Bofutsushosan, a traditional Chinese formulation, inhibits pancreatic lipase activity *in vitro* and suppresses the elevation of plasma triacylglycerols after oral administration of lipid emulsion

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Pancreatic lipase activity measured as fatty acid liberation from lipid emulsion was shown to be inhibited by the addition of Bofutsushosan (BOF) or Daijokito (DJT) extracts at >30 mg/ml. The extracts of Orengedokuto (OGT), Chotosan (CTS), Boiogito (BOT) or Shimbuto (SBT) were ineffective in inhibiting pancreatic lipase activity up to 60 mg/ml. Mice were orally administered with a lipid emulsion in the presence of BOF extracts at 750 and 2250 mg/kg and subsequent elevation of plasma triacylglycerols (TAG) was significantly suppressed as compared with that in the mice which received lipid emulsion alone. However, the addition of DJT extracts did not suppress the elevation of plasma TAG after oral administration of lipid emulsion. Our results suggest that BOF suppresses the absorption of ingested fats and this effect could account for the anti-obese effects of BOF.

Key words Kampo formulation, pancreatic lipase, plasma triacylglycerols, lipid emulsion, Bofutsushosan.

Introduction

Bofutsushosan (BOF) is a traditional Chinese formulation (Kampo formulation in Japanese), which contains 18 kinds of crude drugs (Table 1). This formulation has originally been used to attenuate symptoms associated with metabolic disturbances due to long-term inappropriate lifestyles. Several investigations have revealed that BOF is effective to improve obesity. In female obese subjects with impaired glucose tolerance, the administration of BOF extracts reduced their body weight.[1] In addition, several animal experiments support the anti-obese effect of BOF by demonstrating its reduction of tissue fats contents in both diet- and drug-induced obesity.[2,3] These animal experiments suggest that the improvement of obesity by BOF is due to its enhancing effects on lipolysis and thermogenesis in adipose tissues through the activation of β-adrenergic systems by ephedrine derived from Ephedrae Herba or the inhibition of phosphodiesterase by Glycyrrhizae Radix,[3] both of which are the constituents of BOF (Table 1). However, since Ephedrae Herba and Glycyrrhizae Radix are contained in many kinds of Kampo formulations other than BOF, the above effects of these constituents may not be explanations for the unique efficacies of BOF for obesity. Furthermore, our recent investigation demonstrated that oral administration of BOF prevented atherosclerotic alterations in artery of rats after balloon endothelial denudation.[4]

Dietary triacylglycerols (TAG) are hydrolyzed to monoacylgycerols (MAG) and free fatty acids (FFA) by pancreatic lipase in small intestine, which are absorbed in the epithelium of this organ. MAG and FFA are utilized again to synthesize TAG in the epithelial cells of small intestine and delivered to lymph ducts and then to circulation as a form of chylomicrons. Subsequently, chylomicrons were processed by lipoprotein lipases to smaller lipoprotein particles, which are incorporated into tissues. Therefore, the rates of fat absorption from the small intestine, degradation of chylomicrons, and tissue incorporation of lipoproteins are the major factors to determine the extent of postprandial elevation of plasma TAG concentration. There are many investigations showing the importance of suppression of postprandial elevation of plasma TAG by the hypolipidemic agents in the therapy of metabolic diseases.[5-7]

Recently, much attention is being paid to the development of agents to inhibit pancreatic lipase activity, because such agents suppress the absorption of dietary lipids from the small intestine and are expected to reduce body weight in obese subjects. On the other hand, a number of natural products including plant extracts[8-12] has already been reported to inhibit lipase activity and to suppress the absorption of dietary fats in the experimental animals. Some of these are constituents in Kampo formulations, although the effects of Kampo formulations on pancreatic lipase activity and lipid absorption have not been investigated. In particular, these effects may account for the anti-obese effects of Kampo formulations such as BOF. Therefore, we here report the effects of BOF and other Kampo formulations on pancreatic lipase *in vitro* and the elevation of plasma TAG after oral administration of lipid emulsion in mice.

Materials and Methods

Kampo formulations. BOF, Boiogito (BOT), Oren-

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Table 1. Constituents of Kampo formulations used in this study

Bofutsushosann (BTS)	ratio	Boiogito (BOT)	ratio	Orengedokuto (OGT)	ratio
Zingiberis Rhizoma	0.3	Zingiberis Rhizoma	1.0	Scutellariae Radix	3.0
Glycyrrhizae Radix	2.0	Glycyrrhizae Radix	1.5	Gardeniae Fructus	2.0
Scutellariae Radix	2.0	Atractylodis Lanceae Rhizoma	3.0	Coptidis Rhizoma	2.0
Gardeniae Fructus	1.2	Zizyphi Fructus	3.0	Phellodendri Cortex	1.5
Gypsum Fibrosum	2.0	Astragali Radix	5.0		
Saposhnikoviae Radix	1.2	Radix Stephaniae Tetrandrae	5.0		
Rhei Rhizoma	1.5				
Natrium Sulfuricum	0.7				
Platycodi Radix	2.0				
Atractylodis Rhizoma	2.0				
Schizonepetae Herba	1.2				
Chuanxiong Rhizoma	1.2				
Angelicae Radix	1.2				
Menthae Herba	1.2				
Ephedrae Herba	1.2				
Forsythiae Fructus	1.2				
Talcum	2.0				
Paconiae Radix	1.2				

Chotosan (CTS)	ratio	Daijokito (DJT)	ratio	Shimbuto (SBT)	ratio
Zingiberis Rhizoma	1.0	Rhei Rhizoma	2.0	Zingiberis Rhizoma	1.5
Glycyrrhizae Radix	1.0	Natrium Sulfuricum	1.3	Atractylodis Lanceae Rhizoma	3.0
Gypsum Fibrosum	5.0	Aurantii Fructus Immaturus	3.0	Paeoniae Radix	3.0
Saposhnikoviae Radix	2.0	Magnoliae Cortex	5.0	Poria	4.0
Poria	3.0			Aconiti Tuber	0.5
Aurantii Nobilis Pericarpium	3.0				
Ophiopogonis Tuber	3.0				
Pinelliae Tuber	3.0				
Ginseng Radix	2.0				
Uncariae Uncis cum Ramulus	3.0				
Chrysanthemi Flos	2.0				

gedokuto (OGT), Chotosan (CTS), Daijokito (DJT) or Shimbuto (SBT) were supplied from Tsumura & Co. (Tokyo, Japan) as a form of spray-dried powder of their hot-water extracts. We chose BOF for possible elucidation of its anti-obese effects. BOT, CTS and SBT were chosen because these contain Zigiberis Rhizoma, which is reported to be inhibitory for pancreatic lipase. OGT and DJT were randomly added in the present study. Human daily doses (g) of BOF, BOT, OGT, CTS, DJT and SBT were 4.5, 3.75, 1.5, 4.5, 3.0 and 2.0, respectively. The constituents of these Kampo formulations were listed in Table 1. Quality of BOF extracts was validated by the analysis of chemical compounds included on reverse-phase high-performance liquid chromatography (HPLC). BOF extracts (1g) were dissolved in mobile phase used for HPLC and filtered through PTFE membrane filters and an aliquot of the filtrates was injected to HPLC system. A column was TSK-gel 80TS (4.5mm X 250mm, Tosoh, Tokyo, Japan) maintained at 40℃ and the elution (1ml/min) was operated in a linear gradient manner using 50mM ammonium acetate buffer (pH 3.6)(A) and acetonitrile (B); the mixture of A and B at 90:10 was used as the initial condition and then the proportion of A and B was linearly changed to 0:100 in 60 min and then held for 20 min. Elution of compounds was monitored continuously at 200-400nm in a photodiode array detector. A three-dimensional profile of major compounds included in BOF was shown (Fig. 1).

Measurement of lipase activity. Lipid emulsion was prepared by sonicating 800mg of safflower oil (NOF, Tokyo, Japan) in 9ml of 0.1 M Tris-HCl buffer (pH 7.4) in the presence of 10mg of phosphatidylcholine (Serdary Research, Korea) and 5mg of sodium taurocholate (Wako Pure Chem, Osaka, Japan). Fifty μl of the lipid emulsion was mixed with 100 μL of 5 mM calcium acetate and 250 μl of suspension of the extracts of Kampo formulations in distilled water. The final concentration of each Kampo formulation was set at the range from 10 to 60 mg/ml. The reaction mixture was preincubated for 5 min at 37℃ and lipase reaction was started by adding 50 μL of 1 mg/ml porcine pancreatic lipase (Sigma, St. Loius) dissolved in 0.5 M Tris-HCl buffer (pH 7.4). Reaction was then continued for 20 min at 37℃ and terminated by adding methanol/chloroform (2:1, v/v) to extract total lipids from the reaction mixture according to the methods of Bligh and Dyer.[13] An

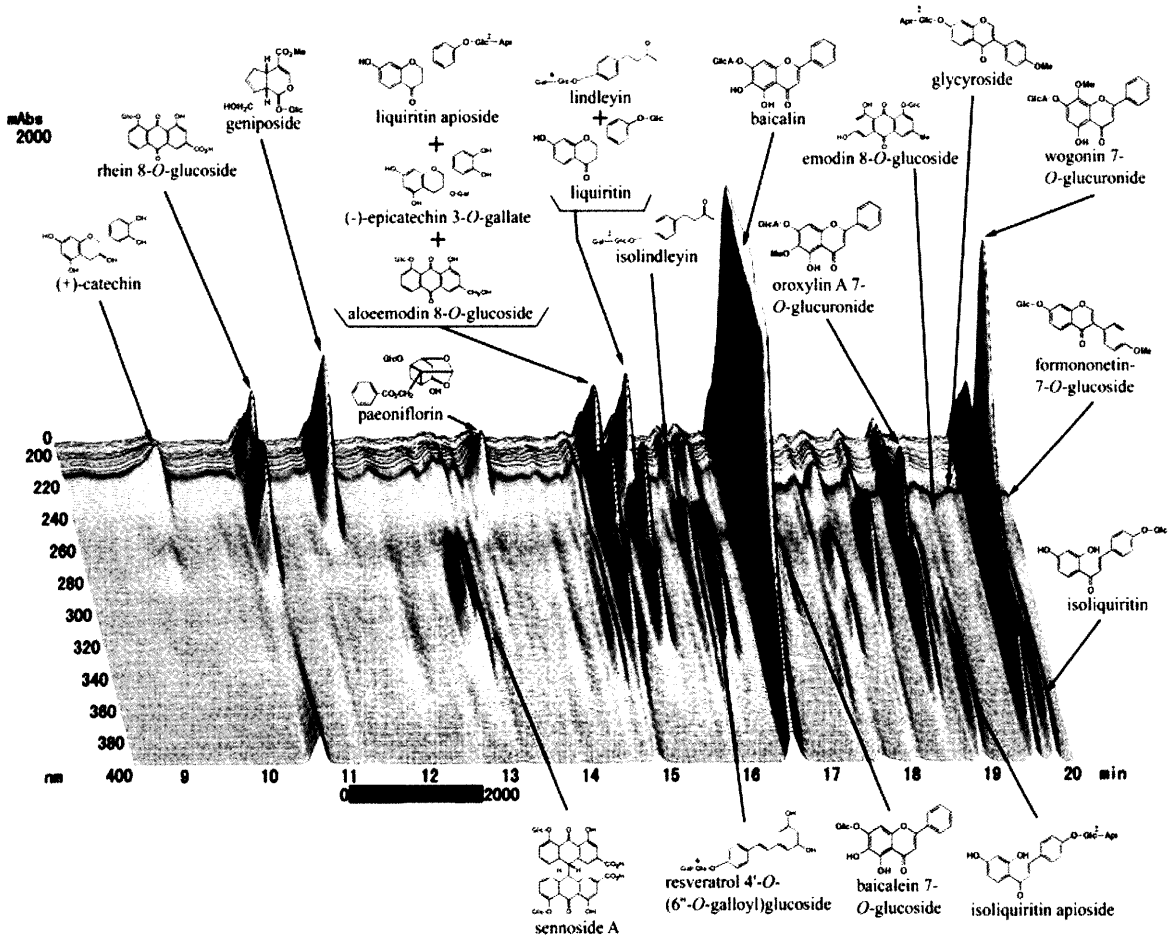

Fig. 1. Analysis of major chemical compounds included in Bofutsusyosan extracts by three-dimensional reverse-phase high-performance liquid-chromatography
Details in the preparation and analysis of BOF extracts were described in Materials and Methods. Peak identification and chemical structures of major compounds were shown.

aliquot of total lipids (1/250) dissolved in chloroform was applied onto silica gel thin-layer chromatography (TLC) plates (Silica gel 60, Merck, Germany). The plates were developed by the mixture of petroleum ether/diethylether/acetic acid (80:30:1, v/v/v) and then dried. The spots of FFA were visualized by immersing the TLC plates into 5% (w/v) $CuSO_4$-4% (v/v) phosphoric acid and heating the plates at 170℃ for 15 min. Scion Image Beta 4.02 software (Scion Corporation, Frederick, MD) was used to incorporate digital images of TLC plates and to determine the density of FFA spots. The amounts of FFA generated by lipase activity were calculated based on the calibration curve developed with known amounts of FFA (palmitic acid). Lipase activity was expressed as the percentages of the control activity measured in the absence of Kampo formulation.

Animals. Male ddY mice at 4 weeks of age were purchased from SLC (Hamamatsu, Japan) and used throughout the experiments. Mice were housed 6-8 mice/cage in an air-conditioned room (temperature at 23±2℃ and humidity at 55±5℃ under a 12h:12h light-dark cycle; the light was turned on at 08.00 h. The animals were maintained in the above conditioned room for 2 weeks before the experiments and fasted for 24hr prior to the administration of lipid emulsion as described below.

Effect of Kampo formulations on the elevation of plasma TAG after oral administration of lipid emulsion. Safflower oil (3ml) was mixed with 7ml of distilled water containing 50 mg of sodium cholate (Wako Pure Chem) in the absence or presence of the extracts of BOF or DJT and this mixture was sonicated. The lipid emulsion was orally administered at 10ml/kg to mice through a gastric tube; the doses were 375, 750 and 2250 mg/kg for BOF extracts and 750 and 2250 mg/kg for DJT extracts. A small volume (~50 μL) of blood was withdrawn by puncturing retroorbital plex with a heparinized glass capillary (Drumond Scientific Company, Broomall, PA) just before (0h), 2, 4 and 6 hr after oral administration of lipid emulsion. Blood was centrifuged at 3,000 rpm for 5 min at 4℃ to obtain plasma. TAG concentration in the plasma was measured by using a commercial assay kit (Triglyceride E-test, Wako). Plasma TAG concentration (mg/dl) at each time point and the incremental area under the curve (AUC) (mg/dl • hr) were calculated. This experiment had been approved by the Committee of Animal Care and Experiments of Toyama Medical and Pharmaceutical University.

Statistical analysis. Data were expressed as the mean ± S.E.M. Stat View-J-5.0 (Abacus Concepts Inc., Berkeley, CA) was used for statistical analysis to compare the control

and treated groups with ANOVA following a Student-Neuman-Kuels post hoc test. The p values below 0.05 were considered statistically significant.

Results

Effect of Kampo formulations on pancreatic lipase activity *in vitro*. We first tried to determine lipase activity by measuring FFA liberated from emulsified lipids by colorimetry based on the Dumcombe's method.[14] However, it was found in the pilot experiments that the addition of Kampo formulations colored considerably organic phase formed during the extraction steps in this assay procedure and FFA liberated during lipase activity could not be measured accurately. Therefore, we measured FFA by densitometry after lipid extraction and TLC separation as described in Materials and Methods. An example for TLC separation and detection of FFA after organic extraction from the assay mixture was shown in Fig. 2. By using these procedures, we found that the addition of BOF extracts inhibited lipase activity in a concentration-dependent manner; significant inhibition was observed at higher than 30 mg/ml (Fig. 3). The addition of DJT extracts also inhibited significantly pancreatic lipase activity at higher than 30 mg/ml although the extent of inhibition was slightly less than that of BOF at 30 and 40 mg/ml. At a higher concentration (50 and 60 mg/ml), the addition of DJT extracts gave similar inhibition to that of BOF at this concentration range. In contrast, OGT marginally augmented lipase activity at lower concentration range (10 and 20 mg/ml) but no further augmentation was shown at the higher concentration range. BOT, CTS and SBT were ineffective in inhibiting pancreatic lipase activity within the concentration range tested.

Elevation of plasma TAG after oral administration of lipid emulsion in mice. As demonstrated above, BOF and DJT were inhibitory for pancreatic lipase activity *in vitro* (Fig. 3) and therefore the effect of oral administration of

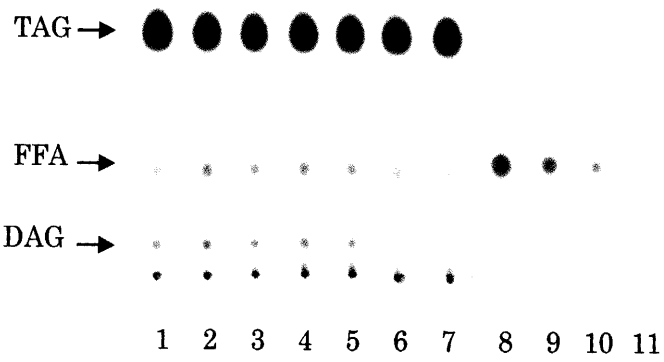

Fig. 2. Separation and detection of free fatty acids by silica-gel thin layer chromatography.
Total lipids extracted from the lipase assay mixture were applied onto silica-gel thin layer chromatography plates and developed in petroleum ether/diethyl ether/acetic acid (80:30:1, v/v)(Lanes 1-7). Triacylglycerols (TAG)(substrate), diacylglycerols (DAG)(intermediate products) and free fatty acids (FFA)(products) were detected in this TLC system. Known amounts of FFA (0.5, 1, 2 and 4 μg) were applied and developed similarly (lane 8, 9, 10 and 11, respectively). The plates were dipped into 5 % $CuSO_4$-4% phosphoric acid and heated at 170℃ for 15 min. The FFA levels were determined densitometrically.

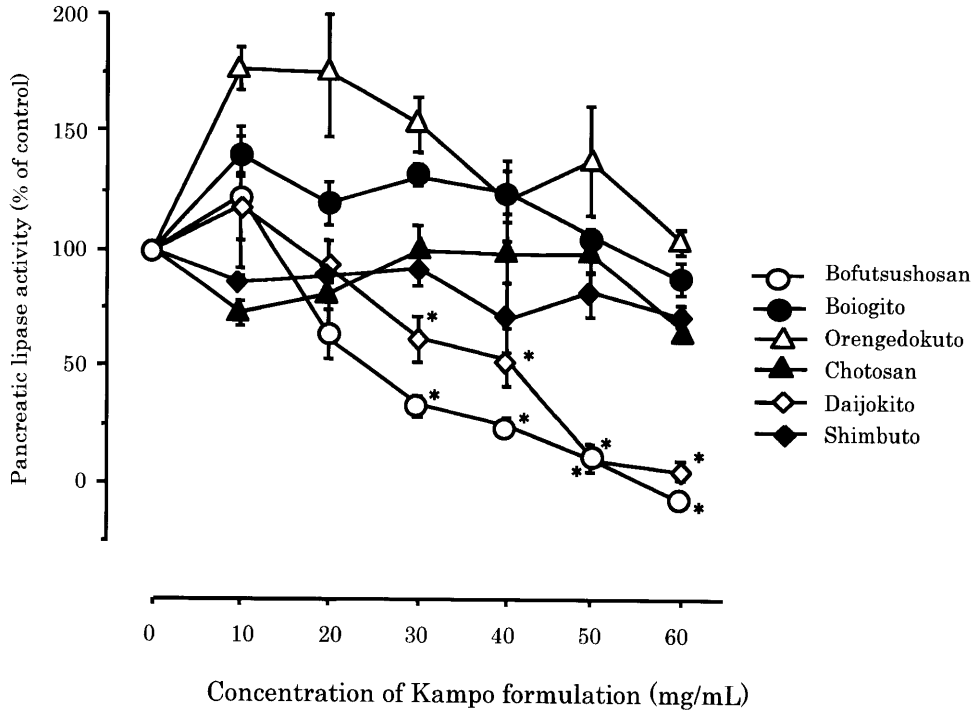

Fig. 3. Effects of the addition of Kampo formulations on the pancreatic lipase activity *in vitro*
The reaction mixture containing emulsified TG and porcine pancreatic lipase was incubated in the absence or presence of various concentrations of each Kampo formulation for 20 min at 37℃. Pancreatic lipase activity was estimated as the amount of FFA liberated during the reaction. Values were expressed as the mean±S.E.M. in triplicate. Statistical analysis was performed with an ANOVA and Student-Neuman-Kuels post hoc test (*$p<0.05$ vs. control).

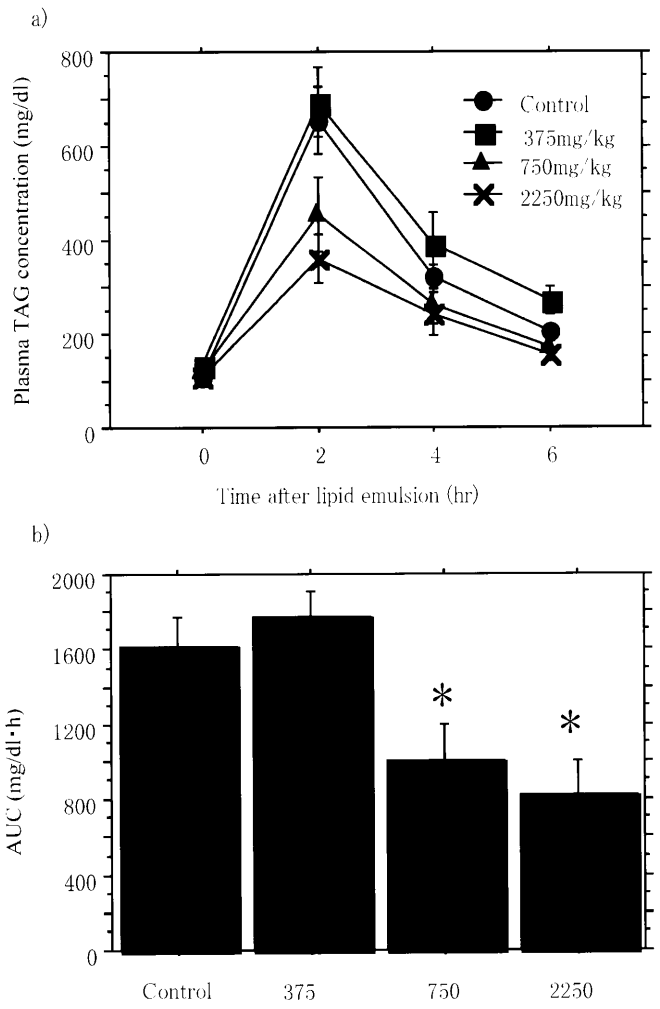

Fig. 4. Effect of Bofutsusyosan on the elevation of plasma TAG after oral administration of lipid emulsion
Lipid emulsion in the presence or absence of Bofutsusyosan (BOF) extracts at the doses indicated was orally administered to mice and blood was obtained just before (0 hr), 2, 4 and 6 hr after the administration. Triacylglycerol (TAG) concentration at each time point was shown in panel a) and the incremental area under the curve (AUC) was shown in panel b). The elevation of plasma TAG after oral lipid emulsion was significantly suppressed by the addition of BOF extracts as compared with the control values in repeated measure ANOVA ($p=0.007$)(a) and in ANOVA with Student-Newman-Keuls post hoc test ($p<0.05$, *) as compared with the control group.

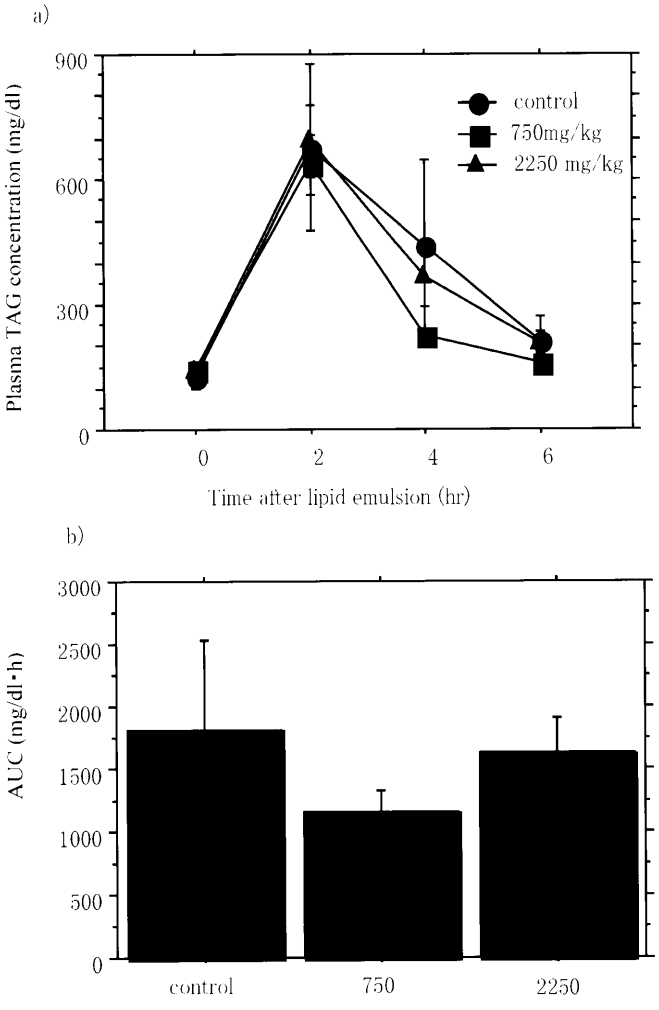

Fig. 5. Effect of Daijokito on the elevation of plasma TAG after oral administration of lipid emulsion
Lipid emulsion in the presence or absence of Daijokito (DJT) extracts at the doses indicated was orally administered to mice and blood was obtained just before (0 hr), 2, 4 and 6 hr after the administration. Triacylglycerol (TAG) concentration at each time point was shown in the panel a) and the incremental area under the curve was shown in the panel b). The elevation of plasma TAG after oral lipid emulsion was not significantly changed by the addition of DJT extracts as compared with the control values.

these formulations on the elevation of plasma TAG concentration after oral administration of lipid emulsion was examined in mice. Plasma TAG concentration was sharply elevated at 2 hr after the oral administration of lipid emulsion, which was followed by the decrease at 4 and 6 hr after administration (Fig. 4a anb 4b). A repeated measure of ANOVA revealed that the addition of increasing amount of BOF extracts significantly reduced the elevation of plasma TAG after oral administration of lipid emulsion ($p=0.007$). When the change in plasma TAG concentration was expressed as AUC after the oral administration of lipid emulsion, it was concluded that the elevation of plasma TAG was significantly suppressed by the addition of BOF extracts at 750 and 2250 mg/kg ($p<0.05$) as compared with the control group. DJT extracts, another inhibitory formulation for lipase activity (Fig. 3) did not significantly change the plasma TAG concentration as well as AUC after oral administration of lipid emulsion at 750 and 2250 mg/kg (Fig. 5a and 5b).

Discussion

Pancreatic lipase acts only at an oil/water interface of micellar form of TAG emulsified with phospholipids or bile salts. Therefore, the compounds to disrupt micelle of substrate TAG can inhibit pancreatic lipase activity. Several kinds of plants extracts prepared from Platycodi radix or Zingiberis Rhizoma are reported to be inhibitory for lipase *in vitro* and also suppress the elevation of TAG under high fat diet.[9,11,12] Since BOF contains both of these plants (Table 1), although Zingiberis Rhizoma is also a constituent of BOT, CTS and SBT, which were not inhibitory for

pancreatic lipase (Fig. 3). Therefore, Platycodi radix may be a constituent responsible for the inhibition of pancreatic lipase activity by BOF. On the other hand, DJT does not contain these saponin-containing constituents but instead contains Rhei Rhizoma and Natrium Sulfuricum, which are also contained in BOF. Accordingly, these constituents may be involved in the inhibition of pancreatic lipase by BOF and DJT. However, Scutellariae Radix, Gardeniae Fructus, Gypsum Fibrosum, and Saposhnikoviae Radix contained in BOF are unlikely to account for its inhibition of pancreatic lipase because they are also contained in OGT and CTS which were inactive to inhibit lipase activity (Fig. 3).

There are many reports showing that the plant extracts with inhibitory activity for pancreatic lipase also suppress the elevation of plasma TAG after high fat loading in the animals.[8-11] However, as shown in the present study, DJT extracts was inhibitory for pancreatic lipase (Fig. 3) whereas its oral administration at 750 and 2250 mg/kg, which corresponds to 15- and 45-fold higher than the human daily dose (50 mg/kg), did not suppress the elevation of plasma TAG after oral lipid emulsion (Fig. 5). These results suggest that only the inhibition of pancreatic lipase does not account for BOF suppression of the elevation of plasma TAG after oral lipid emulsion. BOF could inhibit directly the uptake of FFA or MAG in the small intestine or alter gastrointestinal functions relating to fat absorption. It has been reported that saponin-containing plants inhibit gastric emptying in mice,[15] which may account for the suppression of fat absorption by BOF. In addition, it is also possible that BOF affects releasability of pancreatic or bile juice, which are supposed to play an important role in fat absorption from the small intestine.

In conclusion, BOF and DJT inhibited pancreatic lipase *in vitro* but BOF was only effective to suppress the elevation of plasma TAG after oral administration of lipid emulsion. Although the relationship between the inhibitory activity for pancreatic lipase and the suppression of plasma TAG elevation is still unclear, these unique properties of BOF may be explanations for anti-obese effects of this formulation BOF.[1-3]

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