Rapid Method for the Preparation of an AGE-BSA Standard Calibrator Using Thermal Glycation

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Estimation of advanced glycation end products (AGEs) by determining fluorescence is based on the use of a standard calibrator prepared by incubating bovine serum albumin (BSA) and glucose at 37°C for 60 days. In the present study we attempted to reduce the duration of incubation to 4 days by increasing the temperature to 50°C. It is noteworthy that incubation at 50°C resulted in the rapid production of an AGE-BSA standard calibrator within 4 days. Amino-guanidine reduced the intensity of the

glycation-induced fluorescence, while the addition of lysine intensified the reaction, as shown by the calibrator incubated at 37°C. The protein carbonyl content was shown to increase in the rapidly-formed standard calibrator. Thus we conclude that a simple increase in temperature and the addition of lysine (0.1M) can accelerate the process of glycation-induced fluorescence. This calibrator can be used effectively in fluorescence assays of AGEs. *J. Clin. Lab. Anal.* 19: 11–15, 2005. © 2005 Wiley-Liss, Inc.

Key words: diabetes; glycation; AGE-BSA; lysine; fluorescence; advanced glycation end products

INTRODUCTION

Free amino groups of proteins initially react with reducing sugars to form Schiff's base, which then covalently bonds to form Amadori rearrangement products. These Amadori products undergo a rearrangement reaction to form a heterogeneous group of protein-bound moieties called advanced glycation end products (AGEs). This is a slow reaction, and commensurate with the duration of diabetes there is an increase in the accumulation of AGEs (1). AGEs play an important role in the structural and functional alterations of proteins that occur in long-term diabetes. The formation of AGEs is regarded as one of the most important pathological processes in the complications of long-term diabetes, such as nephropathy, neuropathy, and retinopathy. It is important to monitor serum AGE concentrations during the treatment of patients with diabetic complications (2,3).

Several assay methods have been proposed to determine AGE levels, such as specific fluorescence generated by the Maillard reaction and browning of the proteins, assays based on protein crosslinking and the formation of dimers and trimers, immunological methods using specific enzyme-linked immunosorbent assay (ELISA) techniques or polyclonal antibodies. High-performance liquid chromatography (HPLC) has also been applied to determine AGEs (4).

Fluorescence determination of AGEs is a rapid and widely accepted method. AGEs exhibit characteristic fluorescence with excitation in the range of 350–390 nm, and fluorescence emissions have been measured at 440–470 nm (5,6). Fluorescence estimation at excitation 370 nm and emission 440 nm has been used in several assays to measure AGEs. Such assays are based on the use of a standard calibrator made by incubating bovine serum albumin (BSA) with glucose at 37°C for 60 days (1,6). We propose a rapid method for obtaining an AGE-BSA calibrator using thermal glycation, which can reduce the duration of incubation to 4 days.

The exogenous administration of AGEs provides an important animal model for the study of complications resulting from diabetes and aging. Previously described methods have used exogenously prepared AGE rat serum albumin (RSA) incubated for 40 weeks (7) or 90 days (8) as a model for studying diabetes-related complications such as retinopathy and nephropathy. In this study we reduced the duration of incubation to

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4 days, for preparation of AGE-BSA, which can make faster formation of AGEs for the induction of diabetic complications.

MATERIALS AND METHODS

Reagents

BSA (Fraction V) was obtained from Hi Media Laboratories (India). Glucose and lysine were obtained from Glaxo and Thomas Baker (India), respectively. All other chemicals used were of analytical or HPLC grade, and obtained from S.D. Fine Chemicals Ltd. (India), Qualigens (India), or Sigma (St. Louis, MO).

Preparation of AGEs

We prepared AGE albumin by incubating BSA (50 mg/mL) with 0.5 M glucose in 0.2 M sodium phosphate buffer pH 7.4 for 60 days. One preparation of control albumin was also incubated under the same conditions but without glucose (1). For thermal glycation, BSA (50 mg/mL) and 0.5 M glucose in 0.2 M phosphate buffer (pH 7.4) were incubated at 50°C, 60°C, and 80°C. The control sample of albumin was also incubated under similar conditions. We prepared lysine-derived AGEs by incubating 0.1 M lysine with BSA (50 mg/mL) and 0.5 M glucose at 37°C for 60 days, as well as at 50°C for 4 days. For aminoguanidine inhibition, BSA (50 mg/mL) and 0.5 M glucose were incubated with 0.1 M aminoguanidine at 50°C. Under similar conditions, control samples of 0.1 M lysine and 0.5 M glucose alone and in combination were also incubated.

All incubations were performed under sterile conditions. A small drop of chloroform was added to the solution, and the corks were moistened with toluene to inhibit bacterial growth.

Analyses

We determined protein concentrations using Lowry's method. We estimated fluorescence by measuring emissions at 440nm upon excitation at 370nm using the Jasco FP-1520 Intelligent Fluorescence Detector (Jasco, Tokyo, Japan). The fluorescence values of the test substances were measured at a protein concentration 1 mg/mL. The results are expressed as AGE units (1 U = 1 µg/mL AGE-BSA standard) as previously described by Kilhovd et al. (9).

Protein-Carbonyl Content

Proteins were precipitated with 20% trichloroacetic acid and centrifuged. The precipitate was resuspended in 2,4 dinitrophenylhydrazine (10 mM) and vortexed at 10-

min intervals for 1 hr at room temperature. The pellets were washed three times with ethanol/ethyl acetate to remove the free reagent before centrifugation. The pellets were then resuspended in 0.6 mL of 6 M guanidine hydrochloride, incubated at 37°C for 15 min in a water bath, and centrifuged at 5,000 g for 3 min. The absorbance of the supernatant was measured spectrophotometrically at 366 nm for carbonyl content, and calculations were performed with an ϵ value of 22,000 M⁻¹ cm⁻¹ (10).

RESULTS

To shorten the incubation time of the AGE-BSA standard calibrator, we incubated BSA and glucose at 50°C. The AGEs formed rapidly and the incubation time was reduced from 60 days to 4 days. The AGE-BSA standard calibrator incubated at 37°C exhibited a characteristic emission at 440 nm on excitation at 370 nm, as previously reported (6). The thermally glycated AGE-BSA calibrator showed similar fluorescence spectra (Fig. 1) in terms of the emission maximum valley (440 nm) and shoulder (>480 nm). Both of these fluorescence spectras showed fluorescence intensities that were higher than those of the respective control BSA samples (incubated without glucose at 37°C and 50°C), indicating protein glycation. The fluorescence values showed a progressive increase when the fluorescence was checked at 24-hr intervals.

We attempted further to intensify this reaction by incubating the samples at 60°C and 80°C; however, the majority of samples showed the formation of a visible solid mass, indicating protein denaturation. After 3 days, the control BSA incubated at 60°C showed denaturation, while incubation at 80°C has shown denaturation in plain BSA as well as in BSA with glucose. Therefore, incubation at 50°C is considered an ideal temperature for preparing an AGE-BSA standard calibrator.

After the rapidly formed AGEs were digested with 6 N HCl and analyzed for fluorescence, the results were similar to that of the samples incubated at 37°C.

AGEs are dicarbonyl derivatives, the presence of which was observed in both samples (Fig. 2). Assessments of protein carbonyl content showed that there were four times more dicarbonyl derivatives in a BSA-AGE calibrator incubated at 50°C compared to one incubated at 37°C.

Aminoguanidine inhibits the formation of AGEs (1). Incubation of BSA with glucose in the presence of aminoguanidine at 50°C also inhibited the formation of AGEs, confirming AGEs formation similar to that of 37°C (Fig. 3).

Fig. 1. Relative fluorescence spectra: BSA (a) and BSA + D-glucose (b) incubated at 37°C for 60 days. BSA (a') and BSA + D-glucose (b') incubated at 50°C for 4 days.

Fig. 2. Carbonyl content in moles/liter. (B-37): BSA incubated at 37°C. (B + G 37): BSA and glucose incubated at 37°C. (B-50): BSA incubated at 50°C. (B + G 50): BSA and glucose incubated at 50°C.

AGEs are formed by a reaction of the epsilon amino group of proteins and glucose. The addition of lysine to the reaction mixture intensifies the formation of AGEs. In confirmation of this property, we observed an increase in fluorescence when the AGEs were incubated with lysine at both 37°C and 50°C (Fig. 3). Control samples of 0.1 M lysine alone and in combination with 0.5 M glucose did not show significant fluorescence.

The present technique showed significant reproducibility in terms of fluorescence (46.75 ± 3.62) and carbonyl content (8.68 ± 1.89) when values were expressed in terms of the mean and standard deviation, as shown in Table 1.

DISCUSSION

Previous studies used AGE-BSA standard calibrators prepared by incubating BSA and glucose for 60 days (1).

Fig. 3. Effect of the addition of 0.1 M lysine on fluorescence. (B-37): BSA incubated at 37°C. (B + G 37): BSA and glucose incubated at 37°C. (B + G 37 + 0.1 M Ly): BSA, glucose, and 0.1 M lysine incubated at 37°C. (B-50): BSA incubated at 50°C. (B + G 50): BSA and glucose incubated at 50°C. (B + G 50 + 0.1 M Ly): BSA, glucose, and 0.1 M lysine incubated at 50°C. (B + G + amino): BSA + glucose + aminoguanidine incubated at 50°C.

In this study we intensified the reaction by increasing the temperature, and produced AGEs in a short span of 4 days. Thermally glycated samples showed a similar fluorescence pattern, and exhibited no difference in terms of carbonyl content when compared with samples incubated at 37°C.

Incubation of glucose with BSA at a higher temperature (55°C) accelerates the reaction, as previously reported by Sharma et al. (11). In our studies, incubation of BSA and glucose at 50°C has been shown to increase fluorescence. However, with further increases

TABLE 1. Fluorescence and carbonyl content

Observation no.	Fluorescence (U/mg protein)				Carbonyl Content (M/L) ^a			
	B 37	B + G 37	B 50	B + G 50	B 37	B + G 37	B 50	B + G 50
1	5.50	35.50	7.50	51.25	0.73	2.68	1.09	9.73
2	9.75	33.25	10.50	49.50	0.77	1.45	1.32	8.36
3	11.50	41.00	6.50	43.50	0.86	2.18	1.27	5.68
4	9.75	34.75	6.50	46.50	1.09	2.41	1.45	10.68
5	8.25	33.25	5.25	43.00	0.91	1.77	1.73	8.95
Mean	8.95	35.55	7.25	46.75	0.87	2.10	1.37	8.68
±SD	2.25	3.20	1.98	3.62	0.14	0.49	0.24	1.89

B 37, BSA incubated at 37°C; B + G 37, BSA and glucose incubated at 37°C; B 50, BSA incubated at 50°C; B + G 50, BSA and glucose incubated at 50°C.

^aExpressed in terms of [(carbonyl content) × 10⁻³].

in temperature (to 60°C or 80°C), protein denaturation precluded us from estimating fluorescence.

Aminoguanidine is a hydrazine-like compound that reacts during an intermediate stage of the advanced glycation process to inhibit the formation of protein-bound fluorescent products and cross links (1). In this study, incubation of BSA and glucose in the presence of aminoguanidine at 50°C inhibited this protein glycation, which further confirmed the rapid formation of AGEs.

Free amino acids, such as lysine, form adducts with glucose at physiological pH and temperature. Lysine reacts with aldehyde groups of glucose through their amino groups, and forms glycosyl amino acid adducts (12). We observed this characteristic phenomenon in both of the samples incubated at 37°C and 50°C, and the addition of lysine caused an increase in fluorescence (Fig. 3).

Carbonyl content is considered an indicator of glycation, and is used as a parameter to evaluate oxidation of proteins (13). When these carbonyls were evaluated in the rapidly formed AGE-BSA calibrator, they showed similar results. There was a significant increase in carbonyl content and fluorescence in the sample incubated with lysine at 50°C when compared with the corresponding sample incubated at 37°C, which further confirmed the formation of AGEs.

Exogenously administered AGEs are considered an important model for the study of diabetes and related complications, as previously used by Vlassara et al. (14) and Xu et al. (7). The rapidly derived AGEs can be used for exogenous administration, which shortens the duration of incubation from 60–90 days to 4 days. However, reports have indicated (15) that thermal processing of the proteins brings about major changes in antigenicity resulting from the formation of neoantigens. Therefore, antigenic studies of these rapidly induced AGE-BSA agents must be conducted before

they can be used as an animal model for diabetic complications.

In conclusion, with a simple increase in temperature, an AGE-BSA standard calibrator can be generated rapidly (in 4 days as compared to the conventional 60 days' incubation). This procedure can be used effectively to determine AGEs in clinical samples as a means of monitoring diabetic complications.

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