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Original Article

Cleavage of glycated protein cross-linking and cleavage of AGEs cross-linking of substances contained in vegetables and herbs

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Abstract

Glycative stress-induced production and accumulation of advanced glycation end products (AGEs) in the body contribute to aging and the development of various diseases. PTB cleaves α-diketone bonds and cleaves cross-links associated with the formation of AGEs in glycated proteins (cleavage of AGE cross-linking; CAC). However, since glucospan, α-diketone, and lysine-dihydropyridinium-lysine are involved in the formation of protein cross-links by glycation, the usefulness of the cross-link cleavage effect must be verified using glycated proteins. Whereas, measurement of cleavage of glycated-protein cross-linking (CGPC) using glycated lysozyme as a model has been reported. In this study, CGPC and CAC of 12 substances contained in vegetables and herbs were measured to examine the possibility of degradation of protein cross-links polymerized by glycation. 12 substances were measured for CGPC and CAC, and a high positive correlation was observed between CGPC and CAC in 6 substances (50 %), indicating that α-diketone bond cleavage may be involved in the degradation of glycated protein cross-links. In contrast, only CGPC was observed for the three substances, and it was possible that they cleaved cross-links different from the α-diketone structure; the CGPC measurement had the potential to evaluate glycated protein cross-link cleavage actions other than α-dicarbonyl bond cleavage.

***KEY WORDS*:** advanced glycation end products (AGEs), glycated lysozyme, cleavage of glycated-protein cross-linking (CGPC), cleavage of AGE cross-linking (CAC)

Introduction

The production and accumulation of advanced glycation end products (AGEs) in the body due to glycative stress are factors in aging and the development of various diseases[1,2]. In particular, the formation of protein cross-links associated with the generation of AGEs leads to tissue stiffening, which is a factor in the deterioration of biological functions. The components involved in the formation of protein cross-links by glycation include glucospan, α-diketone, and lysine-dihydropyridinium-lysine[3]. *N*-phenacylthiazolium bromide (PTB) cleaves α-diketone bonds and cleaves cross-links associated with the AGE formation in glycated proteins (cleavage of AGE cross-linking: CAC)[4]. The CAC of a sample is determined by measuring the amount of benzoic acid released when the sample breaks down the intramolecular α-dicarbonyl bond using 1-phenyl-1, 2-propanedione (PPD) as a model compound for α-diketone bonding. CAC active substances include rosmarinic acid[5], elagitannin[6], and flavonoid[7], which are found in plants. On the other hand, in a study in which diabetic rats were treated with PTB, a substance with CAC, the glycated protein cross-links in skin and tail tendon collagen were not degraded[8]. Therefore, the usefulness of CAC in cleaving glycated protein cross-links must be verified using glycated proteins.

When lysozyme is glycated, it forms dimers and trimers by generating cross-linking AGEs, *e.g.*, pentosidine, vesperlysine, pyrropyridine, and crossline[9]. Polyacrylamide gel electrophoresis (SDS-PAGE) can be used to verify the polymerization of glycated lysozyme. Cleavage of glycated-protein cross-linking (CGPC) using glycated lysozyme as a model has been reported in a 50 % ethanol extract of edible purple chrysanthemum[10].

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In the present study, CGPC and CAC of 12 substances in vegetables and herbs were measured to verify the possibility of degradation of protein cross-links polymerized by glycation.

Materials and Methods

1) Reagents

The reagents used were purchased from the following manufacturers; lysozyme hydrochloride (from egg white), rosmarinic acid, 1-phenyl-1,2-propanedione (PPD), and benzoic acid from Fujifilm Wako Pure Chemical Industries (Osaka, Japan); 2-40% Mini-Protean TGX Precast gel from Bio-Rad (Hercules, California, USA); *N*-phenacylthiazolium bromide (PTB) from Sigma-Aldrich Japan (Meguro-ku, Tokyo, Japan). (-)-epicatechin and (-)-epigallocatechin gallate from Nagara Science (Gifu, Japan); 7-hydroxyflavone, 7,4'-dihydroxyflavone, 7,4'-dihydroxyflavanone (Liquiritigenin), 5,7-dihydroxyflavone 7-glucuronide (Chrysin), 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl) propan-1-one (Phloretin), rosmarinic acid (ester of caffeic acid and 3,4-dihydroxyphenyllactic acid), 3,3',4',5,7-pentahydroxyflavone 3-rutinoside (Rutin) from Extrasynthese (Genay Cedex, France); punicalagin (2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose) from Santa Cruz Biotechnology (Heidelberg, Germany); 4',5,7-trihydroxyisoflavone (Genistein) from LC Laboratories (Woburn, Massachusetts, USA); rosemary extract (AGE Breaker)[5] from A2P Science (Lyon, France); Other reagents were purchased from Fujifilm Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan) and used as special or HPLC grade reagents.

2) Samples

Samples were collected from 12 substances known to be present in vegetables, herbs and other plants; flavone, 7-hydroxyflavone, 7,4'-dihydroxyflavone, 7,4'-dihydroxyflavanone, 5,7-dihydroxyflavone 7-glucuronide, 3,3',4',5,7-pentahydroxyflavone 3-rutinoside,(-)-epicatechin, (-)-epigallocatechin gallate, 4',5,7-trihydroxyisoflavone, 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one, rosmarinic acid, and punicalagin (***Table 1***). PTB was used as a positive control for CGPC and CAC.

3) Preparation of glycated lysozyme

With reference to a previous report [10-12], glycated lysozyme was prepared by the reaction of 2 mg/mL lysozyme with 0.1 mol/L phosphate buffer (pH 7.4) containing 0.2 mol/L glucose at 60 °C for 40 hours, followed by the removal of

Table 1. Relationship between CGPC and CAC of substances contained in vegetables and herbs.

No	Substances	Typical herbs containing ingredients	CGPC (%)	CAC (%)
1	flavone	chamomile, green tea, parsley	ND	ND
2	7-hydroxyflavone	berries, green tea, citrus	ND	ND
3	7,4'-dihydroxyflavone	broad beans, fenugreek	16.6 ± 4.7	ND
4	7,4'-dihydroxyflavanone (Liquiritigenin)	nuts, winged beans, leafy vegetables	8.3 ± 1.3	ND
5	5,7-dihydroxyflavone 7-glucuronide (Chrysin)	passion fruit, honey, propolis	54.7 ± 4.9	ND
6	3,3',4',5,7-pentahydroxyflavone 3-rutinoside (Rutin)	apple, buckwheat, green tea	13.6 ± 1.9	2.1 ± 0.9
7	(-)-epicatechin	green tea, apple, berries, cacao	24.6 ± 5.4	6.9 ± 0.2
8	(-)-epicatechin gallate	green tea, cacao	96.8 ± 2.1	42.0 ± 3.9
9	4',5,7-trihydroxyisoflavone (Genistein)	soy, broad beans	ND	ND
10	3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (Phloretin)	apple, pear	19.8 ± 1.3	1.1 ± 0.1
11	rosmarinic acid (ester of caffeic acid and 3,4-dihydroxyphenyllactic acid)	rosemary, lemon balm, perilla	33.6 ± 0.6	27.4 ± 0.1
12	punicalagin (2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose)	pomegranate	83.3 ± 2.0	22.8 ± 0.4
ref	PTB (*N*-phenacylthiazolium bromide)	—	5.6 ± 0.4	23.3 ± 0.2

Data are shown as mean ± SD, n = 3; Sample concentration; 0.5 mmol/L (CGPC), 2.5 mmol/L (CAC); ND, less than 1 %; CGPC, cleavage of glycated-protein cross-linking; CAC, cleavage of AGE cross-linking, ref; positive control; SD, standard deviation.,

low molecular weight substances by 3kDa ultrafiltration membrane (Amicon Ultra-0.5 mL centrifugal filters Ultracel-3K; Merck, Darmstadt, Germany).

4) CGPC

CGPC was calculated as the cleavage ratio of the ratio of glycated lysozyme dimer to monomer upon addition of the sample, referring to a previous report [10]. To measure CGPC, a 1 : 1 solution of 2 mmol/L sample mixed with 0.05 mol/L phosphate buffer containing 0.5 mg/mL glycated lysozyme; (S), and a solution of only sample lysate added instead of sample in S; (R) were prepared and incubated at 37 °C for 16 hours.

The gel was stained with CBB Stain One (Nacalai Tesque), the gel images were imaged with the Pharos FX System (Bio-Rad, California, USA), and the band intensity of the monomer (M) and dimer (D) were analyzed with ImageJ (NIH, Maryland, USA) [13, 14]. For CGPC, an glycated-protein cross-link cleavage rate of 1% or more was considered to be actionable.

Glycated protein cross-link cleavage rate (%)
$= [\ 1 - \{(D_S/M_S)/(D_R/M_R)\}\ ] \times 100$
S; Lysozyme band intensity at the time of sample addition, R; Band intensity of lysozyme when only sample solution is added, M; monomer band, D; Dimer band.

5) CAC

CAC was measured by using PPD as a cross-link model substance and measuring the cleavage rate of α-diketone bonds of PPD by samples according to a previous report [4, 6, 15]. Briefly, 5 mmol/L sample, 10 mmol/L PPD, and 0.2 mol/L phosphate buffer (pH 7.4) were mixed at a ratio of 5 : 1 : 4 and allowed to react at 37 °C for 8 hours. After the reaction was stopped by adding 0.7 N HCl, the amount of benzoic acid; (A) released by the cleavage of the α-diketone bond of PPD by the sample was measured under the same HPLC conditions as previously reported [6]. The AGE cross-link cleavage rate was calculated using the formula below, based on the fact that 1 mol of PPD in the reaction solution yields 1 mol of benzoic acid when the α-diketone bond of PPD; (C) is cleaved. For CAC, an AGE cross-link cleavage rate of 1% or more was considered to be actionable.

AGE cross-link cleavage rate (%) = { (A – B) / C } × 100
A; Amount of benzoic acid in the reaction solution (mol),
B; Amount of benzoic acid in the sample (mol),
C; Amount of PPD used in the reaction (mol).

Statistical analysis

Measurements were expressed as the mean ± standard deviation (SD) of triplicate measurements. Pearson's product-rate correlation coefficient was used to analyze the correlation between measurements. Statistical analysis results were considered significant at a risk rate of less than 5% ($p < 0.05$). Statistical analysis was performed using the statistical analysis software BellCurve for Excel (Shakai Joho Service, Tokyo).

Results

1) CGPC

CGPC was found in 9 out of 12 (75%) of the samples at a concentration of 0.5 mmol/L in the reaction solution (***Table 1***). The percentage of glycated protein cross-link cleavage of the 9 CGPC-containing substances was 35.7 ± 30.4% (mean ± SD) with (-)-epigallocatechin gallate (96.8 ± 2.1%) having the highest value and 7,4'-dihydroxyflavanone (8.3 ± 1.3%) showed the lowest value. An 11.7-fold difference observed for the nine CGPC substances. (-)-Epigallocatechin gallate showed a 17.3-fold stronger effect than PTB, a positive control.

2) CAC

CAC actions were found in 6 of 12 substances (50%) at a concentration of 2.5 mmol/L in the reaction solution (***Table 1***). Six substances with CAC showed a glycated protein cross-link cleavage rate of 17.9 ± 14.0%, with (-)-epigallocatechin gallate (42.0 ± 3.9%) having the highest value and 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl) propan-1-one (1.1 ± 0.1%) showed the lowest value. A 38.2-fold difference was observed in the CACs of the six substances. (-)-Epigallocatechin gallate was 1.8 times stronger than PTB, which was compared as a positive control.

3) CGPC measurement of rosemary extract and PTB

The CGPC assay showed an attenuation in the band intensity of lysozyme. In particular, when 1 ~ 5 mg/mL of rosemary extract was added, the monomer band intensity of lysozyme attenuated to 88.5 ~ 33.5% compared to 100% when no extract was added (***Fig. 1, 2***). CGPC measurements of PTB showed an attenuation of the monomer band intensity, similar to that of rosemary extract. However, the attenuation was 93.6% at the maximum (5 mmol/L). The intensity of the lysozyme dimer band attenuated with increasing concentrations of rosemary extract and PTB.

Discussion

1) Relationship between CGPC and CAC

Of the 12 substances tested, 3 (flavone, 7-hydroxyflavone, and 4',5,7-trihydroxyisoflavone) were not found to be associated with either CGPC or CAC. In addition, 3,3',4',5,7-pentahydroxyflavone 3-rutinoside, (-)-epicatechin, (-)-epigallocatechin gallate, 3-(4-hydroxyphenyl)-1-(2,4,6-(trihydroxyphenyl)propan-1-one, rosmarinic acid, and punicalagin were found to act on both CGPC and CAC. trihydroxyphenyl)propan-1-one, rosmarinic acid, and punicalagin shouwed to act on both CGPC and CAC. A high positive correlation was observed between CGPC and CAC for the six substances with both of these effects ($p < 0.05$), suggesting that α-diketone bond cleavage may be involved in the degradation of protein cross-links (***Fig. 3***).

In contrast, three substances (7,4'-dihydroxyflavone, 7,4'-dihydroxyflavanone, and 5,7-dihydroxyflavone 7-glucuronide) were found only in CGPC. In particular, the

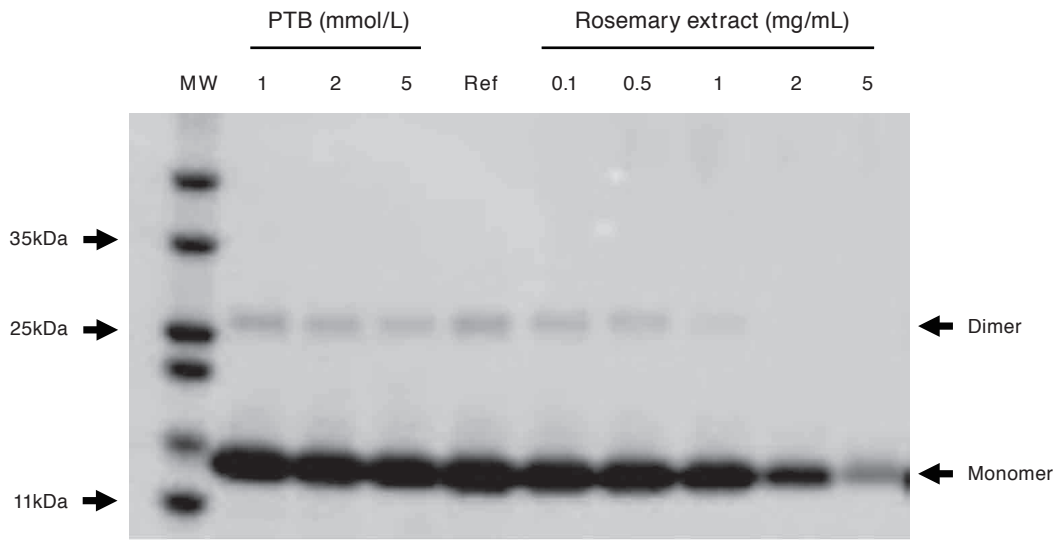

Fig. 1. **CGPC by PTB and rosemary extract and the change of glycated lysozyme band intensity.**

Sample and 0.5 mg/mL glycated lysozyme were incubated at 37 °C for 16 hours, n = 1; SDS-PAGE was conducted using 2 ~ 40 % acrylamide gels. Stained with CBB stain one; MW, molecular weight markers; Ref, incubation without sample (with 50 % ethanol); PTB, *N*-phenacylthiazolium bromide, a positive control; CGPC, cleavage of glycated-protein cross-linking.

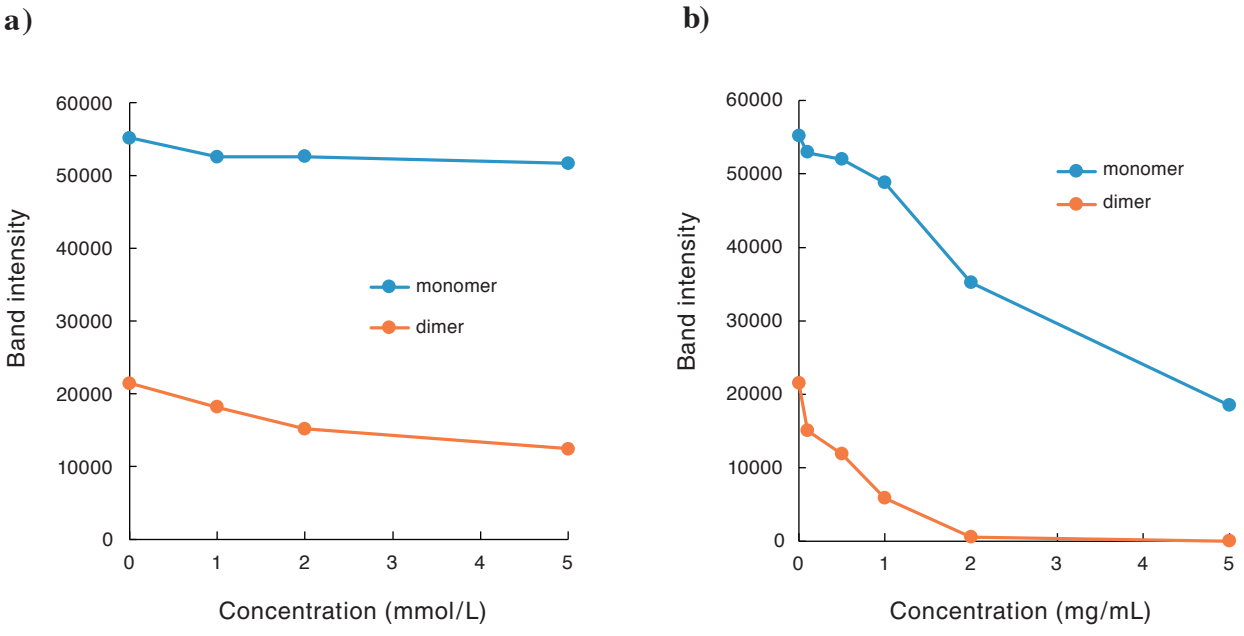

Fig. 2. **Band intensity of PTB and rosemary extract on lysozyme dimer in the glycated lysozyme.**

a) *N*-phenacylthiazolium bromide (PTB), **b)** rosemary extract. Sample and 0.5 mg/mL glycated lysozyme were incubated at 37 °C for 16 hours, n = 1. SDS-PAGE was conducted using 2 ~ 40 % acrylamide gels, stained with CBB stain one. MW, molecular weight markers; Ref, incubation without sample (with 50 % ethanol).

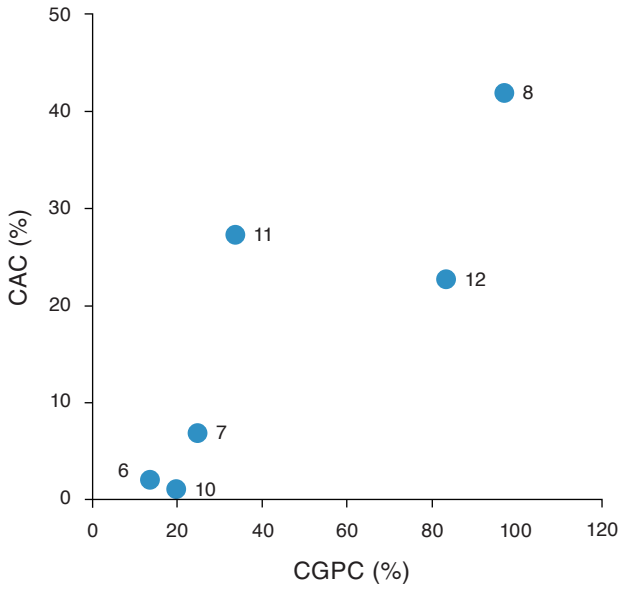

Fig. 3. **Correlation of six substances with both CGPC and CAC.**

Each point indicates average value in triplets of CGPC and CAC. Statistical analysis, Spearman's rank correlation coefficient, $p < 0.05$; Sample concentration, 0.5 mmol/L (CGPC), 2.5 mmol/L (CAC). Numbers in the graph indicate substance number in ***Table 1***. CGPC, cleavage of glycated-protein cross-linking; CAC, cleavage of AGE cross-linking.

protein cross-link cleavage rate of 5,7-dihydroxyflavone 7-glucuronide (64.5 ± 17.7%) was the third most potent among the nine substances with CGPC. No relevance was suggested between the presence or strength of CGPC/CAC and substance structure for the 12 substances. In addition to α-diketone bonds, protein cross-links by glycation include those by glucospan and lysine-dihydropyridinium-lysine [3]. The cross-links that are cleaved by these three substances may differ from the α-diketone structure. These results suggest that the CGPC assay may be able to evaluate the effect of glycated protein cross-link cleavage other than α-dicarbonyl bond cleavage. However, the structure of the protein cross-link formed in the glycated lysozyme dimer is not known, and the mechanism could not be inferred.

2) Challenges in CGPC measurement

In the CGPC assay, the intensity of the monomeric lysozyme band in SDS-PAGE attenuated to 33.5% at the maximum (5 mg/mL) when rosemary extract was added at 1 mg/mL or more, compared to 100% when no extract was added. Protein staining with CBB Stain One is a staining method in which the dye is adsorbed to the amino group of the protein by electrostatic interaction [16]. Insufficient staining was presumably due to a charge change at the dye binding site caused by the coexistence of glycated lysozyme and rosmarinic acid, or other unknown substances, contained in rosemary extract.

In the cross-link formation by protein glycation, reactive aldehyde groups of glucose react with reactive amino groups of protein chains to form glycation intermediates through the formation of Schiff bases [1,2]. These intermediates, subsequently, attack the lysine-amino groups of Amadori products and proteins to form stable cross-links that bind protein chains together. However, when the α-dicarbonyl linkage is cleaved, the protein does not return to its original protein structure, but becomes a single protein molecule with modifications [9,17]. This may have weakened the electrostatic interaction between the protein and the dye, resulting in insufficient CBB staining.

Since CGPC is calculated as the cleavage rate as the ratio of lysozyme polymerized by protein glycation back to monomer when the sample is added to the reaction system, insufficient staining of lysozyme is a cause of measurement error. The CGPC assay of edible purple chrysanthemum extract calculated the protein cross-link cleavage rate based on the reduction rate of only the dimer band intensity of glycated lysozyme [10]. In this case, no attenuation of the lysozyme monomer band was observed. Comparing the CGPC of rosemary extract, which attenuates the lysozyme monomer band, between the previous and present method, the values by the previous methods [10] was calculated to be 1.4-3.5% higher than the present results.

The SD of the CGPC values measured in this experiment ranged from 0.4 to 5.4%, therefore, the difference in analysis methods did not mislead as to the presence or absence of the action. However, in the CGPC measurement of samples attenuating lysozyme staining, care should be taken to use a formula adapted to this verification so that the reduction in band intensity does not become a factor in overestimating the protein cross-link cleavage effect.

Research limitations

In this study, CGPC and CAC were verified using 12 substances found in herbs and other plants as samples. Further comparison and validation of a larger number of substances is needed to determine the relevance of CGPC and CAC of various substances and their effect on protein staining in the evaluation of CGPC.

Conclusion

CGPC and CAC of 12 substances in vegetables and herbs were measured. Six substances (50%) showed a high positive correlation between CGPC and CAC, indicating that α-diketone bond cleavage may be involved in the degradation of glycated protein cross-links. Whereas, only CGPC was observed for the three substances, and it was possible that they cleaved cross-links different from the α-diketone structure. The CGPC assay may be able to evaluate glycated protein cross-link cleavage actions other than α-dicarbonyl bond cleavage.

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Declaration of conflict of interest

There are no conflicts of interest in the conduct of this study.

Reference

1) Ichihashi M, Yagi M, Nomoto K, et al. Glycation stress and photo-aging in skin. *Anti-Aging Med*. 2011; 8: 23-29.
2) Yagi M, Yonei Y. Glycative stress and anti-aging: 1. What is glycative stress? *Glycative Stress Res*. 2016; 3: 152-155.
3) Furber JD. Extracellular glycation crosslinks: prospects for removal. *Rejuvenation Res*. 2006; 9: 274-278.
4) Vasan S, Zhang X, Zhang X. et al. An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. 1996; *Nature*. 382: 275-278.
5) Jean D, Pouligon M, Dalle C. Evaluation in vitro of AGE-crosslinks breaking ability of rosmarinic acid. *Glycative Stress Res*. 2015; 2: 204-207.
6) Yagi M, Mitsuhashi R, Watanabe A, et al. Cleaving effect of pomegranate (*Punica granatum*) extract on crosslink derived from advanced glycation endproducts. *Glycative Stress Res*. 2015; 2: 58-66.
7) Takabe W, Mitsuhashi R, Parengkuan L, et al. Cleaving effect of melatonin on crosslinks in advanced glycation end products. *Glycative Stress Res*. 2016; 3: 38-43.
8) Yang S, Litchfield JE, Baynes JW. AGE-breakers cleave model compounds, but do not break Maillard crosslinks in skin and tail collagen from diabetic rats. *Arch Biochem Biophys*. 2003; 412: 42-46.
9) Aldini G, Vistoli G, Stefek M, et al. Molecular strategies to prevent, inhibit, and degrade advanced glycoxidation and advanced lipoxidation end products. *Free Radic Res*. 2013; 47(Suppl 1): 93-137.
10) Yagi M, Hara H, Mifuru R, et al. Suppression of glycated protein cross-linking formation and cross-linking cleavage reaction of edible purple Chrysanthemum flower extract. *Glycative Stress Res*. 2022; 9: 7-14.
11) Perera HKI, Ranasinghe HASK. A simple method to detect plant based inhibitors of glycation induced protein cross-linking. *Asian J Med Sci*. 2015; 6: 28-33.
12) Perera HKI, Handuwalage CS. Analysis of glycation induced protein cross-linking inhibitory effects of some antidiabetic plants and spices. *BMC Complement Altern Med*. 2015; 15, 175.
13) Rasband WS. Image J. U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.
14) Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012; 9: 671-675.
15) Yagi M, Sakiyama C, Miyata Y, Kamiya S, Yonei Y. Antiglycative effect of genipin and crocetin. *Glycative Stress Res*. 2021; 8: 156-161.
16) Steinberg TH. Protein gel staining methods: An introduction and overview. *Methods Enzymol*. 2009; 463: 541-563.
17) Furber JD. Extracellular glycation crosslinks: Prospects for removal. *Rejuvenation Res*. 2006; 9: 274-278.