Isoflavone-Free Soy Protein Prepared by Column Chromatography Reduces Plasma Cholesterol in Rats

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To know whether isoflavones are responsible for the hypocholesterolemic effect of soy protein, the effect on plasma cholesterol of isoflavone-free soy protein prepared by column chromatography was examined in rats. Five-week-old male Sprague−Dawley rats were fed cholesterol-enriched AIN-93G diets containing either 20% casein (CAS), 20% soy protein isolate (SPI), 20% isoflavone-free SPI (IF-SPI), 19.7% IF-SPI + 0.3% isoflavone-rich fraction (isoflavone concentrate, IC), or 20% CAS + 0.3% IC for 2 weeks. Plasma total cholesterol concentrations of rats fed SPI and IF-SPI were comparable and were significantly lower than that of rats fed CAS. The addition of IC to the CAS and IF-SPI did not influence plasma cholesterol level. Fecal steroid excretion of the three SPI groups was higher than that of the two CAS groups, whereas the addition of IC showed no effect. Thus, a significant fraction of the cholesterol-lowering effect of SPI in rats can be attributed to the protein content, but the isoflavones and other minor constituents may also play a role.

KEYWORDS: Soy protein; isoflavone; plasma cholesterol; fecal steroid excretion; casein; rat; column chromatography

In addition to its high nutritional value and excellent processing properties, soy protein has a hypocholesterolemic potential when compared with animal protein and thus may reduce the risk of atherosclerosis (*1*, *2*). Heated and extruder-treated soy foods are effective in reducing human plasma cholesterol levels (*3*). In addition to the protein component per se, a variety of soy components such as amino acids and peptides, saponins, phytic acid, trypsin inhibitors, fiber, and isoflavones may affect cholesterol metabolism either individually or in combination (*2*). However, the exact mechanism by which soy components decrease blood cholesterol level is not totally understood.

Recently, physiological effects of soy isoflavone have been attracting interest, that is, anticarcinogenic (*4*, *5*), antioxidative (*6*), and antiosteoporotic (*7*) effects. In addition, isoflavones are thought to be an important hypocholesterolemic component in soy protein. Because soy protein that was washed with alcohol to remove isoflavones has no cholesterol-reducing effect in rhesus monkey, it is concluded that the effect of soy protein is attributable to isoflavones (*8*, *9*). In addition, the hypocholesterolemic effect of soy protein free of isoflavones was inferior to that of intact soy protein in mice (*10*) and rats (*11*), respectively. However, because alcohol extraction results in the loss of components other than isoflavones such as saponin and in the denaturation of protein, this procedure may not appropriate to evaluate the plasma cholesterol-lowering effect of isoflavones.

Additionally, the effect of isoflavone supplementation to soy protein on plasma cholesterol was not well understood. The addition of the alcohol extract to soy protein isolate did not influence its serum cholesterol-lowering potential in gerbils (*12*). The isoflavone extract had no influence on plasma cholesterol in cynomolgus monkey (*13*, *14*). In contrast, both ethanol−acetone-extracted and untreated soy protein lowered serum total cholesterol to a similar extent in rats (*15*). Thus, it remained unclear which component(s), in particular isoflavones, in soy protein may be the determinant for plasma cholesterol level.

We developed a novel procedure to remove isoflavones almost quantitatively from soy protein isolate by passage through a hydrophobic adsorbent column without the alcohol treatment. Using this procedure, we currently studied the effects not only of soy protein isolate free of isoflavones but of the isoflavone-rich fraction separated on plasma lipids and fecal steroid excretion in rats.

MATERIALS AND METHODS

All animals were treated in accordance with the guidelines established by the Japanese Society of Nutrition and Food Science (Law 105 and Notification 6 of the Japanese government).

Preparation of Test Materials. Soy protein isolate (SPI, Fujipro; Fuji Oil Co., Osaka, Japan), dissolved in water, was used for the

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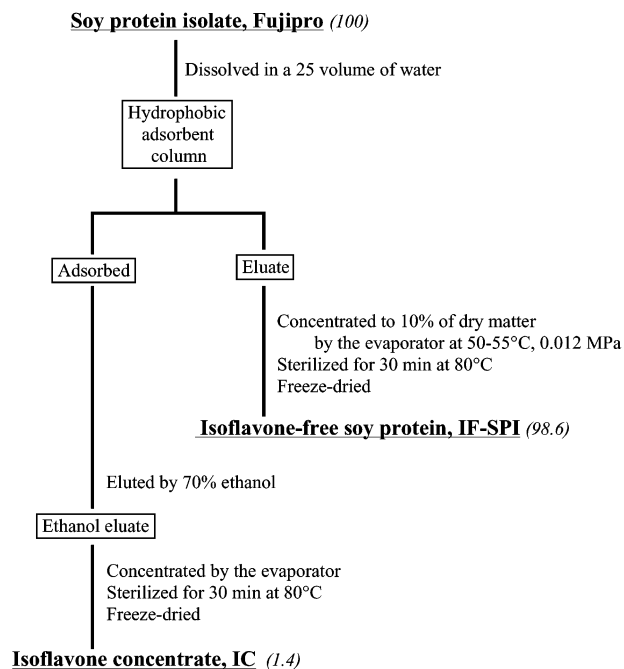† New Ingredients Research Institute.
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Soy protein isolate, Fujipro *(100)*

Figure 1. Schematic diagram for the preparation of test materials. Values in parentheses show the yield of each fraction.

Table 1. Composition and Isoflavone Contents of the Test Substances

	CAS	SPI	IF-SPI	IC
moisture (%)	6.5	1.2	1.6	5.3
crude protein (%)	89.1	85.1	87.2	25.8
ash (%)	1.7	5.6	5.8	5.5
isoflavone content (mg/g)				
total isoflavones		3.91	0.01	284.6
total daidzin[a]	ND[h]	1.13	ND	100.7
total genistin[b]	ND	2.55	ND	170.5
total glycitin[c]	ND	0.23	0.01	13.4
isoflavone compostition (%)				
conjugated[d]		20.8	0	27.6
malonyl[e]		71.2	0	66.2
acetyl[f]		0.3	0	4.8
aglycons[g]		7.7	100	1.4
saponin (mg/g)	ND	3.9	1.9	146

[a] Total daidzin = daidzin + malonyldaidzin + acetyldaidzin + daidzein. [b] Total genistin = genistin + malonylgenistin + acetylgenistin + genistin. [c] Total glycitin = glycitin + malonylglycitin + acetylglycitin + glycitin. [d] Conjugated = (daidzin + genistin + glycitin)/(total isoflavones) × 100. [e] Malonyl = (malonyldaidzin + malonylgenistin + malonylglycitin)/(total isoflavones) × 100. [f] Acetyl = (acetyl-daidzin + acetyl-genistin + acetyl-glycitin)/(total isoflavones) × 100. [g] Aglycones = (daidzein + genistein + glycitin)/(total isoflavones) × 100. [h] ND, not detected.

preparation of isoflavone-free SPI (IF-SPI) and isoflavone concentrate (IC) (**Figure 1**). The IF-SPI fraction was prepared by passing the solution through the hydrophobic adsorbent column (Diaion HP20, Sumitomo Chemical Co., Tokyo, Japan) at room temperature, and the eluate was concentrated to 10% of dry matter by using the evaporator at 50−55 °C and 0.012 MPa, sterilized for 30 min at 80 °C, and freeze-dried. The yield of IF-SPI was 98.6%. As summarized in **Table 1**, IF-SPI was virtually free of isoflavone, 0.01 mg/g. Adsorbed material in the hydrophobic column was eluted by 70% ethanol and then concentrated using the evaporator, sterilized, and freeze-dried (isoflavone concentrate, IC). The IC corresponded to 1.4% of the starting material. Casein (vitamin-free casein, CAS) was purchased from Oriental Yeast Co., Tokyo, Japan. Content of crude protein was measured by using the Kjeldahl method (N × 6.25, Kjeltec Auto 1030 analyzer, Nippon General Trading Co., Tokyo, Japan). Isoflavones were analyzed by a modification of the method of Kudou et al. (16). In brief, test materials were extracted three times with 10 volumes of 70%

Table 2. Composition (Percent) of the Experimental Diets

	CAS	SPI	IF-SPI	IF-SPI + IC	CAS + IC
protein source					
casein[a]	20.0				20.0
soy protein		20.0			
IF-SPI			20.0	19.72	
eluted IC				0.28	0.28
L-cystine	0.3	0.3	0.3	0.3	0.3
dextrinized cornstarch	13.2	13.2	13.2	13.2	13.2
cornstarch	39.1	39.1	39.1	39.1	38.8
sucrose	10.0	10.0	10.0	10.0	10.0
soybean oil[b]	7.0	7.0	7.0	7.0	7.0
cellulose powder	5.0	5.0	5.0	5.0	5.0
mineral mixture[c]	3.5	3.5	3.5	3.5	3.5
vitamin mixture[c]	1.0	1.0	1.0	1.0	1.0
choline bitartrate	0.25	0.25	0.25	0.25	0.25
cholesterol	0.5	0.5	0.5	0.5	0.5
sodium cholate	0.125	0.125	0.125	0.125	0.125

[a] Oriental Yeast Co., Tokyo, Japan. [b] Includes 0.02% of *tert*-butylhydroquinone. [c] AIN-93G mixture, Oriental Yeast Co., Tokyo, Japan.

ethanol for 30 min at room temperature. After centrifugation, the supernatant was used for a quantitative HPLC analysis of isoflavones in a YMC-pack ODS-AM-303 column (250 × 4.6 mm), using a linear gradient of acetonitrile (15−35%) for 50 min at 35 °C by measuring at 254 nm. The solvent flow rate was 1 mL/min. Saponin was analyzed by referring to the paper of Kitagawa et al. (17). In brief, the methanol extract was concentrated and quantitatively analyzed by thin layer chromatography.

Animals and Diets. Specific pathogen-free 5-week-old male Sprague−Dawley rats were purchased from Japan SLC Co. (Hamamatsu, Japan) and housed individually in stainless steel cages under controlled circumstance (temperature = 23 ± 1 °C, humidity = 55 ± 5%, light from 7:00 a.m. to 7:00 p.m.). After acclimation for 5 days on commercial chow (CRF-1, Oriental Yeast Co., Tokyo, Japan), rats were divided into five groups with the same average body weight in each group and then given experimental diets with free access for 14 days. There were six rats in each group. The protein level in experimental diets was maintained at 20%, except for the IF-SPI + IC group, in which the level (19.72 + 0.28%) was adjusted on the basis of the yield of both components as shown in **Table 2**. Experimental diets contained 0.5% cholesterol and 0.125% sodium cholate. The other components were based on the AIN-93G formula (18). Food intake and body weight were recorded daily. On day 15, after 6 h of food deprivation (from 7:30 a.m. to 1:30 p.m.), blood was withdrawn from the aorta abdominalis into a heparinized syringe under intraperitoneal sodium pentobarbital anesthesia (15 μmol/100 g of body weight). Tissues and epididymal and perirenal fat pads were excised, rinsed, and weighed. Plasma was separated by centrifugation at 1900*g* for 15 min at 5 °C and frozen at −30 °C until analyzed.

Analyses. Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, total protein, albumin, urea nitrogen, aspartate aminotransferase, alanine aminotransferase, glucose, and triglyceride were measured enzymatically using a Dry Chem 5500 analyzer (Fuji Film Co., Tokyo, Japan). Feces were collected for the last 3 days of the feeding period and then freeze-dried and powdered. Fecal neutral and acidic steroids were measured by gas−liquid choromatography according to the methods of Miettinen et al. (19) and Grundy et al. (20), respectively.

Statistical Analyses. The results were expressed as means ± SE. Statistical analyses were performed with standard statistical software (SPSS 10.0J for Windows, SPSS Japan Inc., Tokyo, Japan). The results were analyzed by one-way ANOVA, followed by Tukey's honestly significant difference test to identify significant differences among the five groups. Differences were considered to be significant at $P < 0.05$.

RESULTS

Weight Gain, Food Intake, Food Efficiency Ratio, and Organ Weights. Table 3 summarizes growth parameters of rats. There were no significant differences in weight gain, food intake,

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Table 3. Body Weight Change, Food Intake, and Food Efficiency Ratio (FER) of Rats Fed Experimental Diets[a]

	CAS	SPI	IF-SPI	IF-SPI + IC	CAS + IC
initial wt (g)	178 ± 3	178 ± 3	178 ± 2	178 ± 2	178 ± 1
final wt (g)	270 ± 8	273 ± 9	280 ± 6	273 ± 10	267 ± 6
total wt gain (g)	93 ± 6	95 ± 8	103 ± 6	95 ± 8	89 ± 5
total food intake (g)	268 ± 10	264 ± 13	287 ± 8	269 ± 12	258 ± 5
FER	0.34 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	0.35 ± 0.02	0.35 ± 0.01

[a] Values are means ± SE for six rats.

Table 4. Tissue Weight of Rats Fed Experimental Diets[a]

	CAS	SPI	IF-SPI	IF-SPI + IC	CAS + IC
body wt at sacrifice (g)	274 ± 9	276 ± 9	281 ± 6	275 ± 9	263 ± 8
tissue wt (g/100 g of bw)					
liver	5.36 ± 0.27	4.80 ± 0.12	4.97 ± 0.19	4.97 ± 0.20	5.45 ± 0.22
kidney	0.34 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.36 ± 0.02
spleen	0.25 ± 0.00	0.22 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.27 ± 0.02
heart	0.36 ± 0.01	0.37 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.42 ± 0.06
lung	0.39 ± 0.02	0.41 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.42 ± 0.02
fat pad					
epididymal	1.19 ± 0.07	1.22 ± 0.09	1.32 ± 0.07	1.24 ± 0.08	1.45 ± 0.13
perirenal	1.53 ± 0.15	1.37 ± 0.16	1.83 ± 0.11	1.78 ± 0.22	1.59 ± 0.13

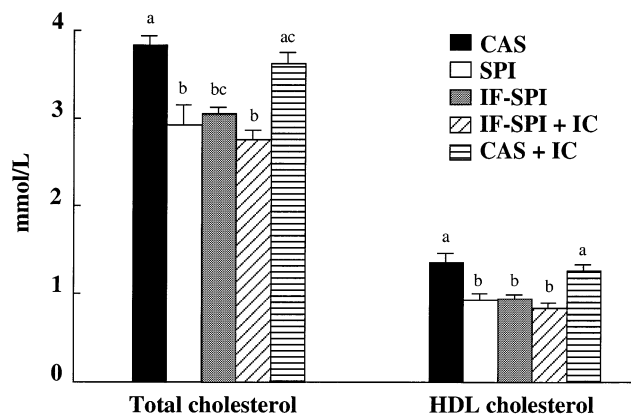[a] Values are means ± SE for six rats.

Figure 2. Plasma cholesterol levels of rats fed experimental diets. Values are means and SE for six rats. Values not sharing a common superscript letter are significantly different at $P < 0.05$ by Tukey's honestly significant test.

and food efficiency ratio among the five groups. **Table 4** shows tissue and visceral fat (epididymal and perirenal fat) weights. There was no difference in the weights of these tissues among the groups.

Plasma Parameters. As shown in **Figure 2**, the plasma total cholesterol concentration of the SPI and IF-SPI groups was significantly lower than that of the CAS group ($P < 0.05$). The addition of IC to the CAS and IF-SPI diets also tended to decrease plasma cholesterol (0.2−0.3 mmol/L), but the reduction was not statistically significant in comparison with the corresponding no-addition groups. The response of plasma HDL cholesterol level was similar to that of total cholesterol, and it was significantly lower in the three SPI groups than in the two CAS groups ($P < 0.05$). **Table 5** shows the results of other plasma chemistries. The alanine aminotransferase activity was significantly higher in the two CAS groups than in the three SPI groups. There were no differences in other plasma parameters analyzed.

Fecal Steroid Excretion. Table 6 shows the results of steroid excretion. Fecal neutral steroid excretion in rats fed soy protein diets tended to be higher than in those fed casein diets, and the

difference between the IF-SPI and the two CAS groups was significant ($P < 0.05$). Dietary manipulation did not influence fecal acidic steroid excretion. Excretion of total steroids showed a pattern similar to that of neutral steroids, but no statistical differences were found among the groups. Thus, the addition of IC in no way stimulated fecal steroid excretion.

DISCUSSION

In this study, we developed a novel procedure to remove isoflavones almost quantitatively from SPI by treating it with a hydrophobic adsorbent column. Because no alcohol treatment was involved in this procedure, the loss of components other than isoflavones and denaturation of protein should be relatively low. The isoflavone-free SPI reduced plasma total cholesterol and stimulated fecal steroid excretion to a similar extent as did intact SPI in rats. This finding differs from the results of previous observations (8−10), in which no cholesterol-reducing effect was observed in animals given soy protein treated with alcohol. The reason for this discrepancy is that alcohol washing causes simultaneous elimination of all of the alcohol-soluble components in addition to isoflavones such as saponin that may partly be responsible for cholesterol reduction by SPI. In fact, saponin was lost almost quantitatively in alcohol-extracted soy concentrate (21), whereas approximately half remained in the IF-SPI we used in the present study. The addition of 1.3% of soybean saponin to the soy protein diet reduced blood cholesterol level in rats (22).

In many of the previous studies, the addition of isoflavones failed to demonstrate the reduction of plasma cholesterol level. The addition of up to 112 mg of isoflavones to 100 g of the diet did not change the plasma cholesterol level in gerbils (12). The addition of 39−56 mg of isoflavones showed no hypocholesterolemic effect in ovariectomized cynomolgus monkeys (13, 14). In the present study, the addition of 80 mg of IC to 100 g of IF-SPI or CAS diets, which were essentially free of IC, tended to reduce plasma cholesterol in rats, but the reduction was not statistically significant.

There is no agreement concerning which component(s) of SPI may be responsible for its hypocholesterolemic effect. Anthony et al. (8, 9) and Kirk et al. (10) insisted that the cholesterol-lowering effect of SPI was not attributed to the

Table 5. Plasma Chemistries of Rats Fed Experimental Diets[a]

	CAS	SPI	IF-SPI	IF-SPI + IC	CAS + IC
total protein (g/dL)	6.32 ± 0.07	6.46 ± 0.14	6.65 ± 0.13	6.46 ± 0.12	6.28 ± 0.21
albumin (g/dL)	3.47 ± 0.05	3.48 ± 0.07	3.51 ± 0.04	3.54 ± 0.05	3.42 ± 0.03
urea-N (mg/dL)	14.5 ± 0.9	13.0 ± 1.0	13.6 ± 1.1	13.3 ± 0.9	12.8 ± 0.7
aspartate aminotransferase (units/L)	81.8 ± 7.6	71.0 ± 3.0	71.6 ± 6.1	69.9 ± 3.8	71.3 ± 6.5
alanine aminotransferase (units/L)	23.5 ± 3.6a	14.2 ± 1.2b	18.3 ± 1.2ab	13.7 ± 1.5b	20.4 ± 1.7ab
glucose (mmol/L)	11.1 ± 0.3	10.7 ± 0.3	11.3 ± 0.4	11.6 ± 0.3	11.2 ± 0.2
triglyceride (mmol/L)	1.65 ± 0.29	1.35 ± 0.22	1.48 ± 0.19	1.71 ± 0.23	1.43 ± 0.12

[a] Values are means ± SE for six rats. Means in the same row not sharing common letters are significantly different at $P < 0.05$ by Tukey's honestly significant difference test.

Table 6. Fecal Steroid Excretion of Rats Fed Experemental Diets[a]

	CAS	SPI	IF-SPI	IF-SPI + IC	CAS + IC
fecal wt (g/day)	1.51 ± 0.07	1.71 ± 0.09	1.80 ± 0.06	1.72 ± 0.11	1.50 ± 0.02
neutral steroids (mg/day)	39.0 ± 2.4a	42.5 ± 3.6ab	51.7 ± 2.8b	42.6 ± 2.0ab	38.9 ± 3.8a
acidic steroids (mg/day)	20.1 ± 1.5	21.6 ± 1.6	18.9 ± 2.3	18.2 ± 2.4	19.8 ± 1.7
total steroids (mg/day)	59.1 ± 2.5	64.1 ± 3.9	70.6 ± 4.2	60.8 ± 3.5	58.8 ± 3.6

[a] Feces were collected for the last 3 days of the feeding period. Values are means ± SE for six rats. Means in the same row not sharing common letters are significantly different at $P < 0.05$ by Tukey's honestly significant difference test.

protein portion but to the isoflavones. On the other hand, Tovar-Palacio et al. (*12*) and Greaves et al. (*13*, *14*) claimed that the interaction of protein with other components such as isoflavone and saponin contributed to the effect. The result of the present study supports the view that the protein component in SPI is a major constituent exerting a plasma cholesterol-lowering effect. In this context, the concentration of plasma total cholesterol was clearly lower in the IF-SPI group (3.05 mmol/L) than in the CAS group (3.84 mmol/L) by 0.79 mmol/L. Moreover, the concentration of the IF-SPI + IC group (2.76 mmol/L) was lower than that of the IF-SPI group by 0.29 mmol/L. The corresponding value between the CAS + IC (3.62 mmol/L) and CAS groups was 0.22 mmol/L. From this estimation it was calculated that about three-fourths of plasma cholesterol reduction is attributable to the protein component in SPI and the rest to the IC fraction.

Hydrophobic peptides in soy protein bind well with bile acids, which has been considered to be an explanation of the cholesterol-lowering effect (*23*). The hydrophobic high molecular fraction (HMF) prepared by enzymatic hydrolysis of soy protein is known to interfere with cholesterol and bile acid absorption, and this fraction is regarded as a principal fraction responsible for the hypocholesterolemic effect of soy protein (*24*). The stimulation of the fecal steroid excretion was in agreement with these observations, as recognized in the IF-SPI group in this study.

In harmony with this observation, the addition of IC to IF-SPI and CAS diets did not stimulate fecal steroid excretion. However, the possible participation of isoflavones in cholesterol lowering may not be ruled out. Isoflavones have structural similarities with estrogen, which has a hypocholesterolemic property in women (*25*). The addition of soy protein extracts to diets reduced hepatic apolipoprotein A-I mRNA levels in gerbils (*12*). Genistein, a representative isoflavone in soy protein, reduced incorporation of [^{14}C]glucose into lipids and increased output of free fatty acids to the medium in a liver perfusion study (*26*).

In conclusion, a significant fraction of the cholesterol-lowering effect of SPI in rats can be attributed to the protein content, but the isoflavones and other minor constituents may also play a role. The results showed that the protein component, but not isoflavones, stimulates fecal steroid excretion. Further

study is needed to elucidate the mechanism underlying the cholesterol-lowering effect of soy protein.

NOTE ADDED AFTER ASAP POSTING

The phrase "aorta abdominal vein" in the original ASAP posting of August 23, 2002, under Animals and Diets, has been changed to "aorta abdominalis" in this posting.

LITERATURE CITED

(1) Anderson, J. W.; Johnstone, B. M.; Cook-Newell, M. E. Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* **1995**, *333*, 276−282.

(2) Potter, S. M. Overview of proposed mechanisms for the hypocholesterolemic effect of soy. *J. Nutr.* **1995**, *125*, 606S−611S.

(3) Kito, M.; Moriyama, T.; Kimura, Y.; Kambara, H. Changes in plasma lipid levels in young healthy volunteers by adding an extruder-cooked soy protein to conventional meals. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 354−355.

(4) Messina, M. J.; Persky, V.; Setchell, K. D. R.; Barnes, S. Soy intake and cancer risk: A review of the in vitro and in vivo data. *Nutr. Cancer* **1994**, *21*, 113−131.

(5) Adlercreutz, C. H. T.; Goldin, B. R.; Gorbach, S. L.; Höckerstedt, K. A. V.; Watanabe, S.; Hämäläinen, E. K.; Markkanen, M. H.; Mäkelä, T. H.; Wähälä, K. T.; Hase, T. A.; Fotsis, T. Soybean phytoestrogen intake and cancer risk. *J. Nutr.* **1995**, *125*, 757S−770S.

(6) Wei, H.; Wei, L.; Frenkel, K.; Bowen, R.; Barnes, S. Inhibition of tumor promoter-induced hydrogen peroxide formation in vitro and in vivo by genistein. *Nutr. Cancer* **1993**, *20*, 1−12.

(7) Potter, S. M.; Baum, J. A.; Teng, H.; Stillman, R. J.; Shay, N. F.; Erdman, J. W., Jr. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl.), 1375S−1379S.

(8) Anthony, M. S.; Clarkson, T. B.; Hughes, C. L., Jr.; Morgan, T. M.; Burke, G. L. Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peri-pubertal rhesus monkeys. *J. Nutr.* **1996**, *126*, 43−50.

Isoflavone-Free Soy Protein Reduces Plasma Cholesterol

J. Agric. Food Chem., Vol. 50, No. 20, 2002 **5721**

(9) Anthony, M. S.; Clarkson, T. B.; Bullock, B. C.; Wagner, J. D. Soy protein versus soy phytoestrogens in the prevention on diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 2524−2531.

(10) Kirk, E. A.; Sutherland, P.; Wang, S. A.; Chait, A.; LeBoeuf, R. C. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J. Nutr.* **1998**, *128*, 954−959.

(11) Ni, W.; Yoshida, S.; Tsuda, Y.; Nagao, K.; Sato, M.; Imaizumi, K. Ethanol-extracted soy protein isolate results in elevation of serum cholesterol in exogenously hypercholesterolemic rats. *Lipids* **1999**, *34*, 713−716.

(12) Tovar-Palacio, C.; Potter, S. M.; Hafermann, J. C.; Shay, N. F. Intake of soy protein and soy protein extracts influences lipid metabolism and hepatic gene expression in gerbils. *J. Nutr.* **1998**, *128*, 839−842.

(13) Greaves, K. A.; Parks, J. S.; Williams, J. K.; Wagner, J. D. Intact dietary soy protein, but not adding an isoflavone-rich soy extract to casein, improves plasma lipids in ovariectomized cynomolgus monkeys. *J. Nutr.* **1999**, *129*, 1585−1592.

(14) Greaves, K. A.; Wilson, M. D.; Rudel, L. L.; Williams, J. K.; Wagner, J. D. Consumption of soy protein reduces cholesterol absorption compared to casein protein alone or supplemented with an isoflavone extract or conjugated equine estrogen in ovariectomized cynomolgus monkeys. *J. Nutr.* **2000**, *130*, 820−826.

(15) Balmir, F.; Staack, R.; Jeffrey, E.; Jimenez, M. D. B.; Wang, L.; Potter, S. M. An extract of soy flour influences serum cholesterol and thyroid hormones in rats and hamsters. *J. Nutr.* **1996**, *126*, 3046−3053.

(16) Kudou, S.; Fleury, Y.; Welti, D.; Magnolato, D.; Uchida, T.; Kitamura, K.; Okubo, K. Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). *Agric. Biol. Chem.* **1991**, *55*, 2227−2233.

(17) Kitagawa, I.; Yoshikawa, M.; Hayashi, T.; Taniyama, T. Characterization of saponin constituents in soybeans of various origins and quantitative analysis of soyasaponins by gas−liquid chromatography. *Yakugaku Zassi* **1984**, *104*, 162−168 (in Japanese).

(18) Reeves, P. G.; Nielsen, F. H.; Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **1993**, *123*, 1939−1951.

(19) Miettinen, T. A.; Ahrens, E. H., Jr.; Grundy, S. M. Quantitative isolation and gas−liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* **1965**, *6*, 411−424.

(20) Grundy, S. M.; Ahrens, E. H., Jr.; Miettinen, T. A. Quantitative isolation and gas−liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **1965**, *6*, 397−410.

(21) Anderson, R. L.; Wolf, W. J. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *J. Nutr.* **1995**, *125*, 581S−588S.

(22) Sugano, M.; Goto, S.; Yamada, Y.; Yoshida, K.; Hashimoto, Y.; Matsuo, T.; Kimoto, M. Cholesterol-lowering activity of various undigested fractions of soybean protein in rats. *J. Nutr.* **1990**, *120*, 977−985.

(23) Iwami, K.; Sakakibara, K.; Ibuki, F. Involvement of post-digestion 'hydrophobic' peptides in plasma cholesterol-lowering effect of dietary plant proteins. *Agric. Biol. Chem.* **1986**, *50*, 1217−1222.

(24) Sugano, M.; Yamada, Y.; Yoshida, K.; Hashimoto, Y.; Matsuo, T.; Kimoto, M. The hypocholesterolemic action of the undigested fraction of soybean protein in rats. *Atherosclerosis* **1988**, *72*, 115−122.

(25) Stampfer, M. J.; Willett, W. C.; Colditz, G. A.; Rosner, B.; Speizer, F. E.; Hennekens, C. H. A prospective study of postmenopausal estrogen therapy and coronary heart disease. *N. Engl. J. Med.* **1985**, *313*, 1044−1049.

(26) Nogowski, L.; Mackowiak, P.; Kandulska, K.; Szkudelski, T.; Nowak, K. W. Genistein-induced changes in lipid metabolism of ovariectomized rats. *Ann. Nutr. Metab.* **1998**, *42*, 360−366.