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Black Tea Polyphenols Suppress Postprandial Hyperglycemia *In Vivo* in Mice and Inhibit α -Glucosidase Activity *In Vitro*

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Abstract

Black tea is reported to have various beneficial effects on health. Activated charcoal-treated black tea (ACBT) did not contain catechins nor caffeine and included small amount of theaflavins (TFs). We had further fractionated ACBT to obtain black tea polymerized polyphenols (BTPP), TFs-poor fraction and TFs-rich fraction and studied *in vitro* and/or *in vivo* effect of the fractions to elucidate the effect of ACBT. Sucrose-loading test in mice showed that ACBT and BTPP at the dose of, 1000 and 560 mg/kg, respectively, suppressed the increase of blood glucose level while secretion of insulin was not affected. We found that this effect is caused by inhibition of α-glucosidase activity. BTPP contained TFs, but the content was not at all enough to explain the activity of ACBT, ^{1}H NMR analysis of BTPP was carried and showed the existence of many benzotropolone ring containing substances as active compounds.

Keywords: Black Tea; α-Glucosidase; Sucrose; Postprandial Hyperglycemia; Benzotropolone Ring

Abbreviations: ACBT: Activated Charcoal-Treated Black Tea; AUC: Area Under the Curve; BMI: Body Mass Index; BTPP: Black Tea Polymerized Polyphenols; CMC・Na: Sodium Carboxymethyl Cellulose; DMSO: Dimethyl Sulfoxide; AcOEt: Ethylacetate; EC: (-)-Epicatechin; ECG: (-)-Epicatechin-3-O-Gallate; EGC: (-)-Epigallocatechin; EGCG: (-)-Epigallocatechin-3-O-Gallate; GLUT: Glucose Transporter; TF: Theaflavin; TF3G: Theaflavin-3-O-Gallate; TF3'G: Theaflavin-3'-O-Gallate; TF3, 3'diG: Theaflavin-3, 3'-di-O-Gallate

Introduction

Recently, overweight and obesity have become an increasingly serious problem in the world. From 1980 to 2013, their population increased in both underdeveloped and developed countries, regardless of gender, reaching 2.1 billion in 2013 [1]. Overweight and obesity are often accompanied by type II diabetes, ischemic heart disease, high blood pressure, various malignant tumors and other health problems [2]. It is necessary to solve these problems.

One of the most effective ways to prevent overweight or obesity is to suppress the degradation of sugars. Oral intake of carbohydrates are followed by digestion in stomach then in intestine, where α-glucosidase on the mucosal epithelia degrades them and produce glucose which is absorbed into the bloodstream. Acarbose, an inhibitor of α-glucosidase, is used to treat type II diabetes and reduce glucose absorption through delayed carbohydrate digestion. This drug therapy is to suppress rapid increase in blood glucose levels after eating, thereby preventing the glycotoxicity. Some studies showed that food ingredients such as salacia reticulata [3] and cacao liquor procyanidin [4] inhibited α-glucosidase (e.g., maltase and sucrase) activity. Therefore, regulation of sugar absorption and metabolism is useful for anti-obesity effects.

Tea, prepared from the leaves of *Camellia sinensis,* are popular beverages consumed all around the world. Teas are classified largely into three groups, green tea, oolong tea and black tea according to the fermentation degrees, non, semi and full fermentation, respectively. Recently, many studies have been done and reported on the effects of the teas and their fractionated products on health including obesity and various health area. Green tea was reported to reduce blood LDL cholesterol, suppress absorption of fat, enhance consumption and burning of energy in adipose tissue [5-7]. Oolong tea also has anti-obesity effects, e.g., inhibition of pancreatic lipase, delay of absorption of triglyceride from lymph duct and suppression of hypertriglyceridemia after meal [8,9]. In addition, anti-stress, anti-diabetes and anti-oxidation effects of the Oolong tea were also reported [10-15]. Black tea is consumed regularly for a long time in the world [6] and its ingredients are effective and beneficial for preventing obesity and hyperglycemia. Black tea was reported to enhance translocation of GLUT4 to cell membrane in muscle of C57BL/6J mice fed a high fat diet for 14 weeks [16]. Also, intake of black tea induced phosphorylation of phosphoinositide 3-kinase (PI3K) and its downstream Akt/protein kinase B was enhanced. PI3K/Akt-pathway and insulin independent AMP-activated protein kinase (AMPK) pathway were activated [17].

These findings suggest that black tea affects not only suppression of intestinal absorption of sugars but also glucose metabolism in muscle and other organs.

Black tea is composed of a few catechins (e.g., EC, ECG, EGC, and EGCG) and several polymerized polyphenols (e.g., TFs and thearubigins). Four major TFs in black tea, namely TF, TF3G, TF3'G and TF3, 3'diG have been identified. Although those amounts are only about 0.3〜2% of dry weight of tea leaves [18], the TFs exhibited suppressive effects of the increasing blood glucose level [19]. However, the TFs was unstable because of oxidative polymerization and decomposition in the extract [18,20]. On the other hand, thearubigins were made by oxidative polymerization of TFs and/or polyphenols having benzotropolone ring and therefore have very complex structures.

To investigate the potential of the black tea ingredients, we isolated the Activated charcoal-treated black tea (ACBT), black tea polymerized polyphenols (BTPP) and their stable TFs by HPLC and NMR studies, and then these fractions were examined the suppression effect of α-glucosidase activity by *in vitro* and *in vivo* studies. Our results suggest that new fractions can be effective for preventing obesity and hyperglycemia.

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Materials and Methods

Chemicals

Theaflavin (TF), theaflavin-3-O-gallate (TF3G), theaflavin-3'-O-gallate (TF3'G), theaflavin-3, 3'-di-O-gallate (TF3, 3'diG) were from Nagara Science, Gifu, Japan.

Intestinal acetone powders from rat (SIGMA, St. Louis, MO, USA), glucose C II test Wako (Wako Pure Chemical Industries, Osaka, Japan), acarbose (SIGMA, St. Louis, MO, USA), distilled water (Otsuka Pharmaceutical Co, Tokyo, Japan), Glutest Neo Sensor (Sanwa Kagaku Kenkyusho, Aichi, Japan), ultra-sensitive mouse insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan), DMSO-d_6 (Euriso Top, France), CMC•Na, maleic acid, sucrose, DMSO and all solvents for HPLC (Nacalai Tesque, Kyoto, Japan) were purchased from the companies shown in parentheses.

Preparation of ACBT: Two hundred grams of black tea leaves (*Camellia sinensis* (L.) Kuntze var. *assamica*) were extracted with 2 L of hot water for 10 min. After filtration, the extract was applied to an 80 ml of activated charcoal column (Kuraraychol, GW-H, Kuraray Chemical Co. Ltd.) whose temperature was kept at 65°C. The eluent in the fractions of 2 to 11 column volumes was concentrated and lyophilized yielding 39 g of ACBT.

Preparation of BTPP: Thirty grams of ACBT was applied to an HPLC column, Daiso SP-120-40/60 ODS-B (110 mm I.D. x 1000 mm), and eluted under monitoring of absorption at 280 nm, at a flow rate of 300 ml/min with 0.2% HCOOH in 15% CH_3CN/aq for 136 min. Elution was continued with 0.2% HCOOH in 80% CH_3CN/aq for 30 min to yield BTPP. The BTPP containing eluent was evaporated and lyophilized twice to remove HCOOH and yielded 8.4 g of dried matter, the yield was 28%.

Preparation of TFs-poor and TFs-rich fraction: Forty nine mg of BTPP was extracted with the mixture of water and ethyl acetate. The resulting aqueous layer yielded, 35.8 mg and the organic layer, 12.7 mg of dried matters. The aqueous layer was named TFs-poor fraction and the organic layer TFs-rich one, respectively.

HPLC analysis of TFs in ACBT, BTPP, TFs-poor and TFs-rich fractions: All fractions were dissolved with 50% CH_3CN and filtrated through 0.45 μm filter (Merck Millipore LH-4) and each was applied to an HPLC. HPLC was conducted using a Develosil ODS-MG-5 (Nomura Chemical, Japan, 4.6 mm I.D. x 150 mm) column and a flow rate of solvent at 1.0 ml/min; the isocratic solvent system used was as follows; 0.04% TFA in 76% H_2O, 21% CH_3CN, and 3% AcOEt. The HPLC was carried out with a photodiode array detector SPD-M10A (Shimadzu Co., Ltd., Japan) detecting absorbance at 375 nm and column temp at 40°C.

NMR analysis of BTPP: Five mg of BTPP was dissolved in 0.55 ml of DMSO-d_6 containing 10 μM of 4-bis (trimethylsilyl) benzene (Sigma-Aldrich) added as an internal standard in 5 mm I.D. NMR tube. ^{1}H NMR spectrum were obtained on AVANCE-3 HD-400 spectrometer (BRUKER BIOSPIN, Germany), with number of scans, 1024; relaxation delay (d1), 20 sec; acquisition time, 4.089 sec.

Measurement of α–glucosidase(sucrase) activity: Enzyme activity was measured essentially following the methods of Yoshikawa et al. [21-23] and Matsuda et al. [24,25]. We determined that intestinal expression of alpha glucosidase between rats and mice was the same from previous reports [26-28].

One gram of acetone powder from rat intestine was suspended in 45 ml of 0.1 M maleic acid buffer (pH 6.0), homogenized and centrifuged at 20,000 × G for 20 min at 4°C. The supernatant removed and diluted twice with the buffer was used as the crude enzyme. The sample in 25 μl of 50% DMSO was added to 50 μl of 74 mM sucrose substrate in 0.1 M maleic acid buffer (pH 6.0). After 3 min, reaction was stopped by adding 400 μl of distilled water and heating for 10 min in boiling water. After leaving on ice for 10 min, an aliquot of 100 μl was put into a well of a 96 well microplate, then 150 μl of glucose CII test Wako was added and left for 10 min to develop color. The amount of D-glucose formed was measured at 510 nm using a microplate reader Filter Max F5 (Molecular Devices, USA). A sample, substrate and crude enzyme were mixed and immediately put into boiling water and left for 10 min to inactivate the enzyme and to serve as a blank. Acarbose was used as a positive control.

Animals

Six-week-old male C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained and acclimatized for a week in an air-conditioned room kept at 23 ± 1.5°C and 55 ± 10% humidity, under a constant 12 h light-dark cycle (light from 7:00 to 19:00). They were fed *ad libitum* on commercial laboratory chow, CE-2 (Clea, Japan) and water. All animal experiments were performed under the guidelines established by the Japanese Society of Nutrition and Food Science (Law No. 105 and Notification No.6 of the Japanese Government).

Sucrose loading experiment: Sucrose was dissolved in distilled water at the concentration of 0.2 g/ml and a dose of 2 g/kg body weight was administered to mice. To dissolve ACBT, a suspension of 1 g/ml of distilled water was heated at 50-60°C, then left at room temperature to cool before use. Solution of 0.5% CMC•Na was added to BTTP at the concentration of 280 or 560 mg/ml, heated at 50-60°C and cooled likewise. The test solutions were orally administered to mice weighing 19-21 g and had been fasted for 16 h. Oral sucrose loading immediately followed. Just before and after 15, 30, 60, 90, 120 min of the administration, ca. 25 μl of blood was obtained from tail vein and submitted to on-the-spot measurement of blood sugar level using Glutest Neo Sensor. Then the blood was centrifuged at 10,000 rpm for 5 min at 4°C and the resulting supernatant was collected and kept at -80°C before measuring insulin concentration using Ultra Sensitive Mouse Insulin ELISA Kit.

Statistical analysis: Values are the mean ± standard error (SEM). After t-test and one-way ANOVA, values were compared by Dunnett-type multiple comparison procedure. All analyses were done using SPSS statistics version 10.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when probability values were less than 0.05.

Results

Analysis of TFs in ACBT, BTPP, TFs-poor and TFs-rich fractions

We prepared ACBT using an activated charcoal column and further fractionated it to obtain BTPP, TFs-poor and TFs-rich fractions as described in Materials and Methods. Amounts of four major black tea TFs in the fractions were analyzed by HPLC and shown in Table 1. Neither caffeine nor catechins were detected in those fractions. Apparently, TFs were not much lost, if any, at each fractionation step. Especially noteworthy is that more TF was recovered in TFs-poor fraction than in TFs-rich fraction. On the other hand, far more of other three TFs were found in TFs-rich fraction than in TFs-poor fraction as expected.

NMR analysis

Five mg of BTTP was submitted to NMR analysis as described in Materials and Methods**.** Signals from hydrogen-bonded OH groups of the benzotropolone ring (Figure 1b) were observed at around 15 ppm in full and expanded ^{1}H NMR spectra of BTPP (Figure 1a and 1c). Presence of six large and several smaller signals (Figure 1a) suggested that in addition to four TFs identified by HPLC, many compounds which have the benzotropolone ring should be present in BTPP.

Inhibition of α−glucosidase (sucrase) activity

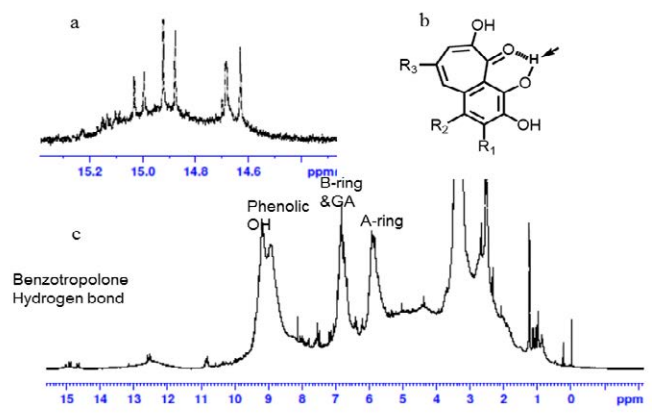
Enzyme inhibition test was performed using crude sucrase preparation from rat intestinal acetone powder. Commercial TFs, e.g., TF, TF3G, TF3'G, TF3, 3'diG were shown to have sucrase inhibitory activity with IC_{50}. Black tea derived fractions, ACBT, BTPP, TFs-poor and TFs-rich fraction were also shown to inhibit sucrase with IC_{50} comparable to that of TFs. Apparently, BTPP and TFs-poor fraction showed similar but about twice stronger IC_{50} than ACBT. Also TFs-poor fraction had somewhat stronger activity than TFs-rich fraction. From the strength of activity and the amount of fraction, total activity unit and contribution of each fraction to the sucrase inhibitory activity of ACBT was calculated and shown (Table 2). It should be noted that, contribution of TFs-poor fraction was almost 4 times more than that of TFs-rich fraction and that four TFs combined together in each of ACBT, BTPP, TFs-poor and TFs-rich fraction contributed less than 1% of the inhibitory activity of each fraction (Tables 2 and 3).

These results clearly showed that although TFs had α−glucosidase (sucrase) inhibitory activity, contribution of TFs to the enzyme inhibitory activity of ACBT or BTPP was very low. Thus, presence of enzyme inhibitory substances other than TFs was strongly suggested. These and NMR results combined, benzotropolone ring containing thearubigins were suggested to be prime candidates for active substances.

		Content of TFs (mg)**				
Fraction	**Weight (g)***	**TF**	**TF3G**	**TF3'G**	**TF3, 3'diG**	**Total**
ACBT	100	64.0	42.2	19.8	84.2	210.2
BTPP	28	59.5	44.6	20.0	87.8	211.9
TFs-poor	20.9	35.8	3.57	0.50	2.91	42.82
TFs-rich	7.1	25.7	61.9	26.7	127.1	241.39

*Weight of dried powder
**One mg of TFs were submitted to HPLC analysis as indicated in Materials and Methods and total amounts in each fraction were calculated.

Table 1: Fractions of black tea and content of tfs in each fraction.



a: An expand spectra of benzotropolone hydrogen bond proton area; b: Structure of benzotropolone group, arrowed H occurs in about δ15; c: A full spectra of BTPP

Figure 1: H NMR spectrum of BTPP in DMSO-*d*6.

	Sucrase Inhibitory Activity		
Fraction	**IC_{50} (mg/ml)**	**Total Activity Unit***	**Contribution (%)**
ACBT	0.516	194,000	100.0
BTPP	0.237	118,000	60.8
TFs-poor	0.239	87,400	45.1
TFs-rich	0.316	22,500	11.6

*Total activity unit is calculated by dividing amount of a material by IC_{50} value of the material.

Table 2: Sucrase inhibitory activity of black tea fractions.

TFs	**IC_{50}(mg/ml)***	**Total Activity Unit in Fractions of****			
		ACBT	**BTPP**	**TFs-poor**	**TFs-rich**
TF	0.479	134	124	75.0	54
TF3G	0.450	94	99	7.9	138
TF3'G	0.400	50	50	1.3	67
TF3, 3'diG	0.307	274	286	9.5	414
Total	—	552	559	93.7	673

*IC_{50} was measured using commercial TFs.
**Total activity unit is calculated as described in the legend of Table 2.

Table 3: Sucrase inhibitory activity of four tfs in black tea fractions.

Sucrose loading test

After 15 and 30 min of simultaneous oral administration of sucrose and ACBT (1000 mg/kg), the rise of blood glucose level was suppressed significantly (Figure 2). The suppression was 16.8% with ACBT when AUC for ACBT and control were compared (Figure 3). On the other hand, blood insulin level measured in the same experiment, was not affected significantly in ACBT group in both analyses (Figures 4 and 5).

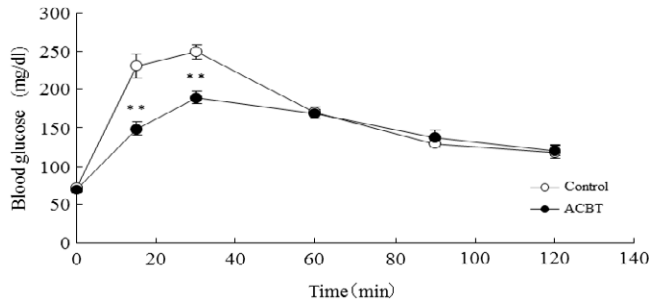
BTPP derived from ACBT given at the dose of 560 mg/kg suppressed significantly the blood glucose level 15 and 30 min after the administration, while at the dose of 280 mg/ml, suppression occurred at 15 min (Figure 6). As for AUC, 560 mg/ml group showed significant 23% suppression while 280 mg/ml group showed suppression tendency by 12.2 % although not significant (Figure 7).

Discussion

It was shown that simultaneous administration of ACBT or BTPP and sucrose to C57BL/6J mice, suppressed the increase of blood glucose level, at the dose of 1000 mg/kg for ACBT (Figures 2 and 3) and 560 mg/kg for BTPP (Figures 6 and 7). On the other hand, no significant difference from control was observed in blood insulin level at any time point after the administration of ACBT at 1000 mg/kg (Figures 4 and 5). Insulin is a hormone known to be released upon increase of blood glucose level resulting from the absorption of glucose from intestine then suppress the blood glucose level. Accordingly, no increment of its blood level indicated that ACBT and BTPP suppressed the increase of blood glucose level by inhibiting the absorption of sugar from intestine. These results, i.e., suppression of blood glucose level without increasing insulin blood level strongly suggested that ACBT and BTPP could be effective in treating diabetics and pre-diabetics who are insulin resistant or insulin secretion incompetent [28].

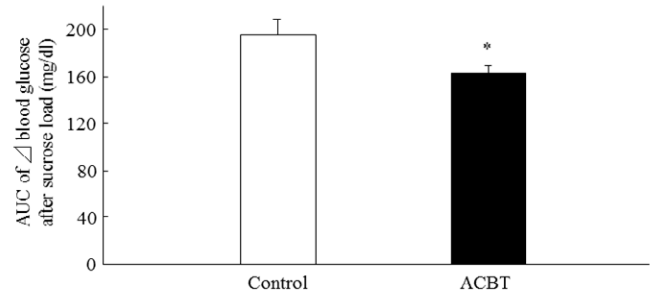
We also showed that, *in vitro,* ACBT, BTPP and fractions derived thereof inhibited a rat intestine α-glucosidase, sucrase. Contribution of TFs-poor fraction was shown to be much more than TFs-rich fraction to the enzyme inhibitory activity of ACBT and BTPP (Table 2).

In the present study, we focused on sucrose and successfully showed that black tea fractionation products, suppressed absorption of sucrose by inhibiting sucrase. In the future studies, it would be interesting to



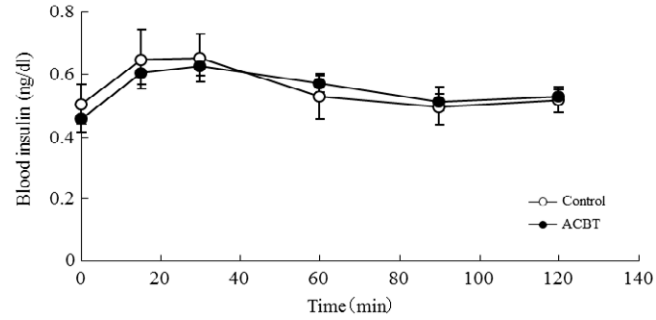
The mice (n = 8 per group) were administered water (control) or ACBT at a concentration of 1000 mg/kg body weight. Blood glucose levels were measured pre (0), 15, 30, 60, 90 and 120 min after sucrose loading. Values are presented as means ± SEM. * * p< 0.01, relative to the control by an unpaired t-test.

Figure 2: Effect of oral administration of ACBT on blood glucose after sucrose loading in C57/BL6 mice.



AUC of blood glucose in the experiment depicted in Figure 2 was calculated and shown. Values are presented as means ± SEM. *p < 0.05, relative to the control by an unpaired t-test.

Figure 3: Effect of oral administration of ACBT on blood glucose AUC after sucrose loading in C57/BL6 mice.



In the experiment depicted in Figure 2, blood insulin levels were measured pre (0), 15, 30, 60, 90 and 120 min after sucrose loading. Values are presented as means ± SEM.

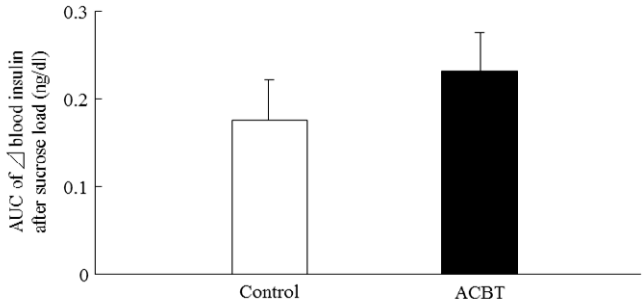
Figure 4: Effect of oral administration of ACBT on blood insulin after sucrose loading in C57/BL6 mice.

test the effectiveness towards other sugars, e.g., starch, maltose and so on, which would lead to better understanding of the mode of action.

Resistant maltodextrin softens the rise of after-meal blood glucose level and thus, in metabolic syndrome patients if taken for 12 weeks with meals improved before-meal blood glucose level and the homeostasis model assessment ratio (HOMA-R), an index of insulin resistance, in comparison with placebo group [29]. Likewise, BTPP which we showed in the present study to suppress the rise of blood glucose level induced by food intake, would affect before-meal blood glucose level and insulin resistance, if taken with each meal for a long time.

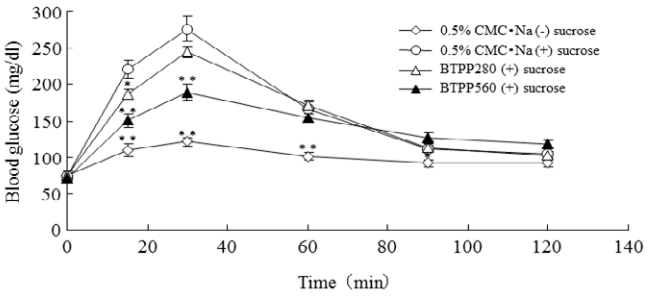
Many studies have been reported to show the relation between obesity and black tea extracts and polyphenols contained. Because many reports are on the effects of black tea extracts on lipid absorption through inhibition of fat degrading enzyme, lipase and on lipid metabolism, possibility that BTPP of our current interest might exert similar effects on after-meal lipids are suggested. Further studies are definitely needed to verify the possibility. As the active substances responsible for the above mentioned anti-obesity effects, catechins, e.g., EC, ECG, EGC and EGCG, TFs and other black tea specific polyphenols are listed [30-33].

Present study focused on sugars, especially sucrose and showed that while the active substances in BTPP were in TFs-poor fraction in addition to TFs, the contribution to the total enzyme inhibitory activity of BTPP was much more from the former than from the latter. TFs-poor fraction is a mixture of, in addition to TF, substances known as thearubigins whose structure is complex and several model compounds are proposed. From ^{1}H NMR analysis of BTPP, presence of many substances having flavan-3-ol and benzotropolone ring [32] was shown indicating the presence of benzotropolone ring containing substances other than TFs (Figure 1). In this respect, it is of interest to note that black tea extract and its fractions are known to have inhibitory activities over α-amylase and lipase, and polymer-like oxidation products are considered to be active compounds [32].



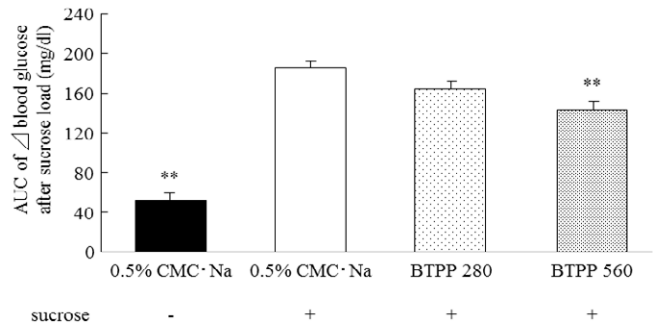
AUC of blood insulin in the experiment depicted in Figure 4 was calculated and shown. Values are presented as means ± SEM.

Figure 5: Effect of oral administration of ACBT on blood insulin AUC after sucrose loading in C57/BL6 mice.



The mice (n = 8 per group) were administered 0.5% CMC•Na as control or BTPP at a concentration of 280, 560 mg/kg body weight. Blood glucose levels were measured pre (0), 15, 30, 60, 90 and 120 min after sucrose loading. Values are presented as means ± SEM. *p < 0.05, * * p< 0.01, relative to the control (one-way ANOVA followed by Dunnett's test).

Figure 6: Effect of oral administration of BTPP on blood glucose after sucrose loading in C57/BL6 mice.



AUC of blood glucose in the experiment depicted in Figure 6 was calculated and shown. Values are presented as means ± SEM. * * $p < 0.01$, relative to the control (one-way ANOVA followed by Dunnett's test).

Figure 7: Effect of oral administration of BTPP on blood glucose AUC after sucrose loading in C57/BL6 mice.

In Figures 4 and 5 BTPP didn't affect insulinemia. However, blood glucose (as AUC) is about 20% reduced by its administration. Because insulin is released in proportion to the variation in blood glucose levels, insulin-independent glucose uptake is expected. Glucagon-like peptide-1 (GLP-1) is secreted from the small and large intestines in specialized intestinal L-cells. It increases following nutrient ingestion, stimulates glucose-dependent insulin release [34,35]. In addition, GLP-1 effects on peripheral tissues other than the pancreas, biological actions of GLP-1 or GLP-1 receptor agonists are mediated via direct (in pancreas, brain, kidney, and heart) or indirect (in stomach, liver, skeletal muscle, and adipose tissue) mechanisms [36]. In further research, we need to measure whether BTPP stimulates releasing GLP-1. In addition, poorly absorbed polyphenols such as flavan-3-ols, especially procyanidins have been reported that activate AMPK in skeletal or gastrocnemius muscle mediated vagal nerve [37]. BTPP may activate AMPK in the non-insulin-dependent manner, enhance translocation of GLUT4 to cell membrane. Further research is required in order to find out this mechanism.

Conclusion

ACBT inhibited sucrose-loading induced increase in blood glucose level, and majority of active substances were in BTPP. Main mechanism of the activity was the inhibition of sucrase resulting in glucose absorption suppression. Large contribution of TFs-poor fraction to the enzyme inhibitory activity and the presence of benzotropolone ring containing substances therein were shown.

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