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Original article

Analysis of anti-glycative components in *Smallanthus sonchifolius* (yacon)

Taiki Yamaguchi, Wakako Takabe, Masayuki Yagi, Yoshikazu Yonei

Anti-Aging Medical Research Center and Glycation Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Abstract

***Aim*:** Glycation, the non-enzymatic reaction between excess blood sugar and proteins, leads to multiple disorders, aging and inflammation of body organs and tissues via the formation and accumulation of advanced glycation end products (AGEs). In this study, we investigated the yacon tuber's inhibitory efficacy against the formation of AGEs and analyzed the anti-glycation compounds in yacon.

***Methods*:** Yacon tuber (*Smallanthus sonchifolius*) was divided into an edible part and a peel part and an extract was prepared by hot water. Human serum albumin (HSA) glycation model were used to evaluate the efficacy of extract against fluorescent AGE formation. We used a reversed phase high performance liquid chromatography (RP-HPLC) system to isolate the compounds in yacon tuber. Mass spectrometry was used for identification of effective compounds in yacon tuber.

***Results*:** Compared to the edible part, the peel part extract (YPE) strongly inhibited fluorescent AGE formation. We observed at least 4 anti-glycative compounds in YPE, however, every compound was shown to be less effective than the whole YPE. Based on mass spectrometry data, we concluded that 3,4- or 3,5- dicaffeoylquinic acid, and 2,3,5- or 2,4,5- tricaffeoylaltraric acid were the principal anti-glycative compounds in yacon.

***Conclusions*:** In yacon tuber, the peel part extract inhibited fluorescent AGE formation. We identified 4 compounds as anti-glycative compounds.

***KEY WORDS*:** glycation, fluorescent advanced glycation end products (AGEs), intermediates of AGEs, yacon (*Smallanthus sonchifolius*), tricaffeoylaltraric acid, dicaffeoylquinic acid,

Introduction

Reducing sugar such as glucose, fructose, etc. is important in life activity. On the other hand, reducing sugar causes glycation, the non-enzymatic reaction between proteins and reducing sugar, and it forms advanced glycation end products (AGEs). Generation and accumulation of AGEs will cause aging and inflammation in body organs and tissues. Nagai *et al*. reported that glycation relates to complications from diabetes, lifestyle diseases, and age-related diseases [1].

To prevent the glycative stress from glycation, multiple ideas have been studied such as I. Inhibition of the AGE formation, II. Promoting degradation of AGEs and III. Preventing the high blood glucose condition [2]. In this study, we are focusing on preventing the formation of AGEs. Several lines of evidence, polyphenolsin plants showed a strong anti-glycation effect [3-6], and we have studied over 500 kinds of plants to evaluate their anti-glycative efficacy [7-11].

Yacon, *Smallanthus sonchifolius*, is a plant originally cultivated in the Andean region, and mainly the tuber is eaten [12]. The yacon tuber contains rich polyphenol compounds such as fructooligosaccharides, chlorogenic acid, caffeic acid, 3,4- or 3,5- dicaffeoylquinic acid (DCQA) and 2,3,5- or 2,4,5- tricaffeoylaltraric acid (TCAA) [12-14]. Fructooligosaccharides are sugars found in plants but never in concentrations as high as in yacon tuber, soluble dietary fibers. Chlorogenic acid has an antioxidant effect and it is included in potatoes, apples, and tomatoes [15]. DCQA has a strong and selective α-glucosidase inhibitory activity [16-18]. TCAA has a strong antioxidative activity and α-glucosidase inhibitory activity [14] and to our knowledge, it has been identified only in yacon. Overall, yacon root contains multiple compounds which have

Corresponding author: Wakako Takabe, PhD
Anti-Aging Medical Research Center and Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University
1-3 TataraMiyakodani, Kyotanabe, Kyoto 610-0321, Japan
TEL & FAX: +81-774-65-6382 eMail: wtakabe@mail.doshisha.ac.jp
Co-authors: Yamaguchi T, ctub2025@mail4.doshisha.ac.jp ;
Yagi M, myagi@mail.doshisha.ac.jp ; Yonei Y, yyonei@mail.doshisha.ac.jp

some health-promoting effects, such as the improvement in the intestinal microflora balance [19-22] and inhibiting the high blood glucose condition due to its α-glucosidase inhibitory activity [14].

In this study, we investigated the compounds effective against glycation in yacon tubers.

Materials & Methods

Materials.

Yacon tuber was purchased from Minamiyamashiro village in Kyoto, Japan in April, 2016. Human serum albumin (HSA) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were analytical grade and obtained from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO), and Dojindo (Kumamoto, Japan).

Preparation of yacon tuber extract.

Yacon tuber was divided into an edible part and a peel part. Each sample was dried and ground, and then 2 g of the powdered samples were mixed with 40 mL of distilled water. After incubation at 80°C for 75 minutes, the extracted samples were centrifuged at 2,500 rpm (570 x g) for 10 minutes and filtered using filter paper. We named the extract from the edible part YEE (Yacon tuber edible part extract) and the peel part YPE (Yacon tuber peel part extract). Five mL of each extract was used for the measurement of solid content by evaporation, and then the appropriate concentrations were adjusted with distilled water.

Preparation of glycated proteins.

The HSA glycation model [7, 8] was used to evaluate the effect of YEE and YPE on glycation. Briefly, 25 μL of the samples were added to 125 μL of 0.1 mol/L phosphate buffer solution (pH 7.4), 25 μL of distilled water, 50 μL of 40 mg/mL HSA, and 25 μL of 2.0 mol/L glucose. The reaction mixture was incubated at 60°C for 40 hours (named A). Distilled water was added instead of samples as a blank (named B). At the same time, using distilled water instead of glucose was also prepared as a blank for each reaction (A' = blank of sample, B' = blank of distilled water). Instead of samples, 1 mg/mL aminoguanidine (AG) [23] was used as a positive control.

Measurement of AGE-derived fluorescence.

AGE-derived fluorescence was measured as reported previously [7, 8]. Briefly, 200 μL of the reaction mixture was used to measure fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 440 nm by a Varioscan® Flash microplate reader (Thermo Scientific, Waltham, MA). The value was calculated using the equation below.

Ratios of Fluorescent AGE formation [%] = 100 × (A – A') / (B – B')

Isolation of the glycation inhibitory compounds in yacon extract.

YPE was isolated using a reversed phase high performance liquid chromatography (RP-HPLC) system (Shimadzu Corporation, Kyoto, Japan), according to the protocol, but slightly modified from Takenaka's report [12], described below. Briefly, 100 μL of YPE were injected in RP-HPLC and fractionated every 0.5 minute. This operation was performed twice, then these fractions were mixed together. Then samples were evaporated by centrifugation evaporator (CC-105 Tomy, Tokyo, Japan) and re-dissolved in 200 μL distilled water and used to determine the inhibitory effect of those fractions on AGE-derived fluorescence formation.
HPLC condition is listed below.
Column: Cosmosil 5C18-MS (4.6 mmϕ × 150 m; Nacalaitesque, Kyoto, Japan), Eluent: (Phase A) 10 mmol/L phosphate acid : acetonitrile (90 : 10), (Phase B) acetonitrile, Gradient: 0 → 49.5% B (0 → 30 min), Column temperature: 40°C, flow rate: 1.0 mL/min, UV detection: 326 nm, injection volume: 100 μL

Analysis of glycation inhibitory compounds in yacon extract.

The glycation inhibitory compounds from YPE were analyzed by liquid chromatography-time-of-flight / mass spectrometer [LC-QTOF/MS] with photo diode array (PDA). The Analytical conditions are listed below.
Column: Wakosil II 5C18 RS (2.0 mmϕ × 150 mm ; Wako), Eluent: (Phase A) 0.1% formic acid, (Phase B) acetonitrile, Gradient: 10 → 95% B (0 → 30 min), Column temperature: 40°C, flow rate- 0.2 mL/min, Ionization method: ESI (negative mode), Capillary voltage: 3,000 V (negative mode), Nebulizer gas: Nitrogen 1.6 bar, Drying gas flow rate: nitrogen 8.0 L/min, Drying gas temperature: 180 °C, Measurement mass range: m/z 50 ∼ 2,000, Measurement wavelength: 190 ∼ 800 nm.

Purification of tricaffeoylaltraric acid fromyacon extract.

Tricaffeoylaltraric acid (TCAA) in yacon was purified from 6 g of dried powder of yacon tuber peel according to the previously described method [24]. The purity of TCAA and the content of those in YEE and YPE were evaluated by HPLC. The purified sample was used for further analysis.

Effect of yacon extract and tricaffeoylaltraric acid against intermediate of AGE formation.

Three kinds of AGE intermediates, 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO) were measured using a RP-HPLC system. Samples were prepared as previously described [25]. Briefly, 100 μL of HSA glycation model reaction mixtures were mixed with 75 μL of 0.2 mmol/L phosphate buffer and 155 μL of distilled water. Then, the samples were deproteinized using 170 μL of 6% perchrolic acid. After centrifugation at 12,000 rpm (13,200 x g) for 10 minutes, 400 μL of supernatant was immediately neutralized with 350 μL of saturated sodium bicarbonate. Then, 3-DG, GO and MGO were labeled with 50 μL of 1 mg/mL 2,3-diaminonaphthalene for 24 hours at 4°C. After centrifugation at 15,000 rpm (20,630 x g) for 10 minutes, supernatants were analyzed by HPLC.
HPLC conditions are listed below.
Column: UnisonUK-Phenyl (3 mmϕ x 75 mm; Imtakt Corp, Kyoto, Japan), Eluent: 50 mmol/L phosphoric acid : acetonitrile = 89:11, Column temperature: 40°C, flow rate: 1.0 mL/min, UV detection: 268 nm, injection volume: 20 μL

Statistics.

The statistical analysis was performed by Dunnett's multiple comparison test. Differences were considered significant at p values less than 0.05.

Results

Evaluation of yacon extracts against fluorescent AGE formation.

The inhibitory efficacy against fluorescent AGEs of YEE and YPE was evaluated using the HSA glycation model (***Fig. 1-a***). Ten % final concentration of YEE and YPE showed the inhibitory efficacy against fluorescent AGE formation, and especially YPE showed a strong inhibitory effect. Without glucose condition, YEE and YPE showed 3 times and twice higher fluorescent AGEs than the reference (water). This result suggested that YEE and YPE might have auto fluorescence in our measurement condition. However, with glucose condition, YPE suppressed about 50% of the fluorescent AGE formation compared to the reference. YEE showed a significant effect, but was less effective than YPE. Therefore, we decided to use YPE for the following experiments in order to identify effective compounds against fluorescent AGE formation.

YPE's 50% inhibition concentration (IC_{50}) against fluorescent AGE formation was 0.093 mg/mL (***Fig. 1-b***). IC_{50} against fluorescent AGE formation of AG, the positive control, was 0.062 mg/mL (***Fig. 1-c***).

Isolation of the glycation inhibitory compounds from yacon extract.

RP-HPLC was used to analyze the compounds in YPE and the flow through was collected every 0.5 minute. Each fraction was evaporated and re-dissolved in distilled water, then it was introduced into a HSA glycation model reaction mixture in order to evaluate the fluorescent AGE formation (***Fig. 2-a***). From these results, several fractions showed strong inhibitory efficacy against fluorescent AGE formation. Next, we selected 4 peaks (#1～4) that have a strong effect and isolated the peaks by hand (***Fig. 2-b***).These peaks were also evaporated and re-dissolved in distilled water, then used to measure the effect of those peaks on fluorescent AGE formation (***Fig. 2-c***). Compared to the reference, all 4 peaks inhibited fluorescent AGE formation, especially peaks #3 and 4 showed a stronger effect than other peaks. However, each peak was less effective than the whole YPE.

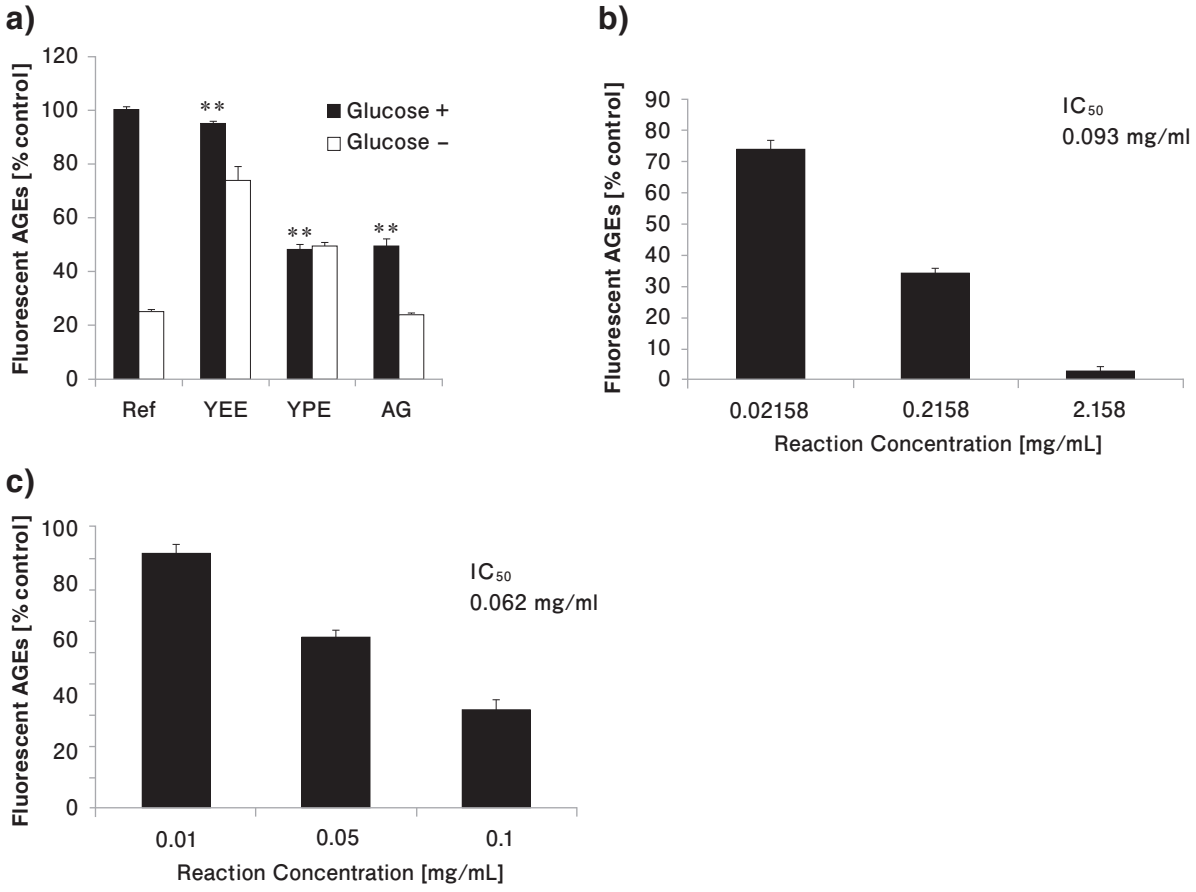

Fig. 1. **1. Effect of yacon extracts and AG on the inhibition of fluorescent AGE formation.**

a) YEE and YPE were introduced into glycation models containing 8 mg/mL HSA and 0.2 mol/L glucose as indicated concentrations of the solid contents. After 40 h incubation at 60°C, fluorescent AGEs were measured by 370/440 nm (AG's final concentration: 0.1 mg/mL). **b)** Three different concentrations of YPE were used to determine its IC_{50} against fluorescent AGE formation. **c)** Three different concentrations of AG was used to determine its IC_{50} against fluorescent AGE formation.** $p < 0.01$ vs ref. YEE, yacon tuber edible part extract. YPE, yacon tuber peel part extract. HSA, human serum albumin. AGEs, advanced glycation end products. AG, aminoguanidine.

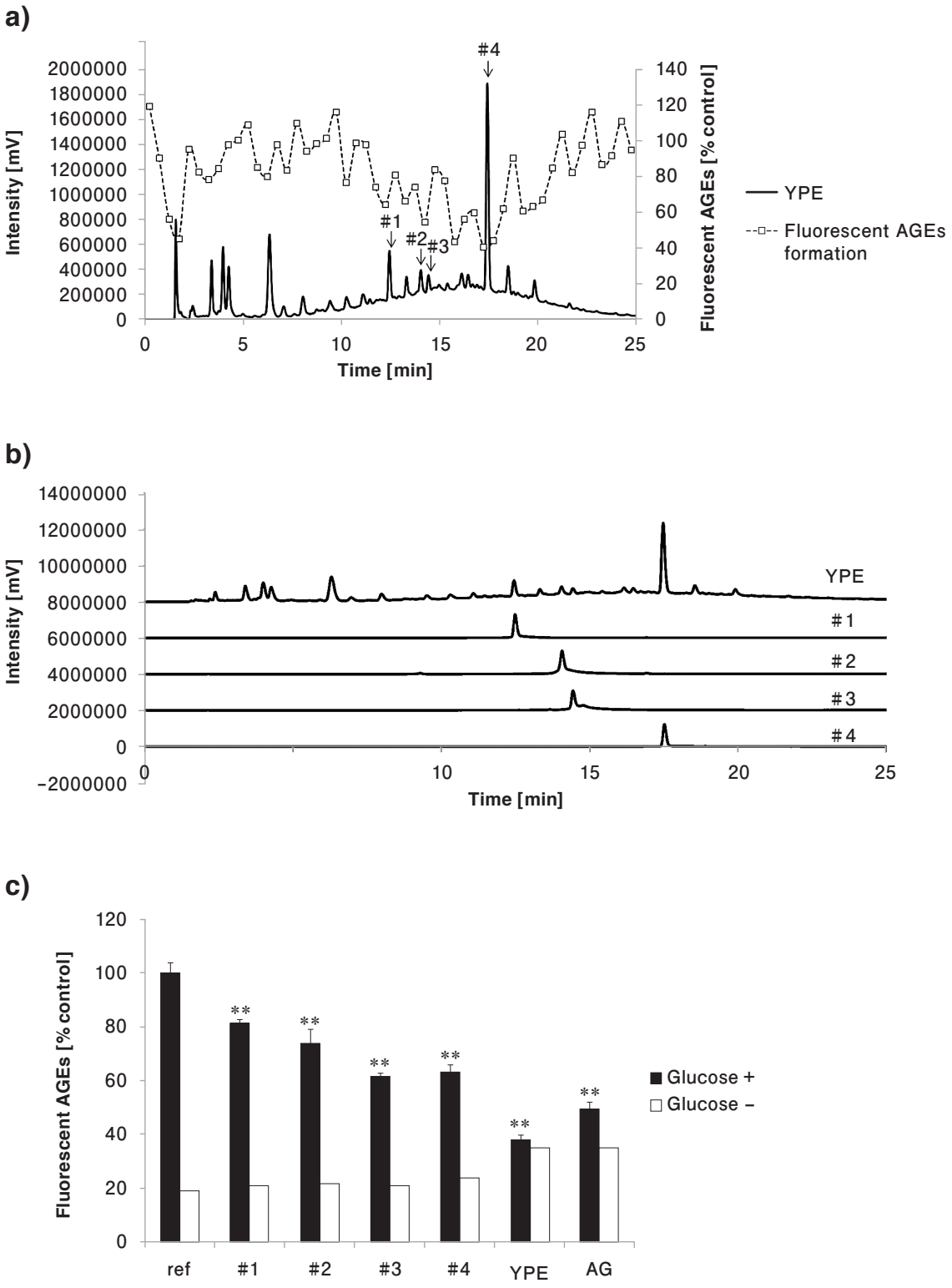

Fig. 2. **Isolation of anti-glycative compounds in yacon extract using RP-HPLC system.**

a) YPE were fractionated by RP-HPLC system. The fractions were added into a HSA glycation model at 60°C for 40 h, then we measured the fluorescent AGEs at 370/440 nm. HPLC condition: Column: Cosmosil 5C18-MS (4.6 mmϕ × 150 m ; Nacalaitesque, Kyoto, Japan), Phase A: 10 mmol/L phosphate acid : acetonitrile (90 : 10), Phase B: acetonitrile, Gradient: 0 → 49.5% B (0 → 30 min), Column temperature: 40°C, flow rate: 1.0 mL/min, UV detection: 326 nm, injection volume: 100 µL. **b)** Verification of isolated peaks by RP-HPLC. **c)** Peaks #1∼4 were added into the HSA glycation model. After 40 h of incubation at 60°C, fluorescent AGEs were measured by 370/440 nm.** $p < 0.01$ vs ref. YPE, yacon tuber peel part extract. HSA, human serum albumin. AGEs, advanced glycation end products.

Identification of glycation inhibitory compounds in yacon extract.

Next, we performed LC-QTOF/MS with PDA analyses to identify those 4 effective anti-glycative compounds in YPE. As shown in ***Table 1***, m/z of those peaks were 533.09 (peak #1), mixture of 515.12 and 533.09 (peak #2), 515.13 (peak #3) and 695.13 (peak #4) at a negative mode. To compare the HPLC chromatograms of these 4 peaks (***Fig. 2-b***) and previous report [12], we expected that these compounds are caffeoylaltraric acid or caffeoylquinic acid. The comparison with chemical standards, we estimated that the peak #1: 2,4- or 3,5-dicaffeoylaltraric acid, peak #2 is the mixture of 3,4-dicaffeoylquinic acid and the isomer of peak #1, peak #3: 3,5-dicaffeoylquinic acid and peak #4: 2,3,5- or 2,4,5- tricaffeoylaltraric acid (TCAA).

Purification of tricaffeoylaltraric acid in yacon extract.

TCAA was purified from YPE and we validated its purity by HPLC using the previously reported method (***Fig. 3***)[12]. Based on the result, one obvious peak is observed. Also, we calculated the concentration of TCAA in YEE and YPE. YEE contained about 0.002 mg and YPE contained about 0.023 mg TCAA in each 1 mg of solid content of YEE and YPE. In addition, we also evaluated the efficacy of TCAA against fluorescent AGE formation, and its IC_{50} was 0.019 mg/mL (***Fig. 4***).

Table 1. The results of analyzing the glycation inhibitory compounds in yacon extract using LC-QTOF/MS with PDA.

Peak #	m/z	Estimation of composition formula
1	533.09	$C_{24}H_{22}O_{14}$
2-1	515.12	$C_{25}H_{24}O_{12}$
2-2	533.09	$C_{24}H_{22}O_{14}$
3	515.13	$C_{25}H_{24}O_{12}$
4	695.13	$C_{33}H_{28}O_{17}$

Peaks #1∼4 were isolated from yacon peel extract and were analyzed by LC-QTOF/MS with PDA. Analytical condition: Column: Wakosil-II5C18 RS (2.0 mmϕ × 150 mm ; Wako Pure Chemical Industries, Osaka, Japan), Phase A: 0.1% formic acid, Phase B: acetonitrile Gradient: 10 → 95% B (0 → 30 min), Column temperature: 40°C, flow rate: 0.2 mL/min, Ionization method: ESI (negative mode), Capillary voltage: 3,000 V (negative mode), Nebulizer gas: Nitrogen 1.6 bar, Drying gas flow rate: nitrogen 8.0 L/min , Drying gas temperature: 180°C, Measurement mass range: m/z 50 ∼ 2,000, Measurement wavelength: 190 ∼ 800 nm.

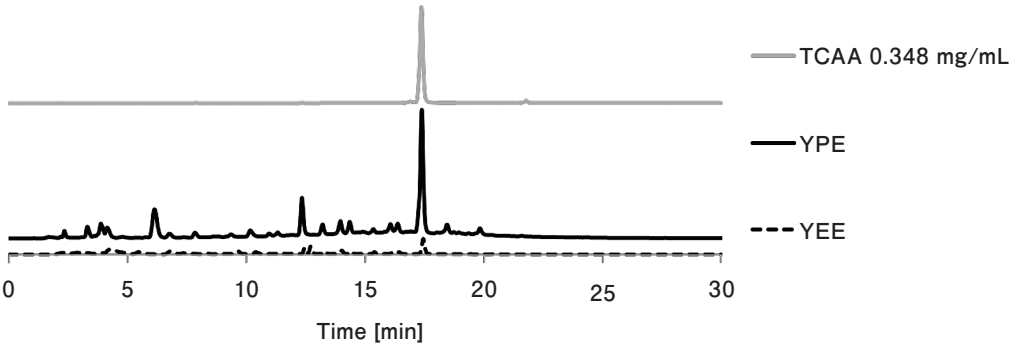

Fig. 3. **Comparison of HPLC chromatogram of yacon extract and tricaffeoylaltraric acid.**

YEE, YPE, TCAA were analyzed by RP-HPLC. HPLC condition: Column: Cosmosil 5C18-MS (4.6 mmϕ × 150 m ; Nacalaitesque, Kyoto, Japan), Phase A:10 mmol/L phosphate acid : acetonitrile (90 : 10), Phase B: acetonitrile, Gradient: 0 → 49.5% B (0 → 30 min), Column temperature: 40°C, flow rate: 1.0 mL/min, UV detection: 326 nm, injection volume: 20 μL.

YEE, yacon tuber edible part extract. YPE, yacon tuber peel part extract. TCAA, tricaffeoylaltraric acid.

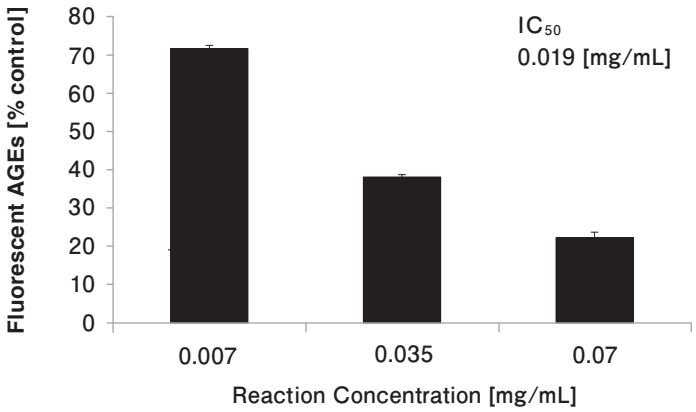

Fig. 4. **Effect of tricaffeoylaltraric acid on fluorescent AGE formation.**

TCAA was used to determine the IC_{50} against fluorescent AGE formation in HSA glycation models. After 40 h of incubation at 60°C, fluorescent AGEs were measured by 370/440 nm. Three different concentrations were used to determine IC_{50} against fluorescent AGE formation. TCAA, tricaffeoylaltraric acid. AGEs, advanced glycation end products. HSA, human serum albumin.

Effect of yacon extract and tricaffeoylaltraric acid against intermediate of AGE formation.

Finally, we also investigated the efficacy of YPE and TCAA on intermediate AGE (3-DG, GO, MGO) formation in HSA glycation reaction model. As shown in ***Fig. 5***, both YPE and TCAA inhibited all 3 intermediates formation. YPE's IC_{50} against 3-DG, GO, MGO were 0.28, 0.046, 0.15 mg/mL, TCAA's IC_{50} against 3-DG, GO, MGO were 0.33, 0.011, 0.024 mg/mL, respectively.

Discussion

In this study, we investigated the anti-glycative compounds in yacon tuber. First, we evaluated the YEE's and YPE's inhibitory efficacy against fluorescent AGE formation using HSA glycation model. YPE has a stronger inhibitory efficacy against fluorescent AGE formation than YEE (***Fig. 1-a***). Thus, we further investigated in order to identify the effective compounds against glycation in YPE (***Fig. 2-a***). We selected 4 peaks (#1～4), especially those that are expected to have strong effect and evaluated the inhibition of fluorescent AGE formation using the HSA glycation model. As shown in ***Fig. 2-c***, peak #1～4 showed a strong inhibitory efficacy against fluorescent AGE formation. However, each peak was less effective than the entire YPE. These results suggested that multiple compounds in yacon tubers contribute to those anti-glycative effect.

Thus, next we tried to identify anti-glycative compounds in YPE. From the ***Fig. 2-b,c*** and the result of structure estimation by LC-QTOF/MS with PDA analyses, DCQA and TCAA highly contribute to anti-glycation effect of YPE. DCQA and TCAA are polyphenols and DCQA is reported to be contained in coffee beans [26, 27], sweet potato's stem and leaf [28], garland chrysanthemum [29], artemisia [30] and TCAA is reported to be contained tuber, leaf and stem in yacon [12]. Based on the RP-HPLC analyses, YPE contains about ten times the TCAA compared to YEE in each 1 mg of solid content (***Fig. 3***). These results indicate that the difference in the amount of TCAA between YPE and YEE may affect their anti-glycative efficacy.

Several lines of evidence indicate that polyphenols have an anti-glycative efficacy [3-6]. We performed the RP-HPLC analyses detected at UV 326 nm which is the typical absorbance of polyphenols. Other than DCQA and TCAA, every YEE peak was smaller than YPE peaks in the chromatograms (***Fig. 3***). We also measured total polyphenol content in yacon tubers using the Folin-Ciocalteu method, which is approved by the National Consumer Affairs Center of Japan (NCAC). YPE contained at least 5 times the amount of polyphenols than YEE in the same solid content (data not shown). This difference may also affect their anti-glycative efficacy.

Traditionally, intake of yacon tubers have been recommended to diabetic patients [31-33]. Especially, fructooligosaccharides which is abundantly existed in yacon tuber are able to resist the hydrolysis of enzymes in the human upper gastrointestinal tract and have a low caloric value [34]. Genta et al. reported that daily intake of yacon syrup obtained from tuber ameliorated body weight, fasting serum insulin and LDL cholesterol in human [33]. Other than that, DCQA and TCAA are reported to have an α-glucosidase inhibitory activity [14, 16-18], which means that intake of yacon tuber may contribute to suppressing increases in blood glucose level. Preventing the high blood glucose condition is one of the ways to prevent glycation. In addition, we demonstrated that DCQA and TCAA in YPE have anti-glycative efficacy. Overall, our findings indicate that anti-glycative effect of yacon tuber itself may partially contribute to those beneficial effects.

Furthermore, we demonstrated that YPE and TCAA have an inhibitory efficacy against all 3 kinds of intermediates of AGE formation in a dose-dependent manner (***Fig. 5***). Because TCAA contains 0.023 mg in 1 mg of solid content of YPE (***Fig. 3***), compare to IC_{50}, TCAA in YPE may strongly

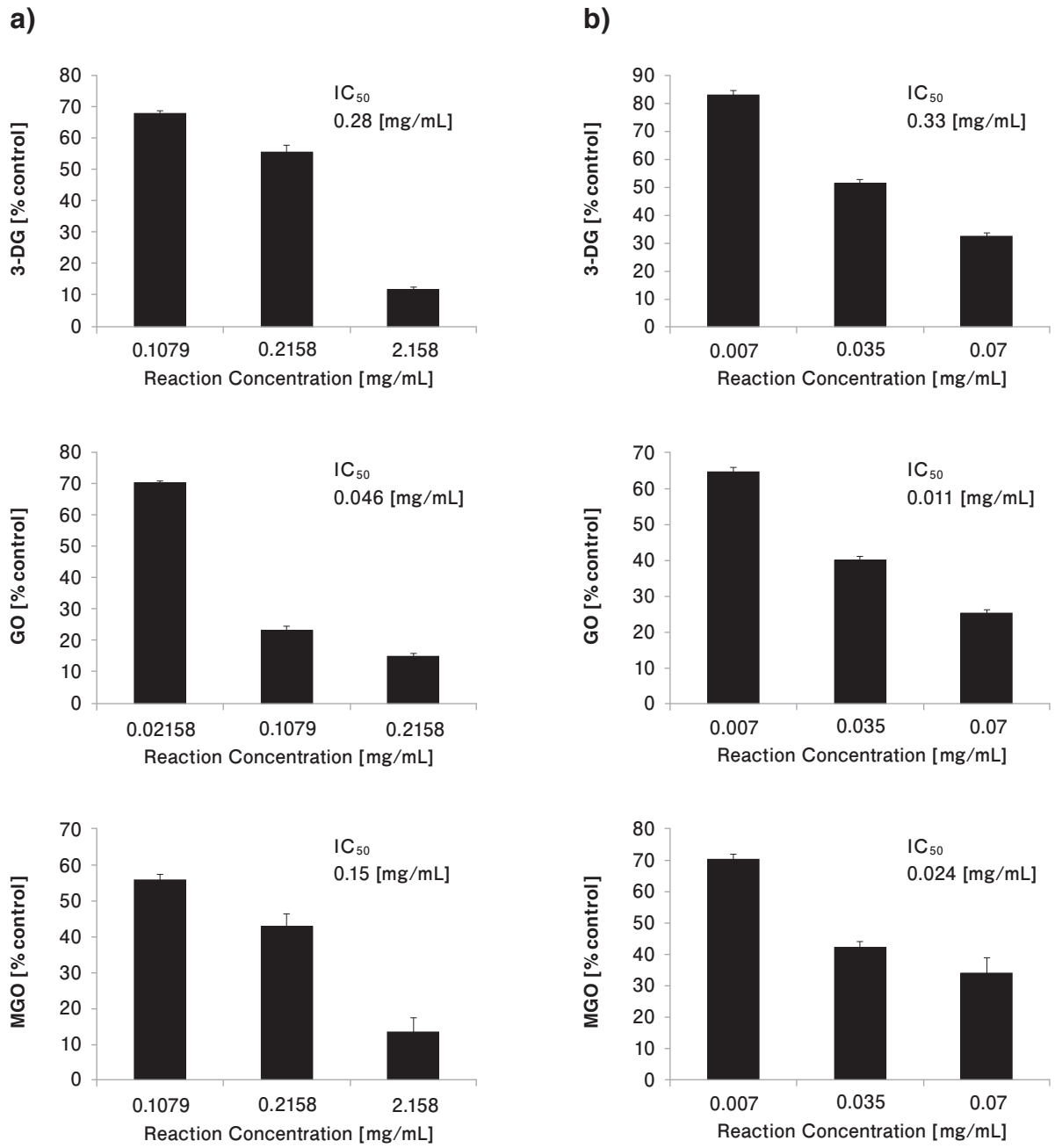

Fig. 5. **Effect of yacon peel extract and tricaffeoylaltraric acid onintermediate of AGE formation.**

YPE **(a)** and TCAA **(b)** were used to determine the inhibitory effect on the intermediates of AGE formation. RP-HPLC analyses were performed to detect 3-DG **(top)**, GO **(middle)** and MGO **(bottom)**. All data were shown as the mean ± SD (n = 3).

HPLC condition: Column- UnisonUK: Phenyl (3 mmϕ x 75 mm; Imtakt Corp, Kyoto, Japan), eluent: 50 mmol/L phosphoric acid : acetonitrile = 89 : 11, Column temperature: 40°C, flow rate: 1.0 mL/min, UV detection: 268 nm, injection volume: 20 µL.

contribute to the inhibition of GO and MGO formation, but not 3-DG. It is reported that AGE intermediates such as 3-DG, GO, MGO are mediating AGE generation pathway [35]. Thus, to inhibit the formation of AGEs, intermediates leads to inhibit the AGE formation.

AG is a specific inhibitor for glycation [23]. IC_{50} of AG against the formation of fluorescent AGEs was 0.062 mg/mL (***Fig. 1-c***). In the previous report, AG's IC_{50} against fluorescent AGE formation was 0.067 ~ 0.100 mg/mL [8, 9].

From the point of view of the inhibition of fluorescent AGE formation, YPE has similar IC_{50} (0.093 mg/mL) and TCAA has smaller IC_{50} (0.019 mg/mL) compared to AG. While AG has shown the preventive effect of nephropathy, retinopathy, and nerve disorder in an animal study [23, 36-38], it will cause adverse effects such as anemia, hepatic disorder and vitamin B6 deficiency. For this reason, AG has not been approved in Japan. In this study, we suggest that TCAA in yacon tuber will come to be used as a new specific inhibitor against glycation. Furthermore, yacon peel is usually disposed of because it's a non-edible part. Using the yacon peels as a source of anti-glycative compounds, might lead to expanding the agricultural resource utilization.

Conclusion

Yacon tuber peel extract (YPE) showed a stronger inhibitory efficacy against fluorescent AGE formation than the edible part yacon tuber extract (YEE). DCQA and TCAA highly contribute to YPE's strong anti-glycation effect. TCAA was able to inhibit not only the formation of fluorescent AGEs, but AGE intermediates.

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Conflict of Interest Statement

The authors claim no conflict of interest in this study.

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