JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Modified Fast Procedure for the Detection and Screening of Antiglycative Phytochemicals

Shih-Hao Wang, Ju-Chun Chang, Rattaphong Pokkaew, Ji-Fang Lee, and Robin Y.-Y. Chiou*

Department of Food Science, National Chiayi University, Chiayi, Taiwan, Republic of China

ABSTRACT: In an attempt to elevate temperature to facilitate glycation, a nonenzymatic reaction by incubation of bovine serum albumin (BSA) and fructose at 50 °C for 24 h has been developed. As conducted and compared to a routine procedure by incubation of BSA and fructose at 37 °C for 168 h, the reactant fluorescence intensities and SDS-PAGE-detected glycated BSA quantities produced by both test temperatures increased with time of incubation. As the Amadori products and α-dicarbonyl compounds during incubation were quantified, both quantities produced at each temperature also increased with an increase of time of incubation, and their trends of changes at both temperatures were similar. In practical application for the detection and screening of the antiglycative phytochemicals, each of 20 peanut root extracts was introduced to a series of BSA−fructose solutions and incubated at 37 and 50 °C for 168 and 24 h, correspondingly. All extracts exhibited notable activities and varied depending on peanut origins. Pair comparison of the resultant antiglycative activities determined at 37 and 50 °C showed that both determined activities for each peanut root extract deviated limitedly. As further analyzed, SDS-PAGE-detected glycated BSA quantities formed at 50 °C were closely proportional to the antiglycative activities determined on the basis of their fluorescence intensities. It is of merit to demonstrate that fluorescence-based determination of BSA−fructose reactant after incubation at 50 °C for 24 h is practical and time-saving in the detection and screening of antiglycative phytochemicals.

KEYWORDS: glycation, bovine serum albumin (BSA), fructose, SDS-PAGE, antiglycative activity, peanut root

INTRODUCTION

Glycation is a nonenzymatic reaction mainly occurring between amino and carbonyl groups of proteins and reducing sugars. Advanced glycation end-products (AGEs) are defined as the irreversible formation of a series of complex end-products through glycation of proteins and are regarded as a post-translational modification of proteins.[1] In blood, a large quantity of AGEs involves structural and bioactivity changes of proteins and is associated with aging and incidence of chronic diseases, such as diabetes mellitus.[2] These products may further interact with proteins to form intra- and intermolecular cross-linkages or degrade to alter protein structures and specific functions. In general, these reactions are slow, without enzyme participation, and take a few hours to a few days to produce AGEs. In the initiation stage, unstable products with a Schiff base are formed by interaction of the amino group of proteins and the carbonyl group of reducing sugars and, then, simultaneously transform to stable ketoamines of Amadori products. In the subsequent secondary stage, as enhanced by oxygen and metallic ions, the Amadori products further interact with the ε-amino group of lysine residues of proteins to form irreversible products. Meanwhile, amines and α-dicarbonyl compounds are formed by rearrangement and dehydration of sugars. In the final stage, the dicarbonyl compounds keep interacting with lysine residues of proteins to form nonfluorescent pyrraline and/or by pentoses interacting with lysine and arginine residues of proteins to form fluorescent pentosidine of AGEs.

From the viewpoint of health care, in addition to taking caution to avoid hyperglycemia, intakes of antiglycative compounds from foods or by dietary supplements may slow glycation and prevent subsequent complications of chronic diseases. Even protein glycation is an inevitable process of aging; the chemical reaction rates are likely to be slowed by the intervention of some sorts of bioactive agents.[3] Thus, natural antiglycative compounds, mainly phytochemicals, have attracted extensive research and development interests of academia and industry. Source exploration of natural antiglycative products is a general goal. To achieve this goal, a fast and reliable procedure for the detection and screening of natural antiglycative phytochemicals and other active compounds is needed. AGEs are featured with absorbance of specific wavelengths and emission of fluorescence.[4,5] The fluorescence emission is unique, and its intensity is instrumentally quantified as a measure of glycation. As reported, most in vitro determinations of antiglycative activity are conducted at 37 °C, human body temperature, for quite long periods.[2,6−12] Measurement under 37 °C is time-consuming, and the elimination of microbial contamination is tedious. On the basis of the fact that glycation is a nonenzymatic reaction, the higher temperature, the higher the chemical reaction rate expected. Accordingly, in this study, in an attempt to develop a time-saving procedure for the detection and screening of natural antiglycative products, a series of reactants of bovine serum albumin (BSA) and fructose were incubated at 37, 45, 50, and 65 °C for various intervals and followed by determination of the fluorescence intensity to quantify AGEs. Meanwhile, the Amadori products and α-dicarbonyl compounds produced during incubation were quantified concurrently. The glycated proteins were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and densitometrically quantified.

Received: March 18, 2011
Revised: May 12, 2011
Accepted: May 14, 2011
Published: May 14, 2011

 © 2011 American Chemical Society

The developed procedure was further applied for antiglycative activity determination of 20 peanut root extracts as a practice for the detection and screening of bioactive phytochemicals.

■ MATERIALS AND METHODS

Chemicals. Methanol, acetic acid, and acetone were purchased from J. T. Baker (Phillipsburg, NJ); potassium diphosphate and dipotassium hydrogen phosphate were purchased from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan); 2-mercaptoethanol and sodium dodecyl sulfate (SDS) were purchased from Merck (Darmstadt, Germany); Acryl/Bis solution, ammonium persulfate (APS), and TEMED were purchased from Amresco Inc. (Solon, OH); other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

AGEs Formation As Affected by Incubation Temperature. Basically, the procedure reported by Yamaguchi et al.[2] in the use of BSA and fructose (Sigma) as glycation reactants was followed with modification. Briefly, BSA (60 mg/mL) and fructose (1.5 M) were respectively prepared in 0.2 M potassium phosphate buffer (pH 7.4, containing 0.06% sodium azide). For each reaction of glycation, 100 μL of BSA solution, 100 μL of fructose solution, and 100 μL of deionized water were deposited in a 1.5 mL microfuge tube (MCT-175-C, Union City, CA). A series of tubes containing the above mixed solution were respectively incubated at 37, 45, 50, and 65 °C (MIR 252, Sanyo, Osaka, Japan) for assigned intervals. After incubation, the reacted solutions were stored frozen at −20 °C until determination of the fluorescence intensity. For determination of the intensity, 100 μL of each reacted solution was withdrawn to a well of a 96-well plate and subjected to intensity determination by a spectrofluorometer (FLx 800, BioTek, Winooski, VT) set at 360 nm of excitation and 460 nm of emission. Basically, the fluorescence intensity of tubes incubated at 37 °C was determined every 24 h for 7 days (168 h). At 45, 50, and 65 °C, their intensities were determined every 6 h, which was set mainly on the basis of results obtained from preliminary experiments. The fluorescence intensity after incubation at 37 °C for 168 h was regarded as a reference in the estimation of the required times of incubation at 45, 50, and 65 °C. After incubation at 65 °C, a cloudy suspension was formed, perhaps due to thermal denaturation of BSA molecules, and for this reason, incubation at 65 °C was not considered further.

For determination of BSA−fructose glycation with a known glycation inhibitor of aminoguanidine (AG) (Sigma), 100 μL of 3 mM AG solution (prepared in deionized water) was introduced in substitution of the deionized water in each of the reactions described above containing BSA and fructose. The tubes were respectively incubated at 37, 45, and 50 °C for specified intervals, followed by fluorescence determination.

Formation of Fructosamine (an Amadori Product) As Affected by Incubation Temperature. The above-described and prepared reactants, namely, BSA−fructose−water and BSA−fructose−AG, were respectively incubated at 37, 45, and 50 °C for specified intervals and subjected to fructosamine determination following the reported procedure[13] with minor modification. Briefly, from each tube after incubation was withdrawn 20 μL into a 1.5 mL microfuge tube and mixed with 160 μL of deionized water; 0.8 mL of 300 μM nitroblue tetrazolium (NBT, dissolved in 100 mM, pH 10.35, sodium carbonate buffer) (Sigma) was then introduced, and the mixture was incubated at ambient temperature (25−27 °C) for 15 min. The absorbance at 530 nm was determined by a spectrophotometer (U-2001, Hitachi Co. Ltd., Tokyo, Japan).

Formation of α-Dicarbonyl Compounds As Affected by Incubation Temperature. The above-described and prepared solutions were respectively subjected to quantification of the α-dicarbonyl compounds by Girard-T assay.[14] Briefly, 20 μL of the incubated solution was mixed with 80 μL of deionized water, 50 μL of Girard-T reagent (500 mM in deionized water), and 850 μL of

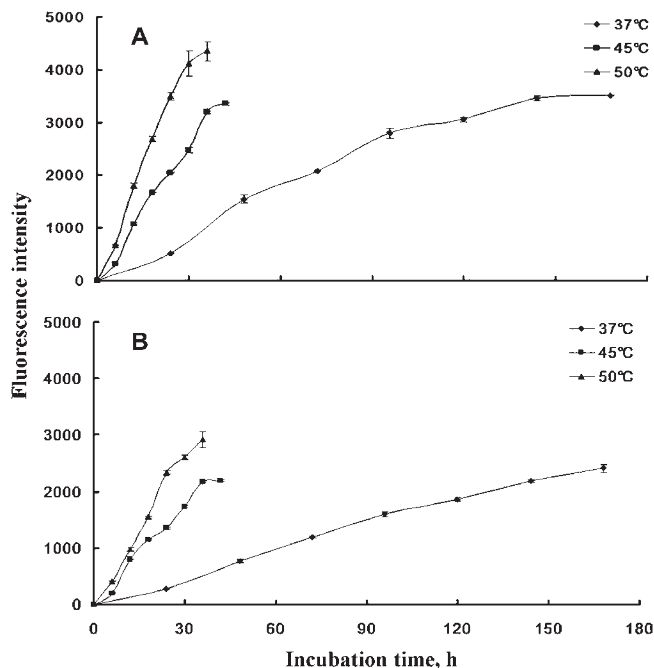

Figure 1. Fluorescence-determined formation of advanced glycation end-products (AGEs) by interaction of bovine serum albumin (BSA) and fructose as affected by incubation temperatures at 37, 45, and 50 °C and introduction of aminoguanidine (AG): (A) BSA−fructose solutions without AG; (B) BSA−fructose solutions with AG. Each value is the mean ± standard deviation of three replicate experiments.

500 mM sodium formate (pH 2.9) in a test tube and incubated at ambient temperature (25−27 °C) for 1 h. Then, the absorbance of the solution at 290 nm was determined with a spectrophotometer (U-2001, Hitachi Co.).

SDS-PAGE Characterization of Glycated Proteins. The reactant solutions with and without AG addition after incubation at 37 and 50 °C for specific intervals were subjected to SDS-PAGE analysis and quantification of the glycated BSA proteins. In each sample preparation, 100 μL of the incubated solution was mixed with 100 μL of 10% trichloroacetic acid (dissolved in cold acetone) and centrifuged at 14000*g* at 4 °C for 20 min (Sigma Labrozentrifugen 2K15, Osterode, Germany). The pellet of protein precipitate was washed with cold acetone twice and then dissolved in 100 μL of SDS-PAGE sample buffer [62.5 mmol/L Tris-HCl (pH 6.8) buffer containing 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, and 0.01 mg/mL bromophenol blue]. After heat treatment at 100 °C for 10 min, 5 μL of the solution was loaded into a well of a prepared SDS-PAGE gel (13.4% polyacrylamide gel containing 0.1% SDS). After electrophoresis (Mini-Protean 3 System, Bio-Rad, Hercules, CA) run at 100 V for 90 min, the gels were stained with Coomassie Brilliant blue R-250 (Sigma) for 20 min and destained with aqueous acetic acid−methanol solution (100 mL of acetic acid and 200 mL of methanol per liter). The gels were scanned by an image scanner (U9909-H7L0, Amersham Biosciences, Uppsala, Sweden) and densitometrically quantified using Fujifilm Multigauge ver. 3.0 Analysis Software (Fujifilm, Tokyo, Japan). Proportional glycated BSA was expressed by dividing the band intensity of the glycated BSA for each test by that of the glycated BSA formed after incubation of BSA−fructose solution at 37 °C for 168 h. The ratio was used as a quantitative index of BSA glycation.

Preparation of Peanut Root Extracts and Determination of Antiglycative Activity. Twenty peanut roots with various breeding lines were provided by the Agricultural Research Institute, Wufen,

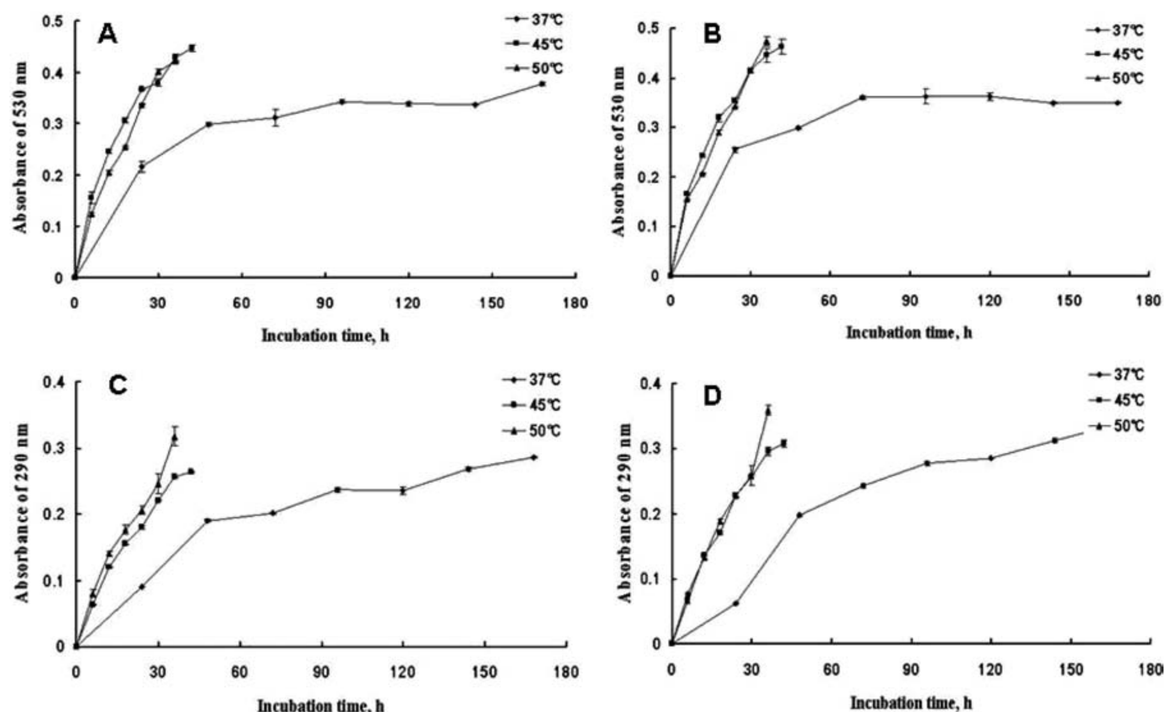

Figure 2. Absorbance-determined formation of fructosamine and α-dicarbonyl compounds by interaction of bovine serum albumin (BSA) and fructose as affected by incubation temperatures at 37, 45, and 50 °C and introduction of aminoguanidine (AG): (A) fructosamine formation by incubation of BSA−fructose solutions without AG; (B) fructosamine formation by incubation of BSA−fructose solutions with AG; (C) formation of α-dicarbonyl compounds by incubation of BSA−fructose solutions without AG; (D) formation of α-dicarbonyl compounds by incubation of BSA−fructose solutions with AG. Each value is the mean ± standard deviation of three replicate experiments.

Taiwan. After harvesting, the roots were collected, cleaned with tap water, and dried under sunshine. The dried roots were ground into a fine powder and stored at −20 °C until used. In the preparation of peanut root extract, 0.5 g of each powder was deposited in a 15 mL centrifuge tube and homogenized with 10 mL of 80% (v/v) methanol at 15000 rpm for 1 min by a polytron with an aggregate probe (PT3000, Kinematica AG, Littau, Switzerland). The tubes were centrifuged at 15000*g* at 4 °C for 20 min. From each tube was withdrawn 5 mL of the supernatant, which was deposited in a centrifuge tube, spin-dried with a sublimator (VaCO I, Zirbus, Bad Grund, Germany), and stored under −20 °C until used. Prior to activity determination, each of the extracted solids was replenished with 5 mL of deionized water to prepare peanut root extract solution. Then, 100 μL of the solution was introduced in substitution of the deionized water in each of the reactant solutions containing BSA and fructose described above. The tubes were respectively incubated at 37 °C for 168 h and at 50 °C for 24 h. Deionized water and 3 mM AG were tested concurrently as a blank and a positive control.

Statistics. Data were expressed as the mean ± standard deviation (SD) of triplicates from three independent experiments and analyzed by the SPSS 12.0 software package. Analyses of variance were performed using ANOVA procedures. Significant differences between means were determined using Duncan's multiple-range test.

RESULTS AND DISCUSSION

Glycation comprises consecutive and sophisticated interactions in blood and body fluids initiated mainly by nonenzymatic reactions between reducing sugars and proteins. The formed α-dicarbonyl compounds further interact with free amino groups through oxidation, dehydration, and cycling reactions to produce fluorescent AGEs. Fluorescence emission of AGEs under UV irradiation renders a unique feature and enables measurement of fluorescence intensity as a quantitative reference of glycation.[5] As reported, most fluorescence quantifications of AGEs are operated by incubation of proteins and reducing sugars at 37 °C for 7 days or longer.[2,6−12] Incubation at 37 °C is mainly adapted from human body temperature. In practice, the procedure is a time-consuming and may be contaminated by growth of microorganisms, and these make it tedious.

For comparison of the nonenzymatic reactions of glycation, BSA and fructose as reactants were respectively incubated at 37, 45, and 50 °C, and their fluorescence intensities were determined successively (Figure 1A). In general, their fluorescence intensities increased with an increase of incubation time. For the reactions at 37 °C for 168 h as a reference quantification of AGEs, the fluorescence intensity was 3511 ± 4 units. At 45 and 50 °C, the fluorescence intensities increased linearly to 3354 ± 27 and 3502 ± 69 units after 42 and 24 h of incubation, respectively. It is obvious that the formation of AGEs after incubation at 50 °C for 24 h is comparable to that with incubation at 37 °C for 168 h. After 96 h of incubation at 37 °C, fluorescence intensities leveled off with further incubation. Underestimation of glycation resulting from incubation at 37 °C for longer than 96 h is very likely. With incubation at 50 °C for 24 h, the fluorescence intensities increased linearly to the same level as obtained by incubation at 37 °C for 168 h.

Aminoguanidine is a known chemical of hydrazine compounds, which is a potent inhibitor of AGE formation. It is effective in the prevention of glycation of vascular collagens interacting with the high blood sugars of diabetic patients.[15−17] When AG was introduced in BSA−fructose solutions at 1 mM (final concentration) and respectively incubated at 37, 45, and 50 °C for successive determinations of fluorescence intensity,

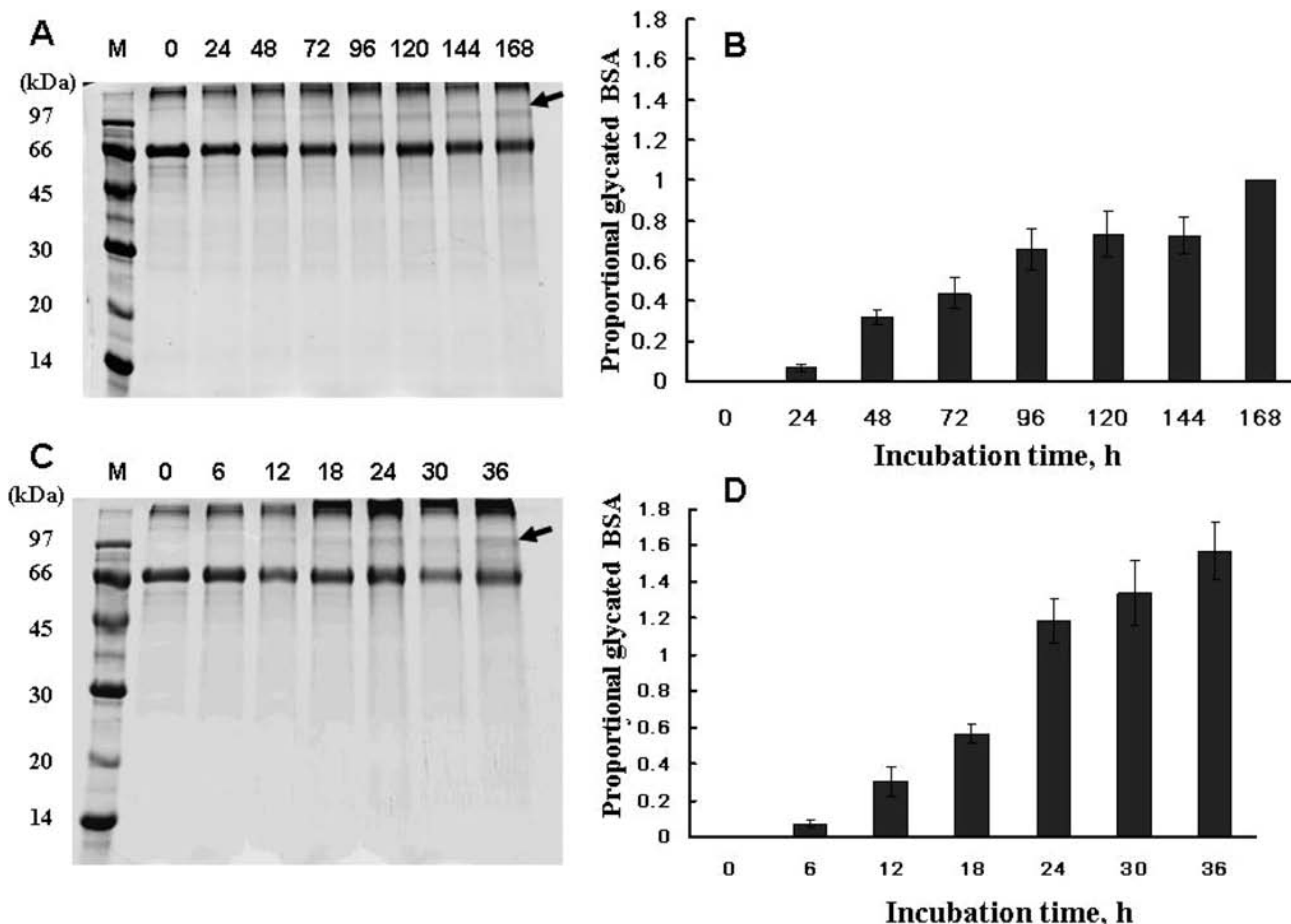

Figure 3. SDS-PAGE analysis of bovine serum albumin (BSA) glycation: (A) glycated BSA formation (indicated with an arrow) by incubation of BSA−fructose solutions at 37 °C for 168 h; (B) proportional glycated BSA formation during incubation at 37 °C; (C) glycated BSA formation (indicated with an arrow) by incubation of BSA−fructose solutions at 50 °C for 36 h; (D) proportional glycated BSA formation during incubation at 50 °C. Each value is the mean ± standard deviation of three replicate experiments. M, protein marker.

their fluorescence intensities increased with time of incubation (Figure 1B). Fluorescence intensities of the solutions after incubation at 37 °C for 168 h, at 45 °C for 42 h, and at 50 °C for 24 h were 2411 ± 71, 2193 ± 12, and 2341 ± 29 units, respectively. As further converted to proportional activity based on the inhibition of AGE formation, the inhibitory activities of AG exhibited at 37 and 50 °C were 31.3 ± 1.9 and 33.2 ± 0.5%, respectively. With the presence of AG as an inhibitor, the fluorescence intensities also increased linearly at 50 °C for 24 h and at 37 °C for 168 h. Thus, incubation of BSA−fructose solutions at 50 °C for 24 h with the presence of an introduced agent was practical for the determination of its antiglycative activity. Even an incubation of <24 h at 50 °C seems likely to be practical as a daily basis of operation.

In addition to fluorescence intensity determination of AGEs, the primary and secondary intermediate products of Amadori and α-dicarbonyl compounds as affected by reaction temperatures have been determined spectrophotometically (Figure 2). The Amadori products were quantified principally on the basis of the reduction of NBT by Amadori products to monoformazan dye, which bears strong absorbance at 530 nm.[18] As shown in Figure 2A, the determined absorbance units all increased with an increase of incubation time. However, a linear increase for incubation at 37 °C was only maintained in the first 24 h. After 24 h of incubation, absorbance units increased slowly and leveled off at 96 h. For the incubations at 45 and 50 °C, both absorbance units increased linearly in the test periods. Absorbance of the reactants after reaction at 37 °C for 168 h and at 50 °C for 24 h were 0.38 ± 0.01 and 0.34 ± 0.01, respectively. Slightly lower contents of Amadori products were detected at 50 °C than at 37 °C, which might be attributed to more dynamic interactions taking place at the former temperature than at the latter. When AG was introduced for incubation and monitored (Figure 2B), as compared on the same basis of reaction time, formation of Amadori products was slightly different from that without AG introduction (Figure 2A). As affected by reaction temperature, a linear increase for incubation at 37 °C was also maintained in the first 24 h. After 24 h of incubation, absorbance units increased slowly and leveled off at 96 h. For incubations at 45 and 50 °C, both absorbance units increased linearly in the test periods. The quantified Amadori product contents were lower at 50 °C incubation than at 37 °C incubation. On the basis of the fact that Amadori products are intermediate products of AGEs, the observed lower Amadori product contents might be due to the more dynamic reactivity undergone at 50 °C than at 37 °C. As observed, incubation at 50 °C for 24 h also provides a reliable measure of the intermediate products of AGEs.

Table 1. Relative Antiglycative Activities of 20 Peanut Root Extracts As Determined by Incubation of Fructose and Bovine Serum Albumin at 37 °C for 168 h and at 50 °C for 24 h

	relative antiglycative activity[a] (%)	
sample[b]	37 °C incubation for 168 h[c]	50 °C incubation for 24 h[c]
aminoguanidine (3 mM)	29.8 ± 2.4	31.0 ± 1.6
1	52.1 ± 1.2	54.5 ± 0.2
2	78.8 ± 2.6*	85.1 ± 1.4**
3	46.0 ± 1.8*	41.8 ± 0.6**
4	75.2 ± 1.4	78.5 ± 4.4
5	49.8 ± 2.5	54.6 ± 1.5
6	56.8 ± 3.6	53.8 ± 1.2
7	40.7 ± 1.4	41.1 ± 2.3
8	45.0 ± 2.1	45.5 ± 1.9
9	33.5 ± 1.3	39.6 ± 2.3
10	74.5 ± 7.6	70.0 ± 0.3
11	70.7 ± 0.7	68.2 ± 3.5
12	42.0 ± 3.5	44.1 ± 1.0
13	53.2 ± 3.2	59.2 ± 1.6
14	55.1 ± 1.7	58.3 ± 4.3
15	57.4 ± 2.0	61.2 ± 2.2
16	53.6 ± 3.5	58.2 ± 2.3
17	54.2 ± 2.8	52.6 ± 2.2
18	64.9 ± 0.4	67.7 ± 1.7
19	51.6 ± 4.7	52.1 ± 2.3
20	50.0 ± 4.4	53.2 ± 0.8

[a] Each value is the mean ± SD (n = 3). Values in the same row with different numbers of asterisks are significantly different ($p < 0.05$). [b] Peanut breeding lines: 1, NI-28; 2, VA-55; 3, NI-30; 4, NI-52; 5, NI-32; 6, NI-73; 7, NI-54; 8, VA-9; 9, NI-75; 10, VA-48; 11, NI-37; 12, NI-9; 13, VA-15; 14, NI-5; 15, NI-20; 16, NI-11; 17, NI-34; 18, NI-50; 19, VA-32; 20, VA-54. [c] Relative antiglycative activity (%) = 100 − [(fluorescence of test sample/fluorescence of control) × 100].

The secondary glycation products of α-dicarbonyl compounds were spectrophotometrically determined at 290 nm after reaction with Girard's reagent T, forming the hydrazone compounds.[14] All determined absorbance units at any incubation temperature increased with an increase of incubation time (Figure 2C). As affected by reaction temperature, a linear increase for incubation at 37 °C was also maintained in the first 24 h. After 24 h of incubation, absorbance units increased slowly and leveled off at 96 h. For the incubations at 45 and 50 °C, both absorbance units increased linearly in the test periods. Absorbances of the reactants after reaction at 37 °C for 168 h and at 50 °C for 24 h were 0.29 ± 0.01 and 0.21 ± 0.01, respectively. Slightly lower contents of α-dicarbonyl compounds were detected at 50 °C than at 37 °C. At 50 °C, temperature-enhanced dynamics of consecutive reactivity in the formation of AGEs might result in lower detected quantities of Amadori (Figure 2A) and α-dicarbonyl compounds as intermediate products than at 37 °C. When AG was introduced and incubated for the determination of α-dicarbonyl compounds (Figure 2D), AG was effective in inhibiting α-dicarbonyl compound formation at 37 °C for 24 h, which was in patial agreement with reported observations.[19,20] As affected by reaction temperature, a linear increase for incubation at 37 °C was maintained in the first 24 h. After 24 h of incubation, absorbance units increased slowly and leveled off at 96 h. For incubations at 45 and 50 °C, both absorbance units increased linearly in the test periods. As affected by reaction temperature, contents of α-dicarbonyl compounds were comparatively lower at 50 °C incubation for 24 h than that at 37 °C for 168 h. The α-dicarbonyl compounds, such as glyoxal, methylglyoxal, and deoxyglucosone, are associated with the physiopathology of diabetic complications, including atherosclerosis, thrombosis, and diabetic microvascular diseases. A dose-dependent decrease in antithrombin III activity has been detected when plasma samples of diabetic patients were incubated with methylglyoxal, forming a thrombus in the vasculature.[21] Thus, the likelihood of incubation of human plasma with fructose or glucose at 50 °C to facilitate glycation to determine the primary Amadori products and the secondary α-dicarbonyl compounds as a clinical approach deserves further research and development.

The AGEs formed after 168 h at 37 °C (Figure 3A) and after 36 h at 50 °C (Figure 3C) were subjected to SDS-PAGE analysis. An obviously glycated protein band of ca. 90 kDa molecular mass (indicated by an arrow) was detected. A similar product with a molecular mass of 91 kDa formed by the interaction of BSA and glucose at 37 °C for 21 days has been reported.[9] When densitometric quantification was made and expressed by proportional glycated BSA of test sample to that of glycated BSA formed at 37 °C for 168 h (Figure 3B,D), glycated BSA formed at 50 °C for 24 h was ca. 1.19-fold higher than that formed at 37 °C for 168 h. This indicates that quantities of the glycated proteins formed at 50 °C for 24 h were close to that formed at 37 °C for 168 h. Similarly, when AG was introduced as an inhibitor, the specified glycated protein formed by BSA−fructose reaction at 50 °C for 24 h was also close to that formed by reaction at 37 °C for 168 h. Because direct quantification of the glycated proteins by SDS-PAGE analysis is a straightforward measure of AGE formation, it is of interest to conduct BSA−fructose reaction at 50 °C for 24 h to achieve a compatible result such as that achieved by reaction at 37 °C for 168 h.

For application in the detection and screening of the natural antiglycative components, 20 peanut root extracts were subjected to activity determination at 37 and 50 °C for 168 and 24 h, respectively. Fluorescence-determined activities are presented in Table 1. All extracts exhibited varied activities against AGE formation depending on peanut breeding lines. This might be relevant to the previous reports that peanut roots contained resveratrol and other antioxidants.[22,23] On the basis of the fact that antiglycative activities determined by incubation at 37 °C for 168 h were comparable to those determined by incubation at 50 °C for 24 h, incubation of BSA−fructose solutions with introduction of peanut root extracts at 50 °C for 24 h is of merit to realize a time-saving. The procedure could be applied for the fast detection and screening of the antiglycative compounds either from peanut roots and from other natural sources of phytochemicals. When the 50 °C incubated solutions were further subjected to SDS-PAGE analysis (Figure 4), formation of the glycated BSA with a molecular mass of ca. 90 kDa inhibited by the peanut root extracts was obvious. Antiglycative activities could be quantitatively evaluated by visualizing the glycated BSA band intensities shown on the electrophoretic gel (Figure 4A) or calculating the proportional glycated BSA of each test sample to a reference of glycated BSA formed by BSA−fructose after incubation at 37 °C for 168 h (Figure 4B). Both evaluations

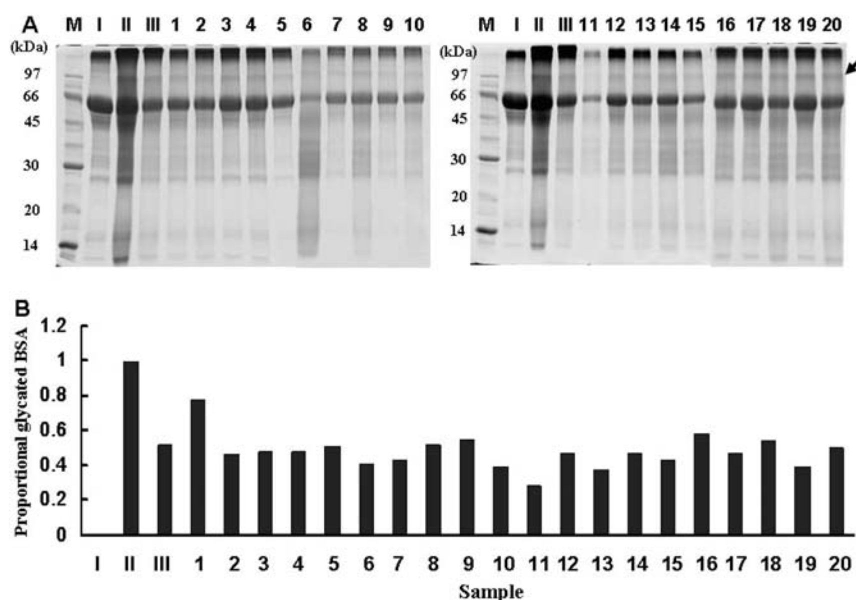

Figure 4. SDS-PAGE analysis of bovine serum albumin (BSA) glycation formed by incubation of BSA—fructose solutions at 50 °C for 24 h as affected by introduction of peanut root extracts with various lines: (A) glycated BSA formation (indicated with an arrow); (B) proportional glycated BSA formation. M, protein marker; I, bovine serum albumin; II, BSA—fructose incubated at 37 °C for 168 h; III, BSA—fructose incubated at 50 °C for 24 h with aminoguanidine (AG). Peanut breeding lines: 1, NI-28; 2, VA-55; 3, NI-30; 4, NI-52; 5, NI-32; 6, NI-73; 7, NI-54; 8, VA-9; 9, NI-75; 10, VA-48; 11, NI-37; 12, NI-9; 13, VA-15; 14, NI-5; 15, NI-20; 16, NI-11; 17, NI-34; 18, NI-50; 19, VA-32; 20, VA-54.

supported that reaction of BSA—fructose at 50 °C for 24 h was practical in the fast detection and screening of the antiglycative phytochemicals.

In conclusion, as supported by quantitative analyses of fluorescence intensity of AGEs and SDS-PAGE monitoring of glycated BSA, incubation of BSA—fructose at 50 °C for 24 h (one day) saves much time in the determination of in vitro glycation. In addition, the procedure also provides a reliable measure in quantification of the Amadori and α-dicarbonyl products. From a realistic viewpoint, the procedure was practical and reliable in the detection and screening of the antiglycative compounds in peanut root extracts. Even an incubation of <24 h at 50 °C seems likely to be a practical operation on a daily basis. In the future, development of a broader spectrum in the fast screening of other bioactive compounds or use in monitoring plasma composition as a health-status surveillance of clinical significance deserves further investigations.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Department of Food Science, National Chiayi University, 300 University Road, Chiayi 60051, Taiwan. Phone: +886-5271-7613. Fax: +886-5277-5524. E-mail: rychiou@mail.ncyu.edu.tw.

Funding Sources

Financial support from the National Science Council, Republic of China (NSC 97-2313-B415-008-MY3), is acknowledged.

ACKNOWLEDGMENT

We thank Dr. K. H. Yang, Agricultural Research Institute, for providing peanut root samples.

REFERENCES

(1) Brownlee, M.; Cerami, A.; Vlassara, H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* **1988**, *18*, 1315–1321.

(2) Yamaguchi, F.; Ariga, T.; Yoshimura, Y.; Nakazawa, H. Antioxidative and anti- glycation activity of garcinol from *Garcinia indica* fruit rind. *J. Agric. Food Chem.* **2000**, *48*, 180–185.

(3) Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advanced glycation end-products: a review. *Diabetologia* **2001**, *44*, 129–146.

(4) Fu, M. X.; Wells-Knecht, K. J.; Blackledge, J. A.; Lyons, T. J.; Thorpe, S. R.; Baynes, J. W. Glycation, glycoxidation, and cross-linking of collagen by glucose. *Diabetes* **1994**, *43*, 676–683.

(5) Lapolla, A.; Traldi, P.; Fedele, D. Importance of measuring products of nonenzymatic glycation of proteins. *Clin. Chem.* **2005**, *38*, 103–115.

(6) Kim, H. Y.; Kim, K. Protein glycation inhibitory and antioxidative activities of some plant extracts in vitro. *J. Agric. Food Chem.* **2003**, *51*, 1586–1591.

(7) Xi, M.; Hai, C.; Tang, H.; Chen, M.; Fang, K.; Liang, X. Antioxidant and antiglycation properties of total saponins extracted from traditional Chinese medicine used to treat diabetes mellitus. *Phytother. Res.* **2008**, *22*, 228–237.

(8) Choi, S. Y.; Jung, S. H.; Lee, H. S.; Park, K. W.; Yun, B. S.; Lee, K. W. Glycation inhibitory activity and the identification of an active compound in *Plantago asiatica* extract. *Phytother. Res.* **2008**, *22*, 323–329.

(9) Wu, J. W.; Hsieh, C. L.; Wang, H. Y; Chen, H. Y. Inhibitory effects of guava (*Psidium guajava* L.) leaf extracts and its active compounds on the glycation process of protein. *Food Chem.* **2009**, *113*, 78–84.

(10) Jung, H. A.; Jung, Y. J.; Yoon, N. Y.; da Jeong, M.; Bae, H. J.; Kim, D. W.; Na, D. H.; Choi, J. S. Inhibitory effects of *Nelumbo nucifera* leaves on rat lens aldose reductase, advanced glycation endproducts formation, and oxidative stress. *Food Chem. Toxicol.* **2008**, *46*, 3818–3826.

(11) Sun, Z.; Peng, X.; Liu, J.; Fan, K. W.; Wang, M.; Chen, F. Inhibitory effects of microalgal extracts on the formation of advanced glycation endproducts (AGEs). *Food Chem.* **2010**, *120*, 261–267.

(12) Zhang, L. S.; Wang, X.; Dong, L. L. Antioxidation and antiglycation of polysaccharides from *Misgurnus anguillicaudatus*. *Food Chem.* **2011**, *124*, 83–187.

(13) Baker, J. R.; Zyzak, D. V.; Thorpe, S. R.; Baynes, J. W. Chemistry of the fructosamine assay: D-glucosone is the product of oxidation of Amadori compounds during the fructosamine assay. *Clin. Chem.* **1994**, *40*, 1950–1955.

(14) Wells-Knecht, K. J.; Zyzak, D. V.; Litchfield, J. E.; Thorpe, S. R.; Baynes, J. W. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in autoxidative modification of proteins by glucose. *Biochemistry* **1995**, *34*, 3702–3709.

(15) Brownlee, M.; Vlassara, H.; Kooney, A.; Ulrich, P.; Cerami, A. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* **1986**, *232*, 1629–1632.

(16) Corbett, J. A.; Tilton, R. G.; Chang, K.; Hasan, K. S.; Ido, Y.; Wang, J. L.; Sweetland, M. A.; Lancaster, J. R.; Williamson, J. R.; McDaniel, M. L. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* **1992**, *41*, 552–556.

(17) Panagiotopoulos, S.; O'Brien, R. C.; Bucala, R.; Cooper, M. E.; Jerums, G. Aminoguanidine has an anti-atherogenic effect in the cholesterol-fed rabbit. *Atherosclerosis* **1998**, *136*, 125–131.

(18) Johnson, R. N.; Metcalf, P. A.; Baker, J. R. Fructosamine: a new approach to the estimation of serum glycoprotein, an index of diabetic control. *Clin. Chim. Acta* **1982**, *127*, 87–95.

(19) Edelstein, D.; Brownlee, M. Mechanistic studies of advanced glycosylation end product inhibition by aminoguanidine. *Diabetes* **1992**, *41*, 26–29.

(20) Chen, H. J.; Cerami, A. Mechanism of inhibition of advanced glycosylation by aminoguanidine *in vitro*. *J. Carbohydr. Chem.* **1993**, *12*, 731–742.

(21) Gugliucci, A.; Menini, T. The botanical extracts of *Achyrocline satureoides* and *Ilex paraguariensis* prevent methylglyoxal-induced inhibition of plasminogen and antithrombin III. *Life Sci.* **2002**, *72*, 279–292.

(22) Chen, R. S.; Wu, P. L.; Chiou, R. Y. Y. Peanut roots as a source of resveratrol. *J. Agric. Food Chem.* **2002**, *50*, 1665–1667.

(23) Wang, K. H.; Lai, Y. H.; Chang, J. C.; Ko, T. F.; Shyu, S. L.; Chiou, R. Y. Y. Germination of peanut kernels to enhance resveratrol biosynthesis and prepare sprouts as a functional vegetable. *J. Agric. Food Chem.* **2005**, *53*, 242–246.