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Hypolipidemic effect of *Shoyu* polysaccharides from soy sauce in animals and humans

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Abstract. Soy sauce (*Shoyu*) is a traditional Japanese fermented seasoning and is available worldwide. We investigated the effects of *Shoyu* polysaccharides (SPS) prepared from soy sauce on hyperlipidemia *in vitro* and *in vivo*. First, SPS inhibited pancreatic lipase. Second, in experiments with animals, it was found that SPS reduced serum triacylglycerol (TG) elevation induced by high-fat diets. Third, in a 2-week placebo-controlled parallel group study, healthy men (TG <150 mg/dl) were treated with 600 mg of SPS (n=5) or placebo (n=5) every day. After 2 weeks, serum TG elevation was significantly (P<0.05) lower in the SPS-treated group than in the placebo-treated group after 6 h of a high-fat diet. Fourth, in a 4-week randomized, double-blind, placebo-controlled parallel group study, hyperlipidemic men (TG >150 mg/dl) were treated with 600 mg of SPS (n=15) or placebo (n=15) daily. After 4 weeks, serum TG levels in the SPS-treated group were significantly (P<0.05) lower than the baseline (0 week). In conclusion, SPS of soy sauce reduce lipid absorption, and soy sauce is a potentially promising seasoning for the treatment of hyperlipidemia through food.

Introduction

Soy sauce (*Shoyu*) is a traditional fermented seasoning from Japan that is available throughout the world (1). In Japanese *shoyu*, soybeans and wheat are the two main raw materials, used in almost the same quantity. Proteins of the raw materials are completely degraded into peptides and amino acids by microbial proteolytic enzymes after fermentation, and no allergens of the raw materials are present in soy sauce (2). In contrast, polysaccharides that originate from the cell wall of soybeans are resistant to enzymatic hydrolysis. These polysaccharides remain in soy sauce even after fermentation and are termed *shoyu* polysaccharides (SPS) (3). Recently, we reported that SPS exhibited potent anti-allergic effects (3), enhancing both macrophage and lymphocyte functions (4) and also iron absorption (5) *in vitro* and *in vivo*. Furthermore, oral supplementation of SPS was an effective intervention for patients with allergic rhinitis (6,7) and enhanced iron absorption in healthy women (5) in two double-blind placebo-controlled clinical studies. However, many of the ingredients of soy sauce are unknown, and the biological activities of soy sauce itself remain to be elucidated. Although soy sauce has been reported to have anti-carcinogenic (8,9), anti-microbial (10), anti-oxidative (11-13), and anti-platelet (14) activities, and to inhibit angiotensin I-converting enzyme (15), further study is necessary to elucidate the biological functions of soy sauce itself and the ingredients it contains.

Recently, in developed countries, the incidence of 'habit-related' diseases (lifestyle-related diseases) such as diabetes, hyperlipidemia, and hypertension have been gradually increasing (16-21). Therefore, dietary therapy is important and could be considered as the first choice treatment. In the present study, in order to clarify the hypolipidemic effect of SPS as a functional dietary component from soy sauce, we examined the effects of SPS on pancreatic lipase activity *in vitro* and on fat absorption induced by feeding high-fat diets *in vivo* using mice and rats. Furthermore, we evaluated the efficacy of SPS in lowering human serum triacylglycerol (TG) levels in two clinical studies.

Materials and methods

Preparation of Shoyu polysaccharides (SPS). Soy sauce (*Shoyu*) was fermented by Higashimaru Shoyu Co., Ltd.

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(Tatsuno, Hyogo, Japan) as described previously (2-7). *Shoyu* polysaccharides (SPS) were prepared according to the method of Kikuchi and Sugimoto (22). SPS were prepared from the dialysate from soy sauce as follows (3-7): 10 ml of soy sauce in seamless cellulose tubing (small size 18, pore size 25 Å, molecular weight cut-off 12,000; Wako Pure Chemical Industries Ltd., Osaka, Japan) was dialyzed overnight in water at 4˚C, and then freeze-dried.

Assay of inhibitory effect on pancreatic lipase. The pancreatic lipase activity was measured using 4-methylumbelliferyl oleate (Sigma Chemical Co., St. Louis, MO, USA) as a substrate according to a previously reported procedure (23). Sample solution (25 μl) dissolved in water and 50 μl of 0.1 mM 4-methylumbelliferyl oleate solution dissolved in a buffer consisting of 13 mM Tris-HCl, 150 mM NaCl, and 1.3 mM $CaCl_2$ (pH 8.0) were mixed in the well of a microplate (Immuno plate, F96 Maxisorp, Nalge Nunc International, Roskilde, Denmark), and 25 μl of the lipase solution (400 U/ml from porcine pancreas; Elastin Products Co., Inc., Owensville, MO, USA) was added to the above buffer to start the enzyme reaction. After incubation at 30˚C for 30 min, 0.1 ml of 0.1 M sodium citrate (pH 4.2) was added to halt the reaction. The amount of 4-methylumbelliferone released by lipase was measured with a fluorometrical microplate reader (Fluostar Galaxy, BMG Labtechnologies GmbH, Offenburg, Germany) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The 50% inhibitory concentration (IC_{50}) was calculated using the mean of 3 observations at each of the 5 concentrations.

Rat long-term dose experiment. The basal diet (CE-2) was purchased, and male SD rats (4 weeks old) were obtained from Clea Japan, Inc. (Tokyo, Japan) and bred in our laboratories. They were housed individually in cages under a 12 h light/dark cycle at a temperature of 23±1˚C and a humidity of 60±5%. In the first week, for preliminary breeding, deionized water and the basal diet were provided daily *ad libitum*. Then, the rats were randomly divided into two groups of six, and deionized water and the high-fat CE-2 diet supplemented with 15% lard (commercial grade, Snow Brand Milk Products Co., Ltd., Tokyo, Japan) were provided daily *ad libitum* for 3 weeks. For 3 weeks, while being fed the high-fat diet, 0.1% SPS were supplemented to the diet. Samples of blood were collected in heparinized tubes from the caudal vein at intervals and were used to determine triacylglycerol (TG) levels. TG was measured with a commercial kit (Wako Pure Chemical Industries Ltd.). After 3 weeks on the diet, the rats were sacrificed, and blood, organs and feces were analyzed as described previously (5).

Mouse short-term dose experiment. Male BALB/c mice (7 weeks old) were obtained from Clea Japan, Inc. and bred in our laboratories. They were housed in cages under a 12 h light/dark cycle at a temperature of 23±1˚C and a humidity of 60±5%. In the first week, for preliminary breeding, deionized water and the basal diet (CE-2) were provided daily *ad libitum*. Then, the mice were randomly divided into three groups of six. To examine the immediate effect of SPS on reduction of TG in the administration of both lard and SPS at the same time, 0.02 g of lard and/or 0.3 mg of SPS were dissolved at 40˚C and orally administered using a metal stomach tube. Three hours after the administration, samples of blood were collected in heparinized tubes from the caudal vein, and were used to determine TG levels with a commercial kit.

Next, to examine the pretreatment effect of SPS on reduction of TG in the administration of lard, the preliminary bred mice were provided with deionized water and the CE-2 diets supplemented with 0.1% SPS daily *ad libitum* for 3 days. Then, 0.02 g of lard was dissolved at 40˚C and orally administered using a metal stomach tube. Three hours after the administration, samples of blood were collected in heparinized tubes from the caudal vein, and were used to determine TG levels with a commercial kit.

Assay of inhibitory effects on intestinal digestion and absorption of lipids. The basal diet (CE-2) was purchased and male SD rats (4 weeks old) were obtained from Clea Japan, Inc., and bred in individual cages. They were housed individually in cages under a 12 h light/dark cycle at a temperature of 23±1˚C and a humidity of 60±5%. During 3 weeks, for preliminary breeding, deionized water and the basal diet were provided daily *ad libitum*. Then, the rats were randomly divided into two groups of six. The rats underwent both portal vein catheterization and a gastrostomy with placement of a swivel apparatus to allow enteral feeding (24). Anesthesia was achieved with 3% halothane in 33% oxygen and nitrous oxide. A fine vinyl catheter (0.5 mm ID, 1.0 mm OD) was introduced into the stomach for injecting the sample, and a fine polyethylene catheter (0.28 mm ID, 0.61 mm OD) was introduced into the portal vein for collecting blood. The external end of the catheter was threaded through a tunnel under the skin, exiting at the scapula, and inserted into the harness and protection coil. The polyethylene catheter was joined to the swivel. The rats were fed the basal diet and water for 24 h after the surgery. Then, they were fed a lipid solution (20% Intralipos, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) at 2.5 ml/kg/h, and 3% SPS solution at 5.6 ml/kg/h through the catheter into the stomach for 360 min. The concentrations of TG and free fatty acid (non-esterified fatty acid, NEFA) from the portal vein blood were assayed with a commercial kit.

Clinical studies. According to the guidelines of the Japan Atherosclerosis Society, the upper limit of the normal range for serum TG is 150 mg/dl. Concentrations above this range are regarded as hyperlipidemic (16). We examined the immediate (Test 1) and prolonged (Test 2) hypolipidemic effects of SPS in two clinical studies. Safety was monitored using clinical history, physical examinations, and routine blood tests, including hepatic and renal function tests, measurements of concentrations of proteins and lipids, and complete blood counts. SPS were prepared from an ethanol precipitate of raw soy sauce, and SPS powder was encapsulated into gelatin capsules as described previously (5-7). The test capsules were constructed with 150 mg of SPS, 60 mg of microcrystalline cellulose, 15 mg of sucrose esters of fatty acids and 5 mg of silicon dioxide per capsule. The placebo capsules were made in the same manner as the test capsules but without SPS. The capsules were indistinguishable between groups. All blood

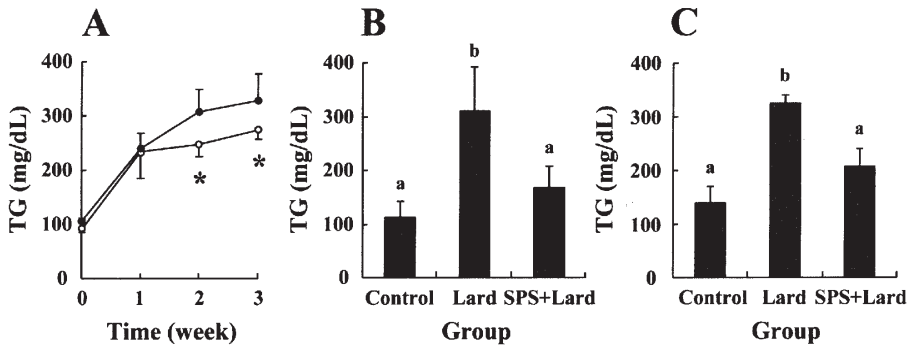

Figure 1. Inhibitory effects of SPS on lipid absorption in rats for long-term dose (A) and in mice for short-term dose (B and C) experiments. For details of SPS preparation, feeding conditions, and assay of serum TG, see Materials and methods. In the rat experiment (A), 0.1% SPS were mixed with 15% lard as a high-fat diet. The diet was administered and serum TG was monitored for 3 weeks. Line with open circles, 0.1% SPS group; line with closed circles, control group (no SPS). Each value is the mean ± SE (n=6). Significantly different from the control at *P<0.05 (Student's t-test). In the mice experiment (B), 0.3 mg of SPS was supplemented with 0.02 g of lard using a metal stomach tube, and serum TG was assayed 3 h after the administration. Each value is the mean ± SE (n=6). Values with different superscripts differ, P<0.05 (Tukey's test). In the next mice experiment (C), 0.02 g of lard was administered using a metal stomach tube after the mice were provided with the diets supplemented with 0.1% SPS for 3 days. Then, serum TG was assayed 3 h after the administration. Each value is the mean ± SE (n=6). Values with different superscripts differ, P<0.05 (Tukey's test).

tests were conducted by BML Inc. (Tokyo, Japan). Routine blood testing, including complete blood cell counts, examinations of hepatic and renal function, and measurements of concentrations of proteins and lipids, were also performed. Notably, serum TG and NEFA of the blood samples were measured by BML Inc. according to an analytical protocol.

Test 1 was conducted to examine the hypolipidemic effect of SPS on the reduction in postprandial triacylglycerolemia and was carried out as follows. In compliance with the principles of the Helsinki Declaration and the study protocol, the purpose and methodology of the study and rights of the subjects were explained in advance, and written consent was obtained from all volunteers. We enrolled 10 apparently healthy men aged 34.3±1.9 years, and their levels of serum TG were <150 mg/dl. Exclusion criteria included hyperlipidemia, diabetes or liver dysfunction, or food allergies. Of the 10 volunteers, 5 were assigned to the SPS group (average age, 34.8±3.4 years) and 5 to the placebo group (average age, 33.8±2.1) in the placebo-controlled parallel group study. For 2 weeks, each volunteer took 4 capsules daily (2 capsules each morning and evening). The SPS dose was 600 mg/day. The day after the 2-week treatment, each volunteer did not eat breakfast in the morning, and completely ate a high-fat diet with 2 capsules of SPS or placebo for lunch. The high-fat diet was designed from a McDonald's hamburger menu; two hamburgers, fried potatoes and coke (total energy, 1644 kcal; total fat, 90.7 g). After the lunch, samples of blood were collected at 3 and 6 h and were used to determine TG and NEFA levels.

Test 2 was conducted to examine the hypolipidemic effect of SPS on hyperlipidemia. The study used a randomized, double-blind, placebo-controlled parallel group design. The protocol was approved by the ethics committees of the participating hospitals. The study was managed and operated by the Institute of General Health Development Co., Ltd. (Tokyo, Japan) in accordance with the Declaration of Helsinki. The trial was carried out for 4 weeks. Exclusion criteria included diabetes, liver dysfunction or food allergies. According to the guidelines of the Japan Society for the Study of Obesity, a body mass index (BMI) >25 is regarded as indicating obesity (16). One hundred and seventy-two male volunteers were asked to complete a meal questionnaire including the TG value and to provide a blood sample 2 weeks before the start of the study. Thirty volunteers with both hyperlipidemia (TG >150 mg/dl) and waist measurements between 85 and 110 cm showing BMIs >25 were registered to participate in the study. Of these 30 volunteers, 15 were randomized into the SPS group (average age, 42.5±6.9 years; BMI, 28.1±3.2) and 15 to the placebo group (average age, 45.9±9.3; BMI, 27.9±2.7). For 4 weeks, each volunteer took 4 capsules daily (2 capsules each morning and evening). The SPS dose was 600 mg/day. During the study, blood samples after fasting were taken every 2 weeks and a meal questionnaire was recorded daily.

Statistical analyses. Data were analyzed using SPSS for Windows 7.5.1 (SPSS Japan Inc., Tokyo, Japan). Differences in data were evaluated between groups with the Student's t-test or Tukey's test. Differences in data were also evaluated within groups with the Dunnett's test. P<0.05 or <0.01 was considered a significant difference, and P<0.10 was considered a significant tendency.

Results

Inhibitory effect of SPS on pancreatic lipase. The inhibitory effect of SPS on pancreatic lipase was ~1 mg/ml of IC_{50}. SPS were water-soluble pectic polysaccharides that originated from soybeans by microbial enzymatic degradation in the brewing process of soy sauce, but heat-treated soybeans had no inhibitory effect. In this assay system, seven types of pectin from citrus and apple had also no inhibitory effect (data not shown). Therefore, the formation of inhibitory substances, SPS, occurred in the brewing process of soy sauce.

Inhibitory effects of SPS on lipid absorption in animals. First, we examined the effects of SPS on serum TG in rats fed a high-fat diet containing lard (Fig. 1A). For 3 weeks, neither food intake nor body weight differed significantly between the two groups. When the high-fat diet was administered

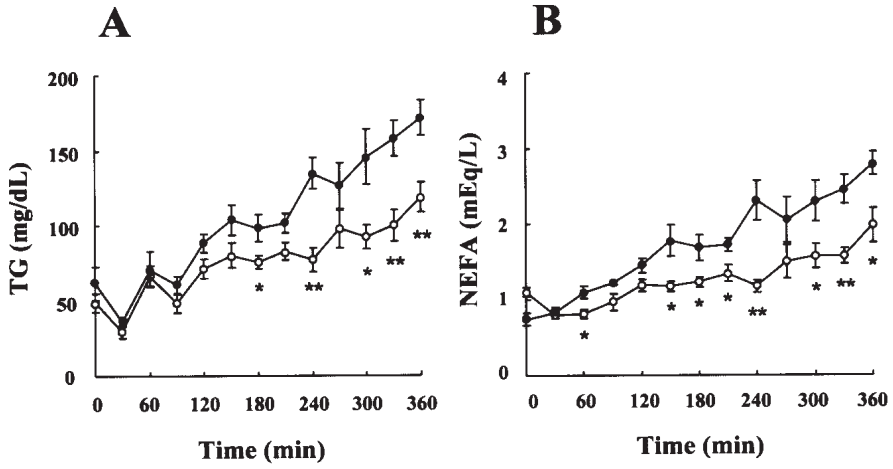

Figure 2. Inhibitory effects on intestinal digestion and absorption of lipids in the catheterized rats. For details of SPS preparation, catheterization, feeding conditions, and assays of TG and NEFA, see Materials and methods. Line with open circles, 3% SPS group; line with closed circles, control group (no SPS). For 360 min, lipid solution was administered at 2.5 ml/kg/h, and 3% SPS solution was administered at 5.6 ml/kg/h through the catheter into the stomach. The concentrations of TG (A) and NEFA (B) from the portal vein blood were assayed. Each value is the mean ± SE (n=6). Significantly different from the control at $^{*}P<0.05$ or $^{**}P<0.01$ (Student's t-test).

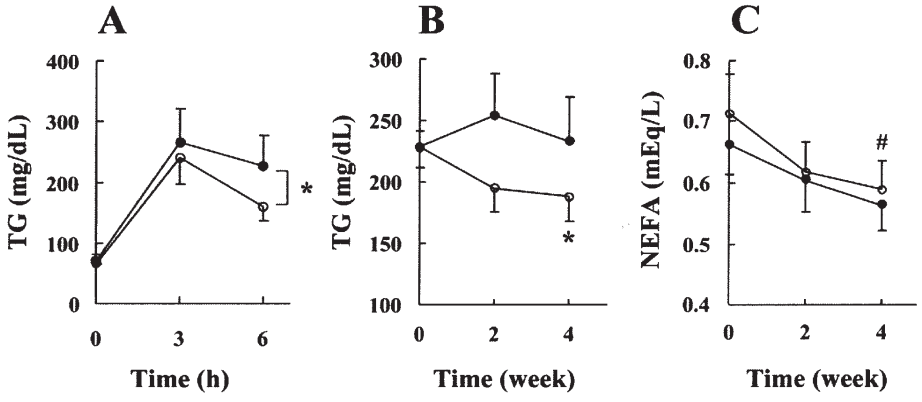

Figure 3. Hypolipidemic effects of SPS in two clinical studies examining immediate (Test 1) and prolonged (Test 2) effects. For details of SPS preparation, test conditions, and assays of serum TG and NEFA, see Materials and methods. Test 1 was conducted as follows: 10 healthy men were enrolled in the placebo-controlled parallel group study (5 in the SPS group and 5 in the placebo group). Line with open circles, SPS group; line with closed circles, the placebo group (no SPS). For 2 weeks, volunteers in the SPS group received 600 mg of SPS per day. The day following the 2-week treatment, each volunteer did not eat breakfast in the morning, and completely ate a high-fat diet with 300 mg of SPS or placebo for lunch. The high-fat diet was designed from McDonald's hamburger menu and consisted of two hamburgers, fried potatoes and coke (total energy, 1644 kcal; total fat, 90.7 g). After the lunch, samples of blood were collected at 3 and 6 h, and were used to determine serum TG levels (A). Each value is the mean ± SE (n=5). Significantly different from the placebo at $^{*}P<0.05$ (Student's t-test). Test 2 was conducted as follows: 30 hyperlipidemic men were enrolled in the randomized, double-blind, placebo-controlled parallel group study (15 in the SPS group and 15 in the placebo group). Line with open circles, SPS group; line with closed circles, the placebo group (no SPS). For 4 weeks, volunteers in the SPS group received 600 mg of SPS per day. During the study, blood samples after fasting were taken every 2 weeks, and were used to determine serum fasting TG (B) and NEFA (C) levels. Each value is the mean ± SE (n=15). From the baseline, $^{*}P<0.05$ was considered a significant difference, and $^{\#}P<0.10$ was considered a significant tendency (Dunnett's test).

for 3 weeks, the serum TG levels greatly increased in a time-dependent manner. In contrast, when the high-fat diet was supplemented with 0.1% SPS, the serum TG levels were significantly lower (P<0.05) than those of the control (no supplementation of SPS) after 2 to 3 weeks. Furthermore, the excreted TG in the feces of SPS-supplemented rats was higher than in control rats (data not shown). Therefore, SPS gradually decreased the rise in serum TG levels after being fed a high-fat diet in the long-term dose experiment.

Second, we examined the immediate effects of SPS on serum TG in mice administrated both lard and SPS at the same time (Fig. 1B). After 3 h of administration of lard only, the serum TG levels in mice were greatly increased. In contrast, the serum TG levels in mice which had been administered both lard and SPS were significantly lower (P<0.05) than in mice which had been administered lard only. Therefore, SPS simultaneously decreased the rise in serum TG levels after the administration of fat in the short-term dose experiment. Furthermore, we examined the pretreatment effect of SPS on serum TG in mice administered lard after 3 days of treatment with SPS (Fig. 1C). After 3 h of administration of lard, the serum TG levels in mice that had been administered SPS were significantly lower (P<0.05) than the serum TG levels in the control group (no supplementation of SPS). Therefore, pretreatment of SPS decreased the rise in serum TG levels after the administration of fat in the short-term dose experiment.

Third, we examined the continuous effects of SPS on lipid absorption in the catheterized rats (Fig. 2). The rats

by enteral lipid with SPS, and portal blood samples ected from the start until 360 min after feeding. The blood concentrations of TG and NEFA in the SPS group of rats were significantly (P<0.05 or <0.01) lower than those in the control group (no supplementation of SPS) after feeding. After 360 min, ΔAUCs (areas under the curves) of both TG and NEFA levels were significantly decreased by 27.1 (P<0.01) and 28.2% (P<0.01), respectively. Therefore, these results indicate that SPS inhibit intestinal lipid absorption by inhibiting pancreatic lipase.

Inhibitory effects of SPS on lipid absorption in humans. We examined the immediate (Test 1) and prolonged (Test 2) hypolipidemic effects of SPS in two clinical studies. First, Test 1 was conducted to determine serum TG levels in 10 healthy men after eating a high-fat diet (Fig. 3A). Six hours after eating the diet, serum TG levels in the SPS group were significantly lower (P<0.05) than those in the placebo group (no supplementation of SPS). Second, Test 2 was conducted to determine serum TG and NEFA in 30 hyperlipidemic men in a 4-week randomized, double-blind, placebo-controlled parallel group study. After 4 weeks of treatment with SPS, there was a significant reduction in serum TG (P<0.05) from the baseline (0 week) (Fig. 3B), and serum NEFA was also reduced (P<0.10) from the baseline (0 week) in the group (Fig. 3C). After 4 weeks of treatment, no significant abnormality was detected in routine blood tests, including hepatic and renal function, and concentrations of proteins and lipids except TG and NEFA (data not shown).

Discussion

Pancreatic lipase is a key enzyme for lipid absorption. It is well known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase (23). Thereby, to suppress serum TG levels and weight gain, it would be effective to reduce fat absorption by lipase inhibition. Orlistat, a specific pancreatic lipase inhibitor, is clinically used for preventing obesity and hyperlipidemia (23). On the other hand, lipase inhibitory materials derived from natural products, such as chitosan (25), pectin (26,27) chondroitin sulfate, and the polyphenolic constituents of *Salacia reticulata* (28), grape seed extract (29) and oolong tea (23), have been reported previously. In animal studies, it has been reported that water-soluble dietary fibers such as glucomannan and pectin, strongly affected lipid metabolism, which led to decreased serum cholesterol and TG levels (30). Furthermore, in human studies, certain food factors, such as ß-conglycinin (16) and soy protein (18) from soybeans, mannooligosaccharides (19) from coffee mannan, and polyphenol-enriched oolong tea (17), suppressed serum lipids and reduced fat storage by mechanisms such as inhibition of fat absorption, excretion of fat, or regulation of lipid metabolism.

In this study, we demonstrated that SPS of soy sauce suppress fat absorption by an inhibition of pancreatic lipase. In animal studies, SPS decreased immediately and gradually serum TG levels induced by high-fat diets in both short and long feedings. Furthermore, pretreatment of SPS decreased serum TG levels induced by high-fat diets in the short feeding, and SPS functioned as both immediate and durative hypolipidemic agents. Recently, Takano *et al* designed an experimental system to monitor the portal concentration of certain nutrients such as glucose and calcium using rats that had undergone portal vein catheterization (24). This method was used to investigate the effect of SPS on lipid absorption in rats with enteral feeding although lymphatic absorption of TG was not directly monitored. In the catheterized rats, SPS also continuously decreased TG and NEFA levels induced by a high-fat diet. Since SPS are pectin polysaccharides degraded from soybeans in the brewing process (3), SPS function by excreting fat just as dietary fibers (30), and also by reducing fat absorption through lipase inhibition as chitosan (25). However, the details of the mechanism (31) underlying the decreased TG synthesis and accumulation prompted by SPS remain unknown. In the first clinical study of healthy men (Test 1), serum TG elevation was significantly (P<0.05) lower in the SPS-administered group than in the placebo-administered group 6 h after a high-fat meal. In the second clinical study of hyperlipidemic men (Test 2), serum TG levels in the SPS-administered group were significantly (P<0.05) lower than the baseline (0 week) after 4 weeks of treatment. In conclusion, SPS of soy sauce are effective in suppressing the serum TG elevation when a high lipid diet is eaten, and SPS reduce high serum TG levels in hyperlipidemia as a dietary treatment. The amount of soy sauce consumed daily in Japan is estimated at ~30 ml per person according to data from the Japan Soy Sauce Brewers Association (1988). Therefore, soy sauce is useful as a hypolipidemic seasoning in daily life, and the SPS from soy sauce are a safe and important food component for the prevention and/or amelioration of visceral fat syndrome, or so-called metabolic syndrome.

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