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Identification of Novel Hypocholesterolemic Peptides Derived from Bovine Milk β-Lactoglobulin

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This study was designed to clarify the mechanisms of hypocholesterolemic action of β-lactoglobuline tryptic hydrolysate (LTH) and to identify the novel hypocholesterolemic peptide derived from LTH by screening using Caco-2 cells and animal studies. Serum and liver cholesterol levels were significantly lower in rats fed LTH than in those fed casein tryptic hydrolysate (CTH). The present study suggests that the inhibition of micellar solubility of cholesterol which causes the suppression of cholesterol absorption by a direct interaction between cholesterol mixed micelles, and LTH in the jejunal epithelia is part of the mechanism underlying the hypocholesterolemic action of LTH. Though no one could trace the hypocholesterolemic peptide to any protein origin, we identified, for the first time, a novel hypocholesterolemic peptide, Ile-Ile-Ala-Glu-Lys (IIAEK). Surprisingly, the present study provides the first direct evidence that a new hypocholesterolemic peptide derived from β-lactoglobuline can powerfully influence serum cholesterol levels and exhibit a greater hypocholesterolemic activity in comparison with that of medicine, β-sitosterol, in animal studies. © 2001 Academic Press

Key Words: **whey protein; β-lactoglobulin; cholesterol; rat; peptide; β-sitosterol; Caco-2 cells; casein; milk; micelle.**

Dietary proteins have been shown to influence serum cholesterol level in many studies (1–4). Essentially most research on the effects of dietary proteins on serum cholesterol levels in animal and human studies has focused on the comparison of soybean protein and milk casein. Only limited data are available concerning the effects of milk whey protein on cholesterol metabolism.

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We previously reported that whey protein exhibited a greater hypocholesterolemic effect in comparison with casein or soybean protein in rats (5, 6). However, the effects of major constituents of whey protein such as β-lactoglobulin have not yet been investigated. Moreover, the mechanism by which whey protein induces hypocholesterolemic action is still unclear. In case of soybean protein peptic hydrolysate (SPH), the hypocholesterolemic action was induced by the inhibition of both cholesterol absorption accompanying the suppression of micellar solubility of cholesterol and ileal reabsorption of bile acids (7).

A prior article (8) has demonstrated the hypothesis that the hypocholesterolemic peptides derived from soybean protein might exist and influence the serum cholesterol level. However, until now, no one could find the hypocholesterolemic peptide in soybean protein nor in any protein. We hypothesized that the peptide derived from bovine milk, β-lactoglobulin, might induce a hypocholesterolemic action.

Thus, in the present study, we attempted to clarify the mechanism of the hypocholesterolemic action of β-lactoglobulin tryptic hydrolysate (LTH) by *in vitro* assays related to both the micellar solubility of cholesterol and the taurocholate binding capacity or *in vivo* studies and to identify the novel hypocholesterolemic peptide derived from LTH by screening using the Caco-2 cells and animal studies.

EXPERIMENTAL PROCEDURES

Preparation of β-lactoglobulin tryptic hydrolysate (LTH). β-lactoglobulin (Laiterie Triballat, Noyal-Sur-Vilaine, France) was dissolved in water 5% (w/v) and heated at 90°C for 10 min. β-lactoglobulin was hydrolyzed by porcine trypsin (Novo Industry, Bagsvaerd, Denmark) at pH 8.0 and 37°C for 3 h. Porcine trypsin was added at the 0.4% level (by weight) to protein. After neutralization with 5% (w/v) citrate, the digest was heated at 95°C for 10 min and then centrifuged at 3000*g* for 10 min. The sediment was discarded. The supernatant was concentrated by ultrafiltration using a 20-kDa mol wt cut-off membrane (GR61PP) (Danish Separation Systems, AS, Nakskov, Denmark). The permeate was electrodialyzed and

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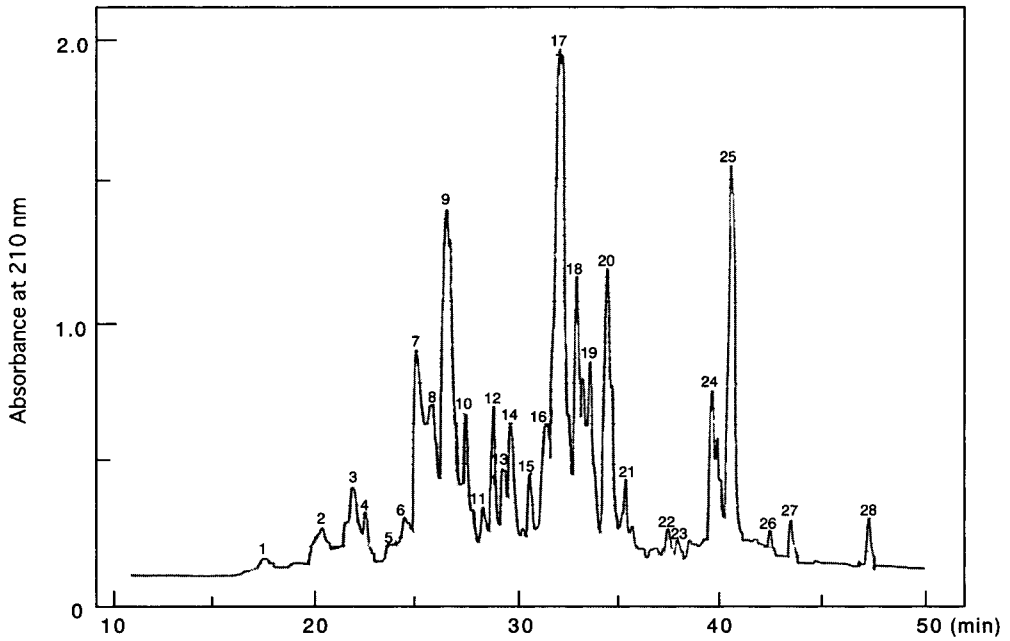

FIG. 1. Purification of the peptides from β-lactoglobulin tryptic hydrolysate (LTH) by reversed phase high performance liquid chromatography (HPLC) on an ODS column.

freeze-dried, and identified as LTH. The chemical composition of LTH was as follows (g/kg): protein, 751; ash, 80; moisture, 48; lipid, 0; sugar, 121.

Preparation of casein tryptic hydrolysate (CTH). Casein (Meiji Milk Products, Tokyo) was dissolved in water 10% (w/v) and hydrolyzed by porcine trypsin (Novo Industry, Bagsvaerd, Denmark) at pH 8.0 and 50°C for 6 h. Porcine trypsin was added at the 0.4% level (by weight) to protein. After the digest was adjusted to pH 4.5 with 1 N HCl, it was centrifuged at 3000*g* for 10 min. The sediment was discarded. The supernatant was applied to a Dowex HCR-W2 column (Muromachi Chemical Industry, Tokyo, Japan). The eluate was freeze-dried and identified as CTH. The chemical composition of CTH was as follows (g/kg): protein, 862; ash, 47; moisture, 66; lipid, 8; sugar, 17.

Purification of peptides. The digest (LTH) was fractionated by reversed phase high performance liquid chromatography (HPLC) on an octadecyl silica (ODS) column (ODS Prep, 20 × 300 mm, Toso, Tokyo, Japan). The column was eluted with a linear gradient of acetonitrile (1%/min), containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 10 ml/min. The usual 80 mg/5 ml digest was applied to the column. The elution was monitored at 210 nm (Fig. 1). Each fraction was dried with a centrifugal concentrator.

Chemical analyses. Protein content was determined by the Kjeldahl method, with an N-to-protein conversion factor of 6.25. Lipids were extracted using chloroform:methanol (2:1, v/v) and weighed. Sugar content was determined by the phenol-sulfonic acid method (9). Moisture was determined as the loss in weight after drying at 105°C for 24 h. Ash content was determined by the direct ignition method (550°C overnight).

Serum, liver, and fecal lipid analyses. Various lipid concentrations were determined using commercially available kits as follows: serum and liver cholesterol with Monotest cholesterol (Boehringer Mannheim Yamanouchi, Tokyo, Japan); HDL cholesterol with HDL-cholestase (Nissui, Tokyo, Japan); serum triglyceride with Triglycolor III (Boehringer Mannheim Yamanouchi, Tokyo, Japan); and serum phospholipid with Phospholipid C-Test Wako (Wako Pure Chemical, Osaka, Japan). Liver lipids were extracted by the method of Folch *et al.* (10), and total lipids were determined gravimetrically as described previously (11). Fecal acidic steroids were measured according to the method of Bruusgaard *et al.* (12) and Malchow-Moller *et al.* (13), while fecal neutral steroids were assayed with trimethylsilyl ether using 1.5% OV-17 with a GC-14A instrument (Shimadzu, Kyoto, Japan) and 5 α-cholestane as the internal standard (14).

Animals and diets. Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) were used in the present animal studies. Room temperature was maintained at 22 ± 2°C with a 12-h cycle of light (8:00–20:00) and dark. The approval of the Gifu University Animal Care and Use Committee was given for our animal experiments. All rats were housed individually in metal cages and were allowed free access to food and water. The detailed procedures of animal experiments were shown in the appropriate sections.

Taurocholate binding capacities of LTH, CTH or cholestyramine measured in vitro. The capacity of LTH, CTH or cholestyramine (Sigma, St. Louis, MO) to bind bile acids *in vitro* was measured according to the methods reported elsewhere (15, 16). LTH, CTH or cholestyramine (10 mg) were incubated by shaking vigorously in 5.0 mL of 0.1 M Tris–HCl 0.1 M NaN_3 buffer, pH 7.4, containing 50 mM taurocholate (Sigma) at 37°C for 30 min. An aliquot of the reaction mixture was dialyzed at room temperature in molecularporous membrane tubing (Spectra/POR, MW cutoff 6000–8000, Spectrum Medical Inc., Los Angeles, CA) against the same buffer for 3 days, and the content of taurocholate in the dialyzate was measured enzymatically (12, 13).

Effects of LTH or CTH on micellar solubility of cholesterol. The micellar solubility of cholesterol with protein hydrolysates *in vitro* was measured by the method of Ikeda *et al.* (17) with some modifications. Micellar solutions (1 ml) containing 10 mM sodium taurocholate (Sigma), 0.4 mM cholesterol (Sigma), 1 mM oleic acid (Sigma), 132 mM NaCl, 15 mM sodium phosphate (pH 7.4), LTH or CTH (10 mg/ml, respectively) were prepared by sonication (Ultrasonic Homogenizer, Model VP-5, Taitec Co. Ltd., Saitama, Japan). Then the mixture was incubated at 37°C for 24 h and ultracentrifuged at 100,000*g* for 60 min at 37°C. The supernatant was collected

for the determination of cholesterol and bile acids as described previously (11).

Effects of LTH or CTH on cholesterol absorption in Caco-2 cells in vitro. Caco-2 cells were acquired from the American Type Culture Collection. The cells were maintained in modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 50 IU/ml of penicillin, and 50 μg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Monolayers were grown in 48-well plastic dishes containing 0.5 mL of FBS supplemented with DMEM as described previously (7, 18), fresh medium being added every 2 days. The experiments described usually used cultures 12–15 days after plating, and were performed in medium-199/Earle's (Gibco, Grand Island, USA) containing 1 mM Hepes. Cell viability, as ascertained by Trypan blue exclusion, was unaffected by any of the experimental procedures. The number of passages of the cell line ranged from 70–85.

[^{14}C]-labelled micellar cholesterol uptake in Caco-2 cells was measured by the method described previously (7, 18). The final concentration of each [^{14}C]-labelled micellar solution (0.2 ml) was as follows: 0.74 kBq [4-^{14}C]-cholesterol (2.1 Gbq/m mol, NEN, Boston, MA), 0.1 mM cholesterol (Sigma), 5 mM phosphatidylcholine (Sigma), 1 mM monoolein (Sigma), 5 mM sodium taurocholate (Sigma), LTH or CTH (1 mg/0.2 ml, respectively). The micellar solution was mixed by ultrasonic vibration.

After 14 days, the cells were rinsed two times with 0.5 ml of PBS. A [^{14}C]-labelled micellar solution (0.2 ml) containing LTH or CTH was then added to the dishes which were incubated at 37°C for 20 min in a CO_2 incubator. After this incubation, the cells were rinsed two times with 0.5 ml of PBS and finally lysed in 0.1% SDS solution. Then 7.5 ml of Aquasol-2 (NEN, Boston, MA) was added, and the radioactivity in the cellular debris was counted to determine the amount of cholesterol associated with the cells.

Effects of LTH or CTH on cholesterol absorption in vivo. After acclimation to a commercial nonpurified diet (MF, Oriental Yeast, Osaka, Japan) for 3 days. Ten-week-old rats weighing 236–248 g were fasted 48 h with free access to water. They were intragastrically intubated with the test solutions containing LTH or CTH using a polyethylene catheter. One hour after the administration of the test solutions, the rats were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture for the analysis of serum. The liver and intestine were excised quickly. The liver was rinsed with ice-cold saline. The luminal content of the small intestine was removed by flushing with ice-cold saline. The test solution consisted of 1 mM monoolein (Sigma), 5 mM taurocholic acid (Sigma) and 37 kBq of [1,2-^{3}H]-cholesterol (1972.1 GBq/m mol, NEN) and LTH or CTH (62.5 mg respectively) in 1 ml of 15 mM phosphate buffer (pH 7.4). These solutions were emulsified by sonication. [^{3}H]-cholesterol incorporated into the serum, liver and intestine was extracted with hexane after saponification of KOH-ethanol as described previously (19). Aliquots of the organic extract were used for scintillation counting.

Effects of dietary LTH, CTH on cholesterol metabolism in rats in vivo. After acclimation to a commercial nonpurified MF diet for 3 days, 5-week-old rats weighing 115–130 g were divided into 2 groups of 6 rats each on the basis of body weight. Each group had free access to one of the respective test diets containing LTH, CTH as the protein source for 14 days. The composition of the diet was according to the formula recommended by the American Institute of Nutrition (20); it contained, in weight percent, CTH, 20; corn oil, 2.0; AIN^{76} mineral mixture, 3.5; AIN^{76} vitamin mixture, 1.0; choline chloride, 0.2; cholesterol, 1.0; sodium cholate, 0.25; sucrose, 24.02; starch, 48.03. 0.23% DL-methionine was added to the diet containing CTH. When LTH was examined, it replaced CTH at an equivalent nitrogen level at the expense of carbohydrates. After 24 h without food, the rats were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture, and the liver removed. Fecal collections (7–9 days) were completed prior to 24 h food restriction and blood sampling. Feces were used for determining fecal steroids.

Effects of fractionated peptides derived from β-lactoglobulin on cholesterol absorption in Caco-2 cells in vitro. Fractionated peptides were used for this experiment. [^{14}C]-labelled micellar cholesterol uptake in Caco-2 cells was measured by the method described previously (7, 18) as shown in the case of CTH or LTH.

Identification of the peptide sequences which suppressed cholesterol absorption in Caco-2 cell study. The amino acid sequence of fractionated peptides which suppressed the cholesterol absorption in Caco-2 cells was determined by a 477A protein sequencer (Applied Biosystems, Tokyo, Japan).

Effects of oral administration of LTH, CTH, β-sitosterol, or peptide (IIAEK) derived from β-lactoglobulin on serum cholesterol level in rats in vivo. After acclimation to a commercial nonpurified MF diet for 3 days, four-week-old rats weighing about 30 g were divided into 4 groups of 7 rats each on the basis of body weight. Each group had free access to the cholesterol diet containing casein during the experimental period. The composition of the casein diet is as follows (%): casein, 20; lard, 5.0; corn oil, 1.0; cellulose, 5.0; AIN^{76} mineral mixture, 3.5; AIN^{76} vitamin mixture, 1.0; cholesterol, 1.0; sodium cholate, 0.25; choline chloride, 0.2; sucrose, 21.02; and starch, 42.03. IIAEK (Purity 95%, Sawady Technology, Tokyo, Japan), CTH, LTH orβ-sitosterol (Tama Biochemical Co., Tokyo, Japan) (200 mg/kg/day, respectively) dissolved in 30% (w/w) ethanol solution was administered orally in rats once a day at 8:00 a.m. for 3 days using a zonde. After a final dosage 24 h without food, the rats were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture. Serum total cholesterol, HDL cholesterol and LDL + VLDL-cholesterol were determined.

Statistical analyses. Results are expressed as means and SEM. The statistical significance of differences was evaluated by Student's *t* test (21) and Duncan's multiple range test (22).

RESULTS

Taurocholate binding capacity of LTH, CTH, or cholestyramine measured in vitro. The taurocholate binding capacity of LTH (47.4 ± 1.5%) was significantly higher than that of CTH (35.1 ± 1.2%) ($P < 0.001$). The value of the taurocholate binding capacity of cholestyramine was 98.5 ± 0.5%.

Effects of LTH or CTH on micellar solubility of cholesterol in vitro. We used cholestyramine as a standard for the micellar solubility of cholesterol in a preliminary test (in mM: cholestyramine, 0.02 ± 0.001; LTH, 0.25 ± 0.02). The micellar solubility of cholesterol was significantly less in the presence of cholestyramine compared with LTH. The micellar solubility of cholesterol in the presence of LTH was significantly lower than with CTH (Fig. 2A). In contrast, no significant effects of protein hydrolysates on bile acid solubility were observed (Fig. 2B).

Effects of LTH or CTH on cholesterol absorption in Caco-2 cells in vitro. Cholesterol uptake from micelles containing LTH (5.70 ± 0.34 p mol/well) was significantly lower than from cholesterol micelles containing CTH (9.50 ± 0.87 p mol/well) ($P < 0.001$).

Effects of the intubation of LTH or CTH on cholesterol absorption in vivo. Final body weights (LTH, 206.6 ± 2.6 g; CTH, 206.3 ± 1.7 g) and relative liver

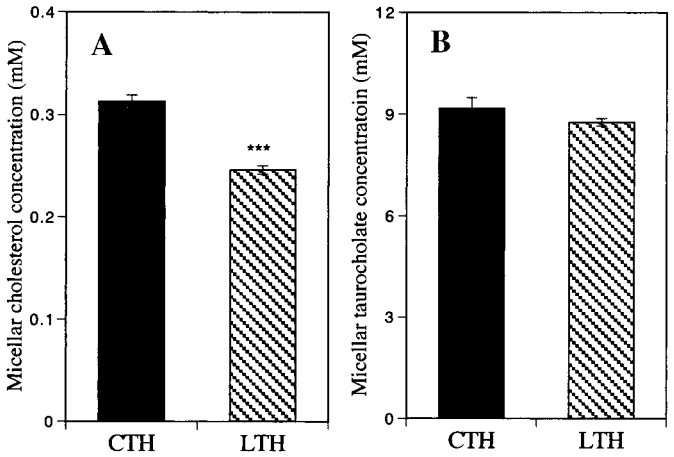

FIG. 2. Effects of casein tryptic hydrolysate (CTH) or β-lactoglobulin tryptic hydrolysate (LTH) on micellar solubility of cholesterol (A) and taurocholate (B) *in vitro.* Each value is expressed as means ± SEM of 3 determinations. Statistical significance compared to CTH by Student's *t* test (***$P < 0.001$).

weights (in g/100 g body weight: LTH, 2.70 ± 0.04; CTH, 2.73 ± 0.10) were unaffected by treatments. The incorporation of [^{3}H]-cholesterol into the serum, liver and intestine was significantly lower in the LTH group than in the CTH group (Table 1).

Effects of dietary LTH, CTH on cholesterol metabolism in rats in vivo. Food intake and body weight gains were unaffected by dietary treatment, but the relative liver weight was significantly greater in the CTH-fed group than in the LTH-fed group (Table 2). Serum total cholesterol levels in the LTH group were significantly lower than in the CTH group. Serum HDL-cholesterol concentration and the atherogenic index in the group fed LTH were significantly higher than in the group fed CTH. Serum triglyceride and phospholipid concentrations were unaffected by dietary treatments (not shown in Table). Liver total lipid and cholesterol concentrations were significantly lower

TABLE 1

Effects of the Intubation of Cholesterol Micelle Containing Casein Tryptic Hydrolysate (CTH) or β-Lactoglobulin Tryptic Hydrolysate (LTH) on the Distribution of [^{3}H]-Cholesterol in Serum, Liver, and Intestine of Rats[a]

	Group	
	CTH	LTH
Serum[b]	0.53 ± 0.08	0.31 ± 0.05*
Liver	1.69 ± 0.28	0.87 ± 0.09*
Intestine	2.45 ± 0.23	1.77 ± 0.19*

[a] Values are means ($n = 6$) ± SEM. Statistical significance compared with CTH group by Student's *t* test. (*$P < 0.05$).
[b] Measured in a 5-ml serum sample.

TABLE 2

Effects of Dietary Casein Tryptic Hydrolysate (CTH) and β-Lactoglobulin Tryptic Hydrolysate (LTH) on Body and Liver Weights, Food Intake, Serum, and Liver Lipids and Fecal Steroid Excretion in Rats[a]

	Diet group	
	CTH	LTH
Body weight gain (g/14 days)	71.2 ± 4.7	68.8 ± 2.5
Liver weight (g/100 g body weight)	5.46 ± 0.22	5.30 ± 0.20
Food intake (day 6, g/day)	17.0 ± 0.8	16.0 ± 1.0
Serum (mg/dl)		
Total cholesterol (a)	136.6 ± 6.5	97.0 ± 3.9***
HDL cholesterol (b)	46.8 ± 1.9	64.3 ± 3.8**
LDL + VLDL cholesterol[b]	89.8 ± 7.9	32.7 ± 4.5***
Atherogenic Index (b)/(a)	0.34 ± 0.03	0.66 ± 0.04***
Liver		
Total lipids (mg/g liver)	102.9 ± 5.8	93.1 ± 5.5
Cholesterol (mg/g liver)	17.6 ± 1.0	8.4 ± 0.6***
Fecal		
Dry weight (g/3 days)	1.73 ± 0.10	2.36 ± 0.04***
Neutral steroids (μ mol/3 days)		
Cholesterol	506.1 ± 50.6	580.3 ± 10.8
Coprostanol	1.4 ± 0.5	53.1 ± 8.7***
Total	507.5 ± 50.3	633.4 ± 11.3*
Acidic steroids (μ mol/3 days)	65.1 ± 4.6	76.4 ± 2.7*
Total steroids[c] (μ mol/3 days)	572.6 ± 54.1	709.8 ± 10.8*

[a] The data are means ± SEM ($n = 6$). Statistical significance compared to CTH group by Student's *t* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
[b] Values were calculated as follows: LDL + VLDL cholesterol = Total cholesterol − HDL cholesterol.
[c] Total steroids = neutral steroids + acidic steroids.

in the LTH-fed group than in the CTH-fed group. Fecal dry weight was significantly higher in the LTH-fed group than in the CTH-fed group. The fecal outputs of total steroids were significantly higher in the LTH group than in CTH-group.

Effects of fractionated peptides derived from β-lactoglobulin on cholesterol absorption in Caco-2 cells in vitro. As shown in Fig. 3, cholesterol uptake from micelles containing fraction 4, 9, 20, 21, 24, or 25 was significantly lower than that from cholesterol micelles containing CTH or LTH. The amounts of other fractions were too enough to evaluate the cholesterol uptake.

Identification of the peptide sequences which suppressed cholesterol absorption in Caco-2 cell study. Fraction 4 was identified as IIAEK which is consistent with residues 71–75 of β-lactoglobulin. Fraction 9 consisted of two kinds of peptides, GLDIQK and ALPMH, which is consistent with residues 9–14 and 142–146 of β-lactoglobulin. Fractions 21 and 25 were identified as VYVEELKPTPEGDLEILLQK which is consistent with residues 41–60 of β-lactoglobulin. As fractions 20 and 24 consisted of peptide mixtures judging from the data of rechromatography, we did not identify them.

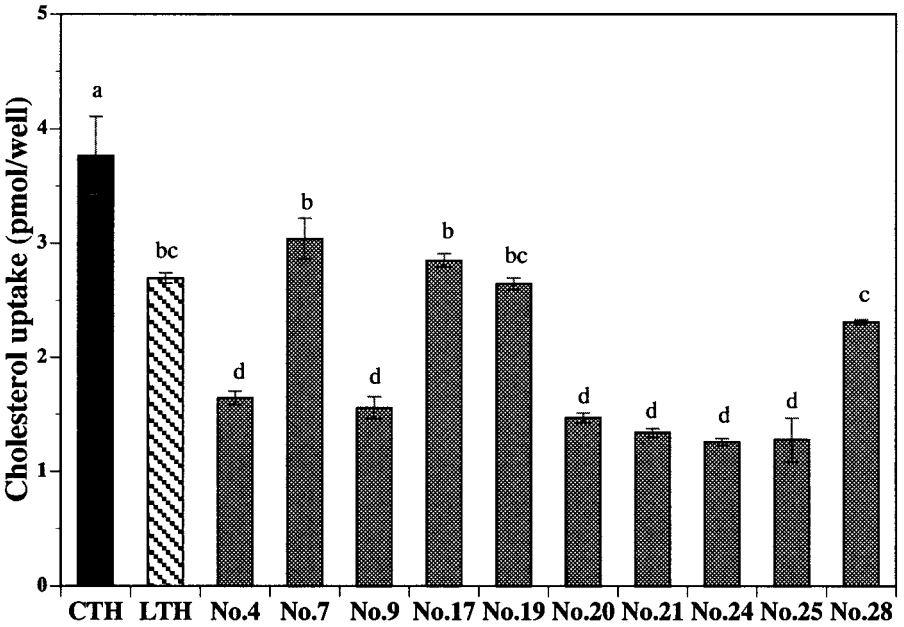

FIG. 3. Effects of fractionated peptides derived from β-lactoglobulin on cholesterol absorption in Caco-2 cells *in vitro.* The digest (LTH) was fractionated by reversed phase high performance liquid chromatography (HPLC) as shown in Fig. 1. After fractionation, cholesterol absorption from micelles containing the peptides from LTH was measured by the method shown in the text.

Effects of oral administration of LTH, CTH, β-sitosterol, or peptide (IIAEK) derived from β-lactoglobulin on serum cholesterol level in rats in vivo. Body weight gains and food intake were unaffected by dietary treatment. Serum total cholesterol level in the IIAEK group was significantly lower than in the CTH, LTH, or β-sitosterol group (Table 3). The lowest serum cholesterol level was observed in rats infused with IIAEK. HDL cholesterol level was significantly increased in rats infused with IIAEK, LTH, or β-sitosterol compared to rats infused with CTH. LDL + VLDL-cholesterol level in IIAEK, LTH, or β-sitosterol was significantly lower than in the CTH group. The atherogenic index was significantly increased in rats infused with IIAEK, LTH, or β-sitosterol compared to rats infused with CTH.

DISCUSSION

We previously reported that whey protein exhibited greater hypocholesterolemic effects in comparison with casein or soybean protein in rats (5, 6). However, the effects of major constituents of whey protein such as β-lactoglobulin have not yet been investigated. More-

TABLE 3

Effects of Oral Administration of Casein Tryptic Hydrolysate (CTH), β-Lactoglobulin Tryptic Hydrolysate (LTH), β-Sitosterol, or IIAEK on Body and Liver Weights, Food Intake, Serum HDL, and LDL + VLDL-cholesterol in Rats[1]

	Group			
	CTH	LTH	β-sitosterol	IIAEK
Body weight gain (g/4 days)	10.4 ± 0.4^{a}	10.5 ± 0.7^{a}	9.5 ± 0.8^{a}	8.7 ± 0.4^{a}
Liver weight (g/100 g body weight)	5.22 ± 0.07^{b}	5.37 ± 0.05$^{a, b}$	5.17 ± 0.10^{b}	5.54 ± 0.11^{a}
Food intake (day 3, g/day)	8.5 ± 0.2^{a}	8.7 ± 0.2^{a}	8.3 ± 0.3^{a}	8.4 ± 0.3^{a}
Serum (mg/dl)				
Total cholesterol (a)	529.3 ± 14.4^{a}	441.3 ± 12.0^{b}	435.4 ± 12.8^{b}	328.7 ± 11.3^{c}
HDL cholesterol (b)	42.6 ± 1.6^{b}	50.5 ± 2.4^{a}	52.1 ± 1.7^{a}	47.5 ± 0.8$^{a, b}$
LDL + VLDL-cholesterol[2]	486.7 ± 26.7^{a}	390.8 ± 22.9^{b}	383.3 ± 24.2^{b}	281.2 ± 20.6^{c}
Atherogenic Index (b)/(a)	0.08 ± 0.01^{c}	0.11 ± 0.01^{b}	0.12 ± 0.01^{b}	0.15 ± 0.01^{a}

[1] The data are means ± SEM (n = 7). Within a row, means with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

[2] Values were calculated as follows: LDL + VLDL cholesterol = Total cholesterol − HDL cholesterol.

over, the mechanisms by which whey protein induced hypocholesterolemic action is still unclear. The present study provides the first direct evidence that β-lactoglobulin tryptic hydrolysate (LTH) has a hypocholesterolemic activity in comparison with casein tryptic hydrolysate (CTH) in rats. It has been postulated that the degree of serum cholesterol-lowering activity depends on the degree of fecal steroid excretion (acidic steroids + neutral steroids) (23). The present study also demonstrated a higher fecal excretion of total steroids in rats fed LTH (Table 2), indicating that the effect is at least in part due to an enhancement of fecal steroid excretion. There have been many studies on the hypocholesterolemic effects of proteins, most of which supported the hypothesis that a peptide with high bile acid-binding capacity could inhibit the reabsorption of bile acid in the ileum and decrease the blood cholesterol level (15). These possibilities may be applicable to the case of LTH on the basis of the evidence of fecal bile acid excretion and taurocholate binding capacity in this study.

In recent studies, monolayers of Caco-2 cell cultures were used as a model system to examine the process of lipid metabolism (24–26). For example, Field *et al.* (24) reported that Caco-2 cells, like the small intestine, had the ability to absorb micellar cholesterol and to express marker enzymes like alkaline phosphatase as small intestinal epithelial cells. We have found for the first time that the cholesterol micelles containing soybean protein peptic hydrolysate (SPH) significantly suppressed cholesterol uptake by Caco-2 cells compared to the cholesterol micelles containing CTH (18). In this study, we found that LTH also directly inhibited the absorption of micellar cholesterol in Caco-2 cells *in vitro.* Cholesterol is rendered soluble in bile salt-mixed micelles and then absorbed (27). The present study indicated that the micellar solubility of cholesterol in the presence of LTH was significantly lower than with CTH. Very interestingly, we have found for the first time that micellar solubility of cholesterol in the presence of SPH was significantly lower than with CTH (7). Sitosterol (17), sesamine (28) or catechin (29) also lowered the micellar solubility of cholesterol in conjunction with the serum cholesterol-lowering effects in rats. These findings including LTH suggest that the suppression of micellar solubility of cholesterol induces the inhibition of cholesterol absorption in the jejunum, and this may be closely related to the lowering action of serum cholesterol. As shown in the cases of LTH and SPH (7), some other dietary proteins or peptides may also affect such solubility. Our preliminary observation indicated that wheat gluten also decreased the micellar solubility of cholesterol accompanying the suppression of cholesterol absorption.

We suggested that the hypocholesterolemic action of LTH was induced by the suppression of cholesterol absorption evidenced by an *in vivo* cholesterol absorption study and an *in vitro* Caco-2 cell study. We tried to identify the active component related to the hypocholesterolemic action of LTH in the present study. We hypothesized that the peptide derived from bovine milk β-lactoglobulin might induce a hypocholesterolemic action. We already reported the relationship between the suppression of cholesterol absorption in Caco-2 cells and the hypocholesterolemic action *in vivo* in the case of SPH (7, 18). Our experimental system to evaluate cholesterol uptake in Caco-2 cells, we called "Caco-2 cell screening", is useful for clarifying the active component underlying the inhibitory effect of peptide on cholesterol absorption from the small intestine. Thus, (1) we first tried to identify the peptide sequences which suppress cholesterol absorption using Caco-2 cell screening in the present study. (2) After Caco-2 cell screening *in vitro,* we then tried to evaluate the hypocholesterolemic activity of new peptides in animal studies *in vivo.* By using Caco-2 cell screening, for the first time we identified four kinds of novel peptide sequences which inhibit cholesterol absorption *in vitro,* i.e., Gly-Leu-Asp-Ile-Gln-Lys, Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys, Ile-Ile-Ala-Glu-Lys and Ala-Leu-Pro-Met-His, which correspond, respectively, to residues 9∼14, 41∼60, 71∼75 and 142∼146 of bovine β-lactoglobulin. In Caco-2 cell study, the degree of the inhibition of cholesterol absorption by LTH was significantly lower than that of an identified hypocholesterolemic peptide. Thus, LTH might be not always consisted of hypocholesterolemic peptides as we completely unidentified the LTH peptide sequences.

A previous article (8) demonstrated the hypothesis that the hypocholesterolemic peptides derived from soybean protein might exist and influence the serum cholesterol level. However, until now no one could find the hypocholesterolemic peptide in soybean protein nor in any protein. Sugano *et al.* (8) suggested that the hypocholesterolemic peptides derived from soybean protein consisted of those peptides of molecular weights between 1 K to 10 K. However, the hypocholesterolemic peptide of soybean protein is unidentified. Although a previous study (30) demonstrated that the hypocholesterolemic effects of papain-hydrolyzed pork meat contained peptides with molecular weights of 3 K or less, the hypocholesterolemic peptide of pork protein is also unidentified.

As we identified four kinds of novel peptide sequences which inhibited cholesterol absorption in Caco-2 cell screening *in vitro,* we tried to evaluate the hypocholesterolemic activity of new peptides in animal studies *in vivo.* IIAEK was chosen for the animal study, because it has no peptic or tryptic digestive site. Thus, IIAEK itself was expected to elicit the hypocholesterolemic action *in vivo.* Though no one could find a hypocholesterolemic peptide from any protein origin, we identified, for the first time, a new hypocholester-

olemic peptide (IIAEK). The present study provides the first direct evidence that the peptide derived from β-lactoglobulin can powerfully influence the serum cholesterol level and that the hypocholesterolemic activity of IIAEK exhibited a greater hypocholesterolemic activity in comparison with the medicine, β-sitosterol, in rats. A novel hypocholesterolemic peptide (IIAEK) induced the suppression of cholesterol absorption evidenced by the Caco-2 cell study. We speculate that IIAEK may decrease the micellar solubility of cholesterol and inhibit cholesterol absorption as in the case of LTH or SPH (7). Myoglobin (31) and haptoglobin (32) are known to decrease the cholesterol solubility in model bile *in vitro*. Ahmed *et al.* (31) suggested that myoglobin decreased the cholesterol solubility through the acceleration of the rate of cholesterol migration from the micellar to the vesicular phase *in vitro*. Thus, IIAEK or LTH may decrease cholesterol solubility by increasing the amount of vesicular cholesterol rather than that of micellar cholesterol. However, the mechanisms of interaction between hypocholesterolemic peptides and cholesterol micelle or cholesterol is unknown at present. The detailed mechanism of the hypocholesterolemic action of this new hypocholesterolemic peptide (IIAEK) is currently being investigated.

This finding of such a novel hypocholesterolemic peptide should play a crucial role in clarifying the mechanisms by which dietary protein induces differential effects on the serum cholesterol level as well as in developing a functional food or an anti-atherogenic drug.

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