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Cholesterol Lowering Mechanism of Soybean Protein Hydrolysate

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Numerous attempts have been made to find the mechanism and component of the cholesterol lowering activity of soybean. In this study, it was proved that the peptides in soybean protein hydrolysate (SPH) made by certain proteases have a hypocholesterolemic effect. Among the mechanisms suggested, that is, blockage of bile acid and/or cholesterol absorption, inhibition of cholesterol synthesis, and stimulation of low-density lipoprotein receptor (LDL-R) transcription, SPH appeared to stimulate LDL-R transcription. When Hep T9A4 cells were incubated with soy protein hydrolysates by using the proteases from *Bacillus amyloliquefaciens* FSE-68, LDL-R transcription was strongly stimulated, but the other mechanisms were not affected. Among the six types of SPH, F1–15, hydrolyzed with the neutral protease to a degree of hydrolysis (DH) of 15%, showed the highest LDL-R transcription. The fractions of molecular weight of 200 and 3000 Da showed LDL-R transcription stimulating activity. The bioactivity is due to soybean peptides because the ethanol extract of soybean protein which contains isoflavones does not stimulate LDL-R transcription. In conclusion, dietary upregulation of LDL-R transcription by soybean may be consequent to an enhanced catabolism or a reduced synthesis of intracellular cholesterol. Therefore, we suggest that soy peptides can effectively stimulate LDL-R transcription in the human liver cell line and reduce blood cholesterol level.

KEYWORDS: Soybean protein; bioactive peptide; cholesterol

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the developed world. Significant research efforts focusing on the prevention and treatment of this disease have identified elevated plasma cholesterol as a primary risk factor for CVD (*1*). There is increasing evidence that the consumption of soybean protein in place of animal protein lowers blood cholesterol level. A meta-analysis of controlled clinical studies concluded that substituting soy protein for animal protein significantly lowered total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides without effecting high-density lipoprotein (HDL) cholesterol (*2*). In 1999, the Food and Drug Administration approved a CVD reduction claim for soybean protein, (*3*) and this has led to an increased interest in the identification of the responsible moiety. However, the component and molecular mechanisms of the soybean protein responsible for these changes have yet to be defined.

It has been argued that there is an interruption in intestinal absorption of bile acids and dietary cholesterol when soybean protein is consumed (*4*, *5*). Lovati et al. postulated that the hepatic metabolism of LDL cholesterol is altered when soybean protein is ingested. Studies in humans (*6*, *7*) and in animal models have suggested that soybean protein might be linked to the direct activation of LDL receptors (LDL-R) in liver cells (*8*, *9*). Other studies in animals have shown that soybean protein consumption changes the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase (*10*).

In this study, we setup a hypothesis that proteases from traditional soybean fermentation starter (*Meju*) are suitable for making cholesterol lowering soybean protein hydrolysates (SPHs). Various types of SPHs were generated by using two proteases derived from *Bacillus amyloliquefaciens* FSE-68 isolated from *Meju* (*11*). Many potential mechanisms for the cholesterol lowering effect for SPH were suggested, and among the mechanisms three approaches were used to clarify the mechanism and to screen cholesterol lowering SPHs; (1) inhibition of bile acid and/or cholesterol absorption, (2) inhibition of cholesterol synthesis, and (3) stimulation of LDL-R transcription. The most active SPH was screened, and its cholesterol lowering mechanism was identified. Finally, possible cholesterol lowering peptides were identified with various chromatography techniques and mass spectrometry from SPHs.

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MATERIALS AND METHODS

Preparation of SPHs. Neutral protease (NPR68) and alkaline protease (APR68) used to make SPHs were originally produced from *B. amyloliquefaciens* FSE-68. A 1% (w/v) solution of isolated soybean protein (SUPRO 1500, Solae, St. Louis, USA) in water was hydrolyzed with NPR68 (enzyme/substrate ratio = 0.1%) at pH 7.0, 45 °C, and APR68 (enzyme/substrate ratio = 0.15%) at pH 9.5, 45 °C. The solution was reacted to degree of hydrolysis (DH) of 5–15% and was heated at 80 °C for 30 min to stop the reaction. The digest was centrifuged at 10000g for 20 min, and then the supernatant was lyophilized. In the case of APR68 treatment, it was neutralized before heat treatment. During the hydrolysis process, the pH level of solution was kept constant with 1 N NaOH by pH-Stat and, the DH was calculated by the amount of consumed NaOH. Six types of SPH were used for this experiment: F1 type SPHs (F1–5, F1–10, and F1–15; hydrolyzed with NPR68 to DH 5, 10, and 15%) and F2 type SPHs (F2–5, F2–10, and F2–15; hydrolyzed with APR68 to DH 5, 10, and 15%).

Ethanol Extract of Isolated Soybean Protein. To obtain ethanol-soluble components from isolated soybean protein (ISP), ISP was processed with a single ethanol extraction. ISP was mixed with 10 vol of 70% (v/v) ethanol and placed in a shaking incubator at 30 °C for 12 h. After filtration, the filtrate was evaporated under vacuum and lyophilized.

Bile Acid Binding Capacities of SPHs. Aliquots of 10 mg of each SPHs, ISP, or cholestyramine were incubated with 1 mL of bile acid solution (1 mM sodium glycocholate in phosphate buffered saline) and placed in a shaking incubator at 37 °C for 2 h (*12*). The mixture was filtered through a 0.1 μm filter, and the content of sodium glycocholate in the filtrate was measured using the 3α-hydroxysteroid dehydrogenase assay (*13*).

Micellar Solubility of Cholesterol. A micellar solution containing 6.6 mM sodium taurocholate, ^{14}C-cholesterol (1 mCi/mmol, Amersham Pharmacia Biotech), 0.5 mM cholesterol, 0.5 mM monoolein, 0.6 mM phosphatidylcholine, 1 mM oleic acid, 132 mM NaCl, and 15 mM sodium phosphate buffer (pH 7.4) was prepared by sonication. One milliliter of micellar solution was mixed with 20 mg of various SPHs, ISP, or cholestyramine, and incubated at 37 °C for 2 h under vigorous shaking. The mixture was filtered using a 0.1 μm filter, the filtrate was mixed with scintillation fluid, and the residual radioactivity of the filtrate was measured with a liquid scintillation counter for the determination of micellar solubility of cholesterol.

Cell Culture. Human hepatocytes (Hep T9A4) obtained from Nestle Research Center (Lausanne, Switzerland) (*14*) were used for in vitro test. Before seeding cells, cell culture dishes or flasks are coated with the appropriate volume of coating solution. Coating solution was prepared with 5 mL of 0.3% collagen solution, 50 mL of 0.1% bovine serum albumin solution, 5 mg of Fibronectin, and 500 mL of Hanks' balanced salt solution (HBSS), and filtered with a 0.1 μm membrane. After incubation of the vessels for at least 15 min, the excess coating is then aspirated off. Cells were grown in 100 mm tissue culture dishes with 10 mL of modified PFMR-4 medium with 3.5% CO_2 at 37 °C. The medium is based on a PFMR-4 (*14*) supplemented with 0.4 mmol/L calcium, 10 mg/L insulin, 5 μg/L EGF, 10 mg/L transferrin, 0.33 nmol/L retinoic acid, 0.2 μmol/L hydrocortisone, 50 nmol/L triiodothyronine, 3% chemically denatured serum (Biofluids, Rockville), 7.5 mg/L bovine pituitary extract, 2 mmol/L glutamine, 5 mL/L P/E stock (Biofluids, Rockville), and 50 mg/L gentamycin.

For subculture, the medium was removed and the cells were washed with HBSS and detached from the culture dish with 0.25% trypsin solution. Heat inactivated fetal calf serum (FCS) (3%) in HBSS was added to stop trypsinization, and the cells were collected by centrifugation at 1000*g* for 5 min The collected cells were subcultured with a split ratio of 1: 10 on every seventh day. For experimental purposes, the cells were plated out in 24-well or 96-well plates.

Measurement of LDH Leakage. Hep T9A4 cells were seeded out in 96-well plates in modified PFMR-4 medium for 24 h and then incubated in modified PFMR-4 containing different preparations of SPH for 24 h at a concentration up to 1000 μg/mL. Lactate dehydrogenase (LDH) activity released from the cells was measured by a cytotoxicity detection kit (Roche molecular biochemicals). To measure maximum value of LDH activity, 1% Triton X-100 was added to the cells, and LDH activity released from the untreated normal cells was used as a blank value. The cytotoxitity was expressed as a percentage of LDH activity released from sample treated cells compared to that from Triton X-100 treated cells.

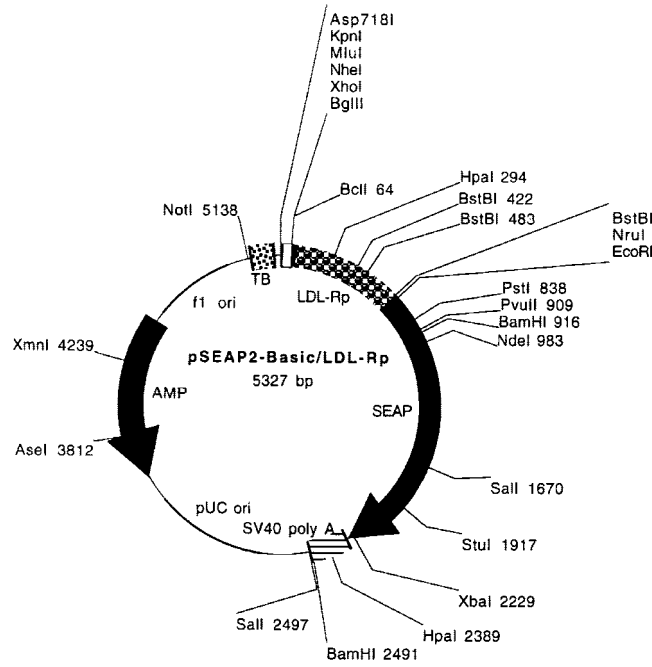

Figure 1. Plasmid map of pSEAP2-Basic/LDL-Rp.

Lipid Synthesis Inhibition. Confluent cells in 24-well plates were preincubated in modified PFMR-4 containing various concentrations of SPH for 2 h. Radioisotope [1-^{14}C] acetate (1 μCi/mL, 1 mM acetate) was then added and further incubated for 20 h. After incubation, the cells were washed 3 times with HBSS and the lipid from cells was extracted with hexane/isopropanol (3:2, v/v). The extracts were dried under nitrogen, and then redissolved in 100 μL of chloroform/methanol (2:1, v/v). The extract was separated by high performance thin layer chromatography (HPTLC, Merck, Germany) using hexane/diethyl ether/acetic acid (75:25:1, v/v). The neo-syntheses of phospholipids, cholesterol, and triglycerides were determined by measuring the [1-^{14}C] acetate incorporation into intracellular lipids with an instant imager (Camberra Packard, Switzerland) and expressed as a percentage of untreated controls.

Protein Quantification. The residual cells were dissolved by incubation at room temperature for 15 min with 0.1 N NaOH. The protein content of cell solution was measured at 595 nm using the Bradford method, (*16*) and bovine serum albumin was used as the standard protein.

DNA Transfection and Reporter Gene Analysis. Genomic DNA extracted from peripheral blood lymphocytes from a healthy donor was used as template for amplification by specific primers of the 625 bp LDL-R promoter (Forward: 5′-TCCTGATTGATCAGTGTCTATT-AGG-3′ and Reverse: 5′-GCTCGCAGCCTCTGCCAGGCAGTGT-3′). After amplification with the high fidelity PFU polymerase (Strategene, Switzerland) the different promoter fragments were subcloned into the pGEM-T vector (Promega). After sequencing, the promoter was cloned into the pSEAP2 basic vector (Clontech, Germany), a reporter system utilizing a secreted form of human placental alkaline phosphatase. The maps of pLDL-R-SEAP2 are shown in **Figure 1**.

Hep T9A4 cells, plated in 24-well plates, were grown to 80–100% confluency. LDL-R gene promoter linked to a secreted alkaline phosphatase gene (pLDL-R-SEAP2) was transiently transfected into Hep T9A4 cells with a pCMV-β-galactosidase vector (β-gal plasmid, Promega) as an internal control of transfection efficiency. Transfection mixture containing 0.5 μg of pLDL-R-SEAP2 and 0.05 μg of β-gal plasmid in Opti-MEM I reduced serum medium (GIBCO BRL, Life Technologies) was prepared using LipofectAMINE Plus reagent (GIBCO BRL, Life Technologies). The mixture (200 μL) was added

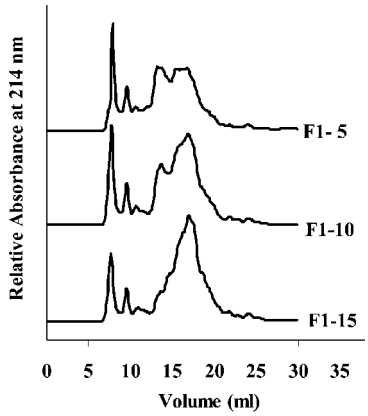

Figure 2. Chromatogram of the F1 type soybean protein hydrolysates obtained by gel permeation chromatography.

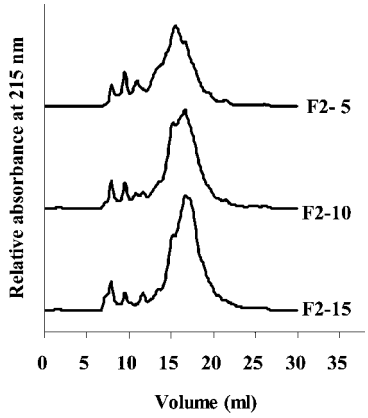

Figure 3. Chromatogram of the F2 type soybean protein hydrolysates obtained by gel permeation chromatography.

to each well and incubated in 3.5% CO_2 at 37 °C for 2 h. The cells were then washed twice with 500 μL of HBSS and resuspended with 300 μL of PFMR-4 containing various SPHs, lovastatin (0.5 μg/ml, as positive control) or 25-OH cholesterol (10 μg/mL, as negative control). After incubation for 48 h, the alkaline phosphatase in the medium was measured using the Phospha-Light chemiluminescent reporter assay system (Applied biosystems) with a luminometer. For β-galactosidase activity, the cells were lysed in 350 μL of 0.025% SDS (w/v), and 30 μL of lysate was incubated at 37 °C for 30 min with 30 μL of assay buffer containing 1.33 mg/mL *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 100 mM β-mercaptoethanol, and 2 mM $MgCl_2$ prepared in 200 mM sodium phosphate buffer (pH 7.3). The reaction was stopped with 100 μL of 1 M sodium carbonate solution, and the amount of *o*-nitrophenol formed was measured at 420 nm. The transcriptional activity of the different promoters was expressed as arbitrary alkaline phosphatase units/β-galactosidase units to normalize the plate-to-plate variations of transfection efficiency.

Statistical Analysis. All values are presented as mean ± SD (standard deviation). Tests for statistical significance of differences were compared by Student's *t*-test for all experiments. A P value < 0.05 was considered significant.

RESULTS

Soybean Peptides by using *B. Amyloliquefaciens* FSE-68 Proteases. The gel permeation chromatography (GPC) patterns of SPHs on Superdex 75 column are shown in **Figures 2** and **3**. F1 type SPHs are characterized by the two high molecular weight peptide peaks, which are over 12.5 kDa and both resistant to neutral protease. The peptides in F1–5 and F1–10, eluted around 14 mL almost disappeared when DH reached 15%. F2 type SPHs had a smaller amount of high molecular weight peptides compared to F1 type peptides, because the alkaline protease has broader specificity than the neutral protease. The molecular weight distribution of F2 type SPHs gradually shifted to the lower molecular weight range during hydrolysis. From these results, it was evidenced that the peptide composition of F1 and F2 type SPHs were significantly different.

Bile Acid Binding Capacities of Soybean Peptides. Bile acid binding capacity of SPHs was less than that of ISP, and the capacities were reduced during hydrolysis steps from 29.1 ± 1.7% (ISP) to 14.9 ± 2.3% (F1–15) and 21.8 ± 1.6% (F2–15). Therefore, the proteases from *B. amyloliquefaciens* strain 68 may hydrolyze the bile acid binding moiety in ISP. The value of the bile acid binding capacity of cholestyramine was 90.5 ± 0.4%.

Micellar Solubility of Cholesterol. Cholestyramine was used as a standard for the micellar solubility of cholesterol in preliminary tests. The micellar solubility was significantly reduced in the presence of cholestyramine (0.8 ± 0.6%) but slightly affected by ISP (79.4 ± 2.9%) and F2–15 (89.3 ± 3.2%). In contrast, F1–15 showed no effect (97.7 ± 4.2%).

LDH Leakage. The cytotoxicity of SPHs was determined by measuring LDH release from the Hep T9A4 cells after incubation for 24 h. F1 type SPHs and isolated soy protein did not significantly affect LDH leakage at a concentration up to 1000 μg/mL, but LDH leakage increased at some concentrations of F2 type SPHs. The latter might be partly resulted from NaCl, because F2 type SPHs contain from 2.3 to 4.5% of NaCl that was formed by the pH stat titration with NaOH solution. Cells treated with 30 μg/mL of NaCl that was equivalent to the amount of NaCl in 1300 μg/mL of F2–5, 900 μg/mL of F2–10 and 670 μg/mL of F2–15, showed an increase in LDH leakage by 13%. Therefore, some LDH leakage by F2 type SPHs might result from NaCl.

Inhibition of Lipid Synthesis. The effects of SPHs on the hepatic lipids synthesis were evaluated. For this purpose, Hep T9A4 cells were incubated with ^{14}C-acetate in the presence or absence of the test compounds. Treatment of 0.05 μL/mL lovastatin decreased cholesterol synthesis of Hep T9A4 by 30.9%, but all SPHs except for F2–10 at a concentration of 500 μL/mL did not change the cholesterol synthesis. In the presence of a high concentration (1000 μg/mL) of each SPH and 500 μL/mL of F2–10, the cells significantly increased the cholesterol synthesis. This was an unexpected effect of SPHs, and it is unsure whether certain components in SPH alter cholesterol synthesis or not. Further study may be required to identify this negative effect of soybean hydrolysate as a cholesterol lowering food. All SPHs did not influence the cellular uptake of ^{14}C-acetate into phospholipids and triglycerides (**Figures 4** and **5**) and did not affect total cell protein amounts significantly.

Stimulation of LDL-R Transcription by Fractionated Soybean Peptides. The importance of the liver LDL receptor (LDL-R) in maintaining normal LDL cholesterol levels is well documented (*17*). The cells containing transiently transfected LDL-R gene promoter linked to a secreted alkaline phosphatase gene (pLDL-R-SEAP2) were incubated with various SPHs, 25-OH cholesterol and lovastatin. After 48 h incubation, the transcription level of LDL-R was compared with untreated controls (**Table 1**). Incubation of cells with a positive control (0.5 μg/mL lovastatin) and a negative control (10 μg/mL 25-OH cholesterol) showed 217.3 ± 12.3% and 1.2 ± 1.3% of LDL-R transcription level, respectively. All SPHs increased the LDL-R transcription level from 126.8 ± 2.2 to 238.0 ± 12.4% at concentration of 200 μg/mL.

To determine whether the isoflavone components of SPHs may play a role in stimulating LDL-R transcription, the effect

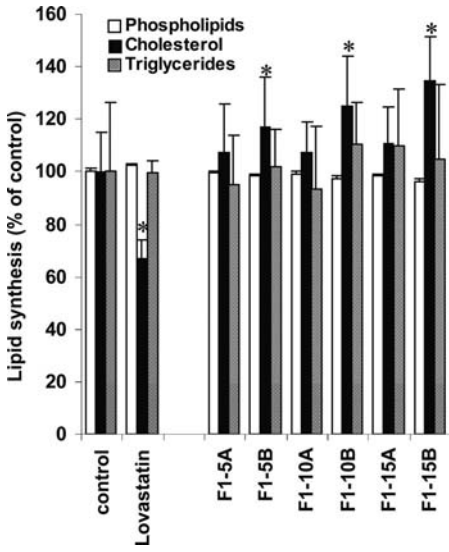

Figure 4. Effect of F1 type SPHs on intracellular lipids synthesis in Hep T9A4 cells. The cells were incubated with lovastatin (0.05 μg/mL, positive control) and two concentration of F1 type SPHs (A: 500 μg/mL and B: 1000 μg/mL). Changes are expressed as % of untreated control (mean $\pm$ SD) ($n = 4$), *$P < 0.05$ vs control.

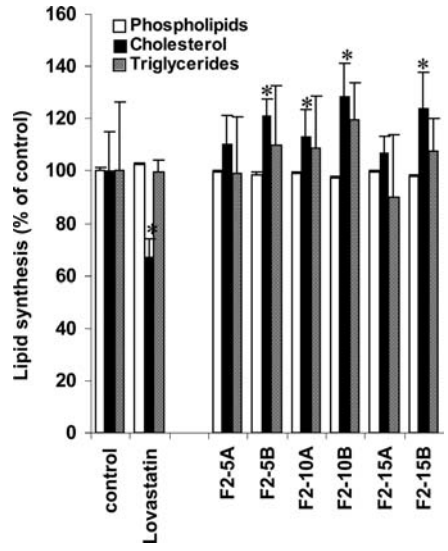

Figure 5. Effect of F2 type SPHs on intracellular lipids synthesis in Hep T9A4 cells. The cells were incubated with lovastatin (0.05 μg/mL, positive control) and two concentration of F2 type SPHs (A: 500 μg/mL and B: 1000 μg/mL). Changes are expressed as % of untreated control (mean $\pm$ SD) ($n = 3$). *$P < 0.05$ vs control.

of ethanol extract of isolated soy protein was evaluated. Intact ISP (200 μg/mL) and ethanol extract of isolated soy protein (7 μg/mL) (3.5% of matter was extracted from ISP by ethanol treatment and it consisted of mainly isoflavones). As shown in **Table 1**, intact ISP and EtOH extact of ISP have no effect on LDL-R transcription. LDL-R transcription was stimulated with increasing DH of SPH, and F1 type SPHs showed greater activity than F2 type SPHs at the same DH. DH and the protease type used vary the effect; therefore, peptides in SPHs may be responsible for stimulating LDL-R transcription. Addition of F1–15 to Hep T9A4 cells at a concentration from 10 to 200 μg/mL caused a significant dose-dependent rise of LDL-R transcription, which increased from 120.4 $\pm$ 3.7% to 238 $\pm$ 2.4%. The stimulation effect did not increase further at a concentration of over 200 μg/mL (**Figure 6**).

Table 1. Effect of Various SPHs and Ethanol Extract of ISP on LDL-R Transcription[a]

samples		LDL-R level (% of control)
control	(no treatment)	100.0 $\pm$ 24.4
25-OH cholesterol	(10 μg/μL)	1.2 $\pm$ 1.3*
lovastatin	(0.5 μg/μL)	217.3 $\pm$ 12.3*
ISP	(200 μg/μL)	66.8 $\pm$ 4.9
EtOH extract of ISP	(7 μg/μL)	81.0 $\pm$ 6.3
F1−5	(200 μg/μL)	148.5 $\pm$ 2.9*
F1−10	(200 μg/μL)	214.5 $\pm$ 23.7*
F1−15	(200 μg/μL)	238.0 $\pm$ 12.4*
F2−5	(200 μg/μL)	126.8 $\pm$ 2.2
F2−10	(200 μg/μL)	152.0 $\pm$ 7.3
F2−15	(200 μg/μL)	200.3 $\pm$ 11.9*

[a] Changes in LDL-R transcription are expressed as % of untreated controls (mean $\pm$ SD) ($n = 4$). 25-OH cholesterol and lovastatin were used as a negative and a positive control, respectively. *$P < 0.05$ vs control.

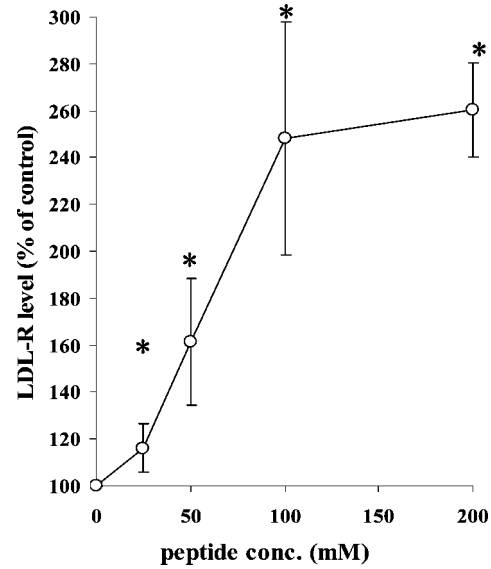

Figure 6. Effect of different concentrations of F1–15 on LDL-R transcription. Changes in transcription level are expressed as % of control (mean $\pm$ SD) ($n = 4$). *$P < 0.05$ vs control.

DISCUSSION

In recent years, major clinical trials have shown a strong correlation between the reduction in plasma cholesterol and the reduction in cardiovascular mortality. Soybean has provided beneficial effects. Numerous attempts have been made to find the mechanism and components of the cholesterol lowering activity of soybean. In this study, a set of experiments was designed to show the cholesterol lowering activity of SPHs prepared with *Meju* protease and to identify the component, particularly soybean peptides. Several potential mechanisms for the cholesterol lowering effect of soybean protein have been speculated. Among the mechanisms, three approaches were used to identify the cholesterol lowering mechanism, the effects of SPHs on inhibition of bile acid or cholesterol absorption, inhibition of cholesterol synthesis, and stimulation of LDL-R.

Various types of SPHs were generated by using two proteases derived from *B. amyloliquefaciens* FSE-68 isolated from *Meju* (*11*). The GPC chromatogram showed that each SPH preparation contains different types of soybean peptides (**Figures 2** and **3**). All SPHs absorbed a low amount of bile acid and cholesterol, so they are neither a good bile acid sequestrant nor an inhibitor of cholesterol absorption. This result conflicts with the results suggesting that hydrophobic peptides in soybean protein bind well with bile acids, (*18*) the hydrophobic high molecular

fraction (HMF) prepared by enzymatic hydrolysis of soybean protein interferes with cholesterol and bile acid absorption, (*4*) and isoflavones-free soybean protein stimulates fecal steroid excretion (*19*). During hydrolysis with *Meju* proteases, hydrophobic domains which can bind bile acids or cholesterol might be destroyed. However, clinical studies by Fumagalli et al. (*20*) showed plasma cholesterol reduction without rising fecal bile acid and neutral sterol excretion in soybean protein-treated patients.

In cholesterol synthesis tests, SPHs did not increase cholesterol synthesis when the level of SPHs in the cell culture medium was lower than 500 μg/mL, but in the presence of a high concentration (1000 μg/mL) of each SPH and 500 μL/mL of F2–10, the cells significantly increased the cholesterol synthesis. It is not certain whether components in SPH are responsible for this effect. If this effect results from certain components in SPHs, it is very important to remove these components from SPHs to increase the cholesterol lowering effect of the soybean product.

To determine the effects of SPHs on the expression of the LDL-R, a cell surface protein that is involved in the control of plasma cholesterol, a LDL-R promoter coupled to reporter gene assay was used. There are several assay methods to check different stages of LDL-R expression: (1) functional cellular LDL binding activity, (2) the amount of LDL-R protein, and (3) LDL receptor mRNA. Compared with the others, the reporter gene assay is a high throughput method and could be automated. In this study, when human hepatic cells were incubated with SPHs, LDL-R transcription was strongly stimulated; therefore, it can be suggested that cholesterol lowering effect of SPHs seems to relate to stimulation of LDL-R transcription. Since the stages of LDL-R expression are associated, the level of LDL-R stimulation is consistent with the level of LDL-R binding activity, LDL-R protein, and mRNA (*21*, *22*). Therefore, stimulation of LDL-R transcription by SPHs may increase LDL cholesterol up-take in liver and eventually reduce blood LDL cholesterol level.

Many scientists have speculated that the isoflavone in soybean is responsible for the blood cholesterol level lowering property of soybean because of its chemical similarity to that of estrogen. However, this study shows that the stimulation of LDL-R by SPHs seemed not to come from isoflavones and other water-soluble components, because there was no significant difference between control and samples (treated with ISP or extract of ISP). Furthermore, LDL-R transcription was further stimulated with an increasing degree of hydrolysis (DH) of SPHs. Only a small amount (0.1%, w/w) of pure proteases was used to prepare for SPHs in this study; therefore, except for the length of soybean protein or peptides, there is no difference among tested samples. This showed that peptides in SPHs are responsible for stimulation of LDL-R. F1 type SPHs have greater activity than F2 type SPHs at the same DH. Using GPC analysis, it was observed that the peptide composition of F1 and F2 type SPHs were significantly different. This result also supports the finding that soybean peptides stimulate LDL-R transcription.

Among the six types of SPHs, F1–15, hydrolyzed with NPR68 to DH of 15%, showed the highest stimulation of LDL-R transcription in a dose-dependent manner.

Expression of LDL-R is predominantly controlled at the level of transcription through a cholesterol or oxysterol-mediated repression (*17*). The regulation involves a family of membrane bound transcription factors called sterol regulatory element-binding proteins (SREBPs) (*23*). In sterol-depleted situations, the 125 kDa SREBP precursor bound to the endoplasmic reticulum or nuclear membrane is cleaved in two proteolytic steps into a soluble 68 kDa fragment that moves into the nucleus, where it stimulates transcription of the SRE-containing gene, including HMG-CoA reductase, HMG-CoA synthetase, and LDL-R. It is possible that soybean peptides may act by interfering with the sterol protection of SREBP proteolysis. On the other hand, soybean peptides have an effect on sterol-independent regulation of LDL-R like several growth factors and hormones (*24*, *25*). It does not seem that LDL-R was indirectly modulated by the peptide because the soybean peptides had no effect on cholesterol biosynthesis at low concentration. Further experiments are required to demonstrate the exact target of these soybean peptides in the hepatic cells.

In conclusion, soy peptides can effectively stimulate LDL-R transcription in the human liver cell line, and dietary upregulation of the LDL-R transcription by soybean may be consequent to an enhanced catabolism or a reduced synthesis of intracellular cholesterol.

LITERATURE CITED

(1) Gould, A. L.; Rossouw, J. E.; Santanello, N. C.; Heyse, J. F.; Furberg, C. D. Cholesterol reduction yields clinical benefit: impact of statin trials. *Circulation* **1998**, *97*, 946–952.

(2) 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.

(3) Food and Drug Administration. Food labeling, health claims, soy protein, and coronary heart disease. *Fed. Reg.* **1999**, *57*, 699–733.

(4) 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.

(5) Makino, S.; Nakashima, H.; Minami, K.; Moriyama, R.; Takii, Y. Bile acid-binding protein from soybean seed: isolation, partial characterization and insulin-stimulating activity. *Am. J. Clin. Nutr.* **2001**, *52*, 803–809.

(6) Baum, J. A.; Teng, H.; Erdman, J. W., Jr.; Weigel, R. M.; Klein, B. P.; Persky, V. W.; Freels, S.; Surya, P.; Bakhit, R. M.; Ramos, E.; Shay, N. F.; Potter, S. M. Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. *Am. J. Clin. Nutr.* **1998**, *68*, 545–551.

(7) Lovati, M. R.; Manzoni, C.; Canavesi, A.; Sirtori, M.; Vaccarino, V.; Marchi, M.; Gaddi, A.; Sirtori, C. R. Soybean protein diet increases low density lipoprotein receptor activity in mononuclear cells from hypercholesterolemic patients. *J. Clin. Invest.* **1987**, *80*, 125–130.

(8) Lovati, M. R.; Manzoni, C.; Corsini, A.; Granata, A.; Fumagalli, R.; Sirtori, C. R. 7S globulin from soybean is metabolized in human cell cultures by a specific uptake and degradation system. *J. Nutr.* **1996**, *126*, 2831–2842.

(9) Lovati, M. R.; Manzoni, C.; Corsini, A.; Granata, A.; Frattini, R.; Fumagalli, R.; Sirtori, C. R. Low density lipoprotein receptor activity is modulated by soybean globulins in cell culture. *J. Nutr.* **1992**, *122*, 1971–1978.

(10) Potter, S. M. Soy protein and cardiovascular disease: the impact of bioactive components in soy. *Nutr. Rev.* **1998**, *56*, 231–235.

(11) Cho, S. J.; Oh, S. H.; Pridmore, R. D.; Juillerat, M. A.; Lee, C. H. Purification and characterization of proteases from *Bacillus amyloliquefaciens* isolated from traditional soybean fermentation starter. *J. Agric. Food Chem.* **2003**, *51*, 7664–7670.

(12) Hirose, N.; Inoue, T.; Nishihara, K.; Sugano, M.; Akimoto, K.; Shimizu, S.; Yamada, H. Inhibition of cholesterol absorption and synthesis in rats by sesamin. *J. Lipid. Res.* **1991**, *32*, 629–638.

(13) Turley, S. D.; Dietschy, J. M. Re-evaluation of the 3-alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid. Res.* **1978**, *19*, 924–928.
(14) Pfeifer, A. M.; Cole, K. E.; Smoot, D. T.; Weston, A.; Groopman, J. D.; Shields, P. G.; Vignaud, J. M.; Juillerat, M.; Lipsky, M. M.; Trump, B. F. Simian virus 40 large tumor antigen-immortalized normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 5123–5127.
(15) Kaighn, M. E.; Kirk, D.; Szalay, M.; Lechner, J. F. Growth control of prostatic carcinoma cells in serum-free media: interrelationship of hormone response, cell density, and nutrient media. *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 5673–5676.
(16) Bradford, M. A. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(17) Brown, M. S.; Goldstein, J. L. A receptor-mediated pathway for cholesterol homeostasis. *Science* **1986**, *232*, 34–47.
(18) Iwami, K.; Sakakibara, K.; Ibuki, F. Involvement of postdigestion 'hydrophobic' peptides in plasma cholesterol-lowering effect of dietary plant proteins. *Agric. Biol. Chem.* **1986**, *50*, 1217–1222.
(19) Fukui, K.; Tachibana, N.; Wanezaki, S.; Tsuzaki, S.; Takamatsu, K.; Yamamoto, T.; Hashimoto, Y.; Shimoda, T. Isoflavone-free soy protein prepared by column chromatography reduces plasma cholesterol in rats. *J. Agric. Food. Chem.* **2002**, *50*, 5717–5721.
(20) Fumagalli, R.; Soleri, L.; Farina, R.; Musanti, R.; Mantero, O.; Noseda, G.; Gatti, E.; Sirtori, C. R. Fecal cholesterol excretion studies in type II hypercholesterolemic patients treated with the soybean protein diet. *Atherosclerosis* **2002**, *43*, 341–353.
(21) Bursill, C.; Paul, D. R.; Bottema, C. D. K.; Pal, S. Green tea upregulates the low-density lipoprotein receptor through the sterol-regulated element binding protein in HepG2 liver cells. *J. Agric. Food. Chem.* **2001**, *49*, 5639–5645.
(22) Pal, S.; Thomson, A. M.; Bottema, C. D. K.; Roach, P. D. Polyunsaturated fatty acids downregulate the low density lipoprotein receptor of human HepG2 cells. *J. Nutr. Biochem.* **2002**, *13*, 55–64.
(23) Horton, J. D.; Shimonura, I. Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. *Curr. Opin. Lipidol.* **1999**, *10*, 143–150.
(24) Kovanen, P. T.; Schneider, W. J. Regulation of the low density lipoprotein (B/E) receptor. *Adv. Vasc. Biol* **1999**, *5*, 165–185.
(25) Makar, R. S.; Lipsky, P. E.; Cuthbert, J. A. Multiple mechanisms, independent of sterol regulatory element binding protein, regulate low density lipoprotein gene transcription. *J. Lipid. Res.* **2000**, *41*, 762–774.

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