Antioxidative and Anti-Glycation Activity of Garcinol from *Garcinia indica* Fruit Rind

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Garcinol, a polyisoprenylated benzophenone derivative, was purified from *Garcinia indica* fruit rind, and its antioxidative activity, chelating activity, free radical scavenging activity, and anti-glycation activity were studied. Garcinol exhibited moderate antioxidative activity in the micellar linoleic acid peroxidation system and also exhibited chelating activity at almost the same level as citrate. It also showed nearly 3 times greater DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity than DL-α-tocopherol by weight in aqueous ethanol solution. In a phenazine methosulfate/NADH−nitroblue tetrazolium system, garcinol exhibited superoxide anion scavenging activity and suppressed protein glycation in a bovine serum albumin/fructose system. Thus, garcinol might be beneficial as a potent antioxidant and a glycation inhibitor under specified conditions.

Keywords: *Garcinia indica; garcinol; antioxidant; glycation*

INTRODUCTION

Garcinol is a polyisoprenylated benzophenone derivative from *Garcinia indica* and other species (Krishnamurthy et al., 1981, 1982; Bakana et al., 1987; Sahu et al., 1989; Iinuma et al., 1996). The dried rind of *G. indica* ('Kokum') is used as a garnish for curry and in traditional medicine in India. 'Kokum' contains 2−3% of garcinol by weight (Krishnamurthy et al., 1981, 1982), and its chemical structure as proposed by Sahu et al. (1989) is shown in Figure 1. Although garcinol is known to be a yellow pigment (Krishnamurthy et al., 1987) and to be an antibiotic reagent (Bakana et al., 1987; Iinuma et al., 1996), its other biological activities are not well-known.

Krishnamurthy reported that garcinol does not react as an antioxidant (Krishnamurthy et al., 1988), but we suspected that it might have antioxidative activity or free radical scavenging activity because the molecule has phenolic hydroxyl groups and a β-diketone structure. Therefore, this study was initiated with the goal of confirming the antioxidative activity of garcinol in the micellar system. To determine the mechanism involved in antioxidative activity, we focused on the chelating and the free radical scavenging activity of the substance.

Many reports have shown a significant role for glycation (nonenzymatic reaction of protein with reducing sugar) in diabetic complications, Alzheimer's disease, and normal aging (Monnier and Cerami, 1981; Wautier et al., 1994; Bucala et al., 1993, 1994; Vitek et al., 1994; Smith et al., 1994). Aminoguanidine and other reagents inhibit glycation by interfering with active carbonyl groups of the intermediates (Brownlee et al., 1986). Such guanidino reagents are thus thought to have a pharmaceutical effect on diabetic complications and other age-related disorders (Li et al., 1996).

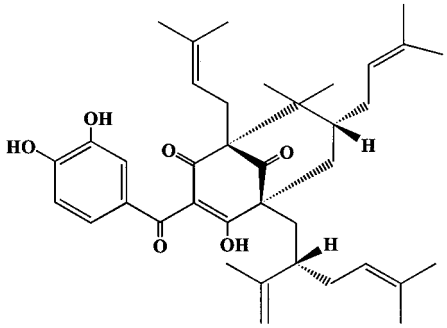

Figure 1. Chemical structure of garcinol.

Oxidative reactions are now known to be included in the later process of glycation (Baynes et al., 1991; Fu et al., 1994; Yaylayan and Huyghues-Despointes, 1994), so antioxidants and/or radical scavengers may prevent this event (Elgawish et al., 1996; Oya et al., 1997). Therefore the anti-glycation activity of garcinol in the in vitro system was tested.

MATERIALS AND METHODS

Reagents. Chemicals were purchased from Wako Pure Chemical industries, Ltd. (Osaka, Japan) unless otherwise noted. DPPH (1,1-diphenyl-2-picrylhydrazyl) and gallic acid were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

Preparation of Garcinol. Garcinol was prepared from *G. indica* rind. In brief, *G. indica* dried fruit rind ('Kokum', purchased from Indo World Trading Co., New Delhi, India) was extracted with ethanol. The extract was fractionated by preparative ODS (octadecyl silica) column chromatography eluted stepwise with 70−80% (v/v) ethanol. The eluate was monitored at UV 254 nm, and the main fractions having absorption at 254 nm eluted at 80% (v/v) ethanol were concentrated and dried by rotary evaporator under 50 °C. The dried material was resolved in hexane and the solution was

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cooled under 5 °C for 2 days. Yellow amorphous powder was collected from the solution and washed with cold hexane on a glass filter. After drying in a vacuum desiccator, the amorphous was solubilized in hot acetonitrile and recrystallized at room temperature; pale yellow crystal needles were obtained from the solvent. The crystals were identified as garcinol (Krishnamurty et al., 1981; Sahu et al., 1989) from the following spectral data: mp 121 °C; $[\alpha]_D^{30}$ −135 ($CHCl_3$); UV λ_{max}^{EtOH} nm (log ϵ) 363 (3.94) and 250 (4.09); IR ν_{max}^{KBr} cm^{-1} 3200−3500, 1730, 1640; ^1H NMR ($CDCl_3$) δ 6.95 (1H, *dd*, J = 9.0 and 2.0 Hz), 6.91 (1H, *d*, J = 2.0 Hz), 6.60 (1H, *d*, J = 9.0 Hz), 4.96, 5.06, 5.10 (1H each, *t*, J = 5.0 Hz), 4.40 (*d*, J = 15.0 Hz), 2.80−1.46 (*m*, 12H, methylene and methyne), 1.78, 1.74, 1.69, 1.62, 1.59, 1.56, 1.21, 1.05 (3H each, *s*); EI-MS *m*/*z* 602 $[M]^+$, 533, 465, 341. The crystal can be dissolved in organic solvents such as ethanol, methanol, acetone, dimethyl sulfoxide, acetonitrile, ethyl acetate, chloroform, and hexane, but it cannot be dissolved in water.

Measurement of Lipid Peroxidation in Micellar System. Measurement of antioxidative activity was done based on the method of Ben Aziz (Ben Aziz et al., 1971). β-Carotene and methyl linoleate were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and Tween 20 was purchased from Sigma (MO).

Methyl linoleate and β-carotene were emulsified with Tween 20 in 1 M Tris-HCl buffer (pH 7.0). A test sample (antioxidant) solution was emulsified in the same way and added to the reaction mixture. The final concentration of methyl linoleate was 1.9 mM and that of β-carotene was 1.1 μM. Garcinol and other antioxidative reagents, (+)-catechin, DL-α-tocopherol, were added at a final concentration of 0.0005% (w/v). The reaction mixture was put into a cuvette; then absorbance at 460 nm at 25 °C was monitored each 30 s during the initial 5 min using a Hitachi 557 spectrophotometer (Hitachi, Ltd., Tokyo). The mean of triplicate measurements was plotted as a function of time, and the color reduction rate was determined by the linear regression of the plotted data.

Measurement of Fluorescent Lipid Peroxidation Products. Measurement of the formation of fluorescent lipid peroxidation products in the presence of amino acid was done based on the method of Shimasaki (Shimasaki, 1994) and slightly modified; 30 mg of methyl linoleate and 9.0 mL of glycine−Tween solution (300 mg of glycine and 300 mg of Tween 20 in 100 mL of 1 M Tris-HCl buffer, pH 7.4) were mixed and emulsified by sonication under ice-cold conditions. A one-hundredth volume of test sample solution (10 mM test sample and 3% w/v Tween 20 in 100 mM Tris-HCl buffer, pH 7.4) was added to the emulsion. The reaction mixture in a screw-capped Pyrex tube was incubated at 50 °C for 1 week, after which the fluorescent product was extracted with a 6-fold volume of ethanol/diethyl ether (3/1) and the fluorescence intensity measured (excitation at 335 nm, emission at 430 nm) with a Shimadzu RF-5000 spectrofluorometer (Shimadzu Co., Kyoto).

Measurement of Fe^{2+} Chelating Activity. Fe^{2+} chelating activity was measured by 2,2′-bipyridyl competing assay; 0.25 mL of $FeSO_4$ solution (1 mM) and an equal volume of test sample solution (both containing 1% sodium dodecyl sulfate) were mixed; 1 mL of Tris-HCl buffer (pH 7.4) and 2,2′-bipyridyl solution (0.1% in 0.2 M HCl) were added to the mixture, respectively, together with 0.4 mL of 10% (w/v) hydroxylamine-HCl and 2.5 mL of ethanol. After filling the reaction mixture up to 5 mL with water, absorbance at 522 nm was measured. All of the reagents were prepared with a degassed pure water Milli-Q SP reagent water system (Millipore, Bedford, MA).

Measurement of DPPH Radical Scavenging Activity. Measurement of DPPH radical scavenging activity was done by the method of Ariga (Ariga, 1990); 100 μM DPPH and 0.0004% (w/v) test sample in 50% (v/v) ethanol solution was incubated at 25 °C for 40 min. The reduction of absorbance at 528 nm was monitored as DPPH radical scavenging activity. Ascorbic acid was adopted as positive control.

Measurement of Superoxide Anion Scavenging Activity. Measurement of superoxide anion scavenging activity was done based on the method described by Nishikimi et al. (1972) and slightly modified; 1 mL of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL of NADH solution (468 μM NADH in 100 mM phosphate buffer, pH 7.4), and 1 mL of sample solution (in methanol) were mixed. The reaction was started by adding 1 mL of phenazine methosulfate (PMS) solution (60 μM PMS in 100 mM phosphate buffer pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured. Compared to the value with no test sample added, the reduction of the absorbance was estimated as superoxide scavenging activity.

Reaction of Proteins and D-Fructose. The procedure of protein and D-fructose reaction followed that of McPherson et al. (1988) and was slightly modified. BSA (bovine serum albumin, fraction V, 20 mg/mL; Sigma, St. Louis, MO) and D-fructose (500 mM) were dissolved in 200 mM potassium phosphate buffer (pH 7.4). These reagents were mixed in autoclaved test tubes after sterilization using a γ-ray radiated membrane filter. The test compound dissolved in ethanol was added to the reaction mixture, and the reaction mixture was incubated for 5 days at 37 °C, after which an equal volume of 10% (w/v) TCA was added and the precipitate was collected by centrifugation. The precipitate was washed twice with ice-cold 5% TCA and resolved in 200 mM potassium phosphate buffer; one aliquot was applied to SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis), and another was applied to fluorescence measurement. The fluorescence was measured at 350 nm excitation and 425 nm emission using a Shimadzu RF-5000 spectrofluorometer. SDS−PAGE was done by the method of Laemmli. Reacted protein solution was mixed with an equal volume of SDS sample buffer (2% w/v SDS, 30% w/v glycerol, 0.25 M Tris hydroxyaminomethane, pH 6.8) and boiled for 2 min; 10 μL of the solution was put on a 10−20% gradient acrylamide slab gel (Multigel 10/20, Daiichi Pure Chemicals, Tokyo) and electrophoresed at a current of 1 mA/lane for 1 h. After that, the gel was fixed with 60% ethanol followed by Coomassie Brilliant Blue (CBB) R-250 staining.

RESULTS AND DISCUSSION

Using the AOAC method, Krishnamurthy observed that garcinol had no antioxidative activity in lipid peroxidation (Krishnamurthy and Sampathu, 1988). We confirmed that their conclusion had equal validity in the micellar system using the method of Ben Aziz et al. (1971). This system was adopted as a model of linoleate photooxidation in an emulsified system. The mechanism of the color reduction of β-carotene was presumed to be the peroxyradical from photooxidized linoleate attacking and bleaching β-carotene. Absorbance of β-carotene was linearly reduced over time; thus the decrease in the rate of color reduction in the presence of antioxidant explained the antioxidative activity. Garcinol exhibited weak antioxidative activity in the system, being at most one-half as strong as DL-α-tocopherol (Toc) and weaker than catechin by weight (Figure 2). Toc was used as a typical example of lipid-soluble natural antioxidant and (+)-catechin (Catechin) as a typical example of a naturally occurring polyphenolic compound. Garcinol might interfere with or scavenge the formation of lipid peroxide, but perhaps at a rate not as fast as Toc or Catechin. Ascorbic acid (AsA), a typical example of a natural water-soluble antioxidant, instead enhanced lipid oxidation in this system (data not shown). It was reported that ascorbic acid reacts as a prooxidant under certain conditions (Ramanathan and Nagaratham, 1993).

The formation of fluorescent lipid peroxidation products was suppressed by the addition of antioxidants. This system was adopted as a model of linoleate autoxidation in an emulsified system. The mechanism

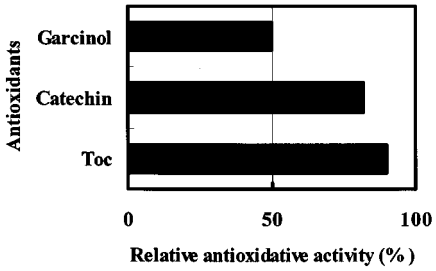

Figure 2. Antioxidative activity of garcinol in a micellar system. The time-dependent degradation of β-carotene (reduction of the absorbance at 460 nm) was recorded, and the reduction rate was calculated as described in the text. The antioxidative activity was defined as the following equation: antioxidative activity = {reduction rate (no sample added) − reduction rate (sample added)}/reduction rate (no sample added); Toc, DL-α-tocopherol; Catechin, (+)-catechin. A typical rate of β-carotene reduction (reduction rate of absorbance at 460 nm) was 8.8×10^{-5}/s.

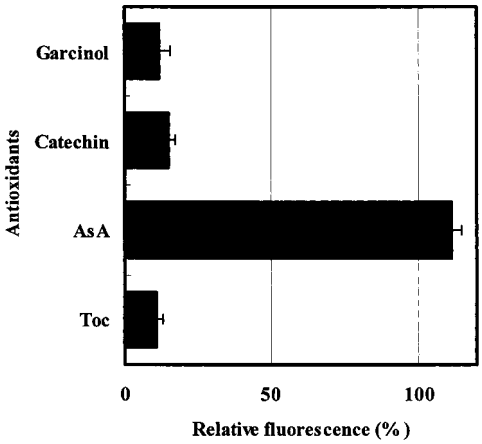

Figure 3. Inhibition of fluorescent product formation from linoleic acid and glycine. Relative fluorescence intensity (ratio to control) was plotted as abscissa. The case with 100% fluorescent intensity has no antioxidative effect. Abbreviations are the same as in the legend to Figure 2; AsA, D-ascorbic acid. All test compounds were added to the reaction mixture at a final concentration of 100 μM.

of fluorescence formation was presumed to be that aldehyde from autoxidized linoleate reacted with glycine and formed the fluorescent aggregate. Garcinol also exhibited the inhibitory activity (Figure 3) and so might interfere with these processes.

2,2′-Bipyridyl can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelator. In this assay, garcinol interfered with the formation of ferrous ion and bipyridyl complex, suggesting that it has chelating activity and captures ferrous ion before bipyridyl. The rate was almost the same as that of citric acid and lower than that of EDTA and DTPA (Figure 4). Garcinol was the only water-insoluble reagent used in this assay, and it was added to the reaction mixture with SDS (3-fold weight of garcinol). This condition might be disadvantageous for the substance. Garcinol reportedly exhibited antibiotic activity (Bakana et al., 1987; Iinuma et al., 1996), and the bacteriostatic activity of the chelating agent is also known (Goldoni et al., 1991). Therefore, chelating activity might be one of the critical mechanisms in the antibiotic activity of garcinol.

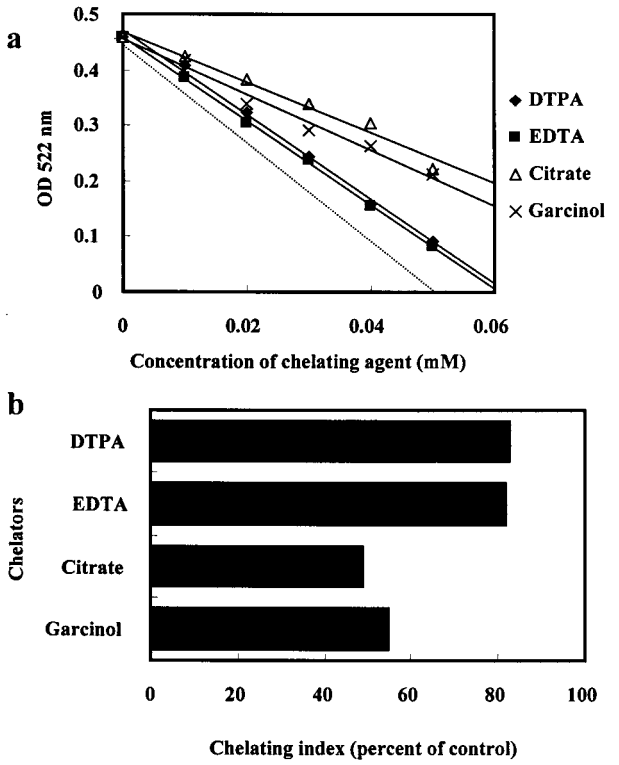

Figure 4. Chelating activity of garcinol with the addition of chelating agent. The absorbance of Fe−2,2′-bipyridyl complex was linearly decreased dose dependently (a). The rate of decreasing was determined from linear regression of the plotted data of the mean of triplicate measurements. The ratio of this rate to hypothetical complete chelation, the inclination of dotted line in (a), was calculated and indicated as index of chelation (b); EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid.

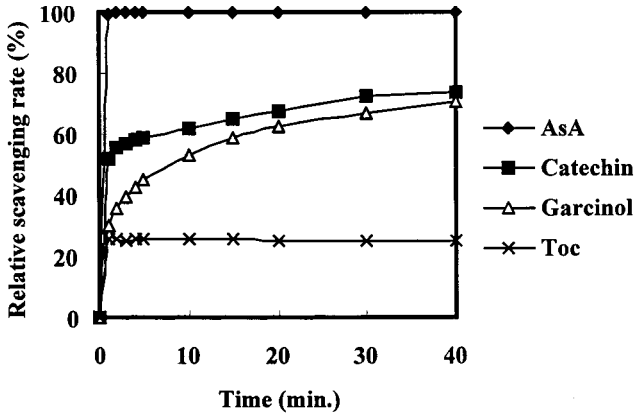

Figure 5. DPPH radical scavenging activity. The decrease of absorbance at 528 nm during 30 min of incubation with ascorbic acid was used as a control. The ratio of the decrease of absorbance with sample to control (%) was plotted according to the time course. The data are the mean of triplicate measurements. A typical decrease of the absorbance using ascorbic acid was from 0.9 to 0.2.

In the DPPH assay, garcinol exhibited potent radical scavenging activity at an extent almost 3 times higher than DL-α-tocopherol and comparable to 85% of the activity of ascorbic acid (Figure 5). But the reaction rate was relatively slow in the early stage, so that this activity may not play a critical role in some cases of lipid peroxidation as Krishnamurthy reported (1988).

In the PMS/NADH−NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT and makes formazan dye. The

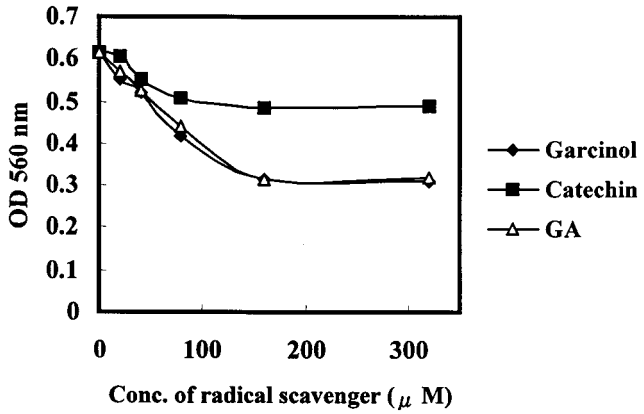

Figure 6. Superoxide anion scavenging activity by the PMS/NADH−NBT method. Measurements were made after 5-min reactions at 25 °C. The data are the mean of triplicate measurements. GA, gallic acid.

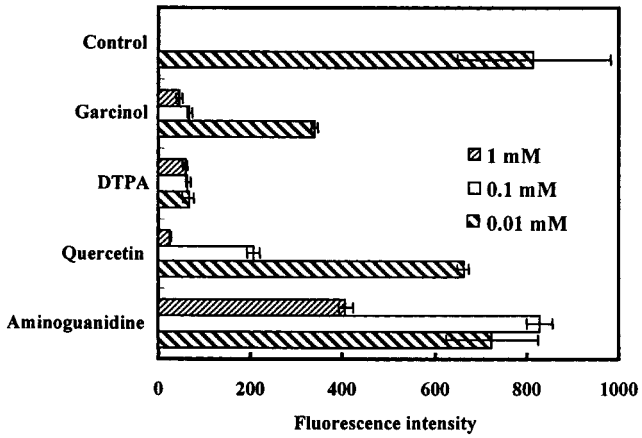

Figure 7. Inhibition of fluorescence formation by garcinol in the fructose−BSA reaction. Each compound was added to the reaction mixture at a concentration of 0.01, 0.1, and 1.0 mM. Garcinol and other water-insoluble compounds were dissolved in ethanol and added to the reaction mixture at 1% (v/v). Ethanol was contained in the control reaction solution (without test compound) at the same concentration. No significant effects were observed in either the presence or absence of 1% ethanol. Error bars indicate standard deviations of duplicate measurements.

decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. A sufficient amount of antioxidant (usually over 300 μM) caused the reduction of the absorbance to reach bottom. Comparison of the ratio of the absorbance reduction confirmed that (+)-catechin and gallic acid were potent superoxide anion scavengers. In this assay, garcinol also exhibited potent superoxide anion scavenging activity almost comparable to that of gallic acid and stronger than that of (+)-catechin (Figure 6). Although ascorbic acid and DL-α-tocopherol exhibited slight activity in this system (data not shown), it might have been because they reduced NBT directly and canceled the reduction of absorbance at 560 nm. A compound that has strong reducing power may react with NBT directly. The bottom level of the reduction of absorbance at 560 nm in this assay might reflect the balance of superoxide anion scavenging and NBT reduction by the tested compound; therefore the validity of the above results might be recognized as limited. The radical scavenging activity of garcinol should be confirmed using the electron spin resonance (ESR) method which can observe the reaction between superoxide anion and radical scavenger more directly.

In conclusion, garcinol has both chelating activity and free radical scavenging activity and is a lipid-soluble superoxide anion scavenger. It may therefore react as an 'antioxidant' in specific systems. Especially, it may prevent metal−ion accelerated lipid peroxidation by its chelating activity and inhibit radical chain reaction by its radical scavenging activity.

The aldehyde group of reducing sugar and amino residues in protein react and result in the formation of specific fluorescent aggregate (advanced glycated end-products, AGEs). This process is referred to as glycation. We adopted an in vitro model system of AGE formation (fructose−BSA system) for evaluating the activity of garcinol as a glycation inhibitor. The reaction rate of fructose and BSA was much faster than that of glucose and BSA (Suárez et al., 1989), and its physiological importance was mentioned by McPherson et al. (1988). Garcinol inhibited the fluorescence formation at a concentration of 0.01 mM as shown in Figure 7. Quercetin was reported to be a potent glycation inhibitor (Morimitsu et al., 1995). Similarly DTPA, a potent metal chelator, inhibited the reaction at the same concentration. Aminoguanidine, however, was not sufficient at any concentration.

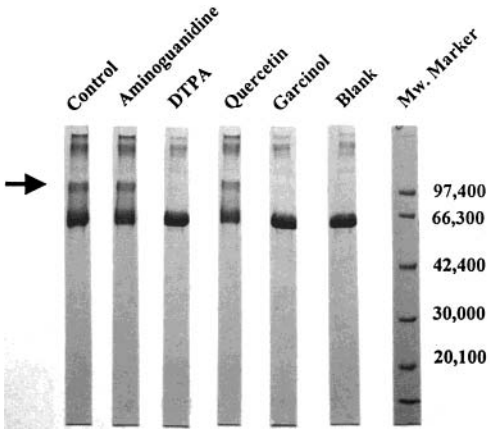

Figure 8. SDS−PAGE profile of glycated protein. Glycated protein (4 mg/mL BSA incubated with 500 mM D-fructose and 0.1 mM test compound in 200 mM pH 7.4 phosphate buffer at 37 °C for 5 days) was precipitated by the addition of 5% TCA (trichloroacetic acid) and centrifuged. The precipitate was washed with 5% TCA two times, resolved in 200 mM potassium phosphate buffer, and applied on SDS−PAGE in a denatured system. Electrophoresis was done as described in the text. Non-fructose-treated BSA was observed at the distance of M_r 64 000 (Blank). In the case of fructose treatment, polymerized material (indicated by arrow) was observed, and its M_r was estimated as 120 000 (Control).

On the SDS−PAGE profile, a band with M_r (relative molecular weight) larger than that of original BSA was observed (indicated by an arrow in Figure 8). The M_r was estimated to be almost twice that of original BSA by comparison with molecular weight markers and was thus determined to be dimeric BSA. Garcinol and DTPA suppressed the formation of such a band. There might be trimer or more polymerized protein present, but we failed to identify such polymerized BSA clearly under this experimental condition.

The band densities of dimeric BSA in SDS−PAGE were measured by densitometry using an ATTO ACD-25DX computing densitometer (ATTO Co., Tokyo) as shown in Figure 9. Increase of dimeric BSA (arrow) was observed during the reaction of D-fructose and BSA. The peak area of dimeric BSA in the densitogram and the

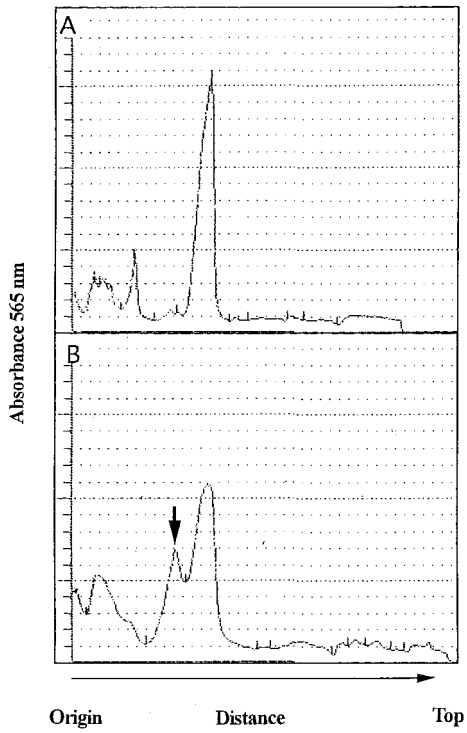

Figure 9. Densitometric profile of glycated protein SDS−PAGE slab gel was scanned at 565 nm. Distance from the origin is indicated as the abscissa, and the absorption is plotted as the ordinate. Arrow indicates the absorption of dimeric BSA: A, profile of blank in Figure 8; B, profile of control in Figure 8.

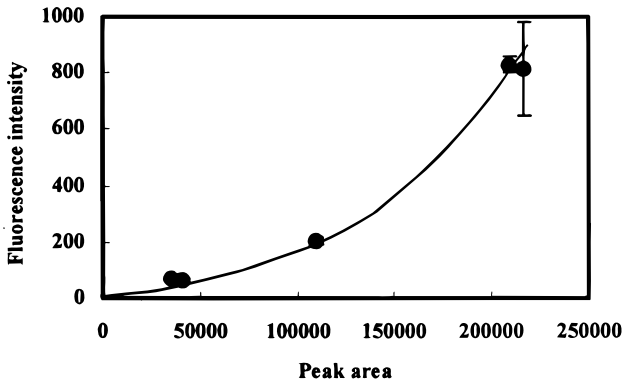

Figure 10. Relationship between fluorescence formation and protein cross-linking. Fluorescence formation indicated in Figure 7 is plotted as the ordinate, and the peak area of cross-linked protein in the densitometric profile (as the arrow in Figure 9) is the abscissa.

fluorescence in Figure 7 were plotted, and a relationship between them was observed (Figure 10). A correlation analysis was done, and a tentative equation was developed as follows: $y = 5.1 \times 10^4\ e^{0.0017x}$ ($r^2 = 0.930$). Actually, the source of fluorescence could not be restricted to dimeric BSA from these data, and it was assumed that the compounds that suppress the fluorescence also suppress the BSA cross-linking, resulting in the formation of AGEs.

Having observed the chelating activity, antioxidative activity, and radical scavenging activity of garcinol as described above, these activities were presumed to contribute to the mechanism of glycation inhibition. DTPA was the most effective among the tested compounds at the concentration of 10 μM; hence the major action of garcinol in the glycation process was presumed

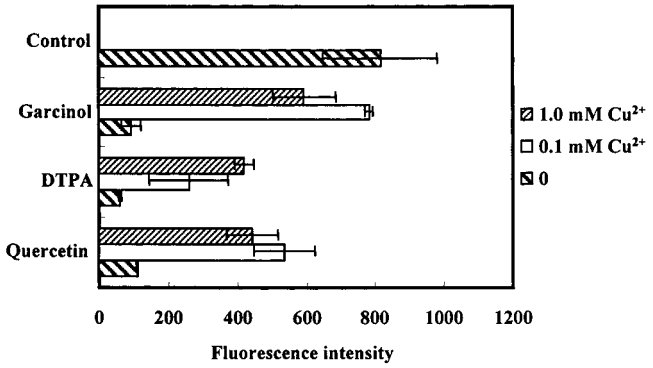

Figure 11. Effect of cupric ion on the inhibition of fluorescence formation by other compounds. Each test compound was added to the reaction mixture at a concentration of 0.1 mM, and cupric ion was added at a concentration of 0 (no addition), 0.1, and 1.0 mM. The reactions were done for 5 days at 37 °C.

to involve chelation of trace metal which is contaminated in phosphate buffer and catalyzes glycation. The importance of metal ion-catalyzed oxidation in glycation was mentioned by Hayase and by Sajithlal (Hayase et al., 1996; Sajithlal et al., 1998). Actually, when Tris buffer was used as a substitute for phosphate buffer, the fluorescence formation was at a very low level (data not shown). To confirm this finding, cupric ion (as sulfate salt) was added to the assay system, and the activity of garcinol was then reduced (Figure 11). The activities of quercetin and DTPA were reduced in the same manner. These observations confirmed that metal ion concentration played a critical role in the control of glycation and that the activity of garcinol was due to its chelating action.

Free radical formation was suggested to be involved in the glycation process (Yim et al., 1995) and radical scavengers to be effective for glycation inhibition (Oya et al., 1997). Therefore, antioxidants (ascorbic acid and DL-α-tocopherol) and hydroxyl radical scavenger (mannitol) were tested in the BSA−fructose system, but none of those compounds exhibited a significant effect on fluorescence formation (data not shown). From these observations, the autoxidative process might not be rate-limiting in glycation.

In conclusion, garcinol exhibited potent glycation-suppressing activity in a phosphate-buffered D-fructose and BSA reaction. It suppressed fluorescence and protein cross-link formation in the reaction system. The mechanism of the activity of garcinol was inferred to be the chelation of trace metal ion that was contaminated in phosphate buffer and which catalyzed the glycation.

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