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Peptides Identified in Soybean Protein Increase Plasma Cholesterol in Mice on Hypercholesterolemic Diets

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ABSTRACT: The in vitro micellar cholesterol displacement assay has been used to identify peptides that may potentially reduce cholesterol in vivo. Two of these peptides, LPYPR and WGAPSL, derived from soybean protein (SP) that have been reported to displace cholesterol from micelles were tested by feeding them as a part of a hypercholesterolemic diet to mice for 3 weeks. Except reduction of very low-density lipoprotein cholesterol (VLDL-C) and triglyceride contents, the peptide-containing diets increased plasma cholesterol content with the increasing dose of the peptides. Mice fed diets supplemented with the peptides also had lower fecal bile acid excretion. Negative correlations between fecal bile acid excretion and plasma total cholesterol content (r = −0.876, P = 0.062) and non-HDL-C content (r = −0.831, P = 0.084) were observed. The mRNA levels of the genes for cholesterol and bile acid metabolism, *CYP51*, *LDLR*, *CYP7A1*, and *LPL*, were up-regulated in mice fed diets supplemented with peptides except the group fed the low dose of WGAPSL. The results suggested that higher plasma total cholesterol content possibly due to lower fecal steroid excretion as well as lower VLDL-C and triglyceride contents might due to the up-regulated expression levels of the genes *CYP51*, *LDLR*, and *LPL*.

KEYWORDS: *soybean protein, micellar solution, peptides, mice, cholesterol, bile acid*

INTRODUCTION

Soybean protein (SP) and its hydrolysates have been shown to affect cholesterol and bile acid metabolism in humans[1] and several animal models such as chickens,[2] rats,[3] mice,[4] hamsters,[5,6] rabbits,[7] and *Cynomolgus* monkeys.[8] The hexapeptide (WGAPSL) derived from SP has been reported to displace cholesterol from bile acid micelles or affect bile acid binding in vitro, suggesting reduction of absorption of cholesterol or reabsorption of bile acids.[9] Peptide (LRVPAGTTFYVVNPDNDENLRMIA)[10] from 7S globulin and peptide (FVVNATSN) from 7S β-conglycinin β-chain[11] have been shown to activate the low-density lipoprotein receptor (*LDLR*) in HepG2 and human hepatocytes (Hep T9A4) cells, respectively, and thus possibly decrease LDL cholesterol (LDL-C) in circulation. Nagaoka et al.[12] recently reported a hexapeptide (VAWWMY) derived from SP that had a high binding affinity to bile acids in vitro and reduced the content of [^{3}H]cholesterol in serum, liver, and intestine of rats gavaged with a micellar solution of [^{3}H]cholesterol compared with SP peptic hydrolysates or casein tryptic hydrolysates. LPYPR, a pentapeptide, from soybean glycinin reduced serum cholesterol in mice after oral administration at a dose of 50 mg/kg for only 2 days.[13] However, although the in vitro studies utilized a widely accepted mechanism of cholesterol homeostasis to identify potentially bioactive peptides, animal studies verifying the activity of cholesterol-lowering peptides are limited.

Peptides LPYPR and WGAPSL, like plant sterols and stanols, displaced cholesterol from mixed micelles in vitro. Because cholesterol displacement is a well-accepted mechanism of action by plant sterols and stanols, it has been widely accepted that the in vitro mixed micellar displacement assay is predictive of cholesterol-reducing potential. However, cholesterol absorption is a multistep process, consisting of the cleavage of sterol/stanol esters, solubilization of unesterified cholesterol by micelles, diffusion of cholesterol through mucosal barriers, uptake of sterols by scavenger receptor (SR-B1), (re)-esterification, incorporation into chylomicrons, and release into the lymph.[14] The use of formulated synthetic mixed micelles compared to natural bile and newly discovered transintestinal cholesterol excretion by plant sterols cannot demonstrate the complexity of the mammalian digestive system. Ikeda et al.[15] showed that sitosterol in emulsions inhibited [^{3}H]cholesterol absorption in rats but did not inhibit absorption in bile cannulated rats infused with [^{3}H]cholesterol and sitosterol in formulated micelles. The micellization of sterols in the small intestine by natural bile components was believed to select sitosterol over cholesterol, resulting in lowered absorption only from emulsions. Recently, it has been shown that the proximal intestine is also able to excrete cholesterol into the lumen without participation of the hepatobiliary system.[16] Plant sterols were shown to increase

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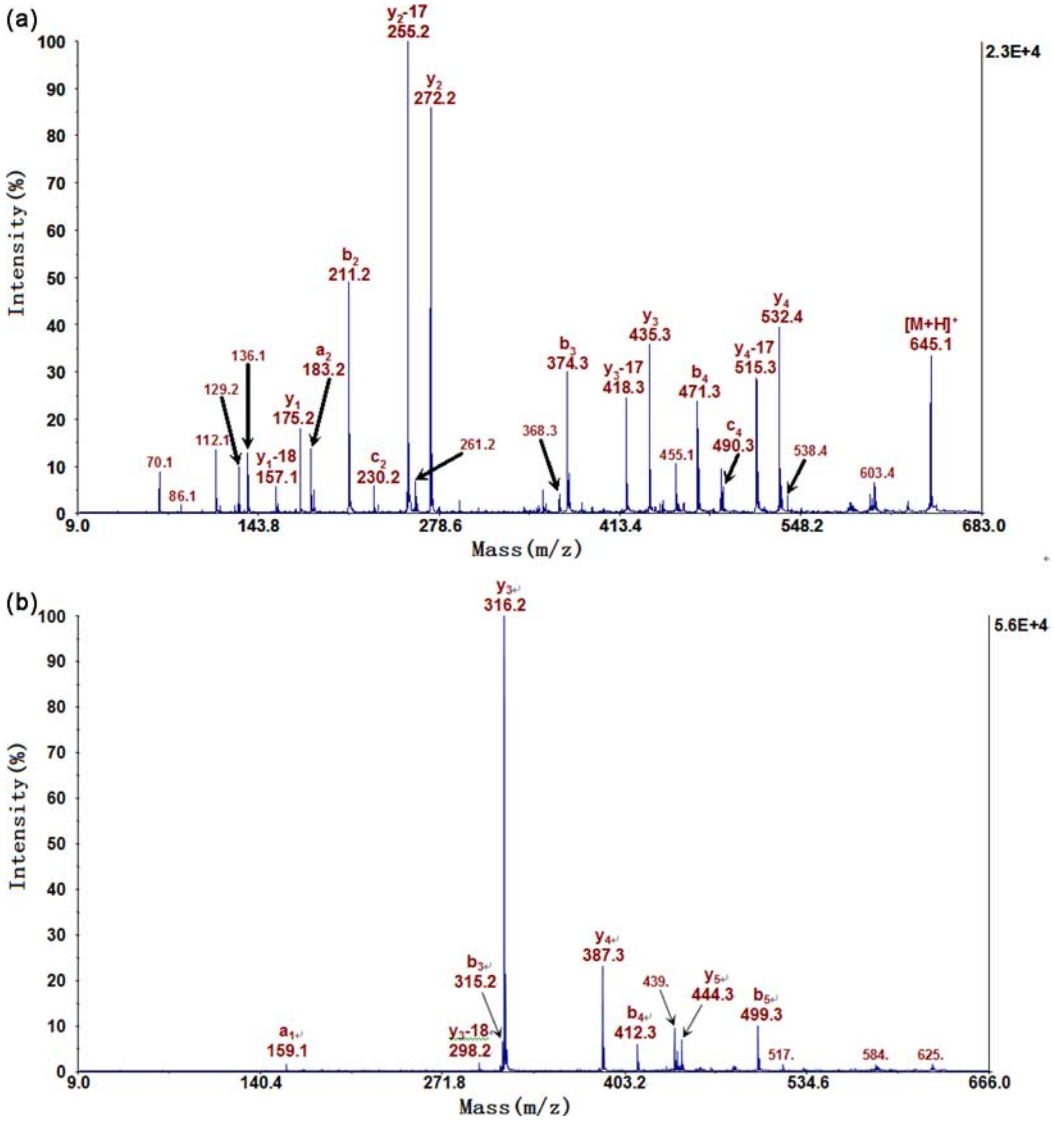

Figure 1. (a) MS/MS-PSD of the protonated peptide ion LPYPR at *m/z* 645.4. Backbone fragment ions are identified by their *m/z* and their corresponding fragment ion type/number. Immonium fragment ions appear at *m/z* 70.1 [P], 86.1 [L], 129.2 [R], and 136.1 [Y]. An internal fragment ion appears at *m/z* 261.2. (b) MS/MS-PSD of the protonated peptide ion WGAPSL at *m/z* 630.3. Backbone fragment ions are identified by their *m/z* and their corresponding fragment ion type/number.

the direct transintestinal cholesterol excretion (TICE) in mice, which was believed to be the primary mechanism of cholesterol lowering, although the mechanism is still not known.[17] Peptides, produced from proteolysis, might also survive digestion and be absorbed by enterocytes and possibly into other tissues.

Small peptides resulting from pancreatic enzyme hydrolysis of proteins are absorbed through the small intestine more readily than free amino acids.[18] Although most ingested proteins are absorbed in the form of small peptides (primarily di- and tripetides), small quantities of larger peptides and small proteins are able to escape further hydrolysis and reach the bloodstream intact to modulate cellular function through their biologic properties.[18] Many factors affect the extent to which peptides reach the blood, such as the digestibility of the peptides, the digestive capacity of pancreatic and intestinal proteases, and the permeability of the mucosa.[18] Therefore, in this study, we investigated the effect of peptides originally identified in soy hydrolysates, LPYPR and WGAPSL, on cholesterol and bile acid metabolism in male mice fed high-fat and -cholesterol diets.

MATERIALS AND METHODS

Materials. SP was purchased from Solae LLC (St. Louis, MO, USA) and extracted by Soxhlet apparatus with ethanol to remove isoflavones. LPYPR and WGAPSL peptides were purchased from GL Biochem Ltd. (Shanghai, China). The purity of each peptide was >90% according to the manufacturer. Peptide sequences were confirmed by tandem mass spectrometry and postsource decay (MS/MS-PSD) using a matrix-assisted laser desorption/ionization time-of-flight-time-of-flight mass spectrometer (4800 MALDI-TOF-TOF) (AB Sciex, Inc., Foster City, CA, USA) in precursor ion suppression mode with α-cyano-4-hydroxycinnamic acid MALDI matrix (Protea Biosciences, Inc., Morgantown, WV, USA). MS/MS-PSD fragment ions were manually assigned using GPMAW software, version 8.01a5 (Lighthouse Data, Odense, Denmark). The mass spectra are shown in Figures1. Buffalo corn starch was purchased from ConAgra Foods Food Ingredients Co., Inc. (Omaha, NE, USA); SP oil, lard fat, and sucrose were obtained from a local supermarket; casein, choline bitartrate, mineral mix, vitamin mix, and microcrystal-

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line cellulose were obtained from Dyets, Inc. (Bethlehem, PA, USA); cholesterol was purchased from Sigma-Aldrich (St. Louis, MO, USA); and DL-methionine was obtained from ICN Biomedicals (Costa Mesa, CA, USA).

Animals and Diets. Male mice (C57BL16J, approximately 15 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). The animals were housed individually in wire-bottom cages in a room maintained at 20−22 °C and 60% relative humidity with a 12 h alternating light−dark cycle. Mice were acclimatized for 1 week and fed a ground commercial chow (Purina Rodent Laboratory Chow). After 1 week, the mice were randomized into a control group, two LPYPR groups (L0.6, L1.2), two WGAPSL groups (W0.45, W0.9), and one SP group with eight mice per group.

Mice were fed high-fat and -cholesterol diets with differing amounts of peptides ad libitum for 3 weeks. Body weights were recorded weekly, and food intake was monitored twice per week. The study was approved by the Animal Care and Use Committee, Western Regional Research Center, USDA, Albany, CA, USA. The diet composition was as follows (g/kg diet): lard fat, 200; SP oil, 25; cholesterol, 1; microcrystalline cellulose, 52.6; casein, 222.2; corn starch, 192.2; sucrose, 304.6; DL-methionine, 3; choline bitartrate, 3; mineral mix, 35; vitamin mix, 10. The contents of LPYPR were 0.6 and 1.2 g/kg diet (L0.6 and L1.2) and those of of WGAPSL were 0.45 and 0.9 g/kg of diet (W0.45 and W0.9). Dose was estimated for LPYPR by estimating 4 g feed intake/day for a 25 g mouse and using 50 mg/kg/day as a minimum level (minimum LPYPR = (50 mg/kg/day) × (0.025 kg/0.004 g/day) = 312 mg LPYPR/kg diet. 2× and 4× were used to improve chances of significant physiological effect. There were no intake data for WGAPSL, and an intermediate dose between 300 and 600 mg/kg diet was selected. SP replaced casein of the control diet, and the SP content of the SP diet was 222.2 g/kg diet.

Blood, Liver, and Feces. Mice were food deprived for 12 h and anesthetized with isoflurane (Phoenix Pharmaceutical, Inc., St. Joseph, MO, USA); blood was collected via cardiac puncture with syringes rinsed with potassium EDTA solution (15% w/v). The blood was transferred to 1.5 mL natural microcentrifuge tubes (containing 5 μL of 15% potassium EDTA), gently rocked for a few minutes, and then stored on ice. The blood was then centrifuged at 10000 rpm for 5 min at 4 °C to separate plasma. Livers were excised, weighed, and separated into two parts. One part was immediately frozen in liquid nitrogen for RNA isolation, and the other part was lyophilized, milled, and stored at −20 °C for lipid extraction. Feces were collected during the last 3 consecutive days of the feeding period and were lyophilized, milled, and stored at −20 °C.

Analysis of Lipoproteins and Triglycerides (TG) in Mouse Plasma. Very low-density lipoprotein cholesterol (VLDL-C), LDL-C, and high-density lipoprotein cholesterol (HDL-C) were separated by size exclusion chromatography and cholesterol concentration was quantitated by postcolumn reaction as previously described.[19] The plasma lipoproteins were eluted with 0.15 M NaCl and 0.02% (w/v) sodium azide at a flow rate of 0.5 mL/min using a Superose 6HR HPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). Cholesterol in the lipoprotein fractions was reacted with a cholesterol reagent (Roche Diagnostic, Indianapolis, IN, USA) at a flow rate of 0.2 mL/min in a postcolumn reactor, consisting of a knitted mixing coil (1615-50 Bodman, Aston, PA, USA) and a temperature-controlled water jacket (Aura Industrials, Staten, NY, USA) using bovine cholesterol lipoproteins as standards (Sigma-Aldrich, St. Louis, MO, USA). Plasma TG was determined using an enzyme assay kit (Genzyme Diagnostics PEI, Inc., PE, Canada).

Hepatic and Fecal Lipids. Freeze-dried, powdered liver and feces were extracted with hexane/isopropanol as described earlier.[20] About 0.5 g was sandwiched between sand layers in an extraction cell and extracted at 60 °C, ∼2175 psig, with 3:2 (v/v) hexane/isopropanol with the Dionex ASE 200. The lipid extract was evaporated to dryness at 37 °C under nitrogen. The lipid residue was brought to constant weight and weighed to determine the total lipid concentration. The entire sample of residue was dissolved in 5:2 (v/v) chloroform/methanol. An aliquot (0.55 mL) was transferred to a vial and solubilized in 2.2 mL of 1% Triton X-100 in chloroform. The mixture was mixed well and evaporated to dryness at 37 °C under nitrogen. One milliliter of deionized water was added to the residue, and the mixture was mixed thoroughly and incubated at 37 °C for 1 h in a water bath. TG concentration of liver and feces was determined with an assay kit, as described above for plasma. Total and free cholesterol concentrations of liver and feces were determined using enzyme assay kits (Wako Chemicals, Richmond, VA, USA).

Fecal Bile Acid. Total fecal bile acids were determined according to an enzymatic method.[21] Briefly, about 50 mg of freeze-dried feces was extracted with 1 mL of 50% aqueous *tert*-butanol for 15 min at 37 °C. At the beginning, in the middle, and at the end of the reaction period, the tubes were vortexed for 10 s. The suspension was then centrifuged at 10000*g* for 2 min. The supernatant (40 μL) was transferred to 13 × 100 mm glass tubes, and the bile acid concentration was determined using the DZ042A-K enzyme recycling rate assay kit (Diazyme, San Diego, CA, USA) and measuring the absorbance at 540 nm using a Nanodrop 2000 C spectrophotometer (Thermo Scientific, USA).

Real-Time PCR. Total RNA from livers was extracted using TRIzol plus RNA purification kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), quantified spectrophotometrically, checked for quality by gel electrophoresis, and used to synthesize cDNA using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) per the manufacturer's protocol. Approximately 1 μL of diluted cDNA (1:10) was used in each real time-PCR with SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and appropriate primers. An Mx3000P instrument (Stratagene, Cedar Creek, TX, USA) was used to perform the PCR. The cycle conditions were as follows: 5 min at 95 °C followed by 40 cycles of incubation at 94 °C for 30 s, then 60 °C for 1 min, and 72 °C for 30 s. PCR reactions were followed by dissociation curve analysis. The results were analyzed using the software provided with the Mx3000P QPCR system. Differences in mRNA expression were calculated using the ΔΔCT method.[22] The sequence of the primer of the lanosterol 14α-demethylase gene (*CYP51*) used for this study was described previously,[23] and the sequences of other primers are shown in Table 1.

Table 1. Primer Sequences

gene	product size (bp)	primer	5′−3′ primer sequence
36B4	199	forward	TCTAGGACCCGAGAAGACCTC
		reverse	GTTGTCAAACACCTGCTGGAT
LDLR	193	forward	TTCCTGTCCATCTTCTTCCCTA
		reverse	GACCATCTGTCTTGAGGGGTAG
LPL	197	forward	CAGGTGGAAGAGCGACTCCTA
		reverse	ACTTCTTCAGAGACTTGTCATGG
CYP7A	153	forward	ACAGCTAAGGAGGACTTCACTCT
		reverse	TTCATCAAGGTACCGGTCGTATT
HL	119	forward	AAGAGAATTCCCATCACCCTG
		reverse	CTGTTTTCCCACTTGAACTTGA

Statistical Analysis. All data are expressed as means ± SE. Differences among groups were determined by one-way ANOVA analysis of variance using the Minitab 15 statistical program (Minitab Inc., State College, PA, USA). Pearson correlation coefficients were calculated for investigating relationships of lipid metabolism with the expression of hepatic genes and determined by the SPSS16.0 statistical program (IBM Inc., New York, NY, USA). Significance was defined at the 95% confidence level.

Table 2. Effects of Different Peptide-Supplemented High-Fat Diets on Body Weight, Food Intake, and Liver and Adipose Tissue Weight[a]

	control	L0.6	L1.2	W0.45	W0.9	SP
body weight (g)	25.4a ± 0.7	24.3ab ± 0.6	23.3b ± 0.8	24.0ab ± 0.7	23.7ab ± 0.7	24.6ab ± 0.4
body weight gain (g)	7.9a ± 0.5	6.5ab ± 0.5	6.0b ± 0.3	6.7ab ± 0.5	6.8ab ± 0.3	7.9ab ± 0.7
food intake (g/day)	4.7b ± 0.1	4.5b ± 0.1	4.9ab ± 0.2	4.7b ± 0.2	4.9 ab ± 0.2	5.1a ± 0.1
liver weight (g)	1.0d ± 0.05	1.2b ± 0.03	1.4a ± 0.06	1.0d ± 0.03	1.2bc ± 0.06	1.1dc ± 0.04
retroperitoneal adipose tissue (g)	0.2a ± 0.04	0.1a ± 0.02	0.1a ± 0.02	0.1a ± 0.03	0.1a ± 0.02	0.1a ± 0.01
epididymal adipose tissue (g)	0.9a ± 0.10	0.8a ± 0.09	0.7a ± 0.05	0.8a ± 0.08	0.7a ± 0.05	0.8a ± 0.06

[a]Data presented as means ± SE. Different letters indicate significant difference at P < 0.05.

Table 3. Effects of Dietary Protein-Supplemented High-Fat Diets on Plasma and Hepatic Lipids[a]

	control	L0.6	L1.2	W0.45	W0.9	SP
plasma lipids						
VLDL-C (mg/dL)	2.9a ± 0.28	2.0bc ± 0.16	1.4c ± 0.08	2.3bc ± 0.20	1.9bc ± 0.10	2.9a ± 0.27
LDL-C (mg/dL)	9.2c ± 0.96	32.1a ± 4.5	41.5a ± 2.9	9.2c ± 1.2	26.3b ± 3.0	22.7b ± 2.5
HDL-C (mg/dL)	85.6bc ± 2.7	97.2a ± 2.1	95.7a ± 1.7	79.8c ± 2.8	94.8b ± 2.4	96.5a ± 1.3
ratio of LDL-C and HDL-C	0.1c ± 0.03	0.33b ± 0.13	0.43a ± 0.11	0.11c ± 0.04	0.28b ± 0.13	0.23b ± 0.09
total cholesterol (mg/dL)	97.7b ± 3.7	131.4a ± 3.3	138.6a ± 4.0	91.3b ± 4.0	123.0a ± 4.0	122.0a ± 3.4
TG (mmol/L)	71.0a ± 5.2	53.8bc ± 3.0	41.4d ± 1.2	57.4b ± 3.0	47.4cd ± 1.5	59.4b ± 5.0
hepatic lipids						
total lipid (%)	15.2b ± 0.6	18.9a ± 1.3	19.6a ± 0.9	17.6ab ± 0.7	18.6a ± 0.8	13.0c ± 0.5
total cholesterol (mg/g liver)	7.3a ± 0.6	6.2ab ± 0.4	5.3 b ± 0.2	7.2a ± 0.4	6.2ab ± 0.5	6.9a ± 0.3
free cholesterol (mg/g liver)	5.2a ± 0.4	4.8ab ± 0.4	3.9b ± 0.3	5.7a ± 0.2	4.7ab ± 0.3	5.0a ± 0.3
TG (mg/g liver)	63.4a ± 2.3	57.5ab ± 5.1	59.7ab ± 2.1	59.8ab ± 4.3	62.8a ± 5.6	46.3b ± 4.3

[a]Data presented as means ± SE. Different letters indicate significant difference at $P < 0.05$.

Table 4. Effects of Different Dietary Protein-Supplemented High-Fat Diets on Fecal Lipid and Bile Acid in Mice[a]

	control	L0.6	L1.2	W0.45	W0.9	SP
total lipid (%)	13.6a ± 0.9	11.3a ± 0.7	10.6a ± 0.87	12.1a ± 1.4	12.1a ± 0.7	13.9a ± 1.3
total cholesterol (mg/day)	2.9a ± 0.2	2.6a ± 0.3	3.2a ± 0.2	2.3a ± 0.4	2.8a ± 0.3	1.2b ± 0.15
free cholesterol (mg/day)	2.8a ± 0.3	2.5a ± 0.4	3.0a ± 0.4	2.2a ± 0.37	2.7a ± 0.18	1.1b ± 0.15
TG (mg/day)	14.8a ± 0.6	15.7a ± 1.3	14.9a ± 3.1	13.9a ± 0.8	15.3a ± 1.5	16.0a ± 0.8
total bile acid (mmol/day)	1.5a ± 0.1	0.89b ± 0.06	0.84b ± 0.07	1.0b ± 0.09	0.83b ± 0.09	0.26c ± 0.02

[a]Data presented as means ± SE. Different letters indicate significant difference at $P < 0.05$.

RESULTS

Body and Organ Weights. Although there was a consistent trend of lower body weight and weight gain by the peptide diets, only the L1.2 diet lowered final body weight (8%) and weight gain (24%) compared with the control (Table 2). The total food intake was similar ($P > 0.05$) in all diet groups except for the higher intake in the SP-supplemented diet group throughout the experimental period. Compared with the control and SP diet groups, the peptides significantly ($P < 0.05$) increased liver weights of the mice except the W0.45 diet group. The liver weights also increased about 17 and 20% as the L and W peptides contents increased. There were no differences of epididymal or retroperitoneal adipose weights among all groups.

Plasma and Liver Lipids. Plasma total cholesterol content was significantly ($P < 0.05$) higher in treatment diet groups (25−41%) except the W0.45 diet group (Table 3).

All peptides reduced plasma VLDL-C (20−50%) and TG contents (19−42%) compared with the control group. The plasma VLDL-C and TG contents were positively correlated ($r = 0.963$, $P < 0.05$) and would be expected because most TG is transported by VLDL. L0.6, L1.2, and W0.9 diet groups had higher hepatic total lipid content, whereas SP lowered the hepatic total lipid content. Generally, total and free hepatic cholesterol levels were lower in treatment animals than in the control, but only the L1.2 diet significantly ($P < 0.05$) reduced the hepatic total and free cholesterol contents and SP significantly ($P < 0.05$) reduced the hepatic TG content compared with the control group (Table 3). The ratio of LDL-C and HDL-C of L0.6, L1.2, and W0.9 diet groups increased 2-, 3-, and 1.5-fold, respectively. In this study, hepatic total cholesterol content was negatively correlated with plasma total cholesterol ($r = -0.88$, $P < 0.05$) and non-HDL-C contents ($r = -0.95$, $P < 0.01$).

Fecal Lipids and Bile Acid. There were no differences of the fecal total lipid content in all diet groups. Compared with the control group, there were no differences of fecal total and free cholesterol contents in all peptide diet groups, whereas the SP diet group had significantly ($P < 0.05$) lower fecal total and free cholesterol contents (Table 4). The fecal bile acid excretion of all treatment diet groups was significantly lower (41−149%) than that of the control group.

Hepatic Gene Expression. Relative expression of *CYP51*, coding for the first committed step of cholesterol synthesis, was 2.5-, 2.5-, 1.5-, and 3.0-fold higher in the L0.6, L1.2, W0.45, and W0.9 diet groups than in the control (Figure 2). The mRNA level of cholesterol 7α-hydroxylase (*CYP7A1*), a gene encoding the rate-limiting enzyme in the classical pathway of bile acid

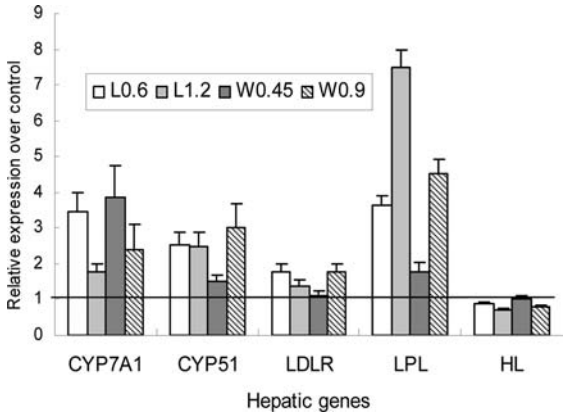

Figure 2. Hepatic mRNA expression of cholesterol and lipoprotein metabolism-related genes including lanosterol 14 α-demethylase (*CYP51*), cholesterol 7 α-hydroxylase (*CYP7A1*), the LDL receptor (*LDLR*), hepatic lipase (*HL*), and lipoprotein lipase (*LPL*) in male mice fed high-fat diets containing peptides of LPYPR and WGAPSL for 3 weeks. Each mRNA was normalized to ribosomal protein 36B4 and is expressed relative to the control level. Data are expressed as the mean ± SE.

synthesis, was up-regulated by 3.5-, 1.8-, 3.8-, and 2.4-fold by L0.6, L1.2, W0.45, and W0.9 diet groups, respectively, compared with the control. In addition, L0.6 and W0.9 diets also induced the expression of *LDLR* (1.8-fold). The mRNA level of lipoprotein lipase (*LPL*), a key enzyme for lipoprotein metabolism, was 3.7-, 7.5-, 1.8-, and 4.5-fold in the L0.6, L1.2, W0.45, and W0.9 diet groups, respectively, compared with the control. The relative mRNA level of hepatic lipase (*HL*), a lipolytic enzyme that hydrolyzes TG and phospholipids in chylomicron remnants, intermediate-density lipoproteins (IDL), and HDL was not significantly different from the control.

DISCUSSION

In this study SP hydrolysate-derived peptides identified by the in vitro micellar cholesterol displacing property were fed to mice on high-fat diets that resulted in increased plasma LDL-C and total cholesterol contents but tended to reduce weight gain.

The effects of these peptides on cholesterol lowering were contrary to expectations, but suggested that the peptides had bioactive properties despite sequence differences. Plant-derived peptides have been shown to have bioactive properties including hypolipidemic properties.[24] Although it is not universally accepted that peptides are absorbed intact into the blood circulation, Lovati et al.[10] postulated that 0.2% of intact peptides and proteins with molecular mass ranging from 3000 to 20000 Da can cross the intestinal wall. Roberts et al.[25] also reported that thyrotropin-reducing hormone (TRH), a tripeptide, luteinizing-hormone releasing hormone (LHRH), a decapeptide, and insulin, a 51 amino acid peptide, could be absorbed intact through the intestine to produce biological effects at tissue levels. Oral TRH produced the same response as intravenous; however, the responses to LHRH and insulin after enteral administration were about 10 and 0.06% of the same amount of intravenous peptides. Oral LHRH activity may be due to its lack of peptic and tryptic digest sites. In this study, LPYPR has both peptic (leucine, L) and tryptic (arginine, R) digest sites, and WGAPSL has a peptic digest site (tryptophan, W), which might lower their physiological activity.

Different species of animals respond in different ways to dietary protein sources. Hayashi et al.[26] reported that SP did not affect the fecal steroid excretion or plasma cholesterol level in three different strains of mice contrary to the rat study. In this study, we also found that mice in the SP diet group had the lowest fecal cholesterol and bile acid excretion. Fecal lipid and total and free cholesterol excretions in mice fed peptide-supplemented diets were not significantly different from the control group, but the fecal bile acid excretion was significantly ($P < 0.05$) lower than that of the control group. Moreover, negative correlations between fecal bile acid excretion and plasma total cholesterol content ($r = -0.876$, $P = 0.062$) and non-HDL-C content ($r = -0.831$, $P = 0.084$) were observed. When hepatic bile acid levels decrease due to fecal excretion, more bile acids are synthesized in the liver from cholesterol, resulting in increased expression of *CYP51* and *CYP7A1*. In this study there was decreased excretion of fecal bile acid accompanied by an anticipated increase in plasma cholesterol, but an unexpected increase in expression of *CYP51* and *CYP7A1* because the latter is sensitive to hepatic levels of bile acid that should have been low. The results are puzzling and suggest that these hydrophobic peptides may be *CYP7A1* agonist. Increasing hepatic synthesis of bile acids would necessitate an increase in cholesterol synthesis and *CYP51* expression.

The hepatic gene expression results suggest that both peptide-supplemented diet groups modulated *CYP7A1* and *CYP51*, genes coding for bile acid and cholesterol synthesis, respectively, although their activity was not dose dependent. The lower levels of *HL* expression at the higher doses of both peptides support the lower levels of plasma TG at the higher doses and may also account for the lower content of VLDL-C and higher content of LDL-C.

Other proteins and peptides have been shown to modulate the activities of hepatic enzymes related to cholesterol and bile acid metabolism.[27,28] Several studies using in vitro micellar cholesterol displacement screening to identify the hypocholesterolemic peptides from SP have shown increased mRNA expression of *LDL-R* and *CYP7A1* in HepG2.[11,29] In this study hepatic and fecal total cholesterol contents were negatively ($r = -0.662$, $P = 0.169$) and positively ($r = 0.706$, $P = 0.147$) correlated, respectively, with the expression of *CYP51*. All diet groups supplemented with peptides had lower fecal bile acid excretion but higher mRNA expression of *CYP7A1* than the control. However, the expression level of *CYP7A1* was positively correlated with fecal bile acid excretion ($r = 0.928$, $P < 0.05$) and would be expected if the excretion resulted in lowered hepatic bile acid content. Lin et al.[8] reported that hepatic hepatocyte nuclear factor 4α (*HNF4α*) deficiency in mice reduced *CYP7A1* mRNA but not protein levels. All peptide treatment diet groups had higher plasma LDL-C content and higher mRNA level of *LDLR* except for the W0.45 diet group. *LDLR* reduces LDL-C content through clearing LDL-C directly from the blood and reduces the production of LDL via uptake of VLDL and VLDL remnants.[30] Autosomal recessive hypercholesterolemia 1 (ARH1), an adaptor protein binding to the cytoplasmic tail of the *LDLR*, is required for efficient internalization of *LDLR* in the liver[31] and therefore for *LDLR*-dependent uptake of LDL, but not for VLDL uptake.[32] The decreased VLDL-C concentration and increased LDL-C concentration might be due to the peptides' impairing the ARH1 binding to *LDLR*. If ARH1 binding to *LDLR* is blocked, LDL would not be internalized and a similar situation may

occur. Another explanation for the lower VLDL-C content and higher LDL-C content might be due to the up-regulation of *LPL*. The mRNA level of *LPL* correlated with plasma VLDL-C ($r = -0.999$, $P < 0.001$) and LDL-C ($r = 0.904$, $P < 0.05$). However, the treatment diet groups had a similar mRNA expression of *HL* compared with the control group. The relative differences might be because *LPL* is the target gene of liver X receptor α (*LXRα*) but *HL* is the target gene of farnesoid X receptor (*FXR*) in humans.[33,34] *HL* is repressed upon influx of cholesterol into the liver.[35] *LXRα* is a nuclear receptor activated by oxysterols that regulates reverse cholesterol transport and cholesterol metabolism through modulation of transcription of many genes involved in these processes.[34] Furthermore, a positive correlation between the mRNA levels of *CYP51* and *LDLR* was observed ($r = 0.865$, $P = 0.067$). *CYP51* and *LDLR* are both the target genes of sterol regulatory element-binding protein 2 (*SREBP2*) and in this case may be possibly regulated similarly.[36]

In summary, we have demonstrated that the SP-derived peptides, LPYPR and WGAPSL, are bioactive and resulted in the reduction of plasma VLDL-C and TG contents and an increase of LDL-C content in mice fed high-fat diets. All of the diet groups supplemented with peptides, except W0.45, had increased plasma total cholesterol and LDL-C contents, possibly due to lower fecal steroid excretion and up-regulated expression level of *LPL*.

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Notes

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