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Identification of LDL-Receptor Transcription Stimulating Peptides from Soybean Hydrolysate in Human Hepatocytes

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Soybean protein and its hydrolysate have been reported to have cholesterol-lowering property, but the responsible components are still largely unknown. In previous study, we found that soybean protein hydrolysate (SPH) prepared with the protease from *Bacillus amyloliquefaciens* FSE-68, strongly stimulates transcription of low density lipoprotein receptor (LDL-R). To identify LDL-R transcription stimulating peptides in human hepatocytes, the SPH was fractionated with gel permeation chromatograpy and the active fraction was further separated by using reverse-phase chromatography. Several peptides in the most active fraction were identified by LC/MS and MS/MS analysis. LDL-R transcription stimulating peptides were synthesized on the basis of identified sequences, and their effect on LDL-R transcription was tested in vitro. Among the synthesized peptides, Phe-Val-Val-Asn-Ala-Thr-Ser-Asn (FVVNATSN) showed the strongest activity, and LDL-R transcription of hepatic cells was increased to 248.8% (compared to 100% of untreated control) by FVVNATSN at a concentration of 100 μM. This study provides direct evidence that peptides derived from soybean protein can influence LDL-R transcription in hepatocytes.

KEYWORDS: Soybean; bioactive peptide; cholesterol; LDL-receptor

INTRODUCTION

Cardiovascular disease (CVD) has become a ubiquitous cause of morbidity and a leading cause of death in most countries (*1*). Although mortality from CVD has fallen substantially in the past three decades, it remains the single leading cause of death for adults worldwide and is expected to remain the leading cause of death and disability in the world. Lloyd et al. (*2*) predicted that 50% of men and 30% of women would develop heart disease over their lifetime. Many studies found a positive relationship between LDL cholesterol level and the rate of new-onset CVD in people who were initially free of CVD or that of recurrent coronary events in people with established CVD (*3–9*). The hepatic LDL receptor (LDL-R) is a cell surface glycoprotein that contains approximately two asparagine-linked (N-linked) oligosaccharide chains of the complex type and approximately 18 serine/threonine-linked (O-linked) oligosaccharide chains. LDL-R is a key regulatory of human plasma LDL cholesterol homeostasis (*10*). Circulating cholesterol in the form of LDL is removed from plasma by the highly specific LDL-R and is internalized via receptor-mediated endocytosis. Therefore, increased hepatic LDL-R expression results in improved clearance of plasma LDL cholesterol (*11*). An immediate result of increased uptake of plasma LDL cholesterol through LDL-R is an elevated intracellular cholesterol concentration. However, high intracellular levels of cholesterol are postulated to lead to the production of cholesterol metabolites such as 25-hydroxycholesterol. The accumulated cholesterol and its metabolites suppresses the transcription of genes involved in cholesterol biosynthesis, such as HMG-CoA reductase as well as the gene for LDL-R in a well-characterized example of end-product feedback repression (*12*). The therapeutic implications of LDL-R studies center on strategies for increasing the production of LDL-R in the liver, thereby lowering plasma LDL-cholesterol levels (*13*).

We previously reported that soybean protein hydrolysate (SPH) exhibited a hypocholesterolemic effect by stimulating LDL-R (*14*). In this study, we hypothesized that peptides in soy protein hydrolysate (SPH) hydrolyzed by neutral protease from *Bacillus amyloliquefaciens* induce hypocholesterolemic action in an in vitro cell culture model and attempted to identify responsible peptides. The SPH was fractionated and LDL-R transcription stimulating peptides in human hepatocytes were identified with various chromatography techniques and mass spectrometry from SPH.

MATERIALS AND METHODS

Preparation of soybean protein hydrolysates (SPH). Neutral protease (NPR68) used to make soybean protein hydrolysate (SPH) was

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originally produced from *Bacillus amyloliquefaciens* FSE-68 (*15*). A 1% (w/v) solution of isolated soybean protein (SUPRO 1500, Protein Technology International, St. Louis, MO) in water was hydrolyzed with NPR68 (enzyme/substrate ratio = 0.1%) at pH 7.0, 45 °C up to a degree of hydrolysis (DH) of 15%. The digest was heated at 80 °C for 30 min to stop the reaction and centrifuged at 10 000*g* for 20 min, and then the supernatant was lyophilized. During the process of hydrolysis, the pH of solution was kept constant with 1 N NaOH and the DH was calculated from the amount of consumed NaOH based on the equation (*16*) DH% = 100 × *BN*(1/*a*)(1/MP)(1/*h*), where *B* is the base consumption in mL, *N* the normality of the base, *a* the average degree of dissociation of the NH_2 groups, MP the mass of protein being hydrolyzed (g), and *h* the total number of peptide bonds in the protein−substrate.)

Cell Culture. Human hepatocytes (Hep T9A4) obtained from Nestle Research Center (Lausanne, Switzerland) (*17*) were used for in vitro test. Before seeding cells, cell culture dishes or flasks were coated with the appropriate volume of coating solution. The 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 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 dish with 10 mL of modified PFMR-4 medium with 3.5% CO_2 at 37 °C. The medium is based on a PFMR-4 (*18*) 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. The number of passages of Hep T9A5 used in this experiment was from 26 to 30.

Measurement of Cytoxicity of SPH. 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 with a cytotoxicity detection kit (Roche molecular biochemicals). To measure the 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 blank value. The cytotoxitity was expressed as percentage of LDH activity released from control cells compared to that from Triton X-100-treated cells.

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′; 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. Hep T9A4 cells, plated in 24-well plates, were grown to 80−100% confluency. The LDL-receptor gene promoter linked to a secreted alkaline phosphatase gene (pLDL-R-SEAP2) was transiently transfected into Hep T9A4 cells by using LipofectAMINE Plus reagent (GIBCO BRL, Life Technologies) with a pCMV-β-galactosidase vector (β-gal plasmid, Promega) as an internal control for transfection efficiency.

The transfected cells were incubated with 300 μL of PFMR-4 containing SPH, lovastatin (0.5 μg/mL, as positive control), or 25-OH cholesterol (10 μg/mL, as negative control), and after incubation for 48 h, the alkaline phosphatase in the medium was measured using the Phospha-Light chemiluminescent reporter assay system (Applied biosystems) using a luminometer or using *p*-nitrophenyl phosphate (pNPP) at 420 nm (*19*). 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.

Purification of LDL-R Transcription Stimulating Soybean Peptides. The SPH was loaded onto a gel permeation chromatography column (Superdex 75 HR 10/30, Amersham Pharmacia Biotech, Sweden) and eluted with 50 mM ammonium acetate buffer (pH 7.0) at a flow rate of 0.5 mL/min. Absorbance was measured at 214 nm. Ovalbumin (MW 43 000), myoglobin (MW 17 600), aprotinin (MW 6500), vitamin B_{12} (MW 1355), and Gly-Gly-Gly (MW 189.2) were used as molecular weight marker. Each fraction was collected and lyophilized and its bioactivity was tested. Active fractions were further separated by reverse-phase chromatography with a Vydac C_8 column (208TP54, 5 μm, 4.6 mm i.d.) using a solvent system of 0.05% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and 80% (v/v) acetonitrile with 0.045% TFA (solvent B) at a flow rate of 1.0 mL/min. Elution was achieved with a linear gradient of solvent B from 0% to 50% for 50 min after 5 min of isocratic elution of solvent A. Detection was determined at 215 nm.

Analysis of Peptides with LC/MS and MS/MS. The peptides purified by a Vydac C_8 column were further separated by using a C_{18} narrowbore reverse-phase column (Vydac 218TP52, 5 μm, 2.1 mm i.d.) with a linear increase of solvent B (0.045% TFA/80% acetonitrile in water) in solvent A (0.05% TFA in water). Elution was achieved with a linear gradient of solvent B from 0% to 50% for 50 min after 5 min of isocratic elution of solvent. The flow rate was 0.2 mL/min and detection was made at 215 nm. LC/MS and MS/MS analysis were performed using a LCQ mass spectrometer (Finnigan Co.) equipped with an electrospray ionization source. The LC/MS and MS/MS data acquisition program was set up to collect ions signal from the eluted peptides using an automatic data-dependent scan procedure of three different scan modes (full scan, zoom scan of major peak, and MS/MS scan). Acquisition and analysis of MS and MS/MS were performed with Xcalibur software V1.2 (Finnigan), including the Bioworks V 1.0 software package for Turbo SQUEST database search.

Soybean Protein Sequences and Database Searches. To determine peptide sequences, the masses and tandem mass spectra of peptides were compared with theoretical peptide masses and reconstructed tandem mass spectra of proteins in major soy protein using the Swiss-Prot database at cross correlation function mode of Turbo SEQUEST. Approximately 90% of the proteins in soybeans exist as storage proteins, which mostly consist of β-conglycinin and glycinin. Basic 7S globulin is a minor storage protein that accounts for less than a few percent, and protease inhibitors (Kunitz trypsin inhibitor and Bowman−Birk inhibitor) are other constituents of soybean protein. The referenced protein names and accession numbers are as follows: 11S glycinin G1 A1aBx (P04776), G2 A2B1a (P04405), G3 A1bB1a (P11828), G4 A5A4B3 (P02858), G5 A3B4 (P04347); basic 7S globulin (P13917); β-conglycinin α-chain (P13916), α′-chain (P11827), β-chain (P25974); Kunitz trypsin inhibitor A/C type (P01070) and B type (P01071); and Bowman−Birk inhibitor (P01055), type C−II (P01063), and type D-II (P01064). The reference sequences contain signal and prosequences, and in vitro limited proteolysis produces several high-molecular mass fragments from the 7S and 11S globulin subunits (*20*). Therefore, the original reference protein sequences above were trimmed to make a database similar to real soybean protein sequence before database searching.

Chemical Synthesis of Peptides. On the basis of peptide sequences identified with LC/MS-MS/MS analysis, potential cholesterol-lowering peptides were synthesized by the Fmoc solid-phase method (*21*) by Peptron Inc. (Daejeon, Korea) and further purified by reverse-phase chromatography with a Vydac C_8 column (208TP54, 5 μm, 4.6 mm i.d.).

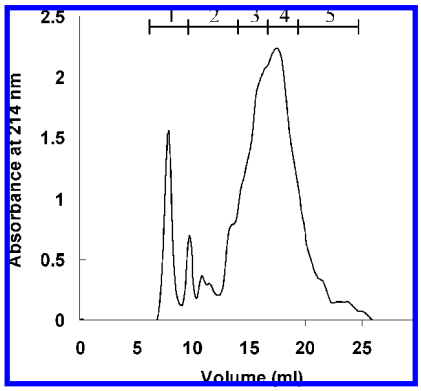

Figure 1. Fractionation of LDL-R transcription stimulation peptides from soybean protein hydrolysate by gel permeation chromatography.

Table 1. Effect of Fractionated Soybean Protein Hydrolysate on LDL-R Transcription[a]

sample	LDL-R level (% of control)
control (no treatment)	100.0 ± 13.3
fraction 1	70.6 ± 1.7
fraction 2	110.8 ± 7.7
fraction 3	167.5 ± 17.2*
fraction 4	157.7 ± 12.2*
fraction 5	107.9 ± 1.2

[a] The HEP T9A4 cells were treated with 60 μg of each fraction. Changes in LDL-R transcription are expressed as percent of untreated controls (mean ± SD) ($n = 4$). *$P < 0.05$ vs control.

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

Purification of LDL-R Transcription Stimulating Soybean Peptides. The SPH was fractionated with gel permeation chromatography (**Figure 1**), and the LDL-R transcription stimulating activity of each fraction was tested in vitro. The 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 (compared 100% of the control), respectively. As shown in **Table 1**, fractions 3 and 4, which eluted between 15 and 19 mL of retention volume, showed the strongest bioactivity, and the estimated molecular weight of the fractions is between 200 and 3000 Da. The active fractions were collected and further purified by using reverse-phase chromatography with a C_8 column. Each fraction was collected, lyophilized, and tested in vitro. Among the fractions, a fraction (RP14) eluted with 21−23% of buffer B showed the highest activity. The RP14 purified from 1000 μg of SPH stimulated the LDL-R transcription by 268.3 ± 8.4% (**Figure 2**).

Identification of Peptides with LC/MS and MS/MS. The selected fractions, RP14, were further separated with a C_{18} narrowbore reverse-phase column (Vydac 218TP52), and MS and MS/MS analysis were performed using a mass spectrometer (**Figure 3**). The collected data were compared with the theoretical mass spectrum in the soybean protein database, and 12 peptides were identified from the peaks of RP14 (**Table 2**). Three peaks (E, H, and K) could not be identified by this approach. The peaks may consist of a nonpeptide component or glycosylated, post-translational modified peptides. The molecular weight of the peptides was in the range from 317.3 to 1560.7 Da and the numbers of amino acid per peptide ranged from 3 to 15.

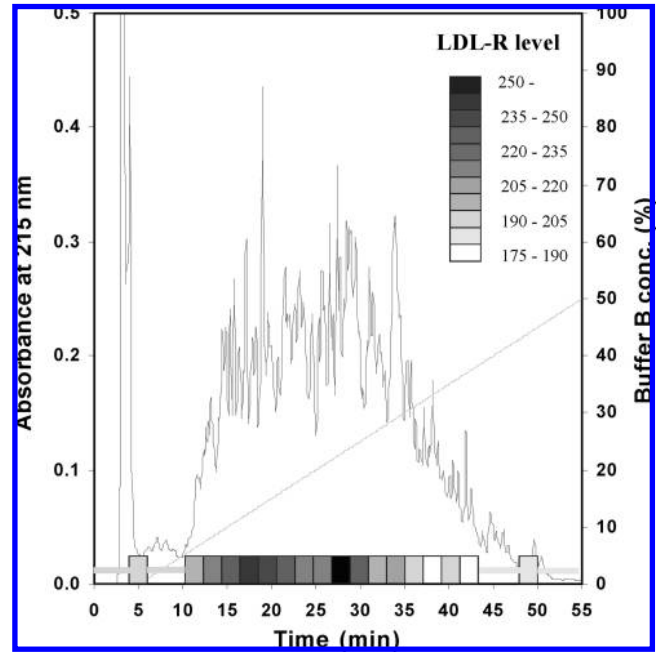

Figure 2. Purification of LDL-R transcription stimulating peptides from GPC fraction of soybean protein hydrolysate by reverse-phase chromatography on a C_8 column. Levels of LDL-R transcription are shown in gray scale bars and expressed as percent of paired untreated controls.

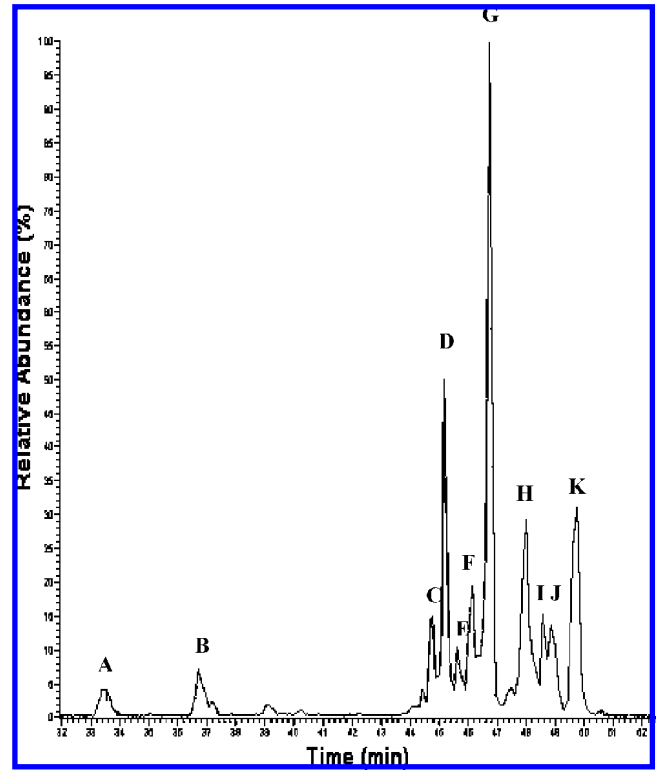

Figure 3. Chromatogram of RP14 on reverse-phase C_{18} column.

Effect of Synthetic Peptides on LDL-R Transcription. Nine peptides were selected from **Table 2** based on peak intensity and were synthesized. Hep T9A4 cells were treated with each of the synthetic peptides, and LDL-R modulation is shown in **Table 3**. When the peptides were tested at a

Table 2. Peptides from RP14 Identified by LC/MS and MS/MS

peak	peptide sequence	mass	origin
A	HDD	385.3	Kunitz trypsin inhibitors
	FVVNATSN	850.9	7S β-conglycinin β-chain
B	LRSRDPIYSNK	1349.5	7S β-conglycinin α-chain
C	FKTNDRPSIGN	1249.4	11S glycinin G2 or G3 basic subunit
	IMLCVGIP	846.1	Kunitz trypsin inhibitors
D	ISSEDKPFN	1037.1	7S β-conglycinin α or α'-chain
E	unidentified		
F	IIVVQGKG	814.0	11S glycinin G5 acidic subunit
G	LQGENEGEDKGAIVT	1560.7	11S glycinin G1 acidic subunit
	LQSGDAL	703.8	7S β-conglycinin α or α'-chain
	IAVPTG	557.7	11S glycinin G1, G2 or G3 acidic subunit
H	unidentified		
I	SSPDIYNPQAGS	1236.3	11S glycinin G1 or G2 basic subunit
K	unidentified		
J	DTPMIGT	734.8	11S glycinin G1 basic subunit

Table 3. Effect of Synthetic Peptides on LDL-R Transcription[a]

peptide sequence	LDL-R level (% of control)
FVVNATSN	248.3 $\pm$ 49.7*
LRSRDPIYSNK	102.2 $\pm$ 5.9
FKTNDRPSIGN	124.7 $\pm$ 15.8*
ISSEDKPFN	91.1 $\pm$ 12.8
IIVVQGKG	105.2 $\pm$ 19.4
LQGENEGEDKGAIVT	101.1 $\pm$ 8.2
IAVPTG	91.2 $\pm$ 13.2
SSPDIYNPQAGS	121.7 $\pm$ 25.9*
DTPMIGT	155.2 $\pm$ 8.4*

[a] The HEP T9A4 cells were treated with 100 μM of each peptide. Changes in LDL-R transcription are expressed as percent of untreated controls (mean $\pm$ SD) ($n = 3$). *$P < 0.05$ vs control.

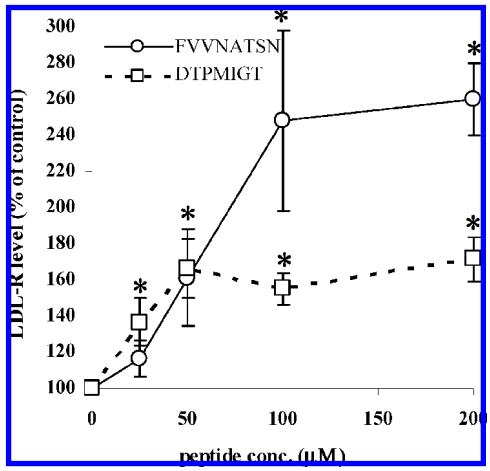

Figure 4. Effect of synthetic soybean peptides on LDL-R transcription. Changes in LDL-R transcription are expressed as percent of untreated controls (mean $\pm$ SD) ($n = 3$). *$P < 0.05$ vs control.

concentration of 100 μM, one peptide (FVVNATSN) strongly increased LDL-R transcription (by 148.3% vs control) and three peptides (FKTNDRPSIGN, SSPDIYNPQAGS and DTPMIGT) showed moderate stimulation of LDL-R transcription (by 24.7%, 21.7%, and 55.2%, respectively vs control). FVVNATSN stimulates LDL-R transcription in a dose-dependent manner ranging up to 100 μM (**Figure 4**). DTPMIGT showed stronger activity at a concentration of 25 μM than FVVNATSN, but had a lower maximum activity; the maximum LDL-R level of DTPMIGT-treated cells was about 155.2% of the control, and that of FVVNATSN-treated cells was about 248.3% of the control.

DISCUSSION

The molecular mechanisms by which soybean influences the cholesterol metabolisms are not clearly defined. One proposed mechanism is that hepatic metabolism of cholesterol or lipoproteins are altered when soybean protein is ingested. Khosla et al. (*22*) found that removal of LDL from circulation is significantly enhanced in rabbits fed soybean protein. In human study, a 7-fold increase in monocyte LDL receptor activity was reported in hypercholesterolemic individuals who consumed soybean protein. They observed that LDL receptor mRNA levels in mononuclear cells were increased by 75% (*13*). 7S globulin of soy protein was found to increase LDL-R activity in human cells (Hep G2) (*23*), and this was associated with 7S recognition by a specific uptake and degradation system (*24*). Among the 7S globulin subunits, the α'-subunit may possibly be responsible for LDL-R activity increase (*25, 26*).

We have previously reported that soybean protein hydrolysate (SPH) exhibited hypocholesterolemic effect by stimulating the transcription of low-density lipoprotein cholesterol receptor. In this study, we have identified the peptides that may be responsible for the cholesterol-lowering effect of SPH. SPH was first fractionated with gel permeation chromatography. Only the peptide fractions in which the molecular weight is between 200 and 3000 Da showed significant LDL-R stimulating activity. Active fractions were further purified with reverse-phase chromatography, and 12 peptides were isolated and finally identified by mass spectrometry.

Among the identified and synthesize peptides, four peptides (FVVNATSN, FKTNDRPSIGN, SSPDIYNPQAGS, and DTPMIGT) showed positive results. Lovati et al. (*27*) and Durnati et al. (*28*) suggested that 7S β-conglycinin, particularly its α'-chain subunit can significantly raise LDL-R expression in vitro and in vivo. In **Table 2**, the RP14 fraction contains fragments of the 7S β-conglycinin α'-chain, but none of them stimulate LDL-R transcription. Except for FVVNATSN, which came from the 7S β-conglycinin β-chain, other active peptides were from the 11S glycinin basic subunit, and FVVNATSN showed the strongest activity. This results shows that preparation method of soy protein hydrolysate is very important to produce bioactive peptide. Lovati et al. (*27*) used pepsin to make soy peptide and got an active one from 7S β-conglycinin; however, we used neutral protein (NPR68), which generates a totally different type of peptide than Lovati's work.

The result of the study does not prove that the identified peptides retain their activity in vivo. However, they do not have any sites for tryptic hydrolysis, lysine (K) and arginin (R), in their sequence except for FKTNDRPSIGN. Therefore, the peptides should be less susceptible to tryptic hydrolysis. Although the concept that peptides are absorbed intact into the blood circulation is not universally accepted, evidence supporting the possibility of tissue utilization of these small peptides is accumulating. Lovati et al. (*27*) postulated that 0.2% of intact peptides with MW ranging from 3000 to 20 000 Da can cross the intestinal wall.

So far, no hypocholesterolemic peptides from soybean protein have been found; however, in this study, LDL-R transcription stimulating new soybean peptides were identified for the first time in human hepatocytes. Recently, Nagaoka et al. (*29*) found hypocholesterolemic peptide (IIAEK) from bovine milk protein hydrolysate in rats. Their peptide inhibited cholesterol absorption in Caco-2 cells, but the peptides from soybean protein hydrolysate stimulate LDL-R transcription in human hepatocytes and may increase uptake of LDL cholesterol from the blood stream. It is suggested that a specific sequence of soybean peptide

effectively stimulate LDL-R transcription in the human liver cell line and may reduce the blood cholesterol level. These identified peptides can be used to understand the mechanisms by which dietary protein or peptide effect the serum cholesterol level and for the development of functional food or hypocholesterolemic drugs.

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