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Effects of Alternatively Prepared *Meju* Methanolic Extracts on Dietary Lipid Digestion

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Abstract

We examined the effect of extracts of *meju* prepared with traditional and standardized methods on pancreatic lipase and the absorption of dietary lipid. Aqueous methanolic (80%, v/v) extracts of *meju* dose-dependently inhibited the activities of porcine pancreatic lipase. The plasma triglyceride levels in Imprinting Control Region mice after a single oral administration of lipid emulsion containing aqueous methanolic extracts from *meju* made by the standardized methods were lower than that of the group given a lipid emulsion containing the extracts of *meju* made by traditional methods. The inhibitory activity of the *meju* extract on dietary lipid digestion appears to be more closely associated with aglycone forms of phenolic compounds such as free isoflavones than with glycosides, since *meju* samples with higher total phenolic or free isoflavone content showed the stronger inhibition against pancreatic lipase. Furthermore, the data suggest that *meju* made using the standardized method, which contains higher levels of total isoflavones relative to traditionally prepared *meju*, could effectively suppress digestion of dietary lipids and therefore have the potential to help ameliorate hyperlipidemia and obesity.

Key words: pancreatic lipase, *meju*, lipid digestion, anti-obesity

INTRODUCTION

Soybean and its products have been reported to have many beneficial health effects, such as improvement of lipid profile and anticarcinogenic activity for some sex-hormone dependent cancers. The major soybean components responsible for health benefits include soy proteins and peptides, isoflavones, saponins, and phytic acid. In particular, soy protein was legally approved to claim health benefits in the cases of heart disease and hypocholesterolemic effects in several countries such as Korea, US, and Japan (1,2). The isoflavones present in soybean that have both weak estrogenic and anti-estrogenic activity may also be partly responsible for the cholesterol lowering and cardioprotective effects. It is not clear how soy protein and other components of soybean act to lower cholesterol and lipid levels. It is possible that their effect on the endocrine system may be partly responsible, since hormones do control lipid metabolism (3,4).

As fermented soy products such as *cheonggukjang*, *meju*, *doenjang*, and *kochujang* undergo extensive enzymatic and chemical changes during fermentation and subsequent aging steps, they usually contain relatively high levels of free forms of phytochemicals and peptides, and, therefore, may be able to exert a stronger bioactive function than unfermented soybeans. In support of this concept, our previous study demonstrated that most of the isoflavones were metabolized into aglycones during the process of *meju* or *doenjang* preparation (5). Aglycone forms of soy isoflavones have been reported to be more effective in suppressing lipid absorption than glycosides (4,6). Because free isoflavones are believed to better compete with glycosides in absorption, methanolic extracts of fermented soy products may have better health benefits than unfermented soybean. We therefore hypothesized that fermented soy products inhibit pancreatic lipase and thereby suppress lipid absorption.

In this study, the effect of aqueous methanolic (80%, v/v) extracts of *meju* made by traditional and standardized methods on pancreatic lipase and the absorption of triacylglycerol in mouse model were investigated.

MATERIALS AND METHODS

Chemicals

Porcine pancreatic α-amylase, α-glucosidase (rat intestinal acetone powder), and *p*-nitrophenyl-α-D-glucopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *p*-Nitrophenyl-α-D-hexa(1→4)-glucopyranoside was purchased from Fluka (Tokyo, Japan). Porcine pancreatic lipase and 4-methylumbelliferyl oleate (4-MU oleate) were also purchased from Sigma. Acarbose (Glucobay), an inhibitor of carbo-

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hydrate-digesting enzymes, was obtained from Bayer Korea Ltd. (Seoul, Korea) and used as the positive control. Orlistat (Xenical), a prescription drug that inhibits pancreatic lipase activity, was provided by Roche Korea Ltd. (Seoul, Korea). Assay kits for blood triacylglycerol and total cholesterol levels were purchased from Asan Pharmaceutical Co. (Seoul, Korea). All other chemicals were of reagent grade.

Preparation of the methanol extracts from the soy products

Traditional *meju* were made as described previously (5) and standardized *meju* was prepared by fermenting steamed soybean (harvested in the Sunchang area) with *Asperigillus oryzae* for various periods of time. Briefly, soybeans were soaked with tap water (25～28°C) for 4 hr, drained, and then steam-cooked at 121°C for 30 min. After cooling, the cooked soybeans were mixed with the conidia of *A. oryzae* var. *oryzae*, a soy sauce koji mold, spread on the tray, covered with two layers of wet cheesecloth, and incubated at the ambient temperature (23～29°C) for 3 days. Each fermented soy sample was subjected to freeze-drying, powdering and then extraction with 80% (v/v) methanol for 24 hr at room temperature. The extracts were filtered through a syringe filter (pore size 0.45 μm) and kept at -20°C prior to use.

Measurement of inhibitory activities against digestive enzymes

The inhibitory activity against pancreatic lipase was assayed using 4-methylumbelliferyl oleate (4-MU oleate) as a substrate at concentrations of 1, 5, 10 mg/mL (7). The reaction mixture consisted of 20 μL of 0.2 mM 4-MU oleate, 30 μL of Mcllvne buffer (0.1 M Na_2HPO_4-citrate, pH 7.4) and 20 μL of sample solution. Adding 30 μL of 0.5 unit lipase, all in a final volume of 100 μL, started the reaction. After incubating at 37°C for 1 hr, 50 μL of 0.1 N HCl and 50 μL of 0.2 M sodium citrate were added to stop the enzyme reaction. The amount of 4-methylumbelliferone (4-MU) released from 4-MU oleate was measured using a fluorometer at an excitation wavelength at 320 nm and an emission wavelength at 450 nm. The inhibitory activities (%) of samples against pancreatic lipase were calculated as follows;

$$\text{Inhibitory activity (\%)} = (1 - \text{fluorescence of sample}/\text{fluorescence of solvent}) \times 100$$

Postprandial blood lipid levels in mice

Eight week-old male ICR mice weighing 40 g were fasted overnight and then given 200 μL lipid emulsion via oral administration with a capillary tube. To prepare the lipid emulsion, 80% methanol extracts of soy products were evaporated, added to 0.9% saline, corn oil, cholic acid, cholesteryl oleate and then sonicated. The composition of lipid emulsion is shown in Table 1. Each animal (5 animals per group) was intubated with the lipid emulsion, with or without *meju* extract, followed by blood sample collection from the tail vein at 0, 30, 60, 120, 180 min using a heparinized capillary tube. The blood samples were then centrifuged (5,500×*g*, 5 min, 4°C). The plasma triglyceride (TG) level was determined using commercial assay kits (Asan Pharmaceutical Co., Seoul, Korea).

Table 1. The composition of lipid emulsion used in this study

Component	Content
Corn oil (μL)	100
Cholic acid (mg)	1.34
Cholesteryl oleate (mg)	3.4
0.9% NaCl (μL)	100
Sample extracts (mg)[1)]	20
Total volume (μL)	200

[1)]80% methanolic extract prepared from *meju* fermented for 6 days by standardized method or 60 days by traditional method.

Isoflavone analysis

The isoflavone profile was analyzed as previously reported (5). Briefly, freeze-dried *meju* was subjected to extraction with acidified acetonitrile, followed by filtering and analysis in an HPLC equipped with a Phenomenex Gemini C18 column and Jasco UV-visible detector (Tokyo, Japan).

Total phenolic contents

Determination of the content of total phenolics was performed according to a previously described method (8). After the extracts had been diluted with water in a stepwise fashion, 100 μL of the diluted solution was pipetted into a 96-well plate. Fifty microliters of Folin-Ciocalteu reagent diluted 5-fold with water and 50 μL of 10% (w/v) sodium carbonate solution were added to each well, and the plate was placed at room temperature for 5 min. The absorbance was read using a microplate reader at 655 nm with DMSO as a blank (Sunrise, Tecan, Australia). A standard curve was established using various concentrations of gallic acid in 95% ethanol. Absorbance values were converted to milligrams of total phenolics per 100 gram of 80% methanolic extract powder.

RESULTS

Effect of aqueous methanolic extracts of *meju* on digestive enzyme activities

The lipase inhibitory activities of two different kinds of *meju* samples, which were prepared by either tradi-

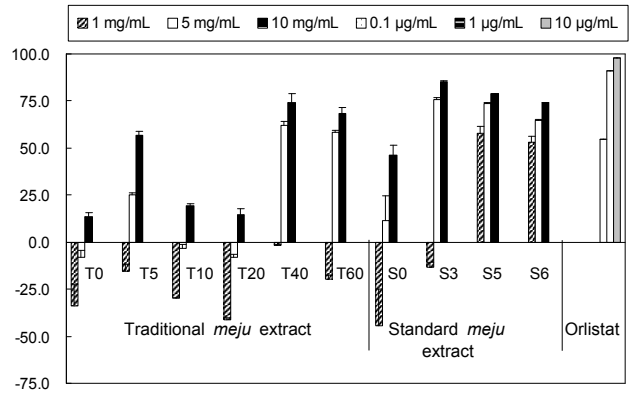

Fig. 1. Inhibitory activity of *meju* extracts against porcine pancreatic lipase (PL). Samples used in the study include 80% methanol extracts of *meju* fermented for 0 (S0), 3 (S3), 5 (S5), 6 (S6) days by standardized method or 0 (T0), 10 (T10), 20 (T20), 40 (T40), 60 (T60) days by traditional method.

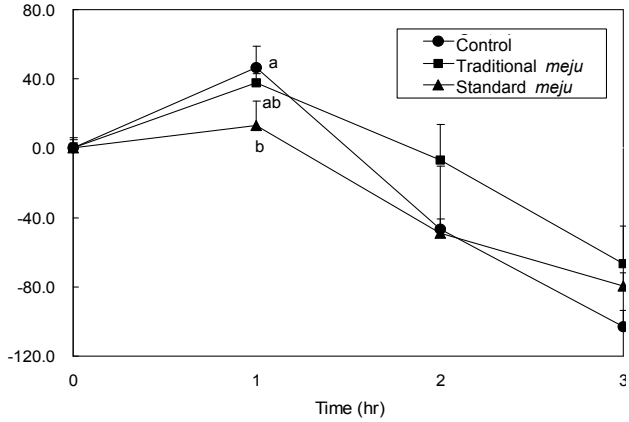

Fig. 2. Effects of *meju* extracts on plasma TG level after oral administration of a lipid emulsion. Lipid emulsion contained 80% methanolic extract made from *meju* fermented 6 days by standardized method or 60 days by traditional method, and its composition was described in Table 1.

tional or standardized methods, were evaluated (Fig. 1). For traditional *meju*, the sample prepared from *meju* fermented for 40 and 60 days showed the highest inhibitory activity against pancreatic lipase. The samples from *meju* fermented for 20 days or less had significantly lower enzyme inhibition than those fermented for 40 and 60 days. Similar results were observed for *meju* samples prepared by the standardized method. That is, 80% methanolic extracts of *meju* fermented for prolonged periods (e.g., 5 or 6 days) inhibited the enzyme more strongly than the samples fermented for 0 or 3 days.

Effect of aqueous methanolic extracts of *meju* on postprandial serum lipid levels in mice

The 80% methanolic extract of *meju* prepared using the standardized method suppressed an increase in plasma TG level after an oral dose of lipid emulsion (Fig. 2). More specifically, plasma TG level was significantly lower in mice at 1 hr after oral administration of lipid emulsion containing standardized *meju* extract, compared to the control group or mice administered lipid containing traditional *meju* extract. However, there was no significant difference in plasma TG level at 2 and 3 hr after oral dose of lipid emulsion (20 mg in 0.2 mL) among experimental groups. The 80% methanolic extracts of traditional *meju* fermented for 60 days did not cause any significant effect on postprandial TG level of mice.

Change of isoflavone profile and total phenolic contents during *meju* preparation

The total isoflavone contents of *meju* prepared either by standardized or traditional methods were reduced in a fermentation time-dependent manner, from 1,842 μg/g in cooked soy to 1,097 μg/g after 6 days of fermentation with *Asp. oryzae* or 582 μg/g after 60 days of fermenta-

Table 2. Change of isoflavone profile during fermentation of *meju* by standard method (Unit: μg/g dw)

Isoflavone derivatives	Fermentation (days)			
	0	3	5	6
Daidzin	420±19[1)]	384±3	260±4	202±11
Glycitin	105±8	85±4	69±2	85±10
Genistin	727±36	666±35	555±57	498±12
M-daidzin	37±6	28±12	13±1	4±1
M-glycitin	30±1	24±2	8±1	9±3
M-genistin	310±10	27±14	14±2	5±1
A-daidzin	63±0	6±1	17±2	13±1
A-glycitin	51±0	35±13	9±1	11±5
A-genistin	69±3	4±1	8±1	5±0
Total glycosides	1,812±55	1,261±63	953±60	832±13
Daidzein	9±0	35±6	83±2	92±5
Glycitein	16±0	32±1	33±2	43±1
Genistein	4±0	20±1	115±5	129±7
Total aglycones	29±0	87±6	231±5	265±3
Total isoflavones	1,842±55	1,348±58	1,185±65	1,097±10

[1)]Values are mean±SD (n=3).

Table 3. Change of isoflavone profile during fermentation of *meju* by traditional method (Unit: μg/g)

Isoflavone derivatives	Fermentation (days)					
	0	5	10	20	40	60
Daidzin	420±19[1)]	398±14	358±17	362±14	212±3	5±1
Glycitin	105±8	100±10	90±1	86±3	66±6	5±1
Genistin	727±36	695±58	676±37	585±54	327±13	84±7
M-daidzin	37±6	32±9	28±3	30±4	24±5	20±8
M-glycitin	30±1	28±3	9±1	11±3	7±1	26±3
M-genistin	310±10	314±24	114±5	64±4	5±1	13±8
A-daidzin	63±0	49±1	11±1	11±1	8±2	9±3
A-glycitin	51±0	45±8	40±7	5±1	6±1	8±7
A-genistin	69±3	67±3	23±1	16±1	18±8	14±3
Total glycosides	1,812±55	1,728±58	1,349±32	1,170±53	672±5	184±15
Daidzein	9±0	11±2	57±6	60±4	67±2	175±21
Glycitein	16±0	16±1	46±6	49±6	42±3	81±5
Genistein	4±0	5±2	20±1	24±1	89±54	141±18
Total aglycones	29±0	32±3	123±12	133±2	197±49	398±8
Total isoflavones	1,842±55	1,760±56	1,472±43	1,303±50	869±46	582±9

[1)]Values are mean±SD (n=3).

Table 4. Total phenolic contents of *meju* prepared by standard and traditional methods

Fermentation period (d)	Standard meju extract[1)]				Traditional meju extract[1)]				
	0	3	5	6	5	10	20	40	60
Total phenolics (GAE g%)[2)]	13±1[3)]	19±1	17±0	18±1	16±1	16±2	15±0	21±2	23±1

[1)]Extracted with 80% methanol.
[2)]GAE represents gallic acid equivalents per 100 g extract powder.
[3)]Values are mean±SD (n=3).

tion in traditional *meju*, respectively (Table 2, 3). In contrast, the levels of total free isoflavones were increased from 29 μg/g in cooked soybean to 265 and 582 μg/g after fermentation for 6 or 60 days in standard and traditional *meju*, respectively. In particular, a significant increase in free isoflavone contents was observed during the last period of fermentation of standard and traditional *meju*. For example, free isoflavones increased from 197 μg/g at the 40th day of fermentation to 398 μg/g at the 60th day of fermentation in traditional *meju* (Table 3). A similarly dramatic change in the free isoflavone profile occurred between the 3rd and the 5th days of fermentation in standard *meju* (Table 2).

A slight increase in total phenolic contents was found during the standard *meju* fermentation process. That is, total phenolic contents increased from 13 g% at day 0 to 18 g% at day 6 of fermentation.

However, the total phenolic contents were significantly increased with extended fermentation time in traditional *meju*, in particular, between the 20th and 40th day of fermentation (Table 4), with values of 15 and 21 g%, respectively.

DISCUSSION

Inhibition of pancreatic lipase is considered one of the most effective ways to control TG absorption, thereby controlling dyslipidemia and obesity. Orlistat, marketed by Roche as a prescription drug under the trade name Xenical, and also known as tetrahydrolipstatin, is one of the drugs developed to treat obesity by preventing the absorption of the fats from the diet (9).

There has been enormous interest in the effect of soy components on lipid metabolism. In particular, soy isoflavones and proteins have been under extensive investigation and have been found to be major components responsible for improving abnormal lipid profiles. More specifically, soy isoflavones and proteins are reported to decrease total circulating cholesterol and LDL-cholesterol, while isoflavones may reduce plasma and tissue cholesterol synthesis by regulating HMG-CoA reductase (10). Although soy proteins and isoflavones have been assumed to upregulate LDL-receptors and promote hepatic scavenging of LDL-cholesterol, as well as inhibit *de novo* biosynthesis of cholesterol by regulating HMG-CoA, their precise mechanism of action to lower plasma cholesterol level remains unclear.

Because *meju* is expected to contain various phytochemicals and their metabolites, and thereby exert a range of bioactive function, the present study focused on the potential of *meju* extract enriched with isoflavones to affect dietary TG digestion and absorption. In particular, the fungal fermentation of soybean that occurs in *meju* preparation is expected to generate free forms of

isoflavones, which usually have stronger biological activity than glycosides.

The results obtained from this current study suggest that the extract of *meju*, prepared by standardized methods, significantly suppresses TG absorption, probably through inhibition of pancreatic lipase (Fig. 1, 2). In fact, aqueous methanolic extract of *meju* was found to effectively inhibit porcine pancreatic lipase using an *in vitro* assay (Fig. 1). Standard *meju* samples collected 5 days after fermentation with *Asp. oryzae* showed the strongest inhibitory activity against porcine pancreatic lipase. It is not clear why standard *meju* at 5 days after fermentation showed the higher inhibitory activity against porcine pancreatic lipase than the other samples. The inhibitory activity seems to be associated with the levels of free isoflavones and total isoflavones, although free isoflavones appear to play more important role than glycoside forms. In fact, the concentration of free isoflavonoids in *meju* made by the standardized method significantly increased from 5 days after fermentation (Table 2), while total isoflavone levels in standard *meju* stay quite high relative to traditional *meju* that was fermented for 40 to 60 days. There is also a possibility that other components in fermented soy products, in addition to the free isoflavones, could contribute to pancreatic lipase inhibition. The observation that methanolic extracts from traditional *meju*, with higher free isoflavones than standard *meju*, showed relatively low inhibitory activity against pancreatic lipase supports that possibility. Thus, small biomolecules present in soybean such as phenolics, carotenoids and saponins, as well as flavonoids, may have an influence on lipid metabolism. As shown in Table 4, the total phenolic contents in *meju* showed positive correlation with inhibitory activity against pancreatic lipase, implicating a potential contribution of phenolic compounds in *meju* extract to the suppression of postprandial TG level. However, phenolic compounds can only partially account for pancreatic lipase inhibition by *meju* extract because the extract of traditional *meju* with the highest phenolics content did not suppress plasma TG increase after oral administration of lipid emulsion, while the sample from standard *meju* with a relatively low phenolics content had a significant effect on the plasma TG level.

It has been well documented that plant saponins, such as ginseng saponins, suppress pancreatic lipase and prevent high fat diet-induced obesity in mice (11,12). Therefore, there is a good possibility that soy saponins may also contribute to the inhibition of TG digestion by lipase.

In conclusion, 80% methanolic extract of *meju* prepared by standardized method caused significant inhibition of porcine pancreatic lipase and also suppressed plasma TG increase after oral administration of lipid emulsion in mice, while the traditional *meju* sample was not effective in controlling plasma TG level. Major factors responsible for lipase inhibition may include the free and total isoflavones, as well as saponins. Thus, our data suggest that *meju* prepared by our standardized method could be a better ingredient for nutritional intervention for the obese or hyperlipidemic people than *meju* prepared using traditional methods.

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