Author's post-print manuscript of the following article
*

Astilbe thunbergii reduces postprandial hyperglycemia in a type 2 diabetes rat model via pancreatic alpha-amylase inhibition by highly condensed procyanidins.

Bioscience, Biotechnology, and Biochemistry, 2017,81(9), 1699-1705.
Check the published version from the bellow link.

Taylers & Francis Online
DOI:10.1080/09168451.2017.1353403
http://www.tandfonline.com/doi/full/10.1080/09168451.2017.1353403

Research Article

***Astilbe thunbergii* reduces postprandial hyperglycemia in a type 2 diabetes rat model via pancreatic alpha-amylase inhibition by highly condensed procyanidins.**

Eisuke Kato[1]*, Natsuka Kushibiki[1], Yosuke Inagaki[2], Mihoko Kurokawa[2], Jun Kawabata[1]

[1]*Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan*

[2]*Q'sai Co., Ltd., Kusagae, Fukuoka, Fukuoka 810-8606, Japan*

*Corresponding author

Tel/Fax: +81 11 706 2496;

E-mail address: eikato@chem.agr.hokudai.ac.jp (E. Kato)

Abstract

Type 2 diabetes mellitus (T2DM) is a common global health problem. Prevention of this disease is an important task, and functional food supplements are considered an effective method. We found potent pancreatic α-amylase inhibition in *Astilbe thunbergii* root extract (AT) and confirmed that AT treatment in a T2DM rat model reduces post-starch administration blood glucose levels. Activity-guided isolation revealed procyanidin (AT-P) as the α-amylase inhibitory component with IC_{50} = 1.7 μg/mL against porcine pancreatic α-amylase. Structure analysis of AT-P revealed it is a B-type procyanidin comprised of four types of flavan-3-ols, some with a galloyl group, and catechin attached as the terminal unit. The abundant AT-P content and its comparable α-amylase inhibition to acarbose, the anti-diabetic medicine, suggests that AT is a promising food supplement for diabetes prevention.

Keywords: pancreatic α-amylase; type 2 diabetes; procyanidin; *Astilbe thunbergii*

Type 2 diabetes mellitus (T2DM) is a significant global problem, with patient numbers increasing every year. Although numerous anti-diabetes medications have been developed and utilized, they are not effective at completely curing the disease [1,2]. Therefore, a method for prevention is as important as a method for treatment of the disease.

Functional food supplements are an effective way of preventing lifestyle-dependent diseases, including diabetes. Components in food display various functions in the digestive tract that have benefits for maintaining health. The inhibition of carbohydrate digestive enzymes is one of these functions with anti-diabetic properties [3].

A. thunbergii is a perennial plant that belongs to the Saxifragaceae family and is distributed widely along mountainous regions in Japan. The plant has been used as an alternative to the *Cimicifuga* genus (*C. dahurica*, *C. foetida*, and *C. heracleifolia*), which is employed in traditional Japanese and Chinese medicine. "Sheng Ma," derived from the *Cimicifuga* genus, has been used as a diaphoretic and antipyretic agent and for prevention of gastroptosis and proctoptosis [4,5]. As an alternative plant, *A. thunbergii* has also been used for these purposes. In addition, scientific studies investigating this plant have identified an anti-obesity effect, such as enhancement of lipolysis, inhibition of pancreatic lipase, and reduction of plasma triacylglycerol levels in high-fat diet-treated rats [6,7].

Obesity is one of the health problems associated with T2DM. Studying the anti-diabetic properties of *A. thunbergii* will concomitantly clarify the anti-obesity effects and will allow the enhanced utilization of this plant for both

anti-obesity and anti-diabetic purposes.

Here, we evaluated the anti-diabetic property of *A. thunbergii* root extract (AT) through *in vitro* α-amylase inhibitory activity assay, and confirmed its effect in a T2DM rat model. Activity-guided isolation revealed procyanidin in AT (AT-P), which is constituted from four types of flavan-3-ols, as the major α-amylase inhibitory component

Materials and methods

General

Chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise noted. Absorbance was measured using a Synergy™ MX microplate reader (BioTek Instruments, Inc.). Bruker AMX 500 (Bruker BioSpin K.K.) was used to obtain nuclear magnetic resonance (NMR) spectra, and residual solvents were used as an internal standard (acetone-d_6: ^{1}H 2.04 ppm). Waters LCT Premier Spectrometer (Waters Co.) was used to obtain mass spectra and was combined with the Waters Acquity UPLC system (Waters Co.) for liquid chromatography-mass spectrometry (LC-MS) analysis.

A. thunbergii extract (AT) powder

AT was a commercial product of BHN Co, Ltd. (Lot No. 100915, Tokyo, Japan). According to the distributer, the extract powder was processed from the root of *A. thunbergii* using 50-60% aq. ethanol as the extracting solvent.

Animal study

Five-week-old male GK:Slc rats supplied from Japan SLC, Inc. (Hamamatsu, Japan) were acclimated at 20–26°C, 12 h light/dark cycle for 1 week with free access to water and a standard rodent diet. All experiments were performed following Hokudo Co., Ltd (Sapporo, Japan) animal experiment regulations.

Sugar tolerance test

Rats were fasted for 18 h and then orally administered each dose of the sample suspended in distilled water, followed by administration of starch, sucrose, or glucose (2 g/kg). Blood samples were obtained from the tail vein, and blood glucose levels were measured by Nipro FreeStyle Freedom Lite (Nipro Co., Osaka, Japan).

Insulin and glucagon-like peptide-1 (GLP-1) levels

Insulin and GLP-1 levels were measured during the first 30 min of the glucose tolerance test. Rats were anesthetized by isoflurane (DS Pharma Animal Health Co., Ltd., Osaka, Japan), and blood samples were obtained from the jugular vein. For the GLP-1 measurement, diprotin A (Peptide Institute, Inc., Osaka, Japan) and protease inhibitor cocktail (Sigma-Aldrich Co., P2714) were added to the blood samples immediately after collection. Insulin levels were measured using a rat insulin ELISA kit (MS301, Morinaga Institute of Biological Science, Inc., Yokohama, Japan). GLP-1 levels were measured using a GLP-1, Active form Assay Kit – IBL (Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan).

Isolation of procyanidin from AT

AT (22.74 g) was suspended in water (1 L) and stirred for 1 hour. The resulting solution was filtered, and the filtrate was applied to a DIAION HP-20 (Mitsubishi Chemical Co.) column. The column was washed with water and eluted with 50% aq. methanol. The 50% aq. methanol eluate was then directly applied to a Sephadex LH-20 (GE Healthcare Japan Co.) column. The column was washed sequentially with 50% aq. methanol and methanol and then eluted with 70% aq. acetone to obtain *A. thunbergii* procyanidin (AT-P, 2.52 g).

Vanillin assay [8]

The AT-P methanol solution (0.5 mL), 35% hydrochloric acid (1.5 mL), and 4% (w/v) vanillin in methanol (3.0 mL) were mixed and incubated for 15 min at room temperature. The reaction mixture was measured for its A_{500} and compared with the standard curve of (+)-catechin.

Analysis of AT-P by gel permeation chromatography (GPC)

AT-P was dissolved in *N*,*N*-dimethylformamide containing 10 mM lithium bromide and analyzed by HPLC equipped with Shodex GF-510 HQ (ϕ7.5×300 mm, Showa Denko K.K., Tokyo). The mobile phase was 10 mM lithium bromide in *N*,*N*-dimethylformamide with a flow rate of 0.4 mL/min at room temperature. Polystylene (Agilent EasiVial Polystylene High MW, Agilent Technologies Japan, Ltd.) was used for the standards.

Thiolysis of AT-P

AT-P (523.4 mg) was dissolved in 0.1 M hydrogen chloride in methanol (40 mL),

and benzyl mercaptan (1 mL) was added. The mixture was stirred for 90 min at 40°C under nitrogen atmosphere. Part of the reaction mixture was dried and analyzed by HPLC (Figure 4). The ratio of each compound was estimated from the peak area after adjustment using commercial catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate as the standards.

The rest of the reaction mixture was evaporated to remove the methanol, diluted with water, and extracted by ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and evaporated. The residue was purified by HPLC using an InertSustain C18 column (GL Science Inc., Tokyo, Japan) with gradient elution (from 40% aq. methanol with 0.1% trifluoroacetic acid [TFA] to 95% aq. methanol with 0.1% TFA for 30 min, flow rate 1.0 mL/min, detection UV 280 nm) to obtain four benzyl mercaptan adducts (**2**–**4**, and **6**). The obtained products were analyzed by ^{1}H-NMR to determine their structures.

Compound **2** (gallocatechin 4-benzylthioether)[9,10]

HR-ESI-MS (positive): $[M+H]^+$ calcd. for $C_{22}H_{21}O_7S$, *m/z* 429.1008, found 429.1011; ^{1}H-NMR (500 MHz, acetone-d_6): 4.07 (1H, d, *J* = 12.5 Hz), 4.13 (1H, d, *J* = 12.5 Hz), 4.08–4.13 (1H, m), 4.37 (1H, d, *J* = 4.1 Hz), 4.88 (1H, d, *J* = 9.8 Hz), 5.81 (1H, d, *J* = 2.2 Hz), 6.01 (1H, d, *J* = 2.2 Hz), 6.51 (2H, s), 7.19–7.42 (5H, m) ppm.

Compound **3** (epigallocatechin 4-benzylthioether)[10]

HR-ESI-MS (positive): $[M+H]^+$ calcd. for $C_{22}H_{21}O_7S$, *m/z* 429.1008; found, 429.1023; ^{1}H-NMR (500 MHz, acetone-d_6): 3.99 (1H, br s), 4.00 (1H, d, *J* = 12.6 Hz), 4.04 (1H, d, *J* = 12.6 Hz), 4.06 (1H, d, *J* = 2.2 Hz), 5.20 (1H, s), 5.89 (1H, d, *J* = 2.2 Hz), 6.01 (1H, d, *J* = 2.2 Hz), 6.55 (2H, s), 7.22 (1H, t, *J* = 7.5 Hz), 7.31 (2H, dd, *J* =

7.5, 7.5 Hz), 7.45 (2H, d, *J* = 7.5 Hz) ppm.

Compound **4** (catechin 4-benzylthioether)[10]

HR-ESI-MS (positive): $[M+H]^+$ calcd. for $C_{22}H_{21}O_6S$, *m/z* 413.1059; found, 413.1077; ^{1}H-NMR (500 MHz, acetone-d_6): 4.07 (1H, d, *J* = 12.3 Hz), 4.12 (1H, d, *J* = 12.3 Hz), 4.14 (1H, dd, *J* = 4.2, 9.8 Hz), 4.38 (1H, d, *J* = 4.2 Hz), 4.94 (1H, d, *J* = 9.8 Hz), 5.81 (1H, d, *J* = 2.2 Hz), 6.02 (1H, d, *J* = 2.2 Hz), 6.81 (2H, m), 6.94 (1H, br s), 7.20 (1H, t, *J* = 7.3 Hz), 7.28 (2H, dd, *J* = 7.3, 7.6 Hz), 7.41 (2H, d, *J* = 7.6 Hz) ppm.

Compound **6** (epicatechin 4-benzylthioether)[10]

HR-ESI-MS (positive): $[M+H]^+$ calcd. for $C_{22}H_{21}O_6S$, *m/z* 429.1059; found, 429.1043; ^{1}H-NMR (500 MHz, acetone-d_6): 3.98 (1H, s), 4.00 (1H, d, *J* = 13.2 Hz), 4.04 (1H, d, *J* = 13.2 Hz), 4.08 (1H, d, *J* = 1.9 Hz), 5.26 (1H, s), 5.89 (1H, d, *J* = 2.2 Hz), 6.02 (1H, d, *J* = 2.2 Hz), 6.76 (1H, dd, *J* = 1.6, 8.2 Hz), 6.79 (1H, d, *J* = 8.2 Hz), 7.03 (1H, d, *J* = 1.6 Hz), 7.22 (1H, t, *J* = 7.3 Hz), 7.31 (2H, dd, *J* = 7.3, 7.3 Hz), 7.45 (2H, d, *J* = 7.3 Hz) ppm.

Pancreatic α-amylase inhibitory activity

The α-amylase inhibitory activity was determined using the method described by Hansawasdi et al [11]. Briefly, 350 μL of starch azure (4 mg/mL, Sigma-Aldrich Co., S7629) suspended in Tris-HCl buffer (0.1 M, pH6.9, 0.01 M $CaCl_2$), 100 μL of sample dissolved in water, and 50 μL of porcine pancreatic α-amylase dissolved in Tris-HCl buffer were incubated for 10 min at 37°C with frequent mixing. The reaction was stopped by the addition of 50% acetic acid (50 μL) and then centrifuged (1,500 × *g*, 5 min), and the A_{595} of the supernatant was measured.

The IC_{50} value was calculated using curve fitting by NIH Image J software.

Statistics

In vivo experiments were performed with n=7 rats/group, and Bartlett's test was used to analyze the homogeneity of variance. Homoscedastic data were analyzed by one-way ANOVA, and heteroscedastic data were analyzed by Kruskal-Wallis test. Data with significant differences were subsequently analyzed by Dunnett multiple comparison test to determine the significance compared with the control. $p<0.05$ was considered significant. *In vitro* experiments were performed in duplicate and repeated at least two times. Average values are shown in the figures. Statistical significance was determined using one-way ANOVA coupled with Dunnett multiple comparison test, and $p<0.01$ was considered significant.

Results and discussion

In vitro α-amylase inhibitory activity assay of AT displayed a 90% inhibition at 0.1 mg/mL indicating its potential anti-diabetic effect. The anti-diabetic effect of AT was confirmed by *in vivo* experiments. Male GK:Slc rats, a T2DM model [12], were orally administered AT. Rats then underwent a sugar tolerance test for either starch, glucose, or sucrose, and blood glucose levels were analyzed (Figure 1). In addition, serum GLP-1 and insulin levels were measured (Figures 2).

Figure 1

Figure 2

When starch was loaded following AT administration (100 or 300 mg/kg), the elevation in blood glucose levels in rats was reduced by approximately 30% at 30 min ($p<0.05$) and 40% at 60 min ($p<0.01$) compared with those of the control (Figure 1A). The area under the curve (AUC) was also decreased by 21% in the 100 mg/kg group and 24% in the 300 mg/kg group ($p<0.01$). These results revealed the efficacy of AT at mitigating the rapid elevation in blood glucose levels following food consumption.

During the sucrose tolerance test, 300 mg/kg AT-administered rats displayed an approximately 25% reduction in blood glucose levels at 90 and 120 min ($p<0.05$), and the AUC decreased by 17% ($p<0.01$) (Figure 1B,C). The glucose tolerance test did not reveal differences between the doses. Serum GLP-1 levels were 1.2–1.6 times higher in the AT-administered group than those of the control group at 30 min post administration ($p<0.01$), but insulin levels did not significantly differ at this time point (Figure 2).

The above results indicate that AT efficiently reduced the rapid elevation in post-meal blood glucose levels. The underlying mechanism is primarily through the inhibition of starch digestion by α-amylase but not through the inhibition of glucose absorption or increased insulin secretion. Inhibition of α-glucosidase and dipeptidyl peptidase IV or enhanced secretion of GLP-1 from L-cells may accompany this mechanism but with relatively minor contributions.

Following the *in vivo* results, the active component in AT that inhibits α-amylase was determined through an activity-guided isolation procedure. Solvent

235 partition of AT between water, 1-butanol, and ethyl acetate gave water layer as
236 the active layer. The water layer was separated by column chromatography
237 employing Diaion HP-20 (Mitsubishi Chemical Co.), Cosmosil 75C_{18}-OPN
238 (Nakalai Tesque, Inc.), and Toyopearl HW-40F (Tosoh Co.) to give an active
239 fraction consisting primarily of procyanidin with presumably large size which was
240 predicted through the elution character in each column and spectroscopic
241 methods.

242 The procedure was thus optimized to achieve an effective separation of
243 procyanidins from AT. The water-soluble portion of AT was absorbed on a
244 DIAION HP-20 column, washed, and eluted with 50% methanol. This was then
245 absorbed on a Sephadex LH-20 column, washed, and eluted with 70% acetone to
246 obtain AT procyanidin (AT-P, yield 11.1% w/w from AT) which has identical
247 character (reverse phase HPLC, GPC and thiolysis product analysis) with the
248 procyanidin isolated through the activity guided fractionation.

249 Figure 3

250 Characterization of the isolated AT-P was performed through several methods. A
251 vanillin assay, a colorimetric method to determine procyanidin content, revealed
252 that 1 mg of AT-P is equivalent to 0.79±0.02 mg of (+)-catechin. A UV spectra of
253 AT-P revealed a peak at 280 nm, which is similar to that of (+)-catechin,
254 indicating that the majority of AT-P consists of flavan-3-ols (Figure 3A).

255 The degree of polymerization of AT-P was analyzed by HPLC analysis employing

a diol column, with the condition suitable to analyze procyanidin with a polymerization degree below n<10. No peak was detected, indicating a high level of polymerization [13,14]. Consistent with this, MALDI and ESI-MS analysis of AT-P did not reveal any distinguishable ions resulting from procyanidin.

To obtain information regarding molecular size, gel permeation chromatography was performed with a Shodex GF-510 HQ column [15]. The chromatogram of AT-P revealed a single peak with an elution volume at the relatively large molecular size region (Figure 3B). The molecular size of the peak was approximately 8,260, estimated from the standard curve of polystylene. This estimation is probably not accurate because of differences in the structure between standards and AT-P. However, this result suggests that AT-P is a highly condensed procyanidin and does not contain lower size molecules.

Figure 4

Next, thiolysis of AT-P was performed to determine the units and estimate the degree of polymerization. AT-P was heated under acidic conditions in the presence of benzyl mercaptan. Analysis of the reaction mixture revealed seven main products (Figure 4). Product **1** was determined to be catechin by comparison with the standard, which is the terminal unit of AT-P. Products **2**–**4** and **6** were isolated and analyzed by ^{1}H-NMR and MS and determined to be gallocatechin 4-benzylthioether (**2**), epigallocatechin 4-benzylthioether (**3**), catechin 4-benzylthioether (**4**), and epicatechin 4-benzylthioether (**6**) (Figure 5)[9,10]. Because of the failure to isolate products **5** and **7**, the reaction mixture

was analyzed by LC-MS, and the two remaining products were determined to be (epi)gallocatechin gallate 4-benzylthioether (**5**) and (epi)catechin gallate 4-benzylthioether (**7**) (Figure 5). From the results of LC-MS, we would like to note that A-type procyanidin-derived products, which are frequently contained in the structure of procyanidins, were not detected. These results gave information regarding the structure of AT-P, which is a B-type procyanidin comprised of four types of flavan-3-ols, some with a galloyl group and catechin attached as the terminal unit. The ratio of the terminal unit and benzyl mercaptan adducts (**2**–**7**) was estimated from the HPLC chromatogram (Table 1). From the estimated ratio, the mean degree of polymerization (mDP) was calculated to be 11.8±0.1. The presence of the four types of flavan-3-ol units (catechin, gallocatechin, epicatechin, and epigallocatechin) is characteristic of AT-P, since previously reported procyanidins are frequently constructed from 1–3 types of flavan-3-ols [16–21].

Figure 5

Table 1

Figure 6

AT-P was analyzed for its α-amylase inhibitory activity (Figure 6). AT-P showed concentration-dependent inhibition of α-amylase with IC_{50} 1.7 μg/mL. When compared at the same concentration, AT-P showed relatively higher activity (85% at 5 μg/mL) than acarbose (61% at 5 μg/mL), the positive control used in the

299 experiment and a medicinally-employed α-amylase inhibitor. The potent activity
300 of AT-P and its high yield from AT clearly indicate that it is the major contributor
301 of α-amylase inhibitory activity in AT.

302 Various procyanidins contained in plants are reported to inhibit α-amylase.
303 Procyanidins from cinnamon (IC_{50} = 4.8 μg/mL) [22], almond (IC_{50} = 2.2 μg/mL)
304 [23], sapodilla (IC_{50} = 4.2 μg/mL) [16], and *Polygonum multiflorum* (IC_{50} = 2.9
305 μg/mL) [17] display comparable α-amylase inhibitory activity to the currently
306 isolated AT-P. In contrast, persimmon peel and leaf (54% at 100 μg/mL and 64%
307 at 240 μg/mL) [18,19], apple (IC_{50} = 1000 μg/mL) [20], and Acacia bark (IC_{50} =
308 38.0 μg/mL) [21] display relatively weaker activity. It is difficult to clearly explain
309 the differences in inhibitory activity from the structures of procyanidins. However,
310 a high mDP and the presence of (gallo)catechin as the extension unit, which are
311 common to the strongly inhibiting procyanidins as well as the current AT-P, may
312 play an important role.

313 In conclusion, *A. thunbergii* potently prevented T2DM. AT decreased post-starch
314 administration blood glucose levels in a T2DM rat model. The procyanidin
315 contained in this material was isolated and characterized as the potent inhibitor
316 of α-amylase, which is responsible for the T2DM preventative effect of *A.*
317 *thunbergii*.

318

319 **Author contributions**

The study was designed by EK, YI, MK, and JK. The *in vivo* experiments were constructed and performed under the responsibility of YI and MK. The *in vitro* experiments were constructed and performed by EK and NK. The results were discussed by all authors. The manuscript was written by EK, and all authors commented on and revised the manuscript.

Disclosure statement

We have no potential conflict of interest to declare.

Acknowledgements

The authors thank Mr. Yusuke Takata and Dr. Eri Fukushi of the GC-MS and NMR Laboratory, Faculty of Agriculture, Hokkaido University for their measurements of mass spectra.

References

1. Krentz AJ, Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* 2005; **65**: 385–411.

2. Kokil GR, Veedu RN, Ramm G a., *et al.* Type 2 Diabetes Mellitus: Limitations of Conventional Therapies and Intervention with Nucleic Acid-Based Therapeutics. *Chem. Rev.* 2015; **115**: 4719–4743.

3. Nakagawa K. Studies Targeting α-Glucosidase Inhibition, Antiangiogenic Effects, and Lipid Modification Regulation: Background, Evaluation, and Challenges in the Development of Food Ingredients for Therapeutic Purposes. *Biosci. Biotechnol. Biochem.* 2013; **77**: 900–908.

4. Komatsu K, Li X-B, Yamaji S, *et al.* Pharmacognostical Studies on the Chinese Crude Drug 'Shengma' (I): General Structure of the Underground Parts of Cimicifuga Plants, Morphological and Anatomical Variations of C. simplex and 'Shengma' Derived from This Species. *Nat. Med.* 1996; **50**: 222–231.

5. Li X-B, Komatsu K, Yamaji S, *et al.* Pharmacognostical Studies on the Chinese Crude Drug 'Shengma' (Part II): 'Shengma' Derived from Genus Cimicifuga Plants. *Nat. Med.* 1997; **51**: 148–161.

6. Han L-K, Miura N, Kawano S, *et al.* Effects of Astilbe thunbergii Rhizomes

Extract on the Postprandial Blood Triacylglycerol Elevation. *Shoyakugaku zasshi Japanese J. Pharmacogn.* 2006; **60**: 68–72.

7. Han L-K, Ninomiya H, Taniguchi M, *et al.* Norepinephrine-Augmenting Lipolytic Effectors from Astilbe t hunbergii Rhizomes. *J. Nat. Prod.* 1998; **61**: 1006–1011.

8. Sun B, Ricardo-da-Silva JM, Spranger I. Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. *J. Agric. Food Chem.* 1998; **46**: 4267–4274.

9. Hashimoto F, Nonaka G, Nishioka I. Tannins and related compounds. LXXVII. Novel chalcan-flavan dimers, assamicains A, B and C, and a new flavan-3-ol and proanthocyanidins from the fresh leaves of *Camellia sinensis* L. var. *assamica* Kitamura. *Chem. Pharm. Bull.* 1989; **37**: 77–85.

10. Cai Y, Evans FJ, Roberts MF, *et al.* Polyphenolic compounds from *Croton lechleri*. *Phytochemistry* 1991; **30**: 2033–2040.

11. Hansawasdi C, Kawabata J, Kasai T. α-Amylase Inhibitors from Roselle (*Hibiscus sabdariffa* Linn.) Tea. *Biosci. Biotechnol. Biochem.* 2000; **64**: 1041–1043.

12. Al-awar A, Kupai K, Veszelka M, *et al.* Experimental Diabetes Mellitus in Different Animal Models. *J. Diabetes Res.* 2016; **2016**: 1–12.

13. Robbins RJ, Leonczak J, Johnson JC, *et al.* Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography-fluorescence detection method for the quantitation of flavanols and procyanidins in cocoa and chocolate containing samples. *J. Chromatogr. A* 2009; **1216**: 4831–4840.

14. Kelm MA, Johnson JC, Robbins RJ, *et al.* High-Performance Liquid Chromatography Separation and Purification of Cacao (*Theobroma cacao* L.) Procyanidins According to Degree of Polymerization Using a Diol Stationary Phase. *J. Agric. Food Chem.* 2006; **54**: 1571–1576.

15. Han KH, Kitano-Okada T, Seo JM, *et al.* Characterisation of anthocyanins and proanthocyanidins of adzuki bean extracts and their antioxidant activity. *J. Funct. Foods* 2015; **14**: 692–701.

16. Wang H, Liu T, Song L, *et al.* Profiles and α-Amylase Inhibition Activity of Proanthocyanidins in Unripe Manilkara zapota (Chiku). *J. Agric. Food Chem.* 2012; **60**: 3098–3104.

17. Wang H, Song L, Feng S, *et al.* Characterization of proanthocyanidins in stems of Polygonum multiflorum thunb as strong starch hydrolase inhibitors. *Molecules* 2013; **18**: 2255–2265.

18. Kawakami K, Aketa S, Nakanami M, *et al.* Major Water-Soluble Polyphenols, Proanthocyanidins, in Leaves of Persimmon (*Diospyros kaki*) and Their α-Amylase Inhibitory Activity. *Biosci. Biotechnol. Biochem.* 2010; **74**:

1380–1385.

19. Lee YA, Cho EJ, Tanaka T, *et al.* Inhibitory activities of proanthocyanidins from persimmon against oxidative stress and digestive enzymes related to diabetes. *J. Nutr. Sci. Vitaminol. (Tokyo).* 2007; **53**: 287–292.

20. Yanagida A, Kanda T, Tanabe M, *et al.* Inhibitory effects of apple polyphenols and related compounds on cariogenic factors of mutans streptococci. *J. Agric. Food Chem.* 2000; **48**: 5666–5671.

21. Kusano R, Ogawa S, Matsuo Y, *et al.* α-Amylase and Lipase Inhibitory Activity and Structural Characterization of Acacia Bark Proanthocyanidins. *J. Nat. Prod.* 2011; **74**: 119–128.

22. Lin G-M, Lin H-Y, Hsu C-Y, *et al.* Structural characterization and bioactivity of proanthocyanidins from indigenous cinnamon (*Cinnamomum osmophloeum*). *J. Sci. Food Agric.* 2016; **96**: 4749–4759.

23. Tsujita T, Shintani T, Sato H. α-Amylase Inhibitory Activity from Nut Seed Skin Polyphenols. 1. Purification and Characterization of Almond Seed Skin Polyphenols. *J. Agric. Food Chem.* 2013; **61**: 4570–4576.

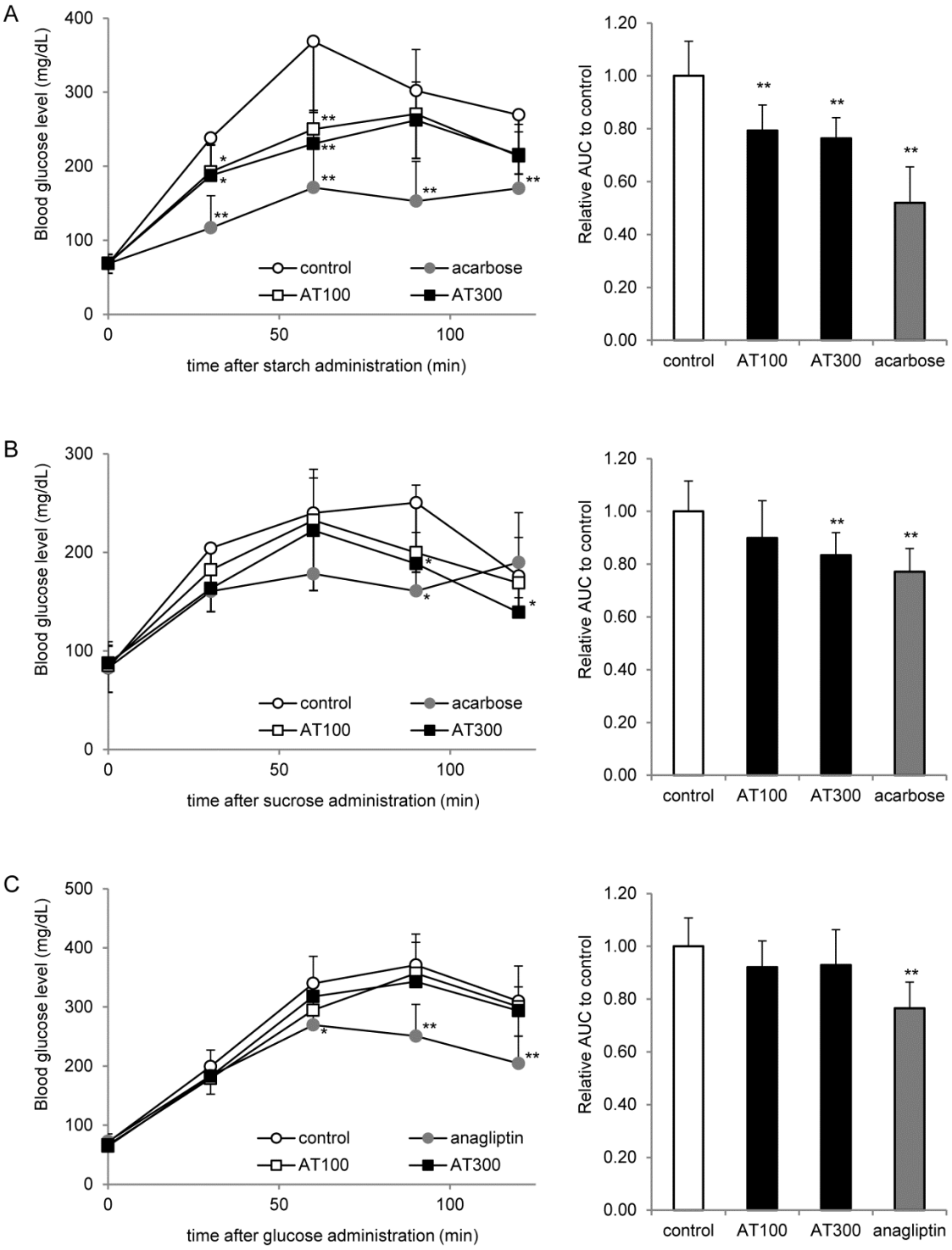

Figure 1. Result of sugar tolerance test with AT administration.

Male GK:Slc rats were orally given AT followed by sugar (A: starch, B: sucrose, C: glucose). AT100: 100 mg/kg AT; AT300: 300 mg/kg AT; acarbose (amylase/glucosidase inhibitor): 10 mg/kg; anagliptin (dipeptidyl peptidase 4 inhibitor): 10 mg/kg. Error bars indicate SD. *$p<0.05$, **$p<0.01$ (Dunnett test).

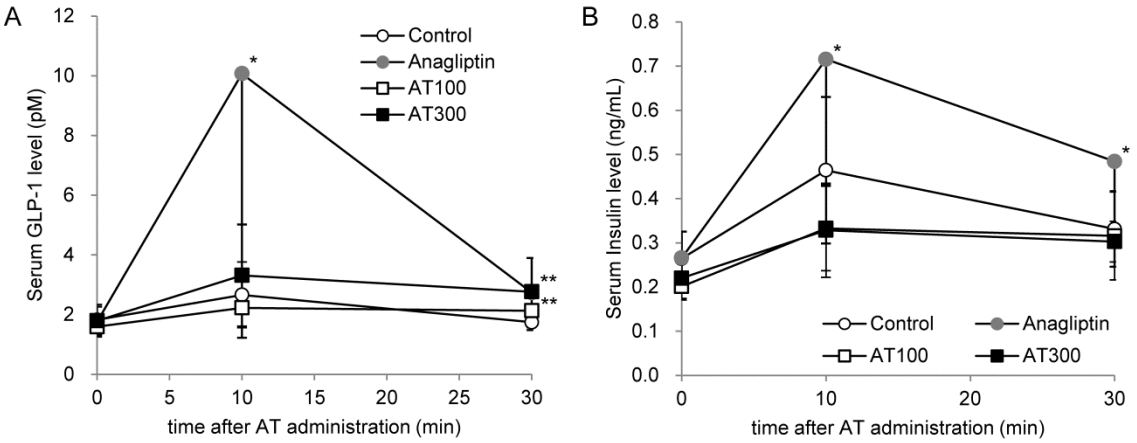

Figure 2. Serum GLP-1 and insulin level after administration of AT.

Male GK:Slc rats were orally given AT and serum level of GLP-1 (A) and insulin was measured (B). AT100: 100 mg/kg AT; AT300: 300 mg/kg AT; anagliptin (dipeptidyl peptidase 4 inhibitor): 10 mg/kg. Error bars indicate SD. *p<0.05, **p<0.01 (Dunnett test).

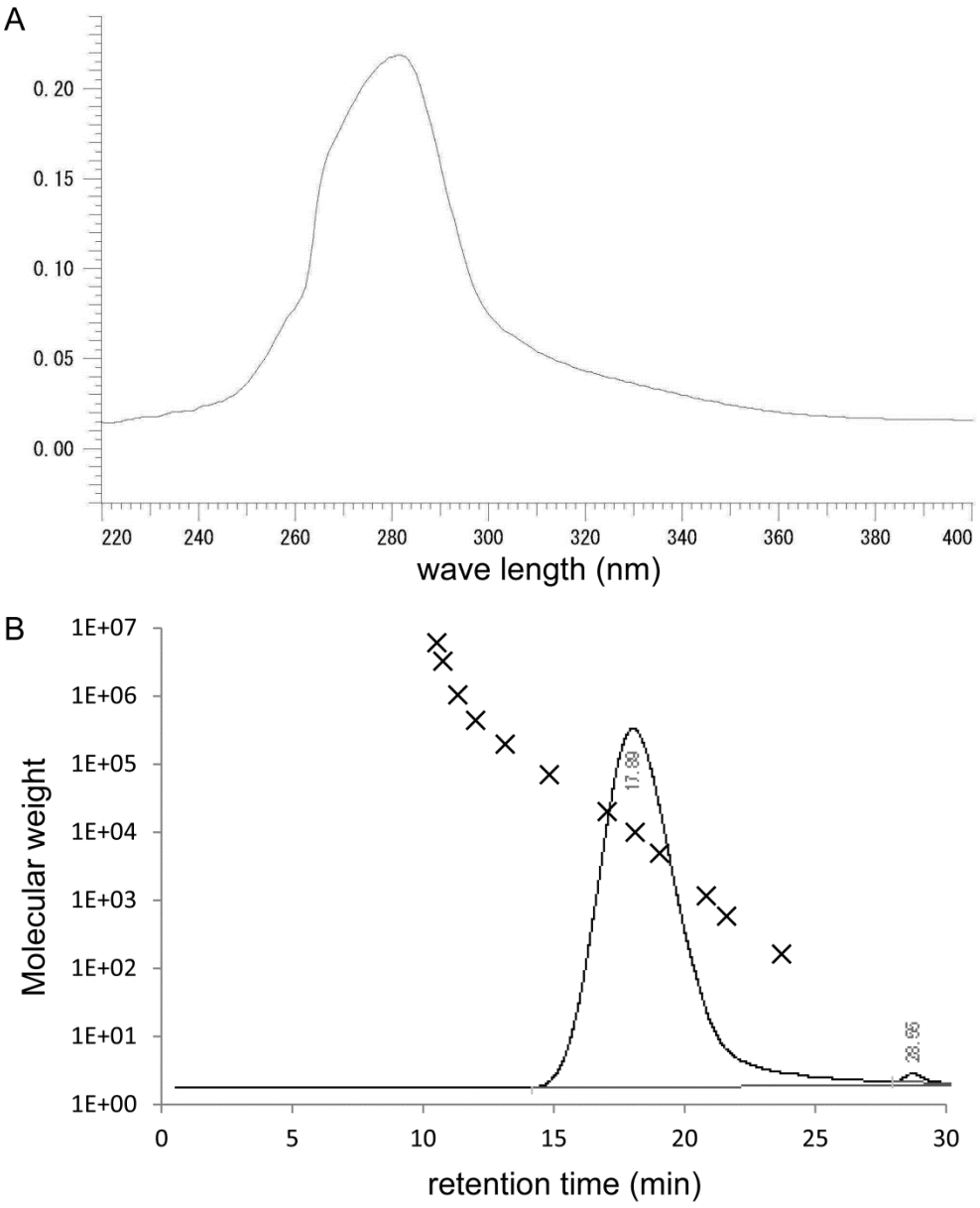

Figure 3. UV absorption and GPC analysis of AT-P.

(A) UV absorption of AT-P; (B) GPC analysis of AT-P with Shodex GF-510 HQ (ϕ7.5×300 mm). X-mark indicates molecular size and retention time of the polystylene standards.

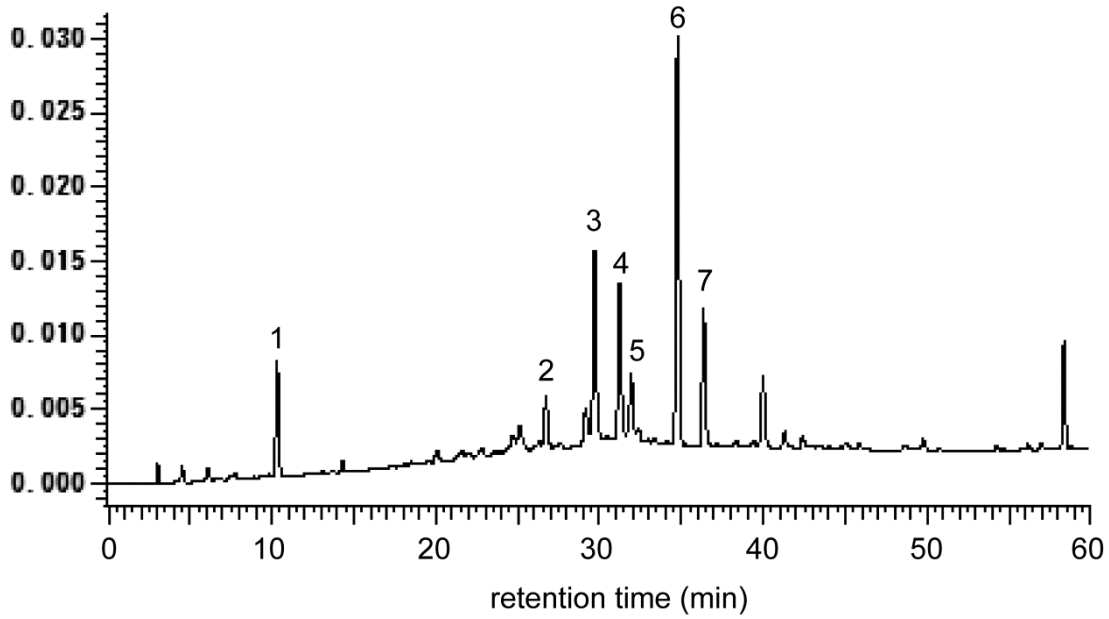

Figure 4. HPLC chromatogram of thiolysis products.
Condition: column InertSustain C18 (ϕ4.6×250 mm), mobile phase gradient elution from 20% aq. methanol containing 0.1% TFA to 95% aq. methanol containing 0.1% TFA with 60 min, flow rate 1.0 mL/min, detection 270 nm. Products: catechin (**1**), gallocatechin 4-benzylthioether (**2**), epigallocatechin 4-benzylthioether (**3**), catechin 4-benzylthioether (**4**), (epi)gallocatechin gallate 4-benzylthioether (**5**), epicatechin 4-benzylthioether (**6**), (epi)catechin gallate 4-benzylthioether (**7**). The stereochemistry of **5** and **7** were not determined.

Table 1. Ratio of AT-P thiolysis products.

position	compound	% mol
terminal	catechin (**1**)	8.5±0.1
extention	gallocatechin 4-benzylthioether (**2**)	9.8±0.2
	epigallocatechin 4-benzylthioether (**3**)	31.6±0.8
	catechin 4-benzylthioether (**4**)	13.2±0.3
	(epi)gallocatechin gallate 4-benzylthioether (**5**)	1.6±0.0
	epicatechin 4-benzylthioether (**6**)	32.4±0.4
	(epi)catechin gallate 4-benzylthioether (**7**)	2.8±0.0
mDP		11.8±0.1

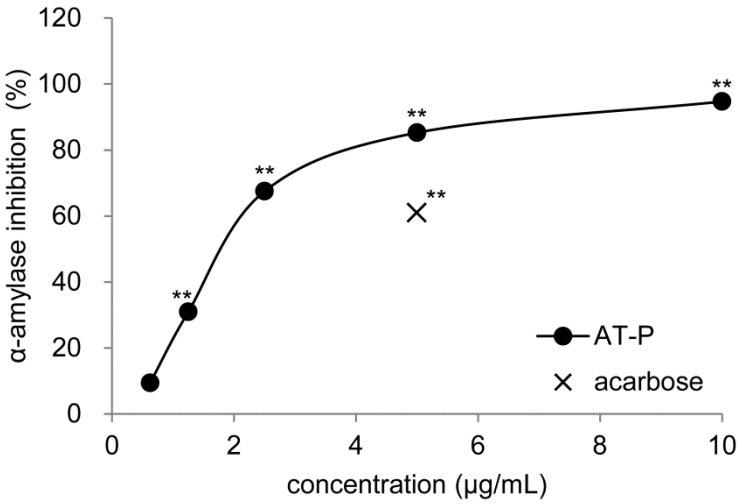

Figure 5. α-Amylase inhibitory activity of AT-P.

Acarbose (5 μg/mL) was used as positive control. Error bars indicate SEM. **p<0.01 (Dunnett test).