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Original Article

Inhibitory effect of rosehip (*Rosa canina* L.) on the formation of advanced glycation endproducts by scavenging reactive carbonyls

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Abstract

The accumulation of advanced glycation endproducts (AGEs) and carbonyl stress *in vivo* are associated with diabetic complications. The objective of this study was to investigate the inhibitory effect of an aqueous extract of rosehip (*Rosa canina* L.) on the formation of AGEs derived from reactive carbonyl species such as methylglyoxal (MGO). A water extract of rosehip exhibited anti-glycation activity in the MGO-BSA assay and a protective effect against reactive carbonyl species in a direct MGO-scavenging assay with IC_{50} values of 3.7 mg/mL and 2.7 mg/mL, respectively. To determine the active substances present in the extract, activity-guided fractionation was performed. The extract was divided into water and butanol layers. The water layer was further fractionated into five subfractions by DIAION HP-20 column chromatography. The twenty-five percent ethanol eluate had high anti-glycation activity (IC_{50} = 1.0 mg/mL) and direct MGO-scavenging activity (IC_{50} = 1.0 mg/mL). HPLC analysis revealed that the eluate was mainly composed of procyanidin glycosides with a number-average molecular weight of 3,500. These results suggest that this aqueous rosehip extract could be beneficial as a natural source of a glycation inhibitor because procyanidin glycosides present in the extract inhibited the formation of AGEs through their direct carbonyl scavenging activities.

***KEY WORDS*:** Glycation, methylglyoxal, *Rosa canina* L., proanthocyanidin, carbonyl scavenger

Introduction

Advanced glycation endproducts (AGEs) are the final products derived from non-enzymatic glycation and oxidation reactions of sugars with the amino groups of proteins, lipids and nucleic acids [1)]. The accumulation of AGEs has been implicated as a major pathogenic process in diabetic complications [2)], normal aging [3)], and many other chronic diseases such as Alzheimer's disease [3)] and atherosclerosis [4)].

Dicarbonyl intermediates such as methylglyoxal (MGO), glyoxal, and 3-deoxyglucosone in the glycation reaction are known as precursors of AGEs because of their high abilities to crosslink with amino groups of proteins to form irreversible AGEs [5)]. Increased levels of MGO have been observed in the blood or tissues of diabetic patients with complications, and have been reported to be an important factor which causes insulin resistance [5)]. The chronic exposure to high concentration of MGO has been thought to induce insulin signaling impairment and apoptosis [5)] and to be linked to the development of diabetic complications [6)].

AGEs inhibitors which possess reactive carbonyl scavenging capacities have been reported to be effective in the prevention of MGO-associated impairment of insulin signaling pathways [5)] and in the amelioration of diabetic complications [1)]. Therefore, inhibiting the formation of AGEs by scavenging reactive carbonyl species (especially MGO) could be a promising therapeutic approach to treat diabetes and prevent diabetic complications.

In traditional medicine, rosehip has been used for the prevention and therapy of the common cold, flu, and diabetes in many European countries [7)]. Biological activities of rosehip which have been reported include anti-oxidative [8)], anti-obesity [9)], anti-inflammatory [10)], and melanin production inhibitory [11)] activities. Rosehip is rich in vitamin C, carotenoids, and phenolic compounds such as quercetin, ellagic acid, hydroxycinnamic acids, and proanthocyanidin aglycones [12, 13)]. Recently, rosehip and its constituent *trans*-tiliroside, a major glycosidic flavonoid isolated from rosehip seed, were reported to exert a blood glucose lowering effect

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after glucose loading in mice [14] and hypoglycemic effects in diabetic rats [15]. However, no study has been conducted to investigate the capacity of rosehip to attenuate carbonyl stress and protein glycation. The objective of this study was to evaluate the anti-glycation and carbonyl scavenging activities of rosehip.

Materials and Methods

Materials

Methylglyoxal (MGO, 40% aqueous solution), aminoguanidine, and polystyrene standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin fraction-V fatty acid-free was from Millipore (Billerica, MA, USA). Procyanidin B2, cyanidin, and pelargonidin were purchased from Extrasynthese (Genay, France). Girard's T reagent was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ammonium ferric sulfate was obtained from Wako Pure Chemical Industries (Osaka, Japan). Delphinidin was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Extraction and fractionation of rosehip

Rosehip powder (50 g) was extracted with 500 mL of distilled water for 1 hour at 95°C after rapid washing with 200 mL of distilled water to remove excess sugar. The extract was mixed with an equal volume of water-saturated *n*-butanol and divided into the water and butanol layers. This liquid-liquid extraction was repeated three times. Each layer was dried under reduced pressure. The water layer dissolved in water was loaded onto a DIAION HP-20 (Mitsubishi Kagaku Co., Ltd., Tokyo, Japan) column. The column was eluted with water, 5% ethanol, 25% ethanol, 50% ethanol, and 99.5% ethanol.

Glycation assay with MGO-BSA

This assay was performed as described previously [16] with slight modifications. Bovine serum albumin (BSA) and MGO were dissolved in 100 mM sodium phosphate buffer (pH 7.4) containing 0.2 g/L sodium azide at a concentration of 20 mg/mL and 60 mM, respectively. BSA solution (50 µL) was reacted with 50 µL of MGO solution in the presence of 50 µL of rosehip samples, aminoguanidine (positive control), or phosphate buffer as a control at 37°C for 7 days. To remove unreacted MGO and interfering substances, 17 µL of 100% w/w trichloroacetic acid (TCA) was added to the reaction mixture. After centrifugation, the TCA precipitate containing fluorescent AGEs-BSA was washed with phosphate buffer and dissolved in 200 µL of alkaline phosphate buffer (pH 10). The fluorescence intensity of this solution was measured using a Synergy H4 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The excitation and emission wavelengths used were 340 nm and 420 nm, respectively. Phosphate buffer was used as the blank. The apparent inhibitory effect was obtained from the following equation: apparent inhibition rate (%) = {1 – (fluorescence of test sample solution) / (fluorescence of control solution)} × 100.

To subtract the contribution of co-precipitated substances with AGEs-BSA from the obtained fluorescence, the quenching rate was then estimated as follows [17]. BSA was reacted with MGO in the absence of rosehip samples as described above. The AGEs-BSA solution obtained was mixed with the rosehip sample for 3 hours at room temperature to allow adsorption to AGEs-BSA. After TCA treatment, the sample was dissolved in alkaline phosphate buffer. The fluorescence intensity of this solution was measured using the microplate reader. The quenching rate was calculated using the following equation: quenching rate (%) = {1 – (fluorescence of test sample solution) / (fluorescence of control solution)} × 100.

The actual inhibition activity was calculated by subtracting the quenching rate from the apparent inhibition rate.

MGO-scavenging assay

The MGO-scavenging capacity was evaluated by measuring MGO consumption after incubating the rosehip samples with MGO [18]. MGO concentration was measured using Girard's T reagent. Phosphate buffer was used as the blank. Twenty-five microliters of 2 mM MGO solution was mixed with 25 µL of test samples, aminoguanidine (positive control), or phosphate buffer as a control under the conditions of 0.1 M sodium phosphate (pH 7.4) at 37°C for 1 hour, followed by the addition of 950 µL of 120 mM sodium borate. An aliquot of the mixture (200 µL) was reacted with 800 µL of 100 mM Girard's T reagent for 10 minutes at room temperature. Absorbance at 326 nm was measured using a UV-VIS spectrometer Evolution 220 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The MGO-scavenging capacity was calculated using the following equation: MGO-scavenging capacity (%) = [1 – {(absorbance of test sample solution with MGO) – (absorbance of test sample solution without MGO)} / (absorbance of control solution)] × 100.

Determination of proanthocyanidin content

To determine the proanthocyanidin content in the rosehip samples, the acid-butanol assay was performed as described previously [11]. Fifty-eight microliters of the samples was mixed with 1 mL of 0.6 N HCl-containing butanol and 16 µL of 0.04 M ammonium ferric sulfate solution. Then, the mixture was incubated for 50 minutes at 95°C. Absorbance at 555 nm was measured. Quantification of proanthocyanidins was achieved with procyanidin B2 as the standard.

Qualitative analysis of proanthocyanidin

To characterize proanthocyanidins present in the rosehip samples, HPLC analysis of the acid hydrolysate of the rosehip sample was performed [19]. One milligram of the rosehip sample dissolved in 1 mL of 1 N HCl was hydrolyzed for 1 hour in boiling water. The hydrolysate was evaporated to dryness and dissolved in methanol. The type of proanthocyanidin in the sample was analyzed by an Agilent 1260 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with an Agilent Poroschell 120 EC-C18 column (4.6 mm × 50 mm, 2.7 µm) and a photodiode array detector at 30°C with anthocyanidins (cyanidin, delphinidin, and pelargonidin) as standards. The elution was achieved under the conditions: linear gradient of 5% to 40% B in 60 minutes with (A) 0.1% formic acid in water, (B) acetonitrile at a flow rate of 0.5 mL/min. The detection wavelength was 550 nm.

Determination of total phenolic content

The amount of total phenolics in the rosehip samples

was determined according to the Folin-Ciocalteu method [20] with procyanidin B2 as a standard. Five hundred microliters of a rosehip sample or standard solution, 250 μL of aqueous solution of Folin-Ciocalteu reagent (1:1, v/v), and 2.5 mL of 0.4 M sodium carbonate were mixed and incubated at 30°C for 30 minutes. Then, the optical density at 765 nm was measured using a UV-VIS spectrometer.

Gel permeation chromatography

To determine the molecular weight profile of proanthocyanidins in the rosehip sample, gel permeation chromatography (GPC) was performed with polystyrene standards and a catechin standard [21]. The rosehip sample dissolved in pyridine-acetic anhydride (1:1, v/v) was acetylated overnight at room temperature. Excess reagent was destroyed by adding water. The resulting precipitate containing acetylated-proanthocyanidins was recovered by centrifugation and dried under reduced pressure. The acetylated-proanthocyanidins were dissolved in tetrahydrofuran (THF) and analyzed by an Agilent 1260 HPLC system equipped with a Shodex KF-804L column (8.0 × 300 mm, Showa Denko K.K., Tokyo, Japan) at 40°C. The elution was isocratic with THF at a flow rate of 0.8 mL/min. The detection wavelength was 254 nm. The number-average molecular weight (Mn), the weight-average molecular weight (Mw), and the polydispersity index (Mw/Mn) of the sample were calculated by a GPC data processing program (SIC μ7 Data Station, System Instruments Co., Ltd., Tokyo, Japan).

Determination of total sugar content

The total sugar content in the rosehip sample was measured by the phenol-sulfuric acid method with glucose as a standard [11]. A 500 μL amount of samples, 500 μL of 5% phenol, and 2.5 mL of sulfuric acid were mixed and incubated for 30 minutes at room temperature. The total sugar concentration was determined by spectrophotometry at a 490 nm wavelength.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons using R (The R Foundation for Statistical Computing, http://www.r-project.org). All p-values < 0.05 were considered as statistically significant.

Results

Anti-glycation and carbonyl scavenging activities of rosehip water extract

To evaluate the anti-glycation activity of rosehip water extract (RE), the MGO-BSA assay was performed. Aminoguanidine (AG), which is a scavenger of α, β-dicarbonyls that prevents the formation of AGEs, was used as a positive control. ***Figure 1A*** indicates that RE inhibited MGO-mediated AGEs formation in a concentration dependent manner (IC_{50} = 3.7 mg/mL). Next, we investigated whether reactive carbonyl species such as MGO interact directly with constituents in rosehip extract.

A concentration dependent MGO-scavenging activity of RE with an IC_{50} value of 2.7 mg/mL was observed as shown in ***Fig. 1B***. These results suggested that RE possesses an inhibitory effect on the formation of AGEs and has direct MGO-scavenging capacity.

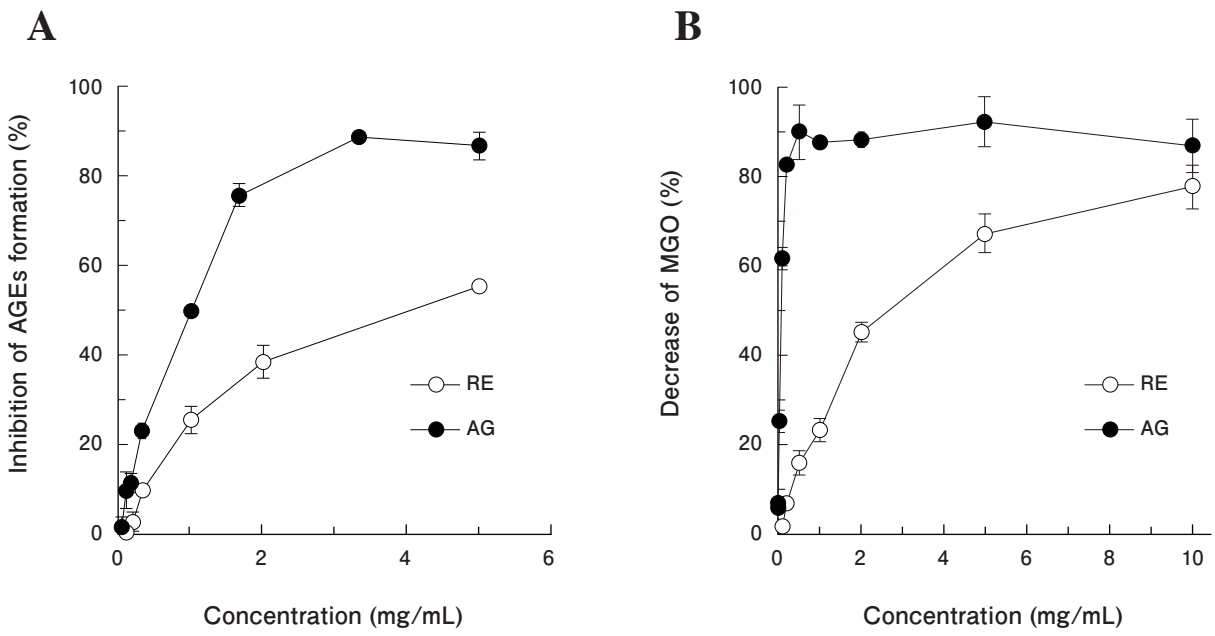

Fig. 1. **Anti-glycation (A) and MGO-scavenging (B) activities of rosehip extract.**
Results are expressed as the mean ± standard deviation of triplicate experiments. MGO, methylglyoxal; AG, aminoguanidine; RE, rosehip water extract.

Activity-guided fractionation of rosehip extract

To determine the active substances in RE, activity-guided fractionation was performed based on two *in vitro* assays (the MGO-BSA and direct MGO-scavenging assays). RE was first partitioned into two layers by the addition of water-saturated *n*-butanol. The water layer showed significantly higher activities compared to the butanol layer with a 53% inhibition in the MGO-BSA assay (***Fig. 2A***) and a 71% decrease in the MGO-scavenging assay (***Fig. 2B***). The butanol layer had only a 16% inhibition in the MGO-BSA assay and an 11% decrease in the MGO-scavenging assay. Subsequently, the water layer was further fractionated by DIAION HP-20 column chromatography. Anti-glycation and MGO-scavenging activities of the 25% ethanol eluate (REW25E) were significantly higher than that of the other eluates, but not significantly higher than that of RE or the water layer (***Figs. 2A*** and ***2B***). The activities of REW25E were concentration dependent, with IC_{50} values of 1.04 mg/mL and 1.02 mg/mL in the MGO-BSA and direct MGO-scavenging assays, respectively (***Figs. 3A*** and ***3B***). These results suggested that active substances are present in REW25E.

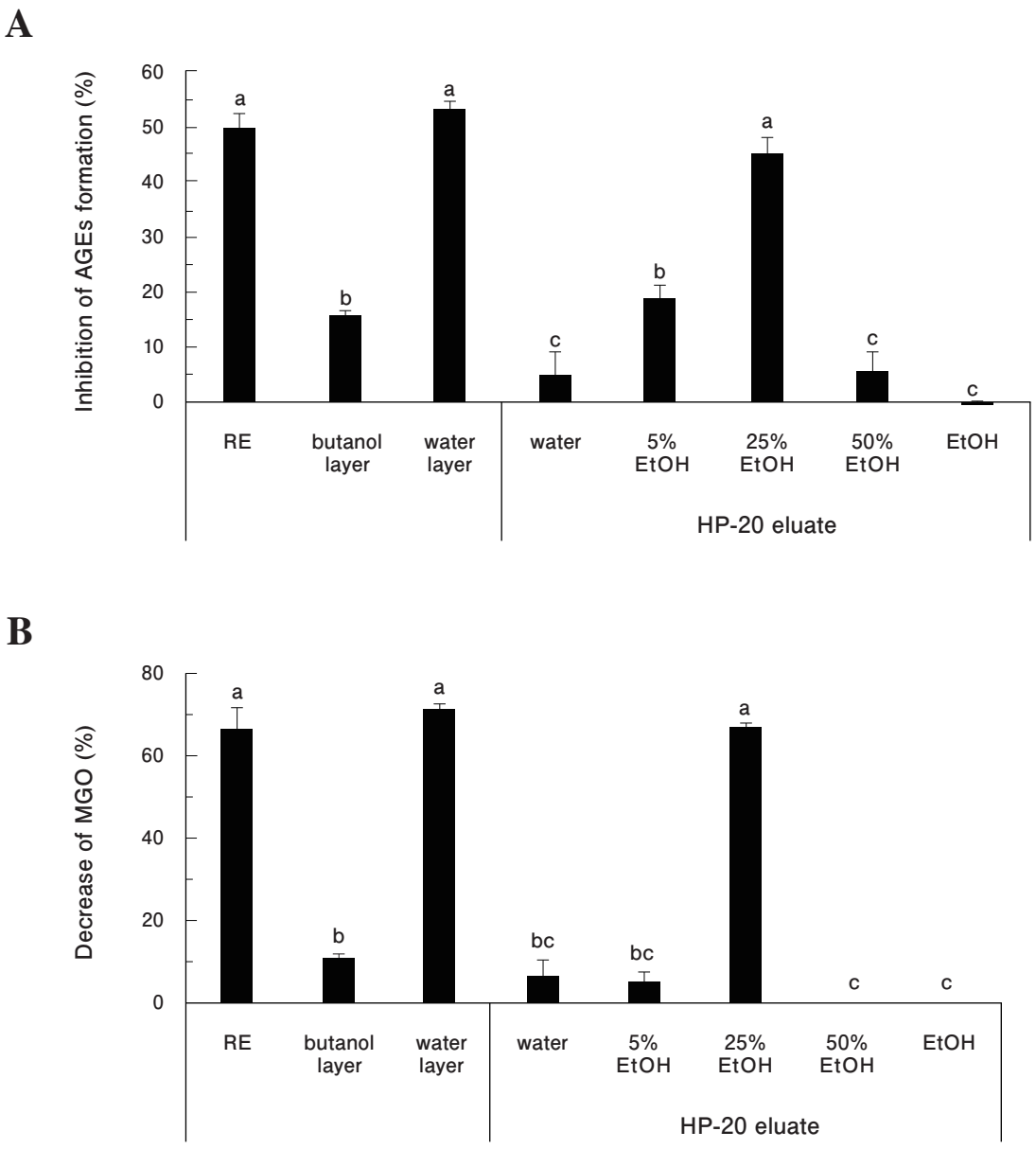

Fig. 2. **Anti-glycation (A) and MGO-scavenging (B) activities of rosehip fractions.**

Results are expressed as the mean ± standard deviation of triplicate experiments. Concentrations assayed were based on the yields of fractions and were 5, 0.37, 4.49, 3.01, 0.21, 0.91, 0.04 and 0.46 mg/mL for RE, butanol layer, water layer, water, 5% ethanol (EtOH), 25% EtOH, 50% EtOH and EtOH, respectively. Different letters above the columns represent a statistically significant difference ($p < 0.05$) by Tukey's test. AGEs, advanced glycation endproducts; MGO, methylglyoxal; RE, rosehip water extract.

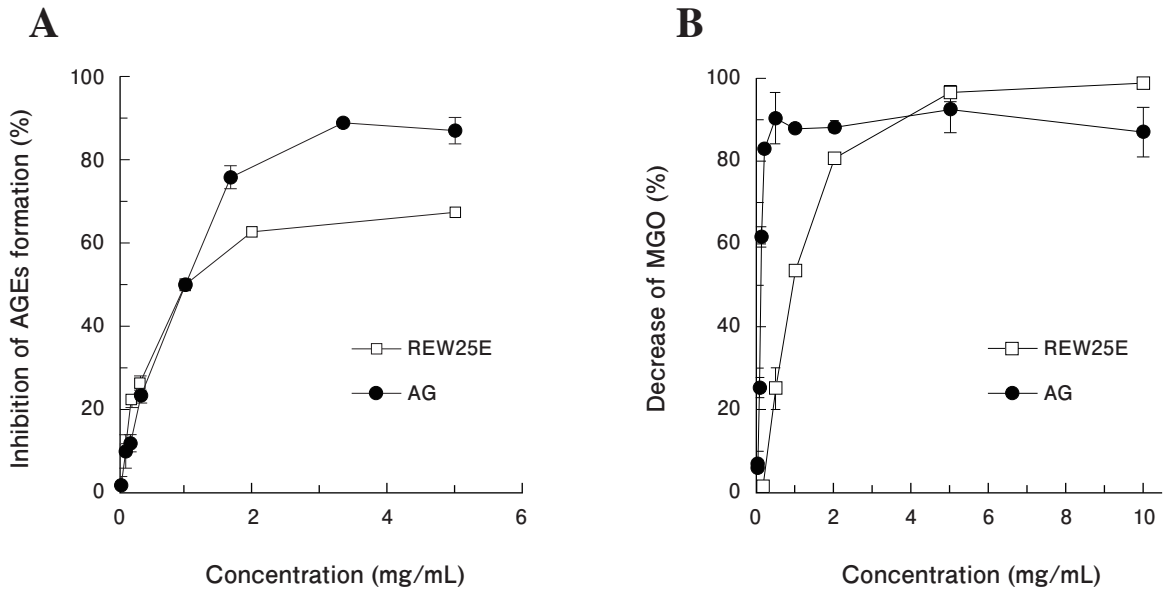

Fig. 3. **Anti-glycation (A) and MGO-scavenging (B) activities of REW25E.**

Results are expressed as the mean ± standard deviation of triplicate experiments. AGEs, advanced glycation endproducts; MGO, methylglyoxal; AG, aminoguanidine; RE, rosehip water extract; REW25E, active fraction of RE.

Compositional properties of the REW25E fraction

Active substances in REW25E were assumed to be hydrophilic phenolic compounds such as proanthocyanidin because of their non-extractability in butanol and adsorption to the aromatic non-polar surface of HP-20 resin that adsorbs aromatic molecules by van der Waal's forces. To clarify the substances, we measured the total phenolics and proanthocyanidins in the fraction (***Table 1***). REW25E contained 61.6% phenolic compounds and 48% proanthocyanidins, indicating that most of the phenolic compounds in REW25E were proanthocyanidins (proanthocyanidins/total phenolics = 78%), as expected. Total sugar analysis showed that REW25E contained 38.8% sugars. These results indicated that the constituents in REW25E are mainly composed of proanthocyanidins and sugars.

Table 1. Constituents of each fraction obtained from RE.

	total phenolics (w/w%)	proanthocyanidins (w/w%)	total sugar (w/w%)
RE	19.9	7.0	
butanol layer	ND	ND	
water layer	17.5	3.4	
water eluate	ND	ND	
5% EtOH eluate	57.3	34.1	
25% EtOH eluate	61.6	48.0	38.8
50% EtOH eluate	58.2	46.8	
EtOH eluate	28.8	10.9	

Results are expressed as the mean of triplicate experiments. ND, not detected; RE, rosehip water extract; EtOH, ethanol.

Qualitative analysis of proanthocyanidins in REW25E

To characterize the proanthocyanidins in REW25E, HPLC analysis of the HCl-hydrolysate of REW25E was performed. HPLC profiles of the hydrolysate (upper) and anthocyanidin standards (lower) are shown in ***Fig. 4***. The single peak observed in the hydrolysate was identified as cyanidin by comparison of the retention time with that of anthocyanidin standards. This result indicated that the proanthocyanidins in REW25E were procyanidin-type, *i.e.* (+)-catechin and/or (–)-epicatechin derivatives.

The molecular weight profiles of REW25E were obtained by GPC. As shown in ***Fig. 5***, REW25E had a molecular weight distribution ranging from about 1,000 to 20,000. The average molar mass (Mw, Mn) and polydispersity (Mw/Mn) of the REW25E proanthocyanidins were 6,000 (Mw), 3,500 (Mn), and 1.7, respectively.

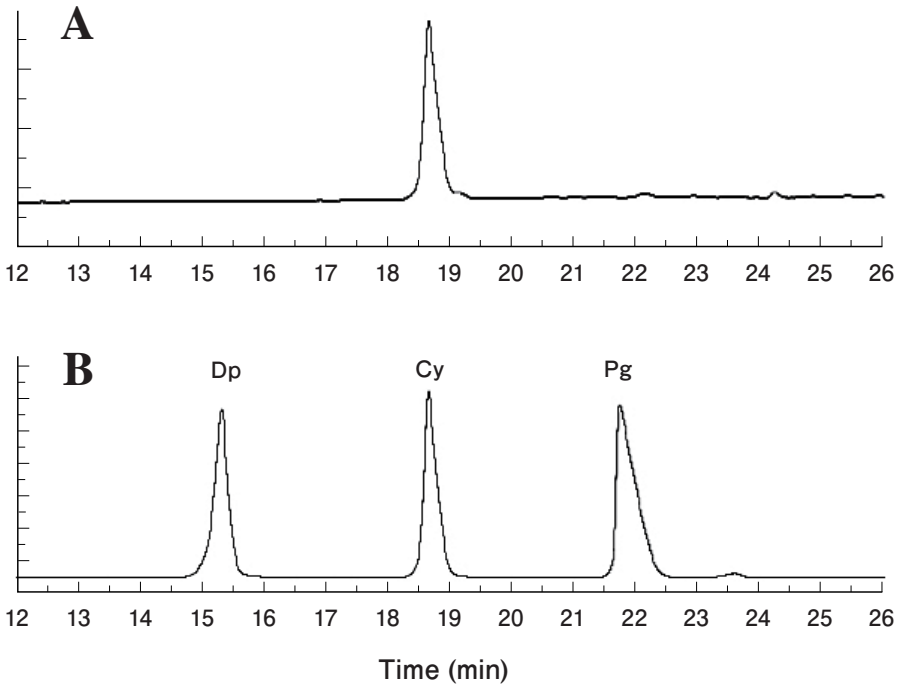

Fig. 4. **HPLC profiles of acid hydrolysate of REW25E (A) and anthocyanidin standards (B).**
Dp, delphinidin; Cy, cyanidin; Pg, pelargonidin. HPLC, high performance liquid chromatography; RE, rosehip water extract; REW25E, active fraction of RE.

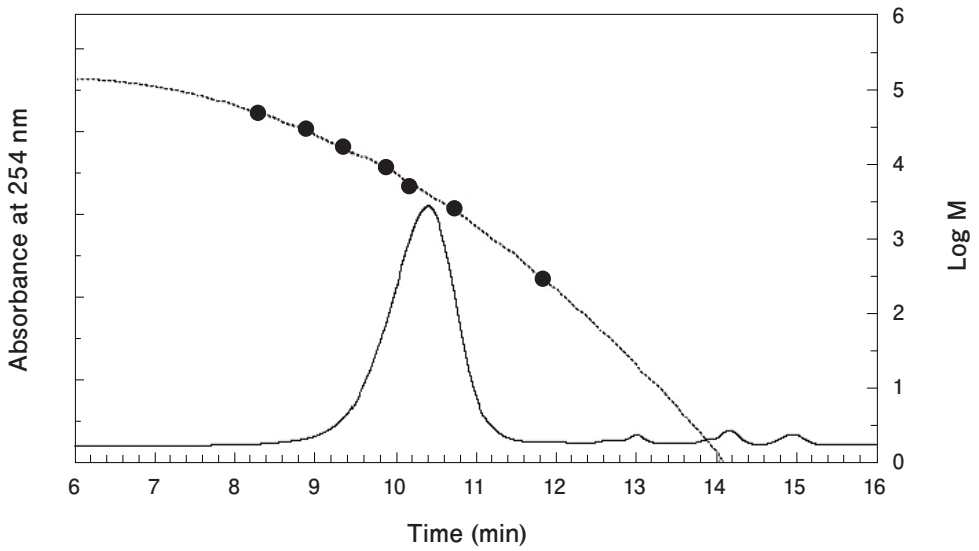

Fig. 5. **Chromatogram of acetylated REW25E and molecular weight calibration curve.**
A molecular weight calibration curve (dashed line) was obtained with six standard polystyrenes (50,000, 30,000, 17,500, 9,000, 5,000, and 2,500 g/mole) and catechin (290 g/mole). M, molecular weight; RE, rosehip water extract; REW25E, active fraction of RE.

Discussion

The accumulation of AGEs and carbonyl stress have been implicated in the pathogenic process of diabetic complications. AGEs inhibitors discovered so far exert their activities through scavenging reactive oxygen species, carbonyl scavenging, metal ion chelating, or AGEs cross-links breakage [22]. Anti-oxidant capacity has been highly correlated with AGEs inhibitory activity *in vitro* [23]. However, recent clinical studies were not able to provide clear evidence for the efficacy of natural anti-oxidant therapy in diabetic patients [24]. In contrast, AGEs inhibitors possessing a carbonyl scavenging capacity demonstrated their efficacy for the treatment of diabetic complications in a clinical report [1].

The main objective of this study was to evaluate whether an aqueous rosehip extract inhibits MGO-mediated protein glycation and scavenges MGO directly and to explore its active substances possessing anti-glycation and MGO-scavenging activities.

The MGO-BSA assay, which is a non-enzymatic protein glycation model, revealed that our aqueous rosehip extract could inhibit MGO-mediated protein glycation. Furthermore, the extract directly trapped MGO in an MGO-scavenging assay. The substances contributing to these activities were identified as procyanidin-type proanthocyanidins. Procyanidins are the most abundant type of proanthocyanidins in plants, while propelargonidins and prodelphinidins are less common [25]. GPC analysis showed that the number-average molecular weight (Mn) of the proanthocyanidins was about 3,500, corresponding to heptamer as the acetylated procyanidin unit equivalent [26]. The active proanthocyanidin-rich (61.6%) fraction also contained a high amount of sugars (38.8%). The free glucose was found only after acid hydrolysis of the fraction (data not shown), indicating that sugars in the fraction were a component of the proanthocyanidin glycosides. Thus, we clarified that the active substances in the fraction were procyanidin glycosides. This finding is consistent with results reported by Fujii *et al.* [11], who found that the active fraction of an aqueous rosehip extract rich in sugar content, exerting inhibitory effect against melanogenesis in mouse melanoma and guinea pig skin, was composed of procyanidin glycosides. The building blocks for proanthocyanidin such as catechin and epicatechin have been reported to react with MGO [24]. In addition, procyanidin B2 and epigallocatechin gallate (EGCG) were also reported to bind to MGO stoichiometrically at an equimolar ratio [24, 27]. Based on structural similarity between procyanidin B2 and proanthocyanidin, polymeric proanthocyanidin has been proposed to react with MGO in a similar manner [24]. Our results conducted in this study support this proposal, whereas the structure in detail remains unclear.

There is considerable interest in the bioavailability of rosehip proanthocyanidins and their bioactivity *in vivo*. However, little is known about the bioavailability of proanthocyanidins present in rosehip. Proanthocyanidin polymer cannot be absorbed from either the stomach or small intestine, whereas procyanidin dimer B2, epicatechin, and catechin have been detected in human plasma after consumption of a flavanol-rich cocoa [28]. Spencer *et al.* reported that procyanidin in a range of trimer to hexamer were hydrolyzed to mixtures of epicatechin monomers and dimers [29]. The absorbed catechin, epicatechin, and procyanidin dimers resulting from the hydrolysis of rosehip proanthocyanidins by intestinal microflora may act as glycation inhibitors and/or carbonyl scavengers *in vivo*.

Conclusions

Our findings demonstrated that an aqueous rosehip extract and its procyanidin glycosides were effective in inhibiting the formation of AGEs. Their abilities to react with MGO were, at least in part, responsible for the inhibition of protein glycation.

Acknowledgments

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Conflicts of Interest Statement

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