Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : January 7, 2023
Accepted : January 16, 2023
Published online : March 31, 2023
doi:10.24659/gsr.10.1_16

Original article

Mass spectrometric and immunological evaluation of AGEs during incubation of ribose with gelatin

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Abstract

Ribose produces advanced glycation end-products (AGEs) more rapidly than glucose. It has also been reported that N^{ε}-(carboxymethyl)arginine (CMA) is formed in collagen, which is the most abundant protein in organisms. In this study, AGEs produced by the reaction of ribose with gelatin, a soluble collagen, were evaluated by immunochemical methods using anti-AGEs antibodies, mass spectrometry, and fluorescence intensity measurements. Measurement by enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed that N^{ε}-(carboxymethyl)lysine (CML), N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) and CMA were significantly increased in the ribose-gelatin sample compared to the gelatin alone sample after a 7-day incubation at 37 °C. The fluorescence intensity at excitation and emission of 370 nm and 440 nm, respectively, was also significantly increased, whereas the AGE structure detected was unknown. In addition, the pericarp hot water extract of *Trapa bispinosa* (TBE) and/or lutein were added to this reaction system to evaluate the inhibitory effect of AGE formation. As a result, inhibition of CML, MG-H1, and CMA formation was observed at ≥ 5 μg/mL of TBE in ELISA and LC-MS/MS. However, no similar inhibitory effect was detected in fluorescence measurements, suggesting that TBE does not inhibit the formation of fluorescent AGEs. Since the inhibition of AGE formation by lutein was not confirmed, it may be a difficult compound to evaluate by *in vitro* studies. This study showed that ELISA and LC-MS/MS measurement can detect three types of AGEs formation in the ribose-gelatin system and that they are useful for screening compounds, *e.g.*, TBE, that inhibit AGE formation.

***KEY WORDS*:** advanced glycation end-products (AGEs), fluorescence, ELISA, LC-MS/MS, *Trapa bispinosa* Roxb.

Introduction

According to a 2020 report by the World Health Organization (WHO), non-communicable diseases (NCDs), including cardiovascular disease, cancer, and diabetes, account for approximately 70 % of global deaths [1)]. These diseases are also referred to as age-related diseases because their onset and progression are associated with aging. Advanced glycation end-products (AGEs) are thought to contribute to the onset and progression of aging-related diseases, and it has been reported that AGEs increase and accumulate in the body with aging, diabetic complications, atherosclerosis, and kidney disease [2-6)]. AGEs are also involved in the inactivation of enzymes and changes in protein conformation [7)]. Therefore, it is thought that suppressing the formation of AGEs in the body can prevent the onset and progression of age-related diseases, and searches for natural compounds to inhibit AGE formation are being conducted worldwide.

To find compounds that inhibit AGEs formation, it is important to establish a model system in which AGEs are rapidly produced and an assay method that can accurately evaluate AGEs content. A mixture of reducing sugars or carbonyl compounds and proteins is incubated, and the level of AGEs is usually measured by the enzyme-linked immunosorbent assay (ELISA). In the conventional methods, glucose, which has the highest physiological concentration among reducing sugars, and albumin have been used. However, glucose requires about 8 weeks or more to produce AGEs when incubated at 37 °C, and albumin is difficult to produce N^{ε}-(carboxymethyl)arginine (CMA), which is produced in large amounts in collagen glycation [8-10)].

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In addition, AGEs have been measured so far by immunochemical methods using anti-AGEs antibodies and fluorescence properties, but in many cases the epitope structure of the antibodies and the AGEs structure detected by fluorescence are unknown[7]. Therefore, it may not be possible to discover the inhibitors for AGEs formation that actually show efficacy in humans. In addition, the serum is occasionally heated at 100 °C for 15 minutes beforehand in measuring blood AGEs, and it has been pointed out that N^{ε}-(carboxymethyl)lysine (CML)[11] and pentosidine[12] are artificially generated by this pretreatment. *Trapa bispinosa* Roxb. is an annual aquatic grass cultivated as an edible and medicinal plant, and has been reported to possess various functions, i.e., antibacterial activity, postprandial hyperglycemia inhibition[13-15]. *In vitro* and *in vivo* studies have also revealed that it has anti-glycation properties, and it is being studied especially from the viewpoint of cataract prevention[16, 17]. Lutein is a type of carotenoid, rich in green and yellow vegetables, and is recognized for its antioxidant capacity[18, 19]. In humans, lutein is present in the lens and retina, and higher serum concentrations have been suggested to decrease the risk of developing ocular diseases such as age-related macular degeneration[20].

In this study, we established a system in which several AGEs are produced in a short incubation period of 7 days using the ribose-gelatin, and used fluorescence measurements, indirect ELISA, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare fluorescent intensity and AGE contents such as CML, N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1), and CMA. Furthermore, this reaction system was used to evaluate the inhibition of AGE formation by the pericarp of *T. bispinosa* hot water extract (TBE) and lutein.

Methods

Sample preparation

The pericarp hot water extract of *T. bispinosa* (TBE) (Hayashikane Sangyo Co., Ltd., Yamaguchi, Japan) and lutein 20 % suspension (Koyo Mercantile Co., Ltd., Tokyo, Japan) were provided by Santen Pharmaceutical Co., Ltd. (Osaka, Japan). Lutein 20 % suspension is a sample of safflower oil containing 20.0 % marigold pigment; therefore, it was regarded that 1 mL of lutein 20 % suspension contains 200 mg of lutein for the experiment. The concentrations of TBE and lutein added in this study were determined based on the data of 10:1 ratio of TBE and lutein in Sante Wellvision (Santen Pharmaceutical Co., Ltd.), a dietary supplement developed with a focus on aging of the eyes and other parts of the body, and previous studies[21, 22].

The samples were prepared with 60 mM D(-)-ribose (Wako Pure Chemical, Osaka, Japan) and 4 mg/mL gelatin from porcine skin, Type A (Sigma-Aldrich, Darmstadt, Germany) in 200 mM phosphate buffer (NaPB, pH 7.2) and sterile filtered through a 0.45 µm filter (DISMIC 25CS045AS, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). TBE and lutein were dissolved in dimethyl Sulfoxide (DMSO, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Then, only TBE was filtered through a 0.45 µm filter (NP-44513-AEF, Tomsic Ltd., Tokyo, Japan), and the ribose and gelatin solutions were mixed in equal volumes in the presence of TBE (final concentrations 0, 1, 5, 10, 50) and lutein (final concentrations 0, 0.1, 1, 5, 10), to make a mixture solution with a final concentration of 30 mM ribose and 2 mg/mL gelatin. A sample of 200 mM NaPB mixed with an equal volume of 4 mg/mL gelatin solution was used as a negative control for AGE formation. After incubation at 37 °C for 7 days, each sample was placed in a cellulose tubing (24/32, EIDIA Co., Ltd., Tokyo, Japan) and dialyzed in 2 L of water at 4 °C for 12 hours. Protein quantification was performed by the bicinchoninic acid (BCA) method.

Fluorescence measurement

Each sample was diluted to 100 µg/mL in phosphate-buffered saline (PBS) and added to 96-well plates (96-well, black, flat bottom, Tecan Trading AG, Männedorf, Switzerland) at 200 µL. Fluorescence intensity was measured using a Microplate Reader (Tecan infinite M PLEX, Tecan Japan Co., Ltd., Kanagawa, Japan) at 370 nm excitation and 440 nm emission.

Indirect ELISA

The assay was conducted based on the previous studies[9, 23]. Briefly, each sample was diluted in PBS to a final concentration of 0.2 or 1 µg/mL. It was added to each well of a 96-well plate (Clear Flat-Bottom Immuno Nonsterile 96-Well Plates, Thermo Fisher Scientific, Waltham, MA, USA) in 100 µL and coated by incubation at 25 °C for 2 hours. The wells were then washed three times with PBS containing 0.05 % Tween 20 (washing buffer). 200 µL of PBS containing 0.5 % gelatin was added to each well and incubated at 25 °C for 1 hour for blocking. After washing three times with washing buffer, 100 µL of primary antibodies, monoclonal anti-CML antibody (0.5 µg/mL)[24], monoclonal anti-MG-H1 antibody (0.5 µg/mL)[25] and monoclonal anti-CMA antibody (1 µg/mL)[10], was added and incubated at 25 °C for 1 hour. The wells were washed three times and incubated with a secondary antibody, at 5,000-fold diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA), for 1 hour at 25 °C. After washing the wells three times, 100 µL of a development solution consisting of 10 mL of citrate-phosphate buffer (pH 5.0), one OPD Tablet (o-Phenylenediamine. 2HCl 5mg/Tablet), and 5.9 mM hydrogen peroxide was added to each well and allowed to react. The reaction was terminated with 100 µL of 1.0 M sulfuric acid, and absorbance at 492 nm was measured using a Microplate Reader.

Pretreatment of samples for AGE measurement by LC-MS/MS

Pretreatment for measurement was based on previous studies[9, 22, 26]. Briefly, 50 µL of sample (25 µg protein) was reduced by adding an equal volume of 100 mM sodium borate buffer (pH 9.1) and one-tenth volume (5 µL) of 2 M sodium tetrahydroborate (Fujifilm Wako Pure Chemical) containing 0.1 M sodium hydroxide (NaOH, Fujifilm Wako Pure Chemical), and allowed to stand at 25 °C for 4 hours. Then, 10 µL of 1 µM [$^2H_2$] CML and [$^2H_3$] MG-H1

(PolyPeptide Laboratories France SAS, Strasbourg, France), 10 µL of 10 µM [$^{13}C_6$] CMA, 5 µL of 1 mM [$^{13}C_6$] lysine and [$^{13}C_6$] arginine (Cambridge Isotope Laboratories, Tewksbury, MA, USA), and 1 mL of 6 M HCl were added to each sample and hydrolyzed by incubation at 100 °C for 18 hours. After drying, the sample was resuspended in 1 mL water, passed over a Strata-X-C column (Phenomenex, Torrance, CA, USA), washed with 3 mL of 2% (v/v) formic acid solution, and eluted with 2 mL of 7% (v/v) ammonia solution. Then, it was dried, resuspended in 1 mL of a solution containing 20% (v/v) acetonitrile and 0.1% (v/v) formic acid, and filtered through a 0.45 µm filter (Millex-LH filter, Merck KGaA, Darmstadt, Germany).

Measurement of AGEs levels by LC-MS/MS

Measurements by LC-MS/MS were performed as described previously [27]. The precursor ions of CMA, [$^{13}C_6$] CMA, arginine, and [$^{13}C_6$] arginine were *m/z* 233, 239, 175, and 181, respectively, the fragment ions were *m/z* 116, 121, 70, and 74, respectively, the collision energy were 16, 16, 23, and 23 V, respectively, detected in positive ion mode. Otherwise, the measurement conditions were similar to previous studies [26]. CML values were normalized to lysine, MG-H1 and CMA values were normalized to arginine values. ***Fig. 3-a***, ***b, c*** show the structural formula of the measured AGEs and the type and site of stable isotope labeling, *m/z* of precursor ions and fragment ions of AGEs and their internal standards.

Statistical analysis

The web application PlotsOfData and Microsoft Excel 2019 were used to draw the measured data [28]. All data are expressed as dots, and means are indicated by horizontal lines. Statistical tests were performed using Dunnett's test, with p-values < 0.05 considered significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [29].

Results

Evaluation of inhibition of AGE formation by fluorescence measurement

The fluorescence intensity of the ribose-gelatin sample, a positive control for AGE formation, was measured at an excitation wavelength (370 nm) and an emission wavelength (440 nm) and was significantly higher than that of the negative control incubated with gelatin alone (A vs B: $p < 0.001$, ***Fig. 1***). The addition of lutein 1 µg/mL alone subsequently resulted in significantly higher fluorescence intensity compared to the positive control (B vs C: $p = 0.001$). Whereas, it was significantly lower in the TBE 5 µg/mL/lutein 0.1 µg/mL and TBE 50 µg/mL/lutein 1 µg/mL groups (B vs G: $p = 0.029$, B vs M: $p < 0.001$).

Evaluation of inhibition of AGE formation by indirect ELISA

ELISA assay showed a significant increase in CML and MG-H1 and CMA all in the ribose-gelatin mixture (A vs B: $p < 0.001$, ***Fig. 2-a, b, c***). In contrast, CML, MG-H1, and CMA were significantly decreased in the TBE 5 µg/mL/lutein 0.1 µg/mL group; TBE 10 µg/mL/lutein 0, 0.1, 1, 5, 10 µg/mL group; and TBE 50 µg/mL/lutein 1 µg/mL group (B vs G, H, I, J, K, L, M: $p < 0.001$, ***Fig. 2-a, b, c,*** respectively). In the TBE 1 µg/mL alone and TBE 1 µg/mL/lutein 0.1 µg/mL groups, only the CMA formation was significantly inhibited (B vs D: $p = 0.027$, B vs E: $p < 0.001$, ***Fig. 2-c***).

Evaluation of AGE formation inhibition by LC-MS/MS

LC-MS/MS clearly detected the peaks of CML, MG-H1, and CMA (***Fig. 3-d, e, f***). The CML, MG-H1, and CMA levels were significantly increased in the ribose-gelatin mixture compared to the gelatin alone sample (A), a negative control for AGE formation (A vs B: $p < 0.001$, ***Fig. 4-a, b, c***). Significant increases in MG-H1 and CMA were detected when lutein 1 µg/mL was added alone compared to the positive control (B vs C: $p < 0.001$, respectively, ***Fig. 4-b, c***). In contrast, CML, MG-H1, and CMA were significantly decreased in the groups with TBE 5 µg/mL/lutein 0.1 µg/mL, TBE 10 µg/mL/lutein 0, 0.1, 1, 5, 10 µg/mL, and TBE 50 µg/mL/lutein 1 µg/mL (B vs G, H, I, J, K, L, M: $p < 0.001$, respectively, ***Fig. 4-a, b, c***). A significant inhibition of CML and CMA formation was detected in the groups with TBE 1 µg/mL/lutein 0, 0.1, 1 µg/mL (B vs D, E, F: $p < 0.001$, respectively, ***Fig. 4-a, c***).

Discussion

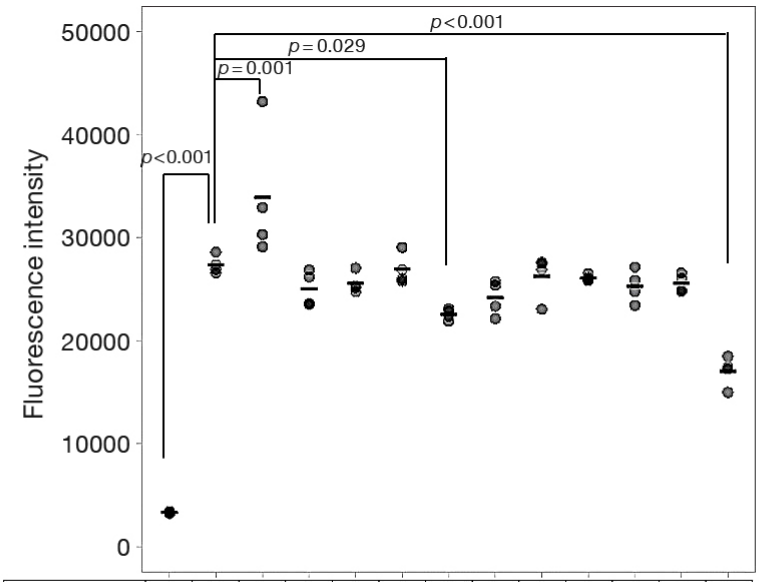
Using a ribose-gelatin system in which AGEs are produced after one week of incubation, we compared the increase in CML, an oxidation-dependent AGE; MG-H1, which is produced *via* methylglyoxal from abnormal carbohydrate metabolism; and CMA, which is produced mostly from collagen, using ELISA and mass spectrometry [10, 30, 31]. Subsequently, changes in fluorescence intensity at excitation and emission of 370 nm and 440 nm, which are often used as a conventional way to evaluate AGEs [32]. ELISA and LC-MS/MS measurements showed that CML, MG-H1, and CMA levels increased significantly after one week of reaction. Fluorescence intensity concurrently increased significantly, suggesting that progression of the Maillard reaction was detectable in three ways.

Using this reaction system, we evaluated the ability of TBE, which has already been reported to inhibit AGE formation, and lutein, which is known to have antioxidant properties, to inhibit AGE formation. As a result, significant inhibition of CML, MG-H1, and CMA formation was observed at TBE levels of 5 µg/mL or higher by ELISA and LC-MS/MS measurements. It has been reported that TBE inhibits ribose-derived MG-H1 generation but not methylglyoxal-derived MG-H1 [22]. Other reports indicate that polyphenols including gallic acid, ellagic acid, and eugeniin are present in TBE, and that ellagic acid inhibits CMA formation *via* antioxidant rather than carbonyl trap [17, 33]. Therefore, it is thought that TBE inhibits the pathway by which CML, MG-H1, and CMA are produced from the

adduct of ribose and protein. Note that even at 1 μg/mL of TBE, significant inhibition of CML and CMA was detected by ELISA and LC-MS/MS, and the difference from the positive control was slight.

Although the Dunnett's test does not allow the comparison of groups with different concentration of lutein, as far as the measurement results of the TBE 1 μg/mL/lutein 0, 0.1, 1 μg/mL group (D, E, F) or the TBE 10 μg/mL/lutein 0, 0.1, 1, 5, 10 μg/mL group (H, I, J, K, L) were compared (***Fig. 2, 4***), the inhibitory effect of lutein on AGE formation was almost negligible. In a previous report in which TBE and lutein were administered to diabetic rats, lutein did not suppress AGEs during short-term administration, while did show a suppressive effect during long-term administration[17]. In a similar case, we previously reported that administration of citric acid inhibited cataracts in diabetic rats, even though citric acid does not inhibit AGE formation *in vitro*[34]. Lutein, a lipophilic carotenoid, is thought to exhibit antioxidative effects *in vivo* by translocation to the retina and lens. Therefore, it is likely that evaluation of the inhibitory effect of lutein on AGE formation *in vitro* is difficult.

Since almost the same inhibitory effect was detected by both ELISA and LC-MS/MS, it is considered that ELISA is sufficient for the first screening of candidate compounds for AGE inhibitors. In contrast to this result, the fluorescence measurement showed a significant decrease in intensity under the conditions of TBE 5 μg/mL/lutein 0.1 μg/mL or TBE 50 μg/mL/lutein 1 μg/mL. Fluorescent AGEs such as pentosidine and crossline are reported[35,36]. Since the formation pathways of each AGE structure are diverse, the inhibitory effect of TBE was also different, which may be the reason for the discrepancy between the fluorescence measurement versus ELISA and LC-MS/MS results. No significant decrease in fluorescence intensity was observed in the group with 10 μg/mL of TBE. It is thought that when sugar and protein are mixed and reacted, fluorescent substances other than AGEs are also generated, and the addition of lutein, which has conjugated double bonds and is fluorescent, affected the fluorescence intensity, resulting in inconsistent results. Aminoguanidine, a known inhibitor of AGEs formation, inhibits the formation of fluorescent AGEs *in vitro*[37], whereas its inhibitory effect on CML and CMA formation was less effective *in vitro*[9]. Therefore, the mechanism of inhibition of AGEs formation by TBE may be different from that of aminoguanidine.

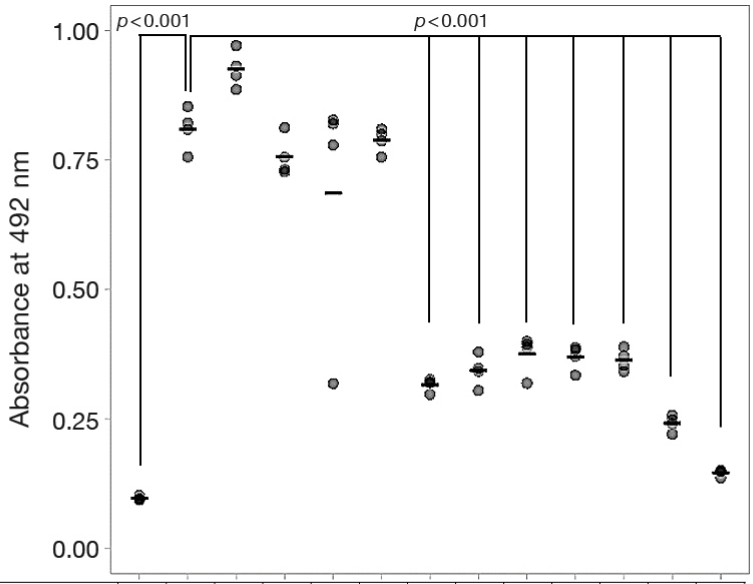


Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1

Fig. 1. **Measurement of fluorescence intensity.**

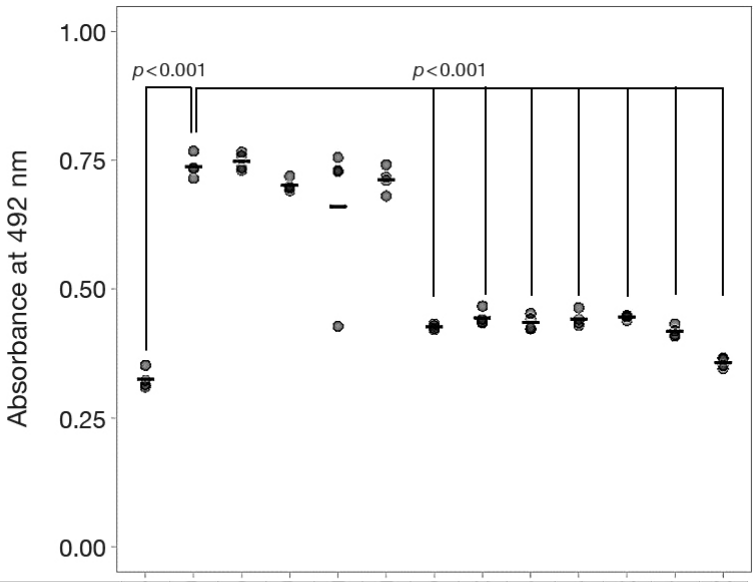
The horizontal axis shows the alphabet identifying each group of samples (groups from A to M, respectively n = 4) and the final concentrations of TBE and lutein. The fluorescence intensity of each reaction solution diluted to 100 μg/mL and measured at an excitation of 370 nm and emission of 440 nm is shown. Negative control for AGE formation (group A) is gelatin alone, while positive control (group B) and TBE-lutein added group (groups from C to M) are ribose-gelatin mixtures. Data are shown as dots, with means indicated by horizontal lines. Means of the positive control for AGE formation (group B) and the other groups were compared by Dunnett's test, and significant differences are shown in the graph. AGE, advanced glycation end-product; TBE, *Trapa bispinosa* extract.

(a)

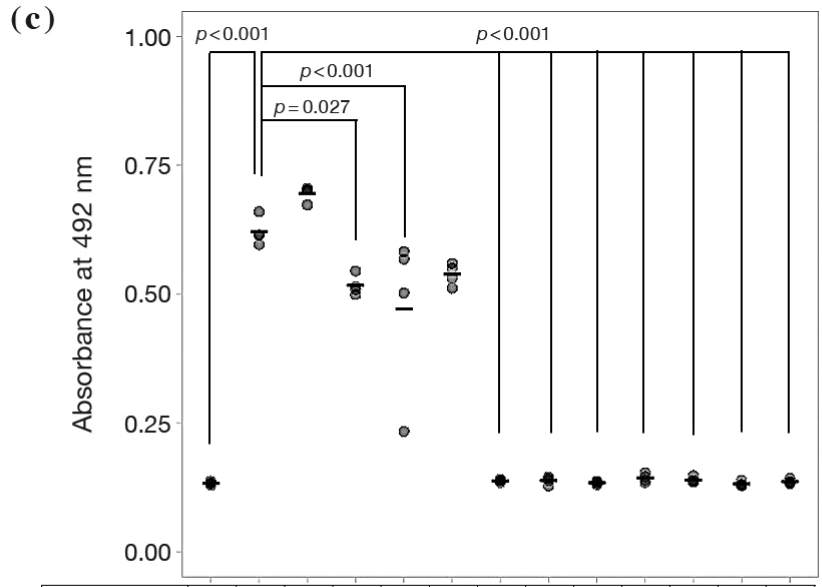


Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1

(b)



Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1



Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1

Fig. 2. **Measurement of AGEs by indirect ELISA.**

The horizontal axis shows the alphabet identifying each group of samples (groups from A to M, respectively n = 4) and the final concentrations of TBE and lutein. The vertical axis shows the absorbance at 492 nm. The negative control for AGE formation (group A) is gelatin only, and the positive control (group B) and the TBE and lutein supplemented groups (groups C-M) are ribose-gelatin mixture. The development time was (**a**) 3 minutes for CML, (**b**) 5 minutes for MG-H1, and (**c**) 7 minutes for CMA. Data are shown as dots, with means indicated by horizontal lines. Means of the positive control for AGE formation (group B) and the other groups were compared by Dunnett's test, and significant differences are shown in the graph. AGEs, advanced glycation end-products; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine; CMA, N^{ε}-(carboxymethyl)arginine; TBE, *Trapa bispinosa* extract.

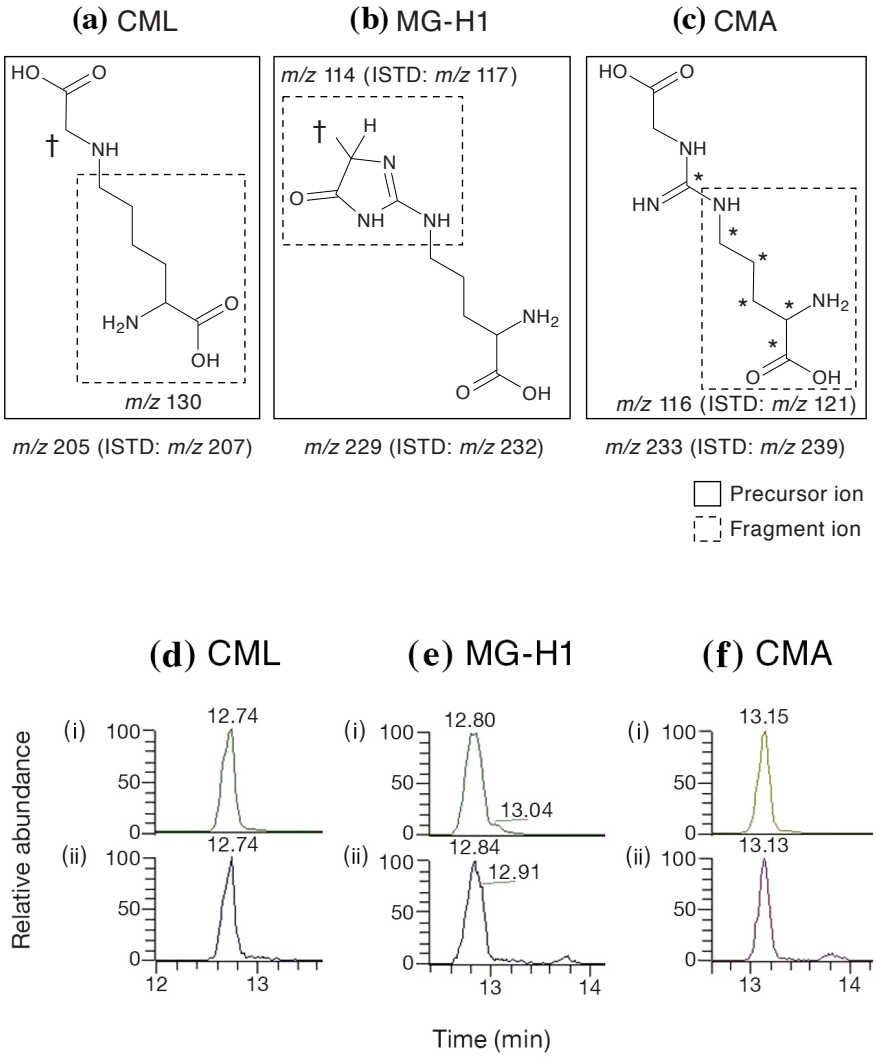
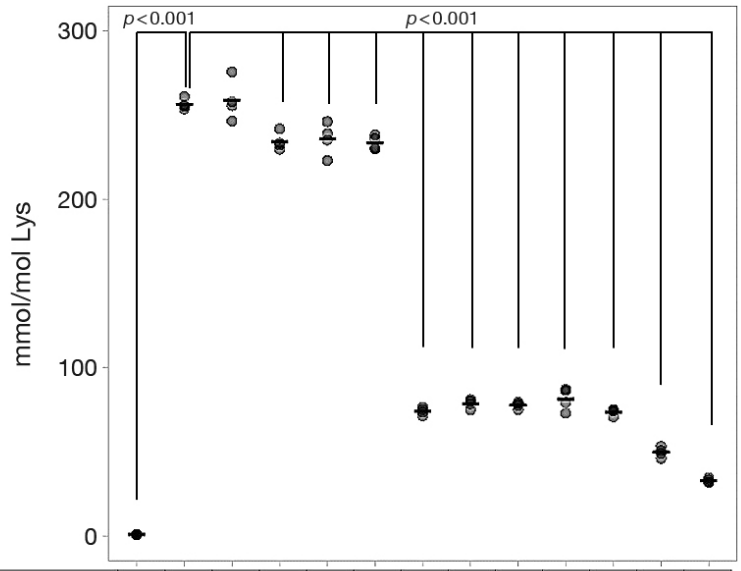


Fig. 3. **Structural formulas and chromatograms of measured AGEs.**

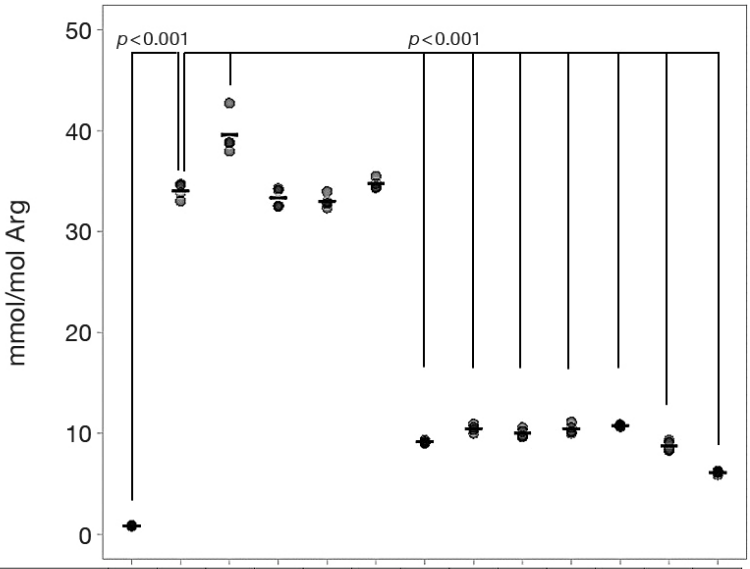
Structural formula of (**a**) CML, (**b**) MG-H1, and (**c**) CMA. Deuterium and carbon-13 in the internal standards are marked by a dagger (†) and asterisk (⋆), respectively. Precursor ions are shown as solid lines and fragment ions as dashed lines. In each sample, fragment ion peaks of (**d**) CML, (**e**) MG-H1, and (**f**) CMA were detected. Typical fragment ion peaks of CML, MG-H1, and CMA detected in the ribose-gelatin mixture sample, which is the positive control for AGEs formation (group B), are shown in (i), each internal standard is shown in (ii), respectively. AGEs, advanced glycation end-products; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine; CMA, N^{ε}-(carboxymethyl)arginine; ISTD, internal standard.

(a)

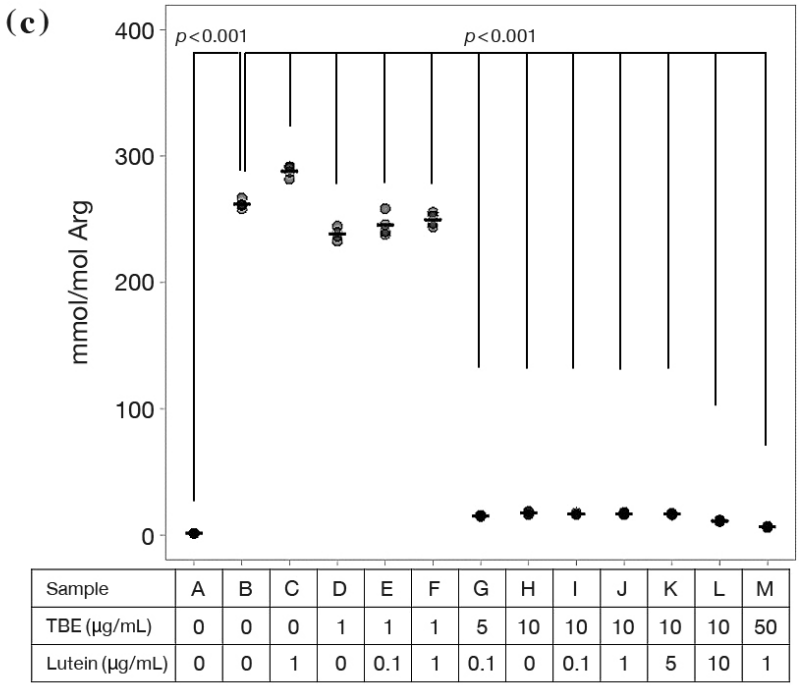


Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1

(b)



Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1



Sample	A	B	C	D	E	F	G	H	I	J	K	L	M
TBE (μg/mL)	0	0	0	1	1	1	5	10	10	10	10	10	50
Lutein (μg/mL)	0	0	1	0	0.1	1	0.1	0	0.1	1	5	10	1

Fig. 4. **Measurement of AGEs by LC-MS/MS.**

The horizontal axis shows the alphabet identifying each group of samples (groups from A to M, respectively n = 4) and the final concentrations of TBE and lutein. (**a**) CML levels normalized to lysine, (**b**) MG-H1 levels normalized to arginine levels, and (**c**) CMA levels normalized to arginine levels in the samples are shown on the vertical axis. Negative control for AGE formation (group A) is gelatin alone, while positive control (group B) and TBE-lutein added group (groups from C to M) are ribose-gelatin mixtures. Data are shown as dots, with means indicated by horizontal lines. Means of the positive control for AGE formation (group B) and the other groups were compared by Dunnett's test, and significant differences are shown in the graph. AGEs, advanced glycation end-products; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine; CMA, N^{ε}-(carboxymethyl)arginine; TBE, *Trapa bispinosa* extract.

Conclusion

This study showed that by using a ribose-gelatin system instead of glucose or BSA, MG-H1, rich in living organisms, was produced in as short a period as 7 days, in addition to CML and CMA, which have been reported previously [9, 38]. Furthermore, the possibility of using ELISA to search for compounds inhibiting AGE formation, such as TBE, was supported by the quantification of AGEs using mass spectrometry.

Conflict of interest declaration

None for this study.

Funding support

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (JSPS KAKENHI #20K05895).

Acknowledgement

The publication of this study was supported by the Isyoku-Dogen Research Foundation.

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