Effect of Lipid Extracted from Tsao-ko (*Amomum tsao-ko* Crevost et Lemaire) on Digestive Enzyme Activity, Antioxidant Activity, Plasma and Liver Lipids, and Blood Glucose Levels of Mice

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Summary Lipids extracted from tsao-ko were separated into three fractions with silica gel column chromatography and fed to mice (3 mo old) for 90 d to clarify their inhibitory activity on digestive enzyme activity. The diets contained the following: control—no tsao-ko, 0.05% total lipid of tsao-ko (TL), 0.0109% chloroform fraction (CF), 0.0245% acetone fraction (AF), or 0.00365% methanol fraction (MeF). Although CF and AF slightly inhibited the activities of α -glucosidase, α -amylase, and lipase, intakes of these fractions had little influence on plasma and liver lipid concentrations when compared with the control diet. MeF did not inhibit α -glucosidase but had DPPH radical scavenging activity and the mice fed this fraction had the most marked reduction in plasma glucose and TBARS concentrations compared with the other diet groups. These results suggest that the fat-soluble polar components of tsao-ko contain an active component that might be associated with decreased plasma glucose and TBARS concentrations in mice.

Key Words tsao-ko, lipids, DPPH, inhibitory activity, glucose

Spices have been utilized in numerous ways throughout history and are amongst the thousands of food flavoring substances used worldwide (1–3). Dietary spices are non-toxic and studies in animal models have suggested that they could be beneficial to human health (3–9). Additionally, in China, many spices have been used in folk medicine. *Amomum tsao-ko* Crevost et Lemaire (tsao-ko), which belongs to the Zingiberaceae family, is used in Chinese cuisine and in traditional and folk medicine for treating stomach disorders and throat infections. In an earlier screening experiment, we demonstrated that 1% tsao-ko can lower triacylglycerol (TG) levels (72.5% of control) and has hypoglycemic (66.2%) and antioxidant activity (71.1%) in mice (9) but the active components of tsao-ko associated with these effects were not identified.

A number of studies have indicated that the oils from

spices have beneficial effects on human health (10–12). For example, garlic oil has been shown to inhibit oxidative damage and bone loss (12–14). It was thought possible that the effective components of tsao-ko might also be contained in a lipid fraction. We previously reported the separation of spice lipids into three fractions, namely, a chloroform eluted fraction (containing TG), an acetone eluted fraction (containing glycolipids), and a methanol eluted fraction (containing phospholipids) using a silica gel column (15). In the present study we separated the lipids of tsao-ko into these three fractions to determine which fraction was most effective in reducing plasma lipids and glucose, and liver lipids in mice.

MATERIALS AND METHODS

Materials. Chloroform, acetone, and methanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were used without further purification. Anhydrous sodium sulfate (Na_2SO_4) and silica gel (C-300; 45–75 mm, 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 March 2004. After peeling the skin of dried tsao-ko

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Abbreviations: AF, acetone fraction; CF, chloroform fraction; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Glu, glucose; MeF, methanol fraction; PL, phospholipids; TBARS, thiobarbituric acid reactive substances; T-chol, total cholesterol; TG, triacylglycerol; TL, total lipids.

fruits, they were reduced to a powder using a disintegrator (Ultra Centrifugal Mill, MRK Co., Ltd., Tokyo, Japan) at room temperature. Lard was supplied by NOF Co., Ltd., Tokyo, Japan. Salt and vitamin mixtures were purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. The α -glucosidase was purchased from Oriental Yeast Co., Ltd. and 2,2-diphenyl-1-picrylhydrazyl (DPPH), α -amylase and lipase were purchased from Sigma (MO, USA).

Extraction and fractionation. The total lipids of tsao-ko were extracted from the powders of tsao-ko by the method of Bligh and Dyer (16). Silica gel chromatography was performed according to the method of Suzuki et al. (15). The total lipid (TL) extract of tsao-ko was applied to a column (25×300 mm) packed with silica gel in chloroform. The chloroform fraction (CF) was removed by elution with 600 mL of chloroform, followed by the acetone fraction (AF) with 500 mL of acetone and then the methanol fraction (MeF) with 600 mL of methanol. After the solvents were reduced by evaporation, each fraction was weighed.

Inhibitory activities of α -glucosidase, α -amylase, and lipase. The various lipid fractions were dissolved in dimethyl sulfoxide (DMSO). The inhibitory activity of α -glucosidase was assayed by method of Tadera et al. (17). The α -amylase inhibitory activity was measured using a Wako-amylase kit (Wako Pure Chemical Industries, Ltd.). The lipase inhibitory activity was measured using a modification of the method of Han et al. (18). A mixture 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) was homogenized with a phycotron (NS-50, Microtec Co., Ltd., Chiba, Japan). This mixture (100 μ L) was incubated with 0.05 mL (2 units) of pancreatic lipase solution and 10 μ L of various concentrations of sample solutions for 30 min at 37°C. The fatty acid released from triolein was measured by the Wako non-esterified fatty acid-C (NEFA-C) test kit (Wako Pure Chemical Industries, Ltd.). All experiments were performed 4–6 times.

Determination of radical-scavenging activity. The DPPH radical scavenging activity of each lipid fraction was assayed by a modification of Bloiss' method (19). Samples of lipid fractions (0.5–15 g/L) in DMSO (10 μ L) were placed in wells of a micro-plate. Twenty micromole DPPH isopropanol solution (200 μ L) was added to each well and the absorbance was measured at 550 nm after 5 min. The IC₅₀ of each lipid fraction was estimated from the linearity point. This experiment 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 salt mixture contained per 100 g: CaHPO₄·2H₂O, 14.56 g; KH₂PO₄, 25.72 g; NaH₂PO₄, 9.35 g; NaCl, 4.66 g; Ca-lactate, 35.09 g; Fe-citrate, 3.18 g; MgSO₄, 7.17 g; ZnCO₃, 0.11 g; MnSO₄·4~5H₂O, 0.12 g; CuSO₄·5H₂O, 0.03 g; KI, 0.01 g. The vitamin mixture contained per 100 g: vitamin A·acetate, 0.1 g; vitamin D₃, 0.25 mg; vitamin

E·acetate, 0.5 g; vitamin K₃, 0.52 g; vitamin B₁·HCl, 0.12 g; vitamin B₂, 0.4 g; vitamin B₆·HCl, 0.08 g; vitamin B₁₂, 0.05 mg; vitamin C, 3.0 g; D-biotin, 2.0 mg; folic acid, 0.02 g; calcium pantothenate, 0.5 g; p-aminobenzoic acid, 0.5 g; niacin, 0.6 g; inositol, 0.6 g; choline chloride, 20 g; cellulose powder, 73.1 g. The four experimental diets contained 0.05% total lipid (TL) of tsao-ko, or 0.0109% CF, or 0.0245% AF, or 0.00365% MeF instead of cornstarch. The amount of each lipid fraction added was equivalent to the experimental diet which contained 1% of tsao-ko powder (9). The control and experimental diets were made once a month, and stored below 5°C.

Animals. Male mice, of the Crlj: 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 five groups of eight animals each and each group was fed on the control or an experimental diet for 90 d. Animals had free access to the experimental or control diets until they were fasted and water until they were killed. 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 the relative humidity at 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 Agriculture Food Research Organization, Japan.

Preparation of plasma, liver homogenates, and whole 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 without perfusion and homogenized with 9 volumes of 1/15 mol/L phosphate-buffered saline (pH 7.4) using a Teflon-glass homogenizer. The mouse body was crushed under liquid nitrogen to extract the total lipids by the method of Bligh and Dyer (16). Plasma samples, liver homogenates, and mouse body samples were stored at -30°C until required for lipid, glucose and body fat analyses.

Plasma glucose, lipids and thiobarbituric acid reactive substances, liver lipids and body fat analyses. Total cholesterol (T-chol), TG and phospholipids (PL) in plasma and liver, and glucose (Glu) in plasma were determined by Wako commercial analytical kits (Wako Pure Chemical Industries, Ltd.). Plasma concentrations of thiobarbituric acid reactive substances (TBARS) were measured by the method of Yagi (20). The lipid contents of the whole body were determined using the method described by Bligh and Dyer (16).

Statistical analyses. All results were expressed as mean±SE. The statistical significance of differences in lipid components and glucose between the experimental tsao-ko fraction groups were determined by one-way

analysis of variance (ANOVA) and Spjotvoll/Soline test using the STATISTICA statistical program package (Stat-Soft Inc., Oklahoma, USA); the significance was set at $p < 0.05$.

RESULTS

Lipid yield from tsao-ko fractions

The total lipid concentration of tsao-ko powders was 51.2 mg/g. Following fractionation of the total lipid the acetone soluble fraction had the highest yield (approximately 48%), followed by the chloroform fraction (approximately 21%) and then the methanol fraction (approximately 7%).

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

The inhibitory activities of each lipid fraction on α -glucosidase, α -amylase, and lipase are presented in Fig. 1. α -Glucosidase activity was suppressed by CF and AF. The greatest inhibition was observed with the AF followed by the CF. In contrast, MeF showed a tendency to enhance the α -glucosidase activity with increasing con-

centrations. The inhibitory action of each lipid fraction on α -amylase activity was less pronounced than that on α -glucosidase activity. For lipase activity, these lipid fractions showed hardly any inhibition. However, a slight inhibition of lipase by MeF was found at the low lipid concentration but it declined, at the higher concentrations, to a level similar to that seen for TL. The DPPH radical scavenging activity of each lipid was least for CF followed by TL, AF and finally MeF (Fig. 2).

Food intake, body weight and body fat

Table 1 shows the average food intake, final body weights, and body fat contents of the mice in each diet group. There were no marked differences in the average food intake between any of the experimental diet groups. At the end of the feeding trial, there were no significant differences in the final body weights between any of the experimental diet groups. The slightly lower values for body fat content in the TL diet groups compared with those of the control and AF diet groups were not significantly different.

Plasma and liver lipids

The plasma and liver T-chol, TG and PL concentrations of mice fed the experimental diets are shown in Fig. 3. Plasma T-chol concentrations were significantly lower in the CF diet group than in the control diet group. There were no significant differences in the plasma TG concentrations between any of the experimental diet groups and the control diet group. The TG concentration was significantly lower in the MeF diet group than in the AF diet group. There were no significant differences between any of the experimental diet groups and the control diet group for plasma PL concentrations.

The content of liver T-chol was not significantly different among the experimental diet groups. TG concentrations of the AF supplemented group were significantly higher than those of the TL and CF diet groups. The liver PL concentrations were significantly lower in mice fed the CF and MeF diets than those from animals fed the control diet. Further, mice fed the CF diet had significantly lower PL concentrations than those fed the AF diet.

Plasma Glu and TBARS

The plasma Glu and TBARS concentrations of mice fed the experimental diets are shown in Fig. 4. Glu concentrations were significantly reduced in the TL, CF, AF

Fig. 1. Effect of total lipids and each lipid fraction from tsao-ko on α -glucosidase, α -amylase, and lipase activities in vitro. Results are expressed as means. TL, total lipid of tsao-ko; CF, chloroform fraction; AF, acetone fraction; MeF, methanol fraction.

Fig. 2. The concentration of total lipid (TL) and lipid fractions (CF, AF and MeF) necessary for a 50% inhibition (IC₅₀) of DPPH free radical scavenging.

Table 1. Average food intake, body weight and body fat content of mice fed the experimental and control diets.

Groups	Control	TL	CF	AF	MeF
Food intake (g/mouse/d)	4.56±0.02	4.58±0.02	4.56±0.02	4.57±0.02	4.53±0.02
Body weight (g/mouse)	47.30±1.18	44.75±0.66	46.24±1.13	48.17±0.47	45.36±1.12
Body fat content (%)	14.45±1.80	11.94±0.99	13.27±0.87	15.6±1.59	13.30±0.87

Values are means±SE. Means without a common letter differ; $p < 0.05$ by the Spjotvoll/Stoline test.

TL, total lipid of tsao-ko; CF, chloroform fraction; AF, acetone fraction; MeF, methanol fraction.

Fig. 3. Total cholesterol, triacylglycerol and phospholipid concentrations of plasma and liver in mice fed experimental and control diets for 90 d ($n=8$ /group). Values are means±SE. Means without a common letter differ; $p < 0.05$ by the Spjotvoll/Stoline test. TL, total lipid of tsao-ko; CF, chloroform fraction; AF, acetone fraction; MeF, methanol fraction.

and MeF diet groups compared with the control diet group. The average plasma Glu concentration in the MeF diet group was significantly lower than that in either the CF or AF diet groups.

Plasma TBARS concentrations were significantly lower in the CF and MeF diet groups than in the control diet group. Additionally, the mean TBARS concentration in the MeF diet group was significantly lower than that of the TL diet group.

DISCUSSION

The present investigation showed that the potency of different lipid fractions of tsao-ko in inhibiting digestive

enzymes varies as do their effects on lipid metabolism in mice. The results support evidence from previous studies that have shown the potential of spice oils to influence various metabolic pathways in ways that may have a positive effect on human health (10–12). A previous study indicated that AF from spices contains the large amount of glycolipids (15). In the present study, an AF percentage of 48 in the tsao-ko lipids was higher than AF percentages of 7.95, 4.61, and 2.59 in the more common spices clove, red pepper, and nutmeg, respectively (15).

The present study of tsao-ko lipids fractions is consistent with previous reports that have indicated that an

Fig. 4. Plasma glucose and TBARS concentrations of mice fed experimental and control diets for 90 d ($n=8$ /group). Values are means $\pm$ SE. Means without a common letter differ; $p<0.05$ by the Spjotvoll/Stoline test. TL, total lipid of tsao-ko; CF, chloroform fraction; AF, acetone fraction; MeF, methanol fraction.

intake of other spices can result in the stimulation or inhibition of the activity of digestive enzymes (4, 5, 21). For example, the activity of α -glucosidase was slightly enhanced by the MeF but strongly inhibited by AF and CF. However, α -amylase and lipase activities were both slightly inhibited by all three lipid fractions. Currently it is not known what components of the various fractions are responsible for the inhibitory actions. Martin et al. showed that tsao-ko contains epicatechin and catechin which have inhibitory activity on the digestive enzyme (23). It is possible that various polyphenolic components, such as catechin, previously identified in tsao-ko may be slightly soluble in the lipid fractions and contribute to the inhibition of enzyme activity (22, 23). The low emulsion drop size enhances the fat digestion by lipase and lipase inhibitory activity of tea catechin is dependent on the emulsion droplet size (24, 25). The decrease in the inhibition of lipase with increasing concentrations of MeF could be due to the variation in emulsive state.

The inhibition of lipase activity contributes to a down-regulation of body weight through the suppression of fat absorption and increases in fat tissue (26–28). However, this study did not show a remarkable inhibitory action of total lipid or lipid fractions on lipase or a significant lowering body weight or fat tissue weight. Martin et al. reported that tsao-ko includes epicatechin and catechin, which decrease body weight and fat mass (23). However, these components dissolve almost entirely in water, but barely dissolve in lipids. It is more likely that the components of tsao-ko which suppressed body weight increases (86.1% comparison of control) in the previous study (9) are non-fat soluble.

In a previous study we showed that an intake of 1% tsao-ko decreased TG levels, but not T-chol levels in mice (9). However, the results from this study suggest that fat-soluble components in tsao-ko may influence

plasma and liver T-chol and TG levels. The CF diet, which includes triglycerides, significantly reduced plasma T-chol levels compared with the control group. The AF diet, which includes glycolipids, significantly raised plasma TG levels compared with the MeF diet group, which includes phospholipids. An intake of the AF also resulted in higher liver TG levels than those observed following an intake of either the CF or TL extract.

Plasma Glu levels were decreased in all experimental diet groups compared with control and this may be related to the inhibitory action of TL and the lipid fractions on α -glucosidase and α -amylase. However, MeF, which resulted in the greatest reduction in plasma Glu, did not inhibit α -glucosidase activity and, thus, MeF probably contains other components that influence glucose metabolism in mice.

Although the plasma TBARS concentrations of mice in each lipid fraction diet group were reduced compared with the control diet group, there was little correlation between these reductions and the DPPH radical scavenging activity. For example, the plasma TBARS concentrations of the AF diet group tended to be higher than those of CF diet group but the DPPH radical scavenging activity was much lower in the CF diet group than the AF group. Perhaps the absorption and/or digestion causes the difference between DPPH radical scavenging activity and plasma TBARS level. In contrast, an intake of MeF resulted in a marked DPPH radical scavenging activity and a significant reduction in plasma TBARS concentration. Martin et al. have shown that strong antioxidant components of tsao-ko include catechins and catechol derivatives (23) and it is possible that the reduction in plasma TBARS in mice fed the MeF is associated with these components. Although these observations strongly support the concept that the antioxidant components of tsao-ko may be fat-soluble polar components, an intake of TL did not significantly reduce the level of plasma TBARS.

The purpose of this study was to clarify the fraction in tsao-ko which suppressed body weight, modified the plasma TG, TBARS, and Glu concentrations as seen in our previous study of tsao-ko. The results showed that the extracted lipid fractions of tsao-ko inhibited digestive enzymes and oxidant activity, either increased or decreased plasma TG, and decreased plasma TBARS and Glu concentrations. It has been shown that polar solvent extracts of various herbs have strong antioxidant activity (29). In the present study the fractions of tsao-ko that markedly influenced plasma Glu and TBARS levels and antioxidant activity were in the MeF, which contains the fat-soluble polar components. The lowering effects of tsao-ko on plasma and liver TG levels and body weight do not appear to reside in the lipid components.

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