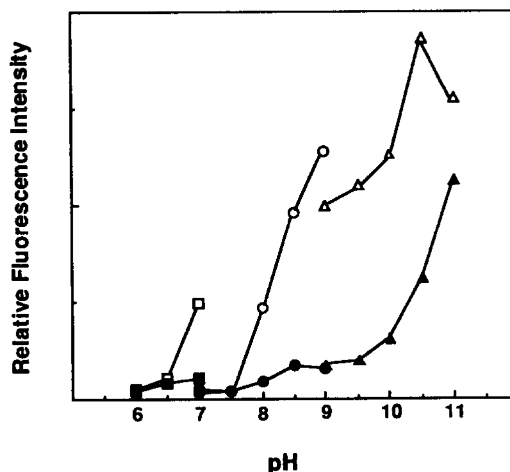

Fig. 4. pH profile of rabbit liver oxoaldehyde dehydrogenase activity toward 3-DG. Detection of activity toward 3-DG was carried out by HPLC quantitation of the DMB derivative of the 3-DGA produced in phosphate buffer (pH 6.0–7.0), Tris-HCl buffer (pH 7.0–9.0), and Na_2CO_3–$NaHCO_3$ buffer (pH 9.0–11.0), respectively. NAD (□, ○, △) or NADP (■, ●, ▲) was used as the coenzyme. The procedure was the same as that described in the text except for the sample incubation time with OAD. The incubation time with OAD was 1 h.

3.3. Application

The production of 3-DG during BSA glycosylation was measured in the absence or presence of aminoguanidine for 41 days. A time course experiment at different concentrations of aminoguanidine showed that the production of 3-DG continued to increase until the end of the incubation; however, the production of 3-DG was fully suppressed by 50 m*M* aminoguanidine (Fig. 5). A more detailed concentration dependency of aminoguanidine was investigated using the same experimental conditions for 41 days. The inhibitory effect of aminoguanidine followed a biphasic curve as indicated in Fig. 6.

4. Discussion

Because monosaccharides are a primary energy source for living organisms, the reaction

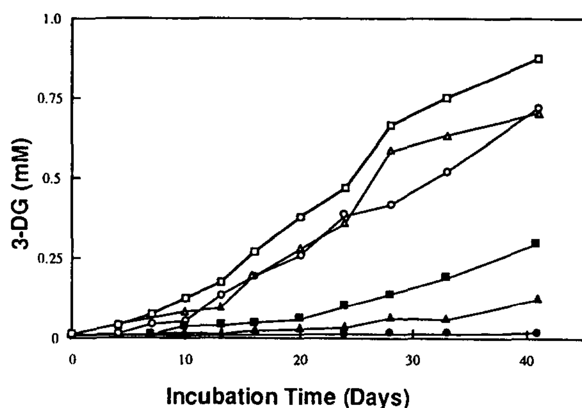

Fig. 5. Effect of aminoguanidine concentration on the glycosylation of BSA with time. BSA was dissolved at a concentration of 20 mg/ml in 0.2 *M* phosphate buffer (pH 7.4) containing 0.5 *M* glucose. Aminoguanidine was added to the solution at the different concentrations (□: 0 m*M*, △: 0.5 m*M*, ○: 1 m*M*, ■: 5 m*M*, ▲: 10 m*M*, ●: 50 m*M*) and the solution was incubated at 37°C for 41 days

between an aldose, such as glucose, and the amino groups in proteins followed by the production of AGEs is inevitable. Under normal circumstances, accidentally modified short-lived proteins will be quickly catabolized and therefore leave no trace of the glycation products. Recent studies show that organisms also have strong defence mechanisms against long-lived proteins such as collagen and lens crystallin [17]. These mechanisms include macrophages, which are able to remove AGEs by receptor-mediated processes, and enzymes, which inactivate deoxyosones and catabolize them to harmless substances.

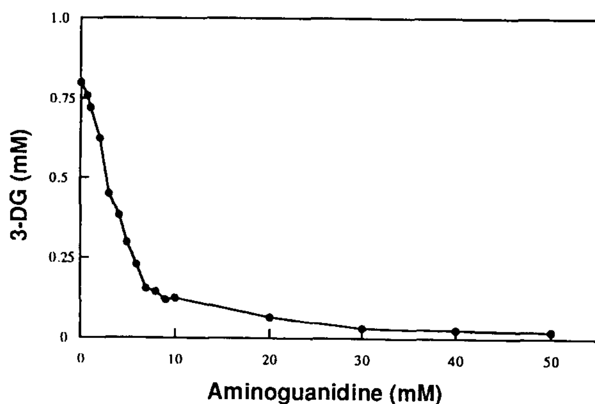

Fig. 6. Inhibition of glycosylation of BSA by various concentrations of aminoguanidine after 41 days of incubation. BSA and glucose were mixed and incubated as described in the text. Aminoguanidine was added to the solution at 16 different concentrations in the range 0–50 m*M*. The level of 3-DGA was measured after 41 days to estimate the inhibition of glycosylation of BSA.

Complete conversion of 3-DG to 3-DGA under the experimental conditions used was confirmed, because the dose–response curves at pH 8.6 and pH 11 coincided and increased in a linear fashion. The crude enzyme used showed a broad pH optimum and a high activity under alkaline conditions as previously reported [16]. Oxoaldehyde reductase and other competing enzymes, which exhaust 3-DG as a substrate, would not react under these conditions. The results indicate that this reaction system could be used for the quantitation of 3-DG. Moreover, for the oxidation of 3-DG rabbit liver homogenate may be used instead of the purified enzyme.

Because 3-DGA is structurally similar to sialic acid, it is not surprising that the DMB derivative of 3-DGA has a retention time very close to that of the DMB derivative of NANA under the HPLC conditions previously reported. However, because sialic acid exists abundantly in living organisms, one should be able to distinguish 3-DGA from sialic acid. The addition of borate to the HPLC mobile phase was effective in separating these derivatives from each other as was expected from our previous experience in separating the structurally related glycopeptides [18,19].

Aminoguanidine is a nucleophilic hydrazine compound which has the ability to block the formation of AGEs by trapping 3-DG and Amadori adducts. There are many reports which show the inhibitory effect of aminoguanidine on glycosylation [8,20,21]. Many of these reports indicate the decrease in fluorescent AGEs or functional amelioration of organs related to diabetic complications. In this report, we were also able to confirm the inhibitory effect of

aminoguanidine on glycation by quantitating the 3-DG that was produced during glycation of BSA. This method for the quantitation of 3-DG is easy and sensitive compared with other methods currently in use. Furthermore, it is important that we are able to detect the prevention of glycation by simply measuring 3-DG in samples using the HPLC method presented here. This method should be useful in the search for drugs that have an effect similar to that of aminoguanidine and for investigating the role of 3-DG and 3-DGA in the etiology of diabetic complications and the aging process.

Acknowledgements

The authors thank Ms. Tsukiko Urata for her excellent technical assistance. We also thank Prof. Bhavanandan of Pennsylvania State University for his appropriate advice during the improvement of this paper and Associate Prof. Kunie Nakamura of Kitasato University for his generous gift of aminoguanidine. This work was supported in part by a Grant-in-aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

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