The Effect of Methanol Extracts of Tsao-ko (Amomum tsao-ko Crevost et Lemaire) on Digestive Enzyme and Antioxidant Activity In Vitro, and Plasma Lipids and Glucose and Liver Lipids in Mice

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Summary Our previous study showed that tsao-ko intake can lower plasma and liver triacylglycerol (TG) concentrations and has hypoglycemic and antioxidant activity in mice. This study involved separating two major fractions (A and B) from the methanol extracts (MeX) of tsao-ko using silica gel column chromatography, and then determining the effect of the fractions in vivo and in vitro to clarify the most effective components of tsao-ko. An intake of MeX and A fraction statistically significantly reduced body lipids and plasma thiobarbituric acid reactive substances (TBARS) concentrations compared with the control and inhibited lipase and α-glucosidase activities. These reductions were not observed in mice fed the B fraction and these inhibitions of B fraction were mild compared with MeX and A fraction. The plasma and liver TG concentrations of each fraction group did not show significant differences compared with the control. The [M–H]+ and maximum UV absorption of the A fraction were 291 m/z and 279 nm, respectively. The peak of A fraction appeared at a similar time to the epicatechin standard in the LC/MS/MS analysis and the MS/MS spectrum of the A fraction was similar to that of the epicatechin standard. It was concluded that the most effective component of tsao-ko for body lipid reduction and hypoglycemic and antioxidant activity was contained in the polar fraction and the evidence suggested that this component could be epicatechin. However, the strongest TG lowering components of tsao-ko may be methanol insoluble.

Key Words epicatechin, tsao-ko, antioxidant activity, in vitro, mice

There is now a growing body of scientific evidence to support the concept that spices have medicinal properties. It is possible that they not only alleviate symptoms of disease, but also help prevent it (1–5). Dietary spices are non-toxic and studies in animal models have suggested that they could be beneficial to human health (3–7). Amomum tsao-ko Crevost et Lemaire (tsao-ko), which belongs to the Zingiberaceae family, is a spice used in Chinese cuisine and as a Chinese traditional medicine. It has been used in folk medicine for the treatment of stomach disorders and throat infections.

In an earlier screening experiment, we demonstrated that 1% tsao-ko can lower triacylglycerol (TG) levels and has hypoglycemic and antioxidant activity in mice (7). However, in a subsequent study, the total extracted lipids from tsao-ko did not significantly decrease TG levels or antioxidant activity, although a significant hypoglycemic effect was still apparent (8). However, significant hypoglycemic and antioxidant activity was evident following an intake of the polar fractions of tsao-ko extracted lipids (methanol fraction) in mice (8). Thus, the most effective components of the total lipids appear to be in the methanol soluble fraction. Therefore, it is inferred that the most effective components of tsao-ko may be polar components. In the present study, we further fractionated the methanol extract of tsao-ko by silica gel chromatography and determined the effect of the fractions on lipase, α-glucosidase, and α-amylase activity in vitro and on plasma lipids and glucose, and liver lipids in mice.

MATERIALS AND METHODS

Materials. n-Hexane, chloroform, and methanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were used without further puri-
fication. The (+)-catechin and (−)-epicatechin were purchased from Funakoshi Co., Ltd., Tokyo, Japan. Anhydrous sodium sulfate (Na₂SO₄) and silica gel (C-300; 45–75 μm, for silica gel column chromatography) were also purchased from Wako Pure Chemical Industries, Ltd. The dried fruits of tsao-ko were purchased at the Liouzhou herbal market, Guangxi, China, in April 2005. Following removal of their skins, the dried fruits of tsao-ko were reduced to powder using a disintegrator (Ultra Centrifugal Mill, MRK Co., Ltd., Tokyo, Japan) at room temperature. The lard was supplied from NOF Co., Ltd., Tokyo, Japan. Salt and vitamin mixtures were purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. All of the materials were kept at 5˚C and used within 1 mo.

**Preparation and fractionation of tsao-ko methanol extract (MeX).** The dried powder of tsao-ko was extracted with 6 times its volume of n-hexane at room temperature for 12 h using a blender. The extract was filtered through 0.45 μm filter paper and the residue further extracted twice with equal volumes of n-hexane. The resultant residue was then extracted in a similar manner with chloroform and then methanol. The extracts were evaporated to dryness using a rotary vacuum evaporator with the water bath heated at 40˚C. The final removal of methanol was carried out by freeze-drying. The two largest fractions (A and B) were obtained following silica gel chromatography of MeX (solvent, chloroform : methanol : water = 6 : 4 : 1).

**Determination of total polyphenols.** The total polyphenol contents of MeX and each sub-fraction of MeX were measured using Folin-Ciocalteu reagent (9). Each diluted fraction of MeX (50 μL of 1 : 20, v/v) and the polyphenol standard (chlorogenic acid) were mixed with Folin-Ciocalteu reagent (500 μL of 1 : 10 with distilled water, v/v) and aqueous Na₂CO₃ (400 μL, 1 M) was then added. The mixtures were left at room temperature for 1 h and the absorbencies measured at 725 nm. The A fraction was also analyzed using an ABI 4000 QTRAP mass spectrometer system (Applied Biosystems, Warrington, Cheshire, UK) fitted with a TurboSpray ESI source interface and separated on a ZORBAX Eclipse Plus C18 column (2.1 mm × 150 mm, 3.5 μm; Agilent Technologies, USA) using a binary gradient (mobile phase A, 0.1% acetic acid in distilled water; mobile phase B, 0.1% acetic acid in acetonitrile) under the following conditions: source temperature, 400˚C; ion spray voltage, 5 kV; collision energy, 10 V; curtain gas (nitrogen), 10 psi; sheath gas (nitrogen), 50 psi; drying gas (nitrogen), 50 psi; and collision gas (nitrogen), high. The scan mode was used for data collection and spectra were scanned over a mass range of 60–900 m/z. The HPLC was coupled to the DAD detector followed by mass spectrometer.

**Determination of antioxidant activity and inhibitory activities of the methanol fractions on lipase, α-glucosidase, and α-amylase activity.** The various fractions were dissolved in dimethyl sulfoxide (DMSO). Their inhibitory action on α-glucosidase was assayed by the method of Tadera et al. (10), on α-amylase inhibitory using a Wako-amylose kit (Wako Pure Chemical Industries, Ltd.) and on lipase using a modification of the method of Han et al. (11). For assay of lipase inhibition, a mixture (100 μL) of triolein (80 mg), phosphatidylcholine (10 mg), bile salt (5 mg), and 1% albumin in 9 mL of 0.1 M Tris buffer (pH, 7.0) homogenized with a phsyocotron (NS-50, Microtec Co., Ltd., Chiba, Japan) was incubated with 0.05 mL (2 units) of pancreatic lipase solution and 10 μL of various concentrations of the sample fractions for 30 min at 37˚C. The fatty acid released from triolein was measured using the Wako non-esterified fatty acid-C (NEFA-C) test kit (Wako Pure Chemical Industries, Ltd.). All experiments were performed between 4 and 6 times. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging of each fraction was assayed by a modification of Bloiss’ method (12). Samples of fractions (0.5–15 μg/L) were placed in wells of a micro-plate and 20 μmol of DPPH isopropanol solution (200 μL) was added to each well and the absorbance measured at 550 nm after 5 min. The IC₅₀ of each fraction was estimated from the linearity point. Each assay was performed 3 times.

**Diets.** The composition of the control diet was as follows: 47.8% cornstarch, 20% casein, 15% sucrose, 6% lard, 5% cellulose powder, 4% salt mixture, 2% vitamin mixture and 0.2% l-methionine. The three experimental diets contained 0.0174% A fraction, 0.0121% B fraction or 0.0407% methanol extract instead of cornstarch. The added amounts of the three fractions were equivalent to those contained in a 1% tsao-ko diet.

The control and experimental diets were prepared once a month, and stored below 5˚C.

**Animals.** Male mice of the Crj:CD-1 (ICR) strain (4 wk old) were obtained from Charles River Japan Inc. (Atsugi, Kanagawa, Japan). All animals were switched from a laboratory chow, MF (Oriental Yeast Co., Ltd.) to the control and experimental diets at 12 wk of age. Mice were randomly divided into 4 groups of 8 animals each and each group was fed on the control or an experimental diet for 90 d. Animals had free access to the experimental diet and water until the fasting period. Body weights were measured once a month. The animals were housed in suspended stainless-steel cages with wire mesh bottoms. The animal room was kept at 24±0.5˚C and a relative humidity of 65±5%. Room lighting consisted of 12-h periods of light and dark. All mice were maintained according to the guidelines for experimental animals of the National Food Research Institute, National Agriculture and Food Research Organization (NARO), Japan.

**Preparation of plasma and liver homogenates and body samples.** At the end of the feeding trials, all mice were fasted for 20 h before being anesthetized with diethyl ether. Blood was then collected from the inferior vena cava with a heparinized syringe and placed in ice-cold tubes. The plasma was separated by centrifugation at 900 × g for 20 min at 4˚C. After collecting the blood, the livers were removed and homogenized with 1/15 mol/L phosphate-buffered saline (pH 7.4) using a Teflon-glass homogenizer. The body fat samples were
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Plasma and liver lipid, and glucose, and body fat analyses. Total cholesterol (T-chol), triacylglycerol (TG), phospholipids (PL) in plasma and liver, and glucose (Glu) in plasma were determined by the Wako commercial analytical kit (Wako Pure Chemical Industries, Ltd.). Plasma concentrations of thiobarbituric acid reactive substances (TBARS) were measured by the method of Yagi (13). The lipid contents of both body and feces were determined using the method described by Bligh and Dyer (14).

Statistical analyses. All results were expressed as mean±SE. The statistical significance of differences in lipid components and Glu between the experimental spice groups were determined by one-way analysis of variance with a modified Tukey’s HSD test ($p<0.05$) using the STATISTICA statistical program package (Stat-Soft Inc., Oklahoma, USA).

RESULTS

Yield of tsao-ko fractions

The amount of MeX was 40.70 mg/g (4.07%) of the dry fruits of tsao-ko powders. The highest yield from MeX following separation on silica gel was the A fraction (1.74%) followed by the B fraction (about 1.21%). The total polyphenol contents of MeX, A and B fractions were 457, 685, and 188 mg/g of tsao-ko powder, respectively. The peak of the A fraction (III) appeared at a similar time as the epicatechin standard (II) (Fig. 1). Further, the MS/MS spectrum of the A fraction (II) was similar to that of the epicatechin standard (I) (Fig. 2). The maximum UV absorption of the A fraction was at 279 nm.

Inhibitory activities of α-glucosidase, α-amylase, and lipase and DPPH radical-scavenging activity

The inhibition of α-amylase, α-glucosidase, and lipase by the tsao-ko fractions is shown in Fig. 3. The extent of inhibition of each enzyme was least for the B fraction; the extent of inhibition increased with MeX and was greatest with the A fraction. None of the three

crushed in liquid nitrogen. Feces collected for 30 d before the end of the feeding trial were dried and ground. All samples were stored at $-30^\circ$C until required for lipid, glucose and body fat analyses.

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fractions resulted in a marked inhibition of α-amylase activity. All three fractions inhibited lipase to a similar extent and α-glucosidase was strongly inhibited by both the MeX and A fractions but not by the B fraction. The concentrations of MeX (0.48 mg/mL) and the A fraction (0.44 mg/mL) required for 50% DPPH free radical-scavenging activity were lower than that for the B fraction (1.40 mg/mL).

Food intake, body weight and body fat

There were no significant differences in the average food intake (g/mouse/d) between any of the experimental diet groups: control group, 4.61 ± 0.02; methanol extract group, 4.56 ± 0.02; A fraction group, 4.56 ± 0.02; and B fraction group, 4.57 ± 0.02. The final body weights and body fat percentages of the mice fed the four diets are shown in Fig. 4. The body weights of the MeX and A fraction diet groups were statistically significantly lower than those of the B fraction diet group and tended to be lower than that of the control diet group. Body lipid percentages were statistically significantly lower in the MeX and A fraction diet groups than in the control and B fraction diet groups. Total lipid content and weight of feces

The weight of feces and their lipid content from control mice and those fed fractions of tsao-ko collected for 30 d up to the end of the feeding trial are given in Fig. 5. The weight of the feces collected decreased in the following order: MeX > A fraction > control > B fraction diet groups, whereas the percentage of lipids in the feces decreased in the order of: B fraction ≈ A fraction > MeX > control diet groups. The excreted lipid content was greatest in mice fed the A fraction followed by MeX, B fraction and then control diets.

Plasma and liver lipids

The plasma and liver T-chol, TG and PL concentrations of mice fed the experimental diets are shown in Fig. 6. Plasma and liver T-chol and PL concentrations were not statistically significantly different between any of the experimental diet groups. The plasma and liver TG concentrations were significantly lower in the A fraction diet group than the B fraction diet group but there were no statistically significant differences between any of the other experimental diet groups. Plasma Glu and TBARS concentrations

The plasma Glu and TBARS concentrations of mice fed the experimental diets are shown in Fig. 7. Plasma Glu concentrations were significantly reduced in the A fraction diet group compared with the control and B fraction diet groups. However, the plasma Glu concentrations of mice fed MeX were not significantly different from any of the other experimental diet groups. Mean plasma TBARS concentrations were significantly lower in A fraction and MeX diet groups compared with the control diet group. Additionally, the mean TBARS concentration in the MeX diet group was significantly lower than that of the B fraction diet group.
DISCUSSION

It has been suggested that the beneficial effects of tsao-ko observed in its use as a Chinese traditional medicine could, at least partially, result from its effects on lipid metabolism (7, 8). Results from the present study indicate that this activity may reside in sub-fractions of methanol extracts of these fruits. The MeX and A fraction, which markedly inhibited lipase and \( \alpha \)-glucosidase activities, contained large amounts of polyphenol compared with the B fraction, which did not exhibit such inhibitory action. Martin et al. has shown that the polar fraction of tsao-ko contains a large amount of catechin or epicatechin with an EI-MS of 290 m/z (15). Other studies have shown that the [M−H]+ and maximum UV absorption of catechin and epicatechin were 291 m/z and 278–280 nm, respectively (16–18). In the present study, the [M−H]+ and maximum UV absorption of major components of A fraction, at 291 m/z and 279 nm, respectively, indicate the presence of catechin or epicatechin and this is reinforced by the 123, 139, and 273 m/z fragment ions. It has also been shown that while catechin and epicatechin inhibit \( \alpha \)-glucosidase activity, their effect on amylase activity is minimal (10). Similar results were also observed with the A fraction in the present study. Furthermore, the peak of the A fraction (III) appeared at a similar time as the epicatechin standard (II). These findings suggested that the active component of methanol extraction from the tsao-ko might be epicatechin and most of it can be extracted in the A fraction.

Results of various animal and human studies have indicated that a catechin intake reduces both body weight and fat by suppressing fat absorption and enhancing fat oxidation (19–22). In the present study, the decreased body weights and lipid contents of mice fed the A fraction and MeX diets compared with those fed the other diets are consistent with these results. The findings that the amount of excreted lipid was greater and the body lipids were lower in these two diet groups suggest that the intake of the A fraction and MeX might be associated with the inhibition of fat absorption.
which is again consistent with catechin being a component of these diets.

Studies have also demonstrated that a reduction in plasma Glu content coupled with the inhibition of α-glucosidase activity, enhancement of fat oxidation and insulin secretion is associated with a catechin intake (10, 20). The significant reduction of the plasma Glu concentration in mice fed the A fraction in our study could be related to an effect of catechin. These results are supported by the significantly lower plasma TBARS concentration in the MeX and A fraction diet groups compared with the control diet group.

We have previously demonstrated that tsao-ko had a TG-lowering effect, hypoglycemic action, and antioxidant action (7). The total lipids extracted from tsao-ko had little influence on TG levels and limited antioxidant action, whereas the polar lipid fraction showed a significant antioxidant effect (8). In the present study, mice fed the MeX and A fraction showed a significant reduction in plasma TBARS content, but not TG, suggesting that polar components of tsao-ko play an important role in the anti-oxidative effects of tsao-ko. It is of interest that, in contrast to our previous study with total tsao-ko (7), an intake of the tsao-ko polar fractions did not influence plasma or liver TG concentrations or final body weight compared with the control. This suggests that tsao-ko fat and methanol insoluble components, such as dietary fiber, could affect these parameters.

In conclusion, our study indicates that polar fractions of tsao-ko have anti-oxidative and hypoglycemic activity in mice. Our results suggest that these activities may be, at least partially, due to the epicatechin found in tsao-ko. Perhaps the polar component plays an important role in the antioxidative activity of tsao-ko. On the other hand, the strongest TG-lowering components of tsao-ko may be methanol insoluble. The usefulness of tsao-ko for a daily dish may be its prevention of lifestyle-related diseases. Furthermore, tsao-ko may prove to be a drug of choice for prevention of diabetes.

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