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Rapid Identification of Methylglyoxal Trapping Constituents from Onion Peels by Pre-column Incubation Method

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Abstract – The methylglyoxal (MGO) trapping constituents from onion (*Allium cepa* L.) peels were investigated using pre-column incubation of MGO and crude extract followed by HPLC analysis. The peak areas of MGO trapping compounds decreased, and their chemical structures were identified by HPLC-ESI/MS. Among major constituents in outer scale of onion, 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone (**2**) was more effective MGO scavenger than quercetin (**6**) and its 4'-glucoside, spiraeoside (**3**). After 1 h incubation, compound **2** trapped over 90% MGO at a concentration of 0.5 mM under physiological conditions, but compounds **3** and **6** scavenged 45%, 16% MGO, respectively. HPLC-ESI/MS showed that compound **2** trapped two molecules of MGO to form a di-MGO adduct and compounds **3** and **6** captured one molecule of MGO to form mono-MGO adducts, and the positions 6 and 8 of the A ring of flavonoids were major active sites for trapping MGO.
Keywords – *Allium cepa* L., Methylglyoxal trapping, Pre-column incubation, Flavonoids

Introduction

Methylglyoxal (MGO), known as one of the most important reactive carbonyl species, is believed to contribute significantly to intracellular advanced glycation end products (AGEs) due to its high reactivity and generation of multiple organs under *in vivo* conditions.[1-4] The dicarbonyl group of MGO can react and modify certain proteins and other biomolecules, which resulted in alterations of their normal functions and finally caused a variety of important diseases associated with diabetes and aging.[5] Moreover, MGO-derived AGEs have been demonstrated to be involved in the pathogenesis of diabetic complications and other conditions such as atherosclerosis and neurodegenerative diseases.[1-5] Recently, MGO scavengers are attracting increased attention because of their possible clinical significance in chronic and age-related diseases. Several pharmacological reagents such as aminoguanidine have been investigated for inhibiting the AGEs formation and developed for diabetic complications by trapping reactive dicarbonyl species. However, these agents have serious side effects.[6] Therefore, natural products with less toxicity may be alternative for the prevention and/or treatment of MGO-associated diseases. Several natural compounds with MGO trapping activities have been also studied, such as phloretin,[7] quercetin,[8] kaempferol,[9] genistein,[3] catechins,[10,11] stilbenes,[2] curcumins,[12] proanthocyanins,[13] anthocyanins[14] and gingerols.[15]

Onion (*Allium cepa* L.) is one of the most important vegetables worldwide and is one of the major sources of dietary flavonoids in many countries. It shows a variety of pharmacological effects such as growth inhibition of tumor and microbial cells, reduction of cancer risk, scavenging of free radicals, and protection against cardiovascular disease, which are attributed to specific sulfur-containing compounds and flavonoids.[16] The major flavonoids found in onion are quercetin and its glycosides.[16,17] Onion peels, containing high levels of flavonoids, also inhibited protein glycation *in vitro* model.[18]

In this study, MGO trapping constituents in the onion peels were investigated by pre-column reaction of MGO and onion extract followed by HPLC analysis. The peak areas of MGO trapping compounds decreased, and their chemical structures were identified by HPLC-ESI/MS.

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Furthermore, purified compounds were also evaluated their MGO trapping activity and their trapping mechanism also explored by HPLC-DAD-ESI/MS.

Experimental

Chemical and reagents – Methylglyoxal (40% aqueous solution), *o*-phenylenediamine, 2-methylquinoxaline, 5-methylquinoxaline and formic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC grade acetonitrile and water, and analytical grade organic solvents were purchased from Daejung Chemical (Gyonggi-do, Korea). All other chemicals used were of analytical grade and from commercial sources.

Preparation of crude extract – Onions were purchased local market, and its peels (100 g) were extracted with ethanol under the reflux for 3 h three times. The combined filtrate was concentrated by rotary evaporator and freeze dried to yield 5.2 g crude extract and then stored at –20 ºC until required.

MGO incubation experiment – The crude onion peel extract (10 mg/mL) was dissolved in PBS (100 mM, pH 7.4) and centrifuged to remove undissolved materials. This crude extract was incubated with 5.6 mM MGO in PBS at 37 ºC and kept for 12 h. After incubation, 500 mL MeOH was added and then analyzed by HPLC. Control experiments with PBS were parallelly carried out for comparison. The chromatographic peaks were identified by online HPLC-DAD-ESI/MS analysis.

HPLC-DAD-ESI/MS analysis – An Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an Advion expression CMS mass spectrometry (Advion, Ithaca, NY) was used to identify major compounds of onionpeel extract. The HPLC conditions were as follows: an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) with an INNO column C18 (2.0 mm × 150 mm, Young Jin Biochrom, Gyeonggi-do, South Korea) was used at a flow-rate of 0.2 ml/min at 40 ºC. The mobile phase consisted of acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B). A linear gradient system was used as follows: 0 – 5 min, 10 – 10% A; 5 – 50 min, 40% A. The chromatographic profile of the effluents was recorded at 280 nm with a spectrum ranging from 210 to 400 nm. The ESI-MS spectra conditions were as follows: negative ion mode; mass range, m/z 100 - 1200; capillary temperature, 250 ºC; capillary voltage, 180 V; source voltage offset, 32 source voltage span, 10; source gas temperature, 250 ºC; ESI voltage, –2.5 kV.

Kinetics of MGO scavenging assay – Considering MGO co-eluted with the mobile phase, this dicarbonyl was allowed to react with *o*-phenylene diamine to form stable 2-methylquinoxaline for reliable quantification by HPLC.[3] Briefly, MGO (0.33 mM) was incubated with PBS (control), or onion extract (0.05, 0.25 and 0.5 mg/ml) or isolated compounds (0.25, 0.5 mM) in PBS (100 mM, pH 7.4) at 37 ºC and kept for 0 – 4 h, respectively. After incubation, *o*-phenylene diamine (derivatization agent, 10 mM) were added to each test solution and shaken by vortex for 5s. The mixtures were kept at room temperature for 30 min for derivatization of the remaining MGO to complete.

HPLC analysis – The levels of 2-methylquinoxaline were analyzed using an Agilent 1260 HPLC system with an Atlantis T3 C18 column (2.1 mm × 150 mm, 3 μm, Waters). The mobile phase consisted of acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B). A linear gradient system was used as follows: 0 – 5 min, 0 – 23% A; 5 – 25 min, 23% A. The injection volume was 10 μl. The chromatographic profile of the effluents was recorded at 226.8 nm and 316 nm.

Result and Discussion

The kinetic curve of trapping MGO by onion peel extract during reaction time (up to 4 h) was investigated (Fig. 1). After reacting with onion peel extract (0.5 mg/ml) and MGO, the relative amount of remaining MGO were from 41.9 to 9.37% at 0.5 h and 4 h, respectively. The onion peel extract showed a significant MGO scavenging effect. After confirmation of MGO trapping

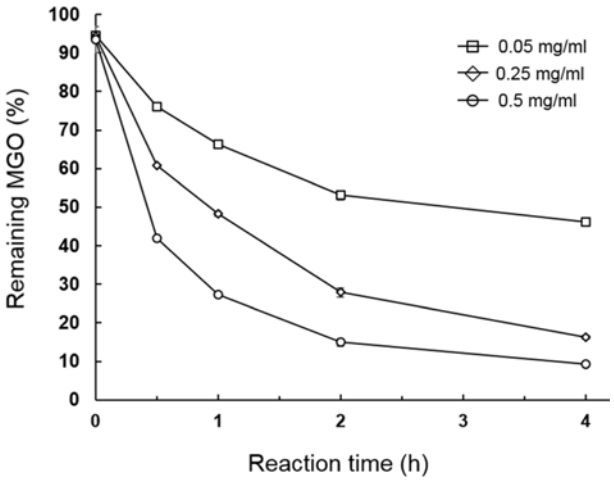

Fig. 1. Scavenging of MGO by onion peel extracts (0.05, 0.25 and 0.5 mg/ml) under physiological conditions (pH 7.4, 37 ºC). Data are presented as the mean ± S.E. of three replications.

Table 1. Peak assignment for the components from onion peel extract

No.	Retention time (min)	Compound identity	()ESI-MS *m/z*	UV $\ddot{e}_{max}$ (nm)
1	6.20	protocatechuic acid	152.7 $[M-H]^-$	259, 293
2	16.6	2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone	316.8 $[M-H]^-$	292
3	30.2	spiraeoside (quercetin 4'-*O*-β-D-glucopyranoside)	463.0 $[M-H]^-$	253, 368
4	35.8	condensation products of quercetin with protocatechuic acid	452.8 $[M-H]^-$	291
5	36.5	condensation products of quercetin with protocatechuic acid	452.8 $[M-H]^-$	292
6	37.7	quercetin	300.9 $[M-H]^-$	255, 367
7	44.2	adduct of quercetin with quercetin 4'-*O*-β-D-glucopyranoside	762.8 $[M-H]^-$	303, 369
8	48.3	4'-*O*-β-D-glucopyranoside of quercetin dimer	762.8 $[M-H]^-$	303, 364

Peak assignment for the components from onion peel extract.
The compounds No. are the same as in Fig. 2.

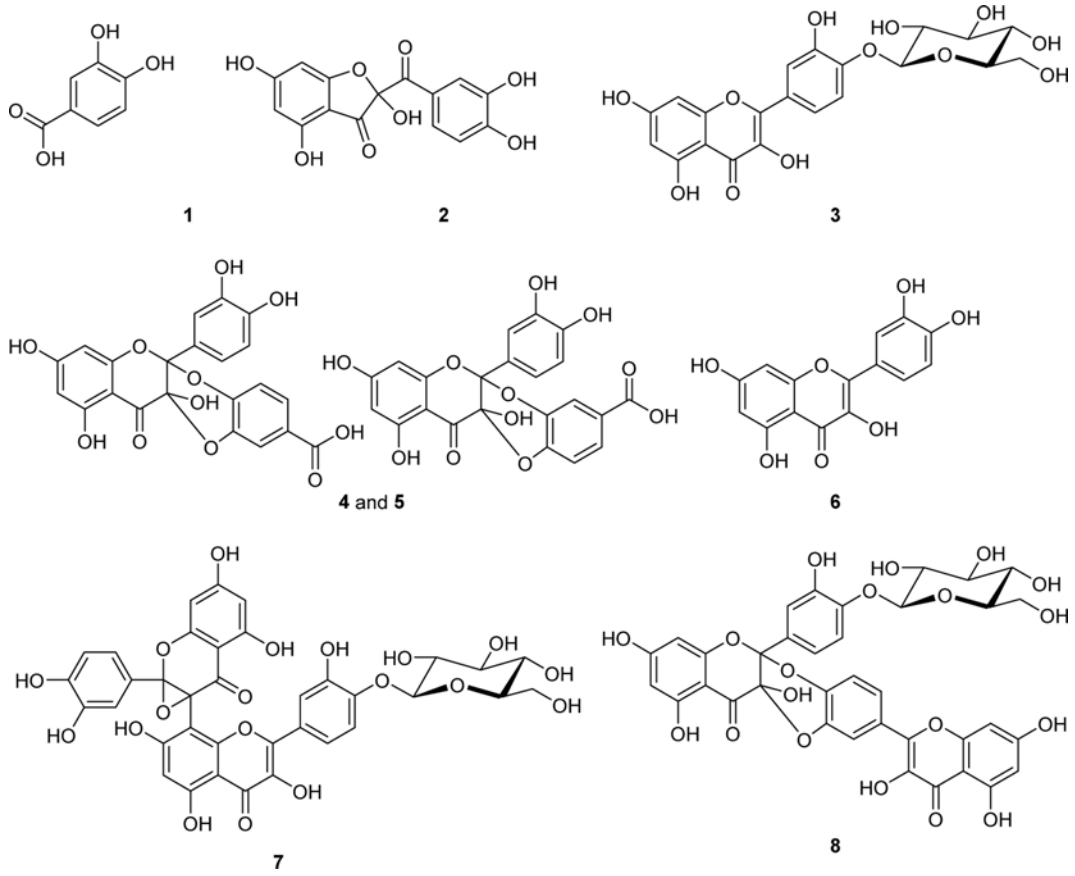

Fig. 2. Chemical structures of the identified components from onion peel extract. The compounds No. are the same as in Fig. 3. and Table 1.

effects, HPLC-DAD-ESI/MS analysis was performed to investigate constituents of onion extract, to led identification of eight compounds by comparing the data of retention times, UV absorption characteristics, and the negative MS values with those in the literatures.[16] The results were listed in Table 1 and an overview of identified compounds was given in Fig. 2.

The aldehyde group in MGO with strong electrophilicity could react target compounds to form new adducts, so such compounds were considered to possess MGO scavenging effect. Once reacted, the amount of both MGO and the target compounds would decrease, while the new MGO adducts would increase accordingly. Based on this, MGO scavengers could be traced in crude extract

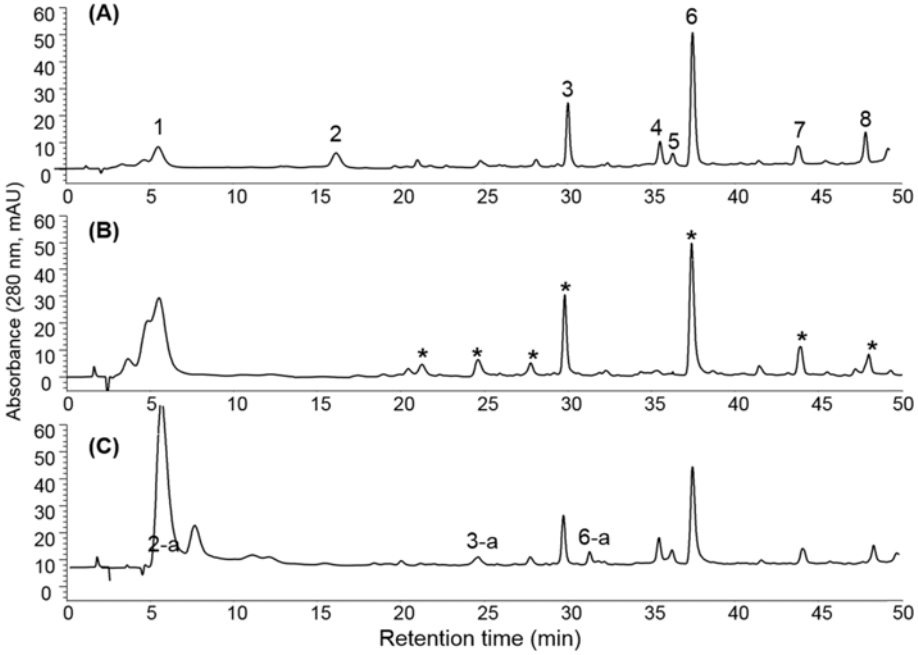

Fig. 3. HPLC-DAD chromatograms of the onion peel extract. Onion peel extract dissolved in methanol (A), after incubation with PBS (100 mM, pH 7.4) for 12 h (B) and 10 mM MGO for 12 h, in PBS (C) at 37 °C. The peaks designated as asterisk (*) were potential MGO scavengers. Peaks 2-a, 3-a and 6-a appeared after reaction.

by pre-column incubation with MGO (or PBS) and crude extract and HPLC peak analysis. In the present study, after incubation of onion peel extract with MGO or blank PBS for 12 h, the reaction product mixtures were analyzed using HPLC-DAD-ESI/MS under the same conditions. Most of the compounds except compound **2** were stable in PBS (100 mM, pH 7.4) at 37 °C for 12 h compared in ethanol solution (Fig. 3A and B). Fig. 3B and C displayed the differences in HPLC-DAD chromatograms monitored at 280 nm after incubation with control PBS (B) or MGO (C) for 12 h. As shown in Fig. 3B., it revealed that the peak areas of compounds marked asterisk (*) significantly decreased or even disappeared with MGO reaction. Therefore, considering the efficiency and effectiveness of such screening assay, in this study, onion extract was incubated with 5.6 mM MGO for 12 h to ensure the discovery of all potential bioactive compounds with stronger MGO scavenging effects. Finally, combined with structural identification data mentioned above, three major flavonoids in an onion peel were regarded as the potential candidates of MGO scavenger. Among these, compounds **2**, **3** and **6** were further isolated and used to evaluate MGO scavenging effects and MGO-adducts experiments, after determination of their chemical structures by NMR spectroscopic data.[16,17] Compounds **2**, **3** and **6** (at 0.5 mM concentration) significantly and time-dependently decreased MGO under physiological conditions, remaining 8.7, 46.7 and 23.8%, respectively, at 4 h of reaction time. Compound **2** showed higher reactivity than others with MGO, as incubated at a concentration of 0.5 mM, only 5% MGO remained at 0.5 h of reaction time. Quercetin (**6**) and its 4'-glucoside, spiraeoside (**3**) also scavenged MGO but reactivity was less than compound **2** (Fig. 4). Then, each reaction mixture of compound **2**, **3** and **6** with MGO (0.5 mM: 0.33 mM) were analyzed by LC/MS. The structural information of these MGO-adducts were obtained using LC/MS analysis. The quasi-molecular ion was obtained *m*/*z* 460.7 $[M-H]^-$, which is 144 mass units higher than that of compound **2** (*m*/*z*318) indicating that this is di-MGO adduct of compound **2** (molecular weight of MGO is *m*/*z* 72). However, incubation of compound **3** or **6** with MGO revealed that addition of 72 mass units, indicating that they are mono-MGO adducts of compounds **3** and **6** (Fig. 5).

Previous studies[19] have confirmed that two unsubstituted positions 6 and 8 in A-ring of flavonoids were the major active sites to trap reactive dicarbonyl species and form mono- and di-MGO adducts, owing to the high electron density upon dissociation of a hydrogen atom from two hydroxyl groups, especially under alkaline conditions. In contrast, the electron density of unsubstituted carbons of B-ring, with two hydroxyl groups substituted at the *ortho*-position, was not strong enough to trap reactive dicarbonyl species in flavonoids. On the basis of these findings, the

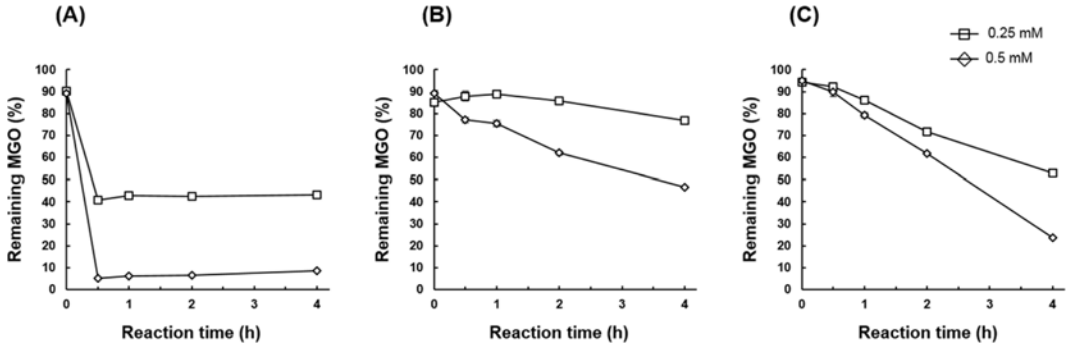

Fig. 4. MGO scavenging capacity of isolated compounds **2**, **3** and **6** under physiological conditions (pH 7.4, 37 °C). Purified compounds **2**, **3** and **6** (0.25 or 0.5 mM) were incubated with MGO (0.33 mM) for up to 4 h. (A) 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone (**2**). (B) spiraeoside (**3**). (C) quercetin (**6**).

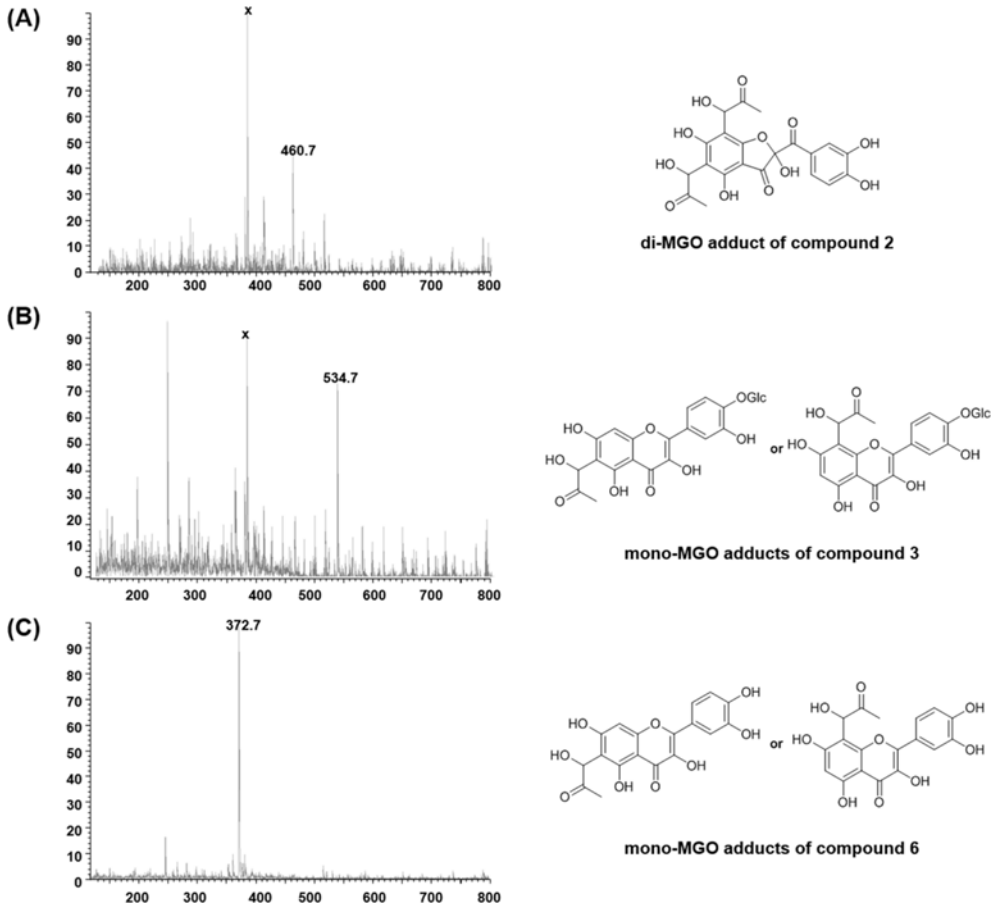

Fig. 5. MS spectra of MGO adducts and proposed structures mono- or di-MGO adducts of compounds **2** (A), **3** (B) and **6** (C) under physiological conditions (pH 7.4, 37 °C).

two unsubstituted carbons at the A-ring (carbons 6 and 8; *meta*-position) were highly possible MGO trapping sites and the reaction mechanisms of flavonoids in onion peels.

The aim of this study is to determine MGO trapping compounds from natural products which consequently reduce accumulation of AGEs. If the active compounds react differently *in vitro* or *in vivo* studies, this study is problematic for application to metabolism in the body. Fortunately, when genistein was administered to mice, MGO-adducts were identified in mice urine samples which exerted the potential for MGO trapping compounds.[20] In addition (+)-catechin trapped MGO in diabetic mice and reduced AGEs accumulation and RAGE expression in diabetic kidneys.[21]

In conclusion, MGO trapping assay by pre-column incubation is useful to identify MGO scavengers from natural products that are effective in reducing AGEs accumulation.

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