Effects of Chebulic Acid on Advanced Glycation Endproducts-Induced Collagen Cross-Links

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Advanced glycation end-products (AGEs) have been implicated in the development of diabetic complications. We report the antiglycating activity of chebulic acid (CA), isolated from Terminalia chebula on breaking the cross-links of proteins induced by AGEs and inhibiting the formation of AGEs. Aminoguanidine (AG) reduced 50% of glycated bovine serum albumin (BSA) with glycolaldehyde (glycol-BSA)-induced cross-links of collagen at a concentration of 67.8±2.5 mM, the level of CA required for exerting a similar antiglycating activity was 38.8±0.5 mM. Also, the breaking activity on collagen cross-links induced by glycol-BSA was potent with CA (IC₅₀=1.46±0.05 mM), exhibiting 50-fold stronger breaking activity than with ALT-711, a well-known cross-link breaker (IC₅₀=72.2±2.4 mM). IC₅₀ values of DPPH· scavenging activity for CA and ascorbic acid (AA) were 39.2±4.9 and 19.0±1.2 µg dry matter (DM) mL⁻¹, respectively, and ferric reducing and antioxidiant power (FRAP) activities for CA and AA were 4.70±0.06 and 11.4±0.1 mmol/FeSO₄·7H₂O/g DM, respectively. The chelating activities of CA, AG and ALT711 on copper-catalyzed oxidation of AA were compared, and in increasing order, ALT-711 (IC₅₀ of 1.92±0.20 mM)<CA (IC₅₀ of 0.96±0.07 mM)<AG (0.47±0.05 mM). Thus, CA could be a breaker as well as an inhibitor of AGE cross-linking, the activity of which may be explained in large part by its chelating and antioxidant activities, suggesting that CA may constitute a promising antiglycating candidate in intervening AGE-mediated diabetic complications.

Key words chebulic acid; Terminalia chebula; chelating activity; collagen cross-link; advanced glycation end-product; antiglycation effect

Non-enzymatic glycation is initiated by a nucleophilic reaction between a carbonyl group from reducing sugars and an amino group from amino acids to form a Schiff base. This reaction is reversible, and is dependent on the concentration of sugar. The labile Schiff base rearranges to more stable Amadori products, which can undergo a series of dehydration rearrangements to form advanced glycation end products (AGEs). A number of studies have shown that the hyperglycemia has an important role in the pathogenesis of diabetic complications by increasing protein glycation and the gradual formation of AGEs in tissues. The AGEs-induced cross-links of proteins such as collagen have been correlated with the severity of diabetic complications.

As preventive or therapeutic approaches for screening antiglycating agents, there are considerable interests in compounds inhibiting or breaking AGEs-protein cross-links. A variety of pharmacological compounds have been studied for their potential to prevent AGE formation or to break AGEs. Aminoguanidine (AG) is a well-known inhibitor of AGEs formation. However, Phase III clinical studies with AG were discontinued because of its side-effects of gastrointestinal disturbance and abnormalities in liver function tests. On the other hand, because the inhibitors of AGEs formation cannot usually affect the AGEs-induced cross-links of proteins once formed, the agents that can break AGEs-induced cross-links would have the potential to attenuate such AGEs-related disease consequences. It is known that ALT-711 is an important protein cross-link breaker. ALT-711 bonds with two carbons are centrally located in an AGE-derived cross-links which makes the complex unstable leading eventually to breakage of bonds and separation of protein. In series of studies in various models of diabetic complications, ALT-711 improved arterial and ventricular function in older rhesus monkeys and reverses large artery stiffness in diabetic rats, but ineffectiveness in blood pressure has been reported in hypertential trials. Thus, the continual searches for new antiglycating agents with higher levels of efficacy and safety in humans are pursued.

As natural products have generally been proven to be relatively safe for human consumption, as compared to synthetic compounds, there has been an increasing interest in the use of natural plant compounds, such as anti-glycating agents. Our research group has attempted to identify compounds having such activity from a variety of plant sources. One of the candidate compounds is chebulic acid (CA), which was isolated from the dried ripe fruit of T. chebula. This plant is used extensively in Ayurveda, and is widely distributed throughout India, Burma and Sri Lanka, and has been used as a traditional medicinal herb. In our previous study, T. chebula extract was determined to have a protective effect against liver damage by monitoring reactive oxygen species production both in vivo and in vitro. The protective role of CA against AGEs-induced endothelial cell dysfunction was reported. However, to the best of our knowledge there has been no previous report on the inhibitory activity of CA against AGEs-induced cross-link and breaking. Hence, the objective of this study is to evaluate inhibitory and breaking activities of CA on AGEs-induced cross-links of collagen.
MATERIALS AND METHODS

Plant Materials and Preparation of CA The medicinal dried ripe fruit of *T. chebula* was purchased from a local market (Kyungdong Herb-Market, Seoul, Korea). Voucher specimens were deposited in the Herbarium at the College of Life Sciences and Biotechnology, Korea University (registration number H-358). The isolation procedures for CA were followed as described previously. The dried ripe fruits of *T. chebula* were ground with a mortar, and were extracted with ethanol at 80°C for 3 h. The extract was lyophilized, resuspended in H2O, and then extracted successively with n-hexane, chloroform and ethyl acetate (EtOAc). The EtOAc-soluble portion was further purified by Sephadex® LH-20 (Amersham Biosciences, Uppsala, Sweden) column chromatography, as a standard single compound, using 20% methanol as the eluent. HPLC analysis with a SymmetryPrep™ C18 column (300×7.8 mm, 7 µm) (Waters, Milford, MA, U.S.A.) was conducted to determine the concentrations of the standard compound, CA (Faces Biochemical Co., Wuhan, China).

Preparation of Glycolaldehyde-Glycated Bovine Serum Albumin (Glycol-BSA) AGEs were prepared by incubating 10 mg/mL of BSA, 10 mM glycolaldehyde, and 1 mM diethylenetriaminepentaacetic acid (DTPA) in 0.1 M potassium phosphate buffer in the presence of 0.02% sodium azide (pH 7.4) at 37°C for 7 d. After 7 d, free glycolaldehyde and low molecular reactants were removed by extensive dialysis against 0.1 M potassium phosphate buffer.

Enzyme-Linked Immunosorbent Assay (ELISA) The inhibitory activity on Glycol-BSA-induced collagen cross-links was measured according to a previously reported method with some modifications. Glycol-BSA (1mg/mL) was incubated in either the presence or absence of AG or CA in 1.5 mg/mL of rat tail tendon type I collagen for 24 h at 37°C. In detail, horseradish peroxidase (HRP)-labeled Glycol-BSA was prepared using a peroxidase labeling kit-NH₂ Unit (Dojin - Molecular Technologies, Inc., Tokyo, Japan) according to the manufacturer’s instruction. Glycol-BSA was incubated in either the presence or absence of AG or CA in 1.5 mg/mL of rat tail tendon type I collagen for 24 h at 37°C. After the unattached Glycol-BSA was washed with 0.05% PBST buffer. Glycol-BSA-induced collagen cross-links were detected using tetra-methylbenzidine (TMB) substrate. Breaking of AGEs-induced cross-links was expressed as the percentage decrease in optical density. The breaking percentage was calculated according to the above equation.

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Statistical Analysis All data were expressed as mean values±S.D. Statistical significance for the difference between the groups was assessed using one-way ANOVA and Duncan’s multiple range tests. SAS software version 9.2 (SAS Institute, NC, U.S.A.) was used to perform all statistical tests. Values of $p<0.05$ were considered to indicate a significant difference.
RESULTS

Inhibitory Effect of CA on Glycol-BSA-Induced Cross-Links to Collagen  We investigated the inhibitory effects of CA on the Glycol-BSA-induced cross-links to collagen using ELISA assay and gel electrophoresis (Fig. 1). CA markedly reduced this Glycol-BSA-induced cross-links to collagen in a dose-dependent manner. CA has 1750-fold stronger inhibition activity on AGEs-BSA induced cross-links than AG (Figs. 1A, B; IC\textsubscript{50} value of CA, 38.8±0.5 μM vs. IC\textsubscript{50} value of AG, 67.8±2.5 mM). In addition, the extent of Glycol-BSA-induced cross-links to collagen was examined by SDS-PAGE under denaturing condition (Fig. 1C). Referring to BSA control in lane 2, Glycol-BSA increased Glycol-BSA band (band a, about 70 kDa) as well as additional bands including band c with lower electrophoretic mobility (Fig. 1C). Whereas AG had little effect on the formations of these bands, the treatment of CA in the incubation mixture showed band (band b) with slightly lower mobility compared with band a, indicating the loss of the positive charge in the Glycol-BSA during the glycation reaction. In addition, less formation with lower electrophoretic mobility on PAGE gel was observed in the reaction mixture with CA.

Breaking Effect of CA on Glycol-BSA-Induced Collagen Cross-Links  To investigate the breaking activities of CA on Glycol-BSA induced collagen cross-links, the strength of breaking cross-links at various concentrations of CA was measured by ELISA (Fig. 2). The treatment with CA resulted in a concentration-dependent decrease in the collagen cross-links. CA (Fig. 2A, IC\textsubscript{50}=1.46±0.05 mM) exhibited 50-fold stronger breaking activity on Glycol-BSA binding to collagen than ALT-711 (Fig. 2B, IC\textsubscript{50}=72.2±2.4 mM). In addition, these breaking activities of inhibitors on the Glycol-BSA-induced cross-links of collagen were analyzed by SDS-PAGE (Fig. 2C). A molecular band (band c) present at the top of the resolving gel was increased in the Glycol-BSA which was cross-linked with collagen in the absence of CA or ALT-77, whereas 2.5 mM CA and ALT-711 remarkably decreased the formation of this band. Glycol-BSA band (band a) was decreased with ALT-711 and CA in dose-dependent manners. Furthermore, in order to confirm whether band a (about 70 kDa), which was formed during glycation with glycolaldehyde, was altered, a monoclonal antibody (anti-AGEs) was used in Western blotting. Results showed that the density of Glycol-BSA was decreased dose-dependently with inhibitors in which the most reduced band density was observed with 5 mM CA (Fig. 2D). It was confirmed that band a of Fig. 2C and that of Fig. 2D have the same mobility. These results suggest that the band a

![Fig. 1. Inhibitory Effect of Chebulic Acid (CA) on Glycolaldehyde-Glycated BSA (Glycol-BSA) Induced Cross-Links to Collagen in Vitro](image-url)

The inhibitions of Glycol-BSA induced cross-links to collagen with CA and aminoguanidine (AG) were measured by ELISA (A) and (B), respectively. All data were expressed as mean±standard deviation of triplicate experiments. **p<0.01 vs. non-treated group (n=3). The inhibitory modulation of Glycol-BSA induced collagen cross-links was revealed by SDS-PAGE (C).
of Fig. 1C, the band a of Fig. 2C and the band a of Fig. 2D are the same one.

**Antioxidant and Chelating Activities of CA** The beneficial effects of test compounds for treatment of cardiovascular and renal malfunctions in diabetes and aging can be largely explained, if not completely, by their antioxidant and chelating activities. The antioxidant activities of CA were evaluated based on the FRAP value and DPPH· $\text{SC}_{50}$ value, showing that the activities of this compound were effective and comparable to those of ascorbic acid, showing DPPH· scavenging activity ($39.2\pm4.9$ vs. $19.0\pm1.2 \mu g/dry\ matter\ (DM)\ mL^{-1}$) and FRAP ($4.70\pm0.06$ vs. $11.4\pm0.1\ mmol\ FeSO_4\cdot 7H_2O/g\ DM$) (Table 1). In addition, we measured the kinetics of oxidation of AA of CA, ALT711 and AG as positive controls. These compounds inhibited the oxidation of AA in dose-dependent manners (Figs. 3A–C). Normalized rates of AA oxidation were plotted as functions of the square roots of the concentrations of CA, ALT711 and AG. (Fig. 3D). The concentrations that produced a 50% inhibitory effect ($IC_{50}$) of three compounds on AA oxidation are shown in Fig. 3D. The compounds displayed $IC_{50}$ values of $0.96\pm0.07\ \mu M$ for CA, $1.92\pm0.20\ \mu M$ for ALT711 and $0.47\pm0.05\ \mu M$ for AG.

**Table 1. Antioxidant Activities of Chebulic Acid**

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<th>$DPPH\cdot SC_{50}^{a}$ ($\mu g/DM\ mL^{-1}$)</th>
<th>$FRAP^{c}$ (mmol $FeSO_4\cdot 7H_2O/g\ DM$)</th>
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<tr>
<td>Chebulic acid</td>
<td>$39.2\pm4.9$</td>
<td>$4.70\pm0.06$</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>$19.0\pm1.2$</td>
<td>$11.4\pm0.1$</td>
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Each value is mean±S.D. of three replicate experiments ($n=3$). $a$ Amount of sample necessary to decrease the initial 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH\cdot$) concentration by 50%. $b$ DM, dry matter. $c$ Ferric-reducing antioxidant power.

Fig. 2. Breaking Effect of CA on Glycol-BSA Induced Cross-Links to Collagen *in Vitro*

The breakings of Glycol-BSA induced cross-links to collagen with CA and ALT711 (alagebrium) were measured by ELISA (A) and (B), respectively. All data were expressed as mean±standard deviation of triplicate experiments. ** $p<0.01$ vs. non-treated group ($n=3$). The breaking modulation of Glycol-BSA induced collagen cross-links was revealed by SDS-PAGE (C). Western bloting of Glycol-BSA with anti-AGEs monoclonal antibody (D). * $p<0.05$ vs. non-treated group ($n=3$).
DISCUSSION

Many reports have alluded to the potential role of AGEs in the development of diabetic complications.\(^{21}\) The AGEs-induced cross-links of collagen have been correlated with the severity of diabetic complications as well as in certain pathological states commonly seen in aging.\(^{22}\) In the present study, CA significantly inhibited 1750-fold more Glycol-BSA induced cross-links to collagen than AG (IC\(_{50}\), 38.8±0.5 µM versus 67.8±2.5 mM; Fig. 1) based on cross-links to collagen, as determined using an ELISA system where the HRP labeled AGEs-BSA induced cross-links of collagen-coated plates was measured in either the presence or absence of CA or AG. However, our previous study showed that the inhibitory activity of CA on glycation-induced protein cross-links was comparable to that of AG, showing that CA and AG had IC\(_{50}\) values of 17.1±0.7 mM and 21.3±1.4 mM, respectively, based on cross-linking assays, which were carried out using a system measuring the incorporation of \([14C]\) lysine into BSA.\(^{23}\) The reaction conditions for inducing protein cross-links may explain the discrepancy between the above results on cross-link inhibitory activities. In the present study, BSA glycated with glycolaldehyde, Glycol-BSA was prepared, and was then employed to induce cross-links to collagen, which was measured using an ELISA system.\(^{18}\) Glycated BSA with glycolaldehyde in the absence of collagen was employed to induce AGEs including cross-links, and the incorporation of \([14C]\)lysine into BSA was used to measure cross-link activity in our previous study. CA showed significantly potent activity in inhibiting cross-links to collagen compared with AG in the present assay, whereas AG as a positive control gave similar magnitude of potency against cross-links in ELISA assay (IC\(_{50}\), 67.8±2.5 mM) and the previously reported radioactivity assay (IC\(_{50}\), 21.3±1.4 mM).\(^{23}\) To the best of our knowledge, it is the first report that such micromolar concentrations of CA have a highly inhibitory effect against AGEs-BSA induced cross-links to collagen, suggesting that CA may constitute a promising intervention agent in diabetic complications.

A limitation of AGE inhibitors is that they cannot affect pre-existing AGE cross-links, and so, another way to reduce levels of AGEs is through the use of the so-called ‘cross-link breakers,’ such as alagebrum chloride and ALT-711, which have been investigated with hypertension trials (Phase II), although those have been terminated due to lack of effectiveness in blood pressure.\(^{12}\) We performed the breaking assay in which the HRP-labeled AGEs-BSA induced cross-links to collagen was incubated for measuring breaking activity in the presence of ALT-711 and CA. The significantly strong activity of CA compared to that of ALT-711 (Figs. 2A, B, IC\(_{50}\), 1.46±0.05 mM vs. 72.2±2.4 mM) was confirmed by SDS-PAGE and Western blotting analysis. Thiazolium salts, such as ALT-711 and N-phenacylthiazolium bromide, are known to selectively add a dicarbonyl AGE cross-links, followed by an internal rearrangement to cleave the dicarbonyl bond, breaking the cross-links.\(^{6}\) However, not a single AGE-cross-link structure, which contains a dicarbonyl structure prone to the proposed action of AGE breakers, has been identified in tissue proteins to date.\(^{22}\) Recently, Nagai et al.\(^{24}\) proposed that the activities of AGE inhibitors and breakers of the AGEs formation and development of diabetes complications may be accounted at large by their chelating activity. The reported IC\(_{50}\) values for of ALT-711 and AG, for the inhibition of copper-catalyzed
oxidation of AA are 80 μM and 2.5 mM, respectively. However, IC̴/₂ values for inhibiting metal-catalyzed oxidation were determined as 1.92 ± 0.20 mM for ALT711, and 0.47 ± 0.05 mM for AG as well as 0.96 ± 0.07 mM for CA, in this study (Fig. 3). Although such discrepancies of IC̴/₂ values remains to be explained, in our experiment we added 2.0 μM CuCl₂ instead of 0.5 μM CuCl₂ to 50 mM chelate-treated phosphate buffer to obtain a linear relationship between time and the logarithmic percentage value of AA remaining. The chelating activities of CA as well as AG and ALT711 on copper-catalyzed oxidation of AA were compared based on our experimental condition, and in increasing order, ALT711 < CA < AG. In addition, the antioxidant activities of CA were comparable to those of AA, showing DPPH· scavenging activity (IC̴/₂ values: 39.2 ± 4.9 vs. 19.0 ± 1.2 μM/dry matter mL⁻¹) and FRAP activity (4.70 ± 0.06 vs. 11.42 ± 0.1 mmol FeSO₄·7H₂O/g DM). On the other hand, the values of ALT711 and AG for DPPH· scavenging and FRAP activities were below the threshold of determination. Because CA lacks functional groups which can cleave dicarbonyl compounds or react with carbonyl intermediates, the high potency of CA on AGEs-BSA induced cross-link breaking can be ascribed to its chelating and antioxidant activities. It is interesting to note that citrate, a nonspecific chelator at the same dose as triethylentetraamine (0.1 g/100 mL of drinking water) yielded comparable protection against cardiac structural and functional changes in the Zucker type 2 diabetic rat, and that citrate inhibited the formation of a well-known AGE, carboxymethyl lysine in the lens.

Our present in vitro studies showed that CA is a more potent inhibitor cross-linking and beaker of collagen cross-linking than the control compounds, such as AG and ALT711. These results suggest that CA could be a breaker as well as an inhibitor of AGE cross-linking, and the activity may be explained in large part by its chelating and antioxidant activities. However the mechanism of action of CA is not clear yet. In vivo studies are needed for the more practical use of CA as an anti-diabetic agent and for use in therapy of diabetic complications.

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REFERENCES


