Screening System for the Maillard Reaction Inhibitor from Natural Product Extracts

Nobuyasu Matsuura,[a] Tadashi Aradate,[a] Chihiro Sasaki,[a] Hiroyuki Kojima,[b] Mitsuharu Ohara,[b] Junichi Hasegawa,[b] and Makoto Ubukata*,[a]

[a]Biotechnology Research Center, Toyama Prefecture University, 5180 Kosugi, Imizu-gun, Toyama 939–0398, Japan and [b]Ichimaru Pharcos Co. Ltd., 318–1 Shinsei, Motosu-gun, Gifu 501–0475, Japan

(Received July 3, 2002; Accepted August 2, 2002; Published online August 8, 2002)

An assay for the Maillard reaction has been developed to screen efficiently inhibitors from natural resources such as extracts of plants. The fluorometric analysis of fluorescent material based on advanced glycation endproducts (AGEs) was applied to measurement of an inhibitory index of the Maillard reaction to detect all of the inhibitors at each step of the complicated reaction. To solve the two major problems, slowness of the reaction rate and the existence of interfering substances such as quencher and fluorescent material in the screening sources, we devised the following procedures. Slowness of the reaction rate was solved by raising the reaction temperature to 60°C at which the conformation of bovine serum albumin (BSA) did not change greatly. To remove the interfering substances, AGEs-BSA was precipitated from the reaction mixture. Thus, an efficient assay system by measuring the fluorescent intensity based on AGEs was established to isolate the glycation inhibitors from natural product extracts.

Key words —— Maillard reaction, advanced glycation endproduct, inhibitor, screening system

INTRODUCTION

Since Maillard reported the nonenzymatic browning reaction by heating a mixture solution of sugar and amino acid, the Maillard reaction has been studied in the field of sitology.[1)] The reaction is roughly divided into two stages, the early and late Maillard reactions. The early stage consists of the process whereby an Amadori rearrangement product is formed *via* 1,2-eminal after Schiff base formation between the amino group of protein and the aldehyde group of the reducing sugar.[2,3)] The late stage involves the following processes: complicated oxidation reaction, dehydrogenation and condensation followed by formation of fluorescent proteins containing advanced glycation endproducts (AGEs) and intra- and intermolecular crosslinkage of the proteins.[4)] AGEs are a generic name for advanced glycation endproducts by the Maillard reaction, and the structures of some AGEs, N^{ε}-(carboxymethyl)lysine,[5)] pyrraline,[4)] pentosidine,[6)] crossline,[7)] *etc*., have been reported. On the other hand, an Amadori rearrangement product as hemogrobin A_{1c} (HbA_{1c}) was identified from the blood of a diabetic subject in 1975.[8)] In addition, it was reported in 1984 that the fluorescent material (FFI) in the AGEs protein accumulated in dura mater collagen with age, and the values of FFI obtained at autopsy from diabetic subjects were found to be higher than those from normal subjects.[9)] The Maillard reaction *in vivo* has attracted attention by these observations. Moreover, glycation of proteins causes structural and functional changes, such as immobilization by crosslinkage,[10)] addition of resistance to protease[11)] and change in the activity of glycated enzyme.[12)] From these phenomena, it seems that the accumulation of AGEs is one cause of diabetic complication, and the prevention and improvement of such the accumulation are possible by inhibiting the formation of AGEs. Aminoguanidine (AG),[13)] aspirin,[14)] vitamin B6,[15)] taurine[16)] and quercetin,[17)] *etc*., have been reported as inhibitors of the Maillard reaction. Since AG was found to be a specific inhibitor of AGEs formation and cross-linking formation of proteins in 1986,[13)] its biological characteristics have been evaluated by various researchers. For example, AG was found to prevent increase in collagen-related fluorescence in the aorta, glomeruli and renal tubles of diabetic rat,[18)] and AG inhibited the accel-

*To whom correspondence should be addressed: Biotechnology Research Center, Toyama Prefecture University, 5180 Kosugi, Imizu-gun, Toyama 939–0398, Japan. Tel.: +81-766-56-7500; Fax: +81-766-56-2498; E-mail: ubukata@pu-toyama.ac.jp

erated diabetic retinopathy in spontaneous hypersensitive rats.[19] These experiments indicate a possibility of the Maillard reaction inhibitor as a diabetic complication drug. However, the effective dose of AG is very high and chronic administration cannot be avoided because of the property of the diabetic complication. Therefore, the development of more potent drugs has been desired. From this viewpoint, Morimitsu *et al*. performed the screening of glycation inhibitors from plant extracts, and quercetin was isolated as an active compound from thyme.[17] In spite of the significance of the Maillard reaction inhibitor as a remedy for the diabetic complication, no report on isolation of the inhibitor from natural product extracts could be found except for the Morimitsu report in our survey of the literature. Then we assumed that there is some drawback in the screening system for natural products. While many detection methods for the Maillard reaction have been developed upto now, the detection methods of the Amadori compound, especially HbA_{1c} as the most fundamental index that shows the glycemic control in the diabetes mellitus, are important.[20–22] In this paper, we report the development of a new detection method for the Maillard reaction, which is useful for isolation of the inhibitor from natural product extracts.

MATERIALS AND METHODS

Chemicals —— Bovine serum albumin (BSA) (fraction V) was obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, U.S.A.). An aqueous solution of D-[6-^{3}H]-glucose (0.74–1.6 TBq/mmol) was purchased from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, England). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan). Alkaline PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) was adjusted to pH 10 with 0.25 N NaOH.

Conformational Changes of BSA —— BSA (800 μg/ml) was dissolved with 50 mM phosphate buffer, pH 7.4 and incubated for 30 hr at various temperatures between 37 and 80°C; successively, the changes in the CD spectrum were monitored at the same temperature with the spectropolarimeter J-720W (JASCO, Japan).

***In Vitro* Glycation** —— 500 μl of reaction mixture was prepared containing 400 μg BSA, 200 mM glucose with or without inhibitor or 10 μl of plant extracts in 50 mM phosphate buffer, pH 7.4, and the reaction mixture was heated at 60°C on a heat block for indicated hours. The sample of blank, unreacting solution without inhibitor or plant extract, was kept at 4°C until measurement. After cooling, aliquots of 100 μl were transferred to new 1.5 ml plastic tubes, and 10 μl of 100 % (w/v) TCA was added to each tube. The supernatant containing glucose, inhibitor and the interfering substances was removed after agitation and centrifugation (15000 rpm, 4°C, 4 min), then the precipitate of AGEs-BSA was dissolved with 400 μl of alkaline phosphate-buffered saline (PBS) to serve for screening. The comparison of fluorescence spectrum (ex. 370 nm) and the change in fluorescence intensity (ex. 370 nm, em. 440 nm) based on AGEs were monitored by using spectrofluorometer RF-1500 (Shimadzu, Japan). The absorbance (350 nm) change based on Schiff base formation was measured by spectrophotometer U-3210 (Hitachi, Japan). The glucose incorporation activity was measured by the following method. D-[6-^{3}H]-glucose (5 μl: 0.185 MBq) was added to the above-mentioned reaction mixture, and the radioactivity incorporated in BSA was measured by using a liquid scintillation counter BECKMAN LS 6000TA (Beckman, U.S.A.) after incubation, TCA precipitation and solubilization with alkaline PBS.

The Preparation of Plant Extracts —— Each of the dried species (1 g) was extracted with 10 ml of 50% ethanol at room temperature for 1 week and then each extract was filtered.

The Measurement of the Maillard Reaction Inhibitory Activity —— Real inhibition activity was estimated by subtracting the quenching effect from the apparent inhibitory activity. Apparent inhibitory activity was calculated by the above method. Quenching effect was measured by using the same sample dissolved with alkaline PBS after TCA treatment of the mixed solution of 2 μl of plant extract and 100 μl of the 30 hr incubated control solution without inhibitor or plant extract.

RESULTS

Conformational Change of BSA Based on Temperature Rise

The rise of the reaction temperature enables us to solve the problem of slowness of the reaction rate and low sensitivity for the detection of AGEs, because the Maillard reaction is a nonenzymatic reaction. However, it may cause thermal denaturation of BSA as a substrate of the reaction. Then, the confor-

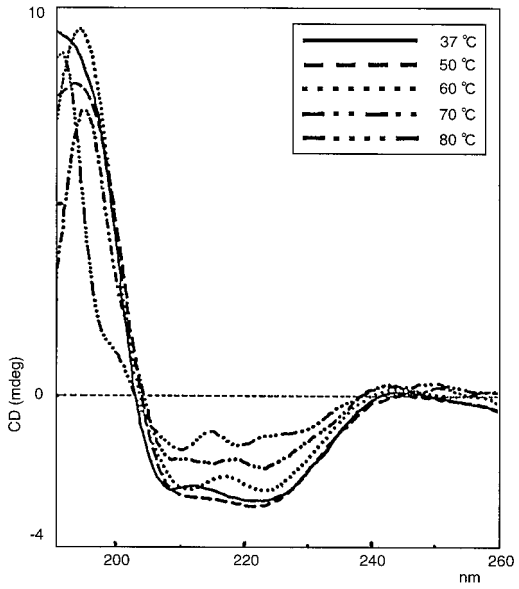

Fig. 1. Conformational Changes of BSA on Temperature Rise

The changes in the CD spectrum of BSA were examined on rising temperature (37, 50, 60, 70 and 80°C). BSA solution in phosphate buffer was incubated at each temperature for 30 hr. CD spectrum was measured at the same temperature.

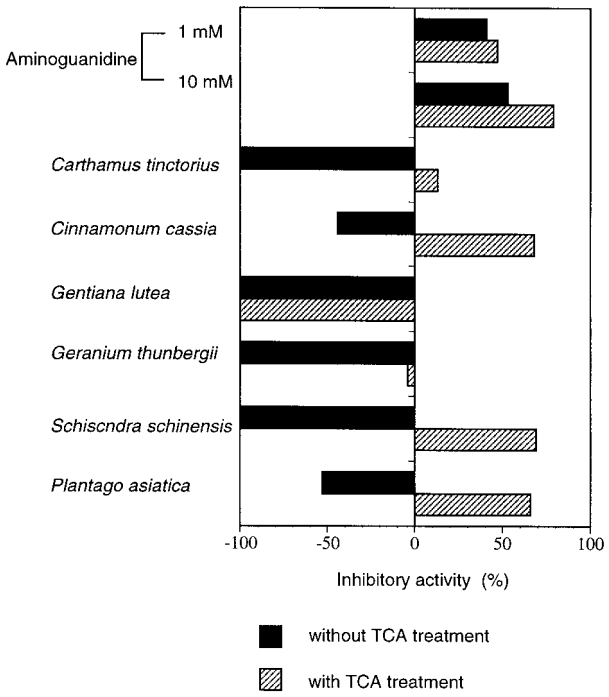

Fig. 2. The Maillard Reaction Inhibitory Activity after the Removal of Fluorometry Interfering Substance

Inhibitory activity in using screening sources with TCA treatment was compared with that without TCA processing.

mational change by heat treatment of BSA was monitored by the change in circular dichroism (CD) spectra. Figure 1 shows the CD spectra of BSA treated at 37, 50, 60, 70 and 80°C. As the result, the spectra at the temperature below 60°C did not show striking change; however, large changes at over 70°C were observed. It is suggested that most of the α-helical structures of BSA are retained at the temperature below 60°C. Thus, we set the reaction temperature in the screening system at 60°C.

Removal of Interfering Substances

Plant extracts used for screening samples contain excessive amounts of coloring matter like chlorophyll and autofluorescent material like phycobiliprotein.[23] Since the fluorescence intensity based on the AGEs generation is very low, it is considered that the quenching effect is very high in the case of the screening samples containing such natural product extracts. Arithmetic removal of the quenching effect has been done by subtracting the fluorescence intensity of the control solution, to which was added the screening sources, from that of the solution after the reaction in the presence of the screening sources. It was impossible to detect the inhibitory activity by the arithmetic removal of the quenching effect in most of the natural product extracts as shown in the data without TCA treatment (Fig. 2). Probably, the components of the natural product extracts were denatured and excessive amounts of the interfering substances were formed under the reaction conditions. Therefore, physical removal of autofluorescent and quenching materials from the reaction mixture was examined. Adding TCA to the solution after the reaction precipitated all proteins including AGEs-BSA, and the supernatant including the interfering substances was removed. Precipitated AGEs-BSA was dissolved with alkaline PBS, and the fluorescence intensity and apparent inhibitory activity were measured. Then, the quenching effect by the nonspecific adsorption to the protein of the screening sources was estimated by measuring fluorescence intensity of the TCA-treated solution of the solution which added the screening source for the solution after the reaction without adding it. Finally, real inhibitory activity was calculated by subtracting the quenching effect from the apparent inhibitory activity. As the result, the effective inhibition activity was detected in some natural product extracts.

Change in AGEs Structure by TCA Treatment

The effect on the structural change in AGEs by TCA precipitation following dissolution with the

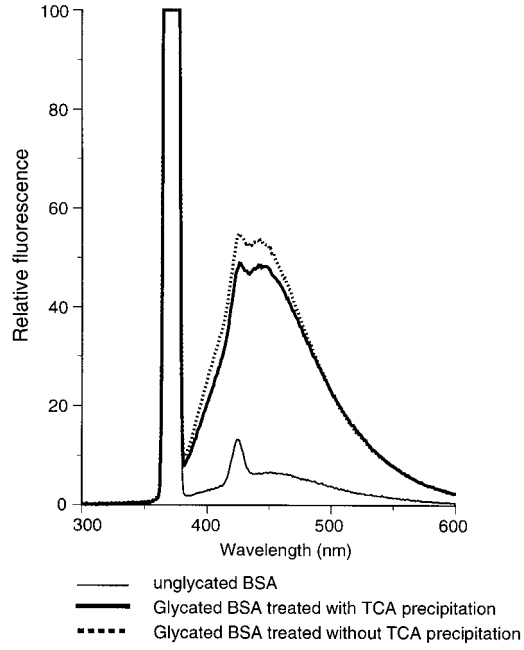

Fig. 3. Change in AGE Structure by the TCA Processing

The change in fluorescence spectrum (ex. 370 nm) of the reaction mixture before reaction, with or without TCA treatment was examined.

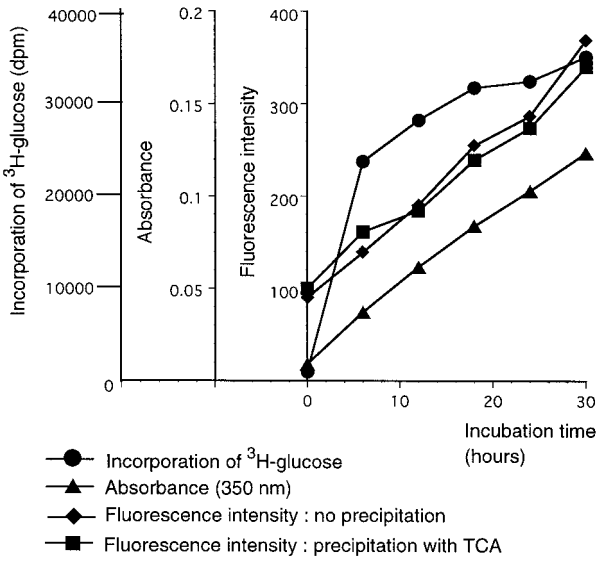

Fig. 4. The Synchronization between TCA Treatment and Other Methods

Time course of fluorescence intensity based on AGEs with TCA method (■) was compared with those of incorporation of ^{3}H-glucose (●), absorbance based on Schiff base (▲) and fluorescence intensity without TCA method (◆). These data were the average of experiments carried out at $n = 3$.

alkaline PBS was examined (Fig. 3). The TCA treatment was done on the control solution after the reaction, and its fluorescence spectrum (ex. 370 nm) based on FFI, one of AGEs[24] was compared with that before the TCA treatment. The fluorescence spectrum of the reaction mixture before TCA treatment showed the formation of AGEs under the reaction conditions, and the pattern of the spectrum was essentially the same as that after TCA treatment. This result suggested that the TCA treatment did not affect the structure of AGEs.

Synchronization between TCA Treatment and Other Methods

Progression of the Maillard reaction detected by the method of TCA treatment, synchronized with those detected by other known methods (Fig. 4). The time course of fluorescence intensity based on AGEs formation using the TCA treatment was compared with those of the incorporation activity of ^{3}H-glucose into BSA, the absorbance based on Schiff base and the fluorescence intensity based on AGEs without the TCA treatment. As the result, its radioactivity, absorbance and fluorescence intensity increased with the progress of the reaction, and the increases were proportional to the incubated time. These were synchronous with the increase of the fluorescence intensity using the method of TCA treatment. Thus, it was confirmed that the assay with the TCA treatment is compatible with other methods for the detection of the Maillard reaction.

The Maillard Reaction Inhibition Activity by AG

AG is a well-known compound as the Maillard reaction inhibitor. The Maillard reaction inhibitory activity by AG was examined using the TCA treatment method (Fig. 5). The inhibitory activity was dose-dependent regardless of with or without the TCA treatment. However, the inhibitory activity in the case of the method with the TCA treatment was higher than that without TCA treatment. A main reason for this difference might be the quenching effect by the decomposed AG in the reaction.

DISCUSSION

In this study, improvement of the conventional assay method was carried out to establish an efficient screening system of the Maillard reaction inhibitor using natural product extracts. In general, the Maillard reaction using a reducing sugar such as glucose and proteins such as BSA or a basic amino acid such as lysine and arginine has been studied in neutralized phosphate buffer. In order to detect its

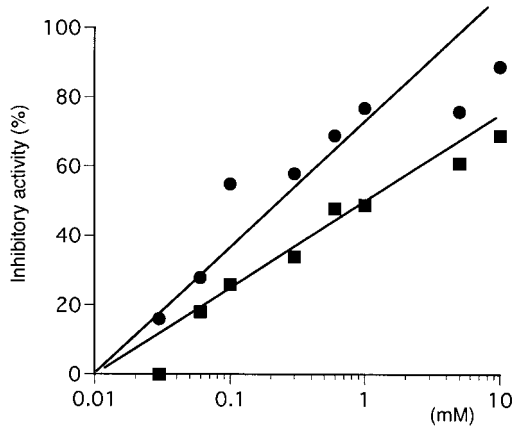

Fig. 5. Maillard Reaction Inhibitory Activity by AG

Inhibition activity of AG with TCA treatment (●) was compared with that without TCA treatment (■). These data were the average of experiments carried out at $n = 3$.

progress over a short period, ribose of which the reducing power is higher than that of glucose and lysine and/or arginine instead of protein has been used.[25] However, the Maillard reaction using such substrate composition would not reflect the result obtained *in vivo*. We therefore used glucose, of which concentration in the blood of a diabetic is controlled,[26] as a reducing sugar, and inexpensive BSA, which is a component of mammalian blood, as a protein substrate. Thus, we improved the reaction conditions and the detection method using these substrates. The conventional assay for the Maillard reaction is generally divided into the following three methods: detection of the radioactive incorporation of $^{14}C/^{3}H$-glucose into protein,[13] the reducing power of Amadori products[27] and the fluorescence intensity based on of AGEs formation.[8] The detection sensitivity of the method using $^{14}C/^{3}H$-glucose is very high, and detection is possible under the physiological condition at 37°C and for a comparative short time (30–60 hr). However, it is possible to detect only the binding activity of glucose with protein, which is the initial stage of the Maillard reaction. Therefore, it might be impossible to detect the inhibitor of successive stages including AGEs formation, even if an effective inhibitor exists in the screening sources. In addition, usable facilities for the isotope have been limited. The detection of an Amadori compound, especially HbA_{1c},[8] has been used as the most fundamental index for the glycemic control in diabetes mellitus. Thus, various detection methods using high performance liquid chromatography,[22] monoclonal antibody,[28] periodic acid[20] and reduction using $Na[^{3}H]_3B_4$[21] have been developed. Especially, the first two methods are clinically used at present. However, it is also impossible to detect the late-stage Maillard reaction inhibitor by this method, because the Amadori compound is an early-stage product. It is possible that the fluorescence intensity method based on AGEs could detect any inhibitor at all reaction stages from binding of glucose with protein to the AGEs formation. However, several weeks are usually needed to detect the weak fluorescence based on AGEs, because the AGEs generation rate is very slow under the physiological conditions.[29] Since the inhibitor of AGEs formation, which must be a cause of diabetic complication, should be detected as much as possible in the screening, we adopted the detection of the fluorescence intensity for our screening system. To use the detection method, the following two problems should be solved. One problem is the slowness of the reaction rate and the other is fluorometry interference by autofluorescences substances and color pigments, which exist in natural products extracts. The reaction rate should depend on the reaction temperature, because the Maillard reaction is a nonenzymatic reaction. Therefore, the problem of slowness of the reaction rate could be solved by using higher reaction temperature. However, Takeda *et al*. reported a conformational change by heat treatment of BSA namely, the α-helical structure began to decrease and convert into a β-structure at over 40°C.[30,31] This result was a little different from our observation that there was no remarkable conformational change up to 60°C. We used Fraction V of BSA, whereas Takeda *et al*. utilized high-pure crystallized BSA, so it seems to show different stability at high temperature. As a result, we could set the reaction temperature at 60°C in our screening system without any trouble.

In natural product extracts as a screening source, a large amount of autofluorescence and coloring substances are included. The fluorescence intensity of these substances was comparatively high; for instance, the intensity in some samples was over 10 times higher than that of the control. From the standpoint of reliability of the inhibitory activity, it was difficult to exclude properly the quenching effect by the subtraction processing. In addition, it was impossible to exclude the quenching effect by subtraction in the screening samples where the structure of the material in the screening sources changed, colored and discolored under the reactions. Thus, the interfering substances in the reaction mixture were removed successfully by adding TCA and the

precipitating protein included the AGEs-BSA complex. Then, the fluorescence intensity of the protein fraction was measured. Among the known AGEs, FFI,[9] pentosidine,[6] pyrraline,[4] crossline[7] and N^{ε}-(carboxymethyl)lysine,[5] only pentosidine was reported to change its fluorescence intensity by the pH fluctuation. In this study, FFI was detected as the major fluorescent material by the fluorescent spectrum. The fluorescent spectrum pattern based on FFI did not change even after TCA treatment. Thus, it was considered that the chromophore of FFI was not affected by TCA treatment. IC_{50} of AG on the Maillard reaction was reported to be 2–10 mM, in spite of the difference in type and concentration of the substrate in the reaction mixture. Although we also obtained a similar result, the inhibitory activity of AG on the Maillard reaction with TCA treatment was higher than that without TCA treatment. In this study, TCA precipitation of BSA-AG was applied to the assay for the Maillard reaction as a key step for the successful inhibitor screening. This method is applicable to high throughput screening using a 96/384 well fluorescence plate reader.[32–34]

REFERENCES

1) Kaanane, A. and Labuza, T. P. (1989) The Maillard reaction in foods. *Prog. Clin. Biol. Res.*, **304**, 301–327.
2) Kato, H., Shin, D. B. and Hayase, F. (1987) 3-Deoxyglucosone crosslinks proteins under physiological conditions. *Agric. Biol. Chem.*, **51**, 2009–2011.
3) Kawakishi, S., Tsunehiro, J. and Uchida, K. (1991) Autoxidative degradation of Amadori compounds in the presence of copper ion. *Carbohydr. Res.*, **211**, 167–171.
4) Hayase, F., Nagaraj, R. H., Miyata, S., Njoroge, F. G. and Monnier, V. M. (1989) Aging of proteins: immunological detection of a glucose-derived pyrrole formed during maillard reaction *in vivo*. *J. Biol. Chem.*, **264**, 3758–3764.
5) Ahmed, M. U., Thorpe, S. R. and Baynes, J. W. (1986) Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J. Biol. Chem.*, **261**, 4889–4894.
6) Dyer, D. G., Blackledge, J. A., Thorpe, S. R. and Baynes, J. W. (1991) Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine *in vivo*. *J. Biol. Chem.*, **266**, 11654–11660.
7) Obayashi, H., Nakano, K., Shigeta, H., Yamaguchi, M., Yoshimori, K., Fukui, M., Fujii, M., Kitagawa, Y., Nakamura, N., Nakamura, K., Nakazawa, Y., Ienaga, K., Ohta, M., Nishimura, M., Fukui, I. and Kondo, M. (1996) Formation of crossline as a fluorescent advanced glycation end product in vitro and in vivo. *Biochem. Biophys. Res. Commun.*, **226**, 37–41.
8) Ratzan, K. R., Giraudo, C., Amado, C., Lauredo, I. and Horowitz, G. (1975) Effect of sodium cyanate upon the function of normal human polymorphonuclaer leukocytes. *J. Infect. Dis.*, **131**, Suppl, S73–S80.
9) Monnier, V. M., Kohn, R. R. and Cerami, A. (1984) Accelerated age-related browning of human collagen in diabetes mellitus. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 583–587.
10) Watala, C., Gwozdzinski, K., Pluskota, E., Pietrucha, T., Walkowiak, B., Trojanowski, Z. and Cierniewski, C. S. (1996) Diabetes mellitus alters the effect of peptide and protein ligands on membrane fluidity of blood platelets. *Thromb. Haemost.*, **75**, 147–153.
11) Niemann, M. A., Bhown, A. S. and Miller, E. J. (1991) The principal site of glycation of human complement factor B. *Biochem. J.*, **274**, 473–480.
12) Sakurai, T., Matsuyama, M. and Tsuchiya, S. (1987) Glycation of erythrocyte superoxide dismutase reduces its activity. *Chem. Pharm. Bull.*, **35**, 302–307.
13) Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P. and Cerami, A. (1986) Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science*, **232**, 1629–1632.
14) Huby, R. and Harding, J. J. (1988) Non-enzymic glycosylation (glycation) of lens proteins by galactose and protection by aspirin and reduced glutathione. *Exp. Eye. Res.*, **47**, 53–59.
15) Booth, A. A., Khalifah, R. G. and Hudson, B. G. (1996) Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: comparison with aminoguanidine. *Biochem. Biophys. Res. Commun.*, **220**, 113–119.
16) Malone, J. I., Lowitt, S. and Cook, W. R. (1990) Nonosmotic diabetic cataracts. *Pediatr. Res.*, **27**, 293–296.
17) Morimitsu, Y., Yoshida, K., Esaki, S. and Hirota, A. (1995) Protein glycation inhibitors from thyme (*Thymus vulgaris*). *Biosci. Biotechnol. Biochem.*, **59**, 2018–2021.
18) Soulis-Liparota, T., Cooper, M., Papazoglou, D., Clarke, B. and Jerums, G. (1991) Retardation by aminoguanidine of development of albuminuria, mesangial expansion, and tissue fluorescence in streptozocin-induced diabetic rat. *Diabetes*, **40**, 1328–1334.

19) Hammes, H. P., Brownlee, M., Edelstein, D., Saleck, M., Martin, S. and Federlin, K. (1994) Aminoguanidine inhibits the development of accelerated diabetic retinopathy in the spontaneous hypertensive rat. *Diabetologia*, **37**, 32–35.
20) Ahmed, N. and Furth, A. J. (1991) A microassay for protein glycation based on the periodate method. *Anal. Biochem.*, **192**, 109–111.
21) Sheikh, M. A. and Robb, D. A. (1993) Measurement of non-enzymic glycosylation with a radiochemical assay. *Int. J. Biochem.*, **25**, 379–384.
22) Sellami, M., Labaied, N., Ghanem, A., Nagati, K. and Fattoum, S. (1995) Analysis of glycosylated A1c hemoglobin by liquid phase chromatography and immunoagglutination. *Arch. Inst. Pasteur Tunis*, **72**, 3–6.
23) Reuter, W., Westermann, M., Brass, S., Ernst, A., Boger, P. and Wehrmeyer, W. (1994) Structure, composition, and assembly of paracrystalline phycobiliproteins in Synechocystis sp. strain BO 8402 and of phycobilisomes in the derivative strain BO 9201. *J. Bacteriol.*, **176**, 896–904.
24) Peterson, C. M. and Jones, R. L. (1977) Minor hemoglobins, diabetic "control," and diseases of postsynthetic protein modification. *Ann. Intern. Med.*, **87**, 489–491.
25) Syrovy, I. (1994) Methods Glycation of albumin: reaction with glucose, fructose, galactose, ribose or glyceraldehyde measured using four methods. *J. Biochem. Biophys.*, **28**, 115–121.
26) Rajbar, S. (1968) An abnormal hemoglobin in red cells of diabetics. *Clin. Chim. Acta*, **22**, 296–298.
27) Johnson, R. N., Metcalf, P. A. and Baker, J. R. (1983) Fructosamine: a new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin. Chim. Acta*, **127**, 87–95.
28) Rumley, A. G., Kilpatrick, E. S., Dominiczak, M. H. and Small, M. (1993) Evaluation of glycaemic control limits using the Ames DCA 2000 HbA1c analyzer. *Diabet. Med.*, **10**, 976–979.
29) Shaw, S. M. and Crabbe, M. J. (1994) Monitoring the progress of non-enzymatic glycation *in vitro*. *Int. J. Pept. Protein Res.*, **44**, 594–602.
30) Takeda, K., Wada, A., Yamamoto, K., Moriyama, Y. and Aoki, K. (1989) Conformational change of bovine serum albumin by heat treatment. *J. Protein Chem.*, **8**, 653–659.
31) Takeda, K., Hamada, S. and Wada, A. (1993) Secondary structural changes of large and small fragments of bovine serum albumin in thermal denaturation and in sodium dodecyl sulfate denaturation. *J. Protein Chem.*, **12**, 223–228.
32) Lynch, B. A., Loiacono, K. A., Tiong, C. L., Adams, S. E. and MacNeil, I. A. (1997) A fluorescence polarization based Src-SH2 binding assay. *Anal. Biochem.*, **247**, 77–82.
33) Pezzuto, J. M. (1997) Plant-derived anticancer agents. *Biochem. Pharmacol.*, **53**, 121–133.
34) Wu, P., Daniel-Issakani, S., LaMarco, K. and Strulovici, B. (1997) An automated high throughput filtration assay: application to polymerase inhibitor identification. *Anal. Biochem.*, **245**, 226–230.