Soy Protein Peptic Hydrolysate with Bound Phospholipid decreases Micellar Solubility and Cholesterol Absorption in Rats and Caco-2 Cells

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ABSTRACT This experiment was designed to evaluate the effects of casein, soy protein, soy protein with bound phospholipids (SP), soy protein peptic hydrolysate (SPH) or soy protein peptic hydrolysate with bound phospholipids (SPHP) on the micellar solubility of cholesterol and the taurocholate binding capacity in vitro. We also evaluated the effects of various proteins on cholesterol metabolism in rats and Caco-2 cells. SPHP had a significantly greater bile acid-binding capacity than that of SPH in vitro. Micellar cholesterol solubility in vitro was significantly lower in the presence of SPHP compared to casein tryptic hydrolysate (CTH). The cholesterol micelles containing SPHP and SPHP significantly suppressed cholesterol uptake by Caco-2 cells compared to the cholesterol micelles containing CTH. Consistent with these findings, the in vivo cholesterol absorption study using radioisotopes, fecal excretion of total steroids was significantly greater in rats fed the SPHP diet compared with those fed the casein, soy protein, SP and SPH diets. Serum total cholesterol was significantly lower in rats fed SPHP than in those fed casein. The concentrations of total lipids and cholesterol in liver were significantly lower in the SPHP-fed group compared with all other groups. These results suggest that the suppression of cholesterol absorption by direct interaction between cholesterol-mixed micelles and SPHP in the jejunal epithelia is part of the mechanism underlying the hypocholesterolemic action of SPHP. SPHP may also inhibit the reabsorption of bile acids in the ileum, thus lowering the serum cholesterol level. J. Nutr. 129: 1725–1730, 1999.

KEY WORDS: serum cholesterol soy protein phospholipid Caco-2 cells rats


In addition, the cholesterol-lowering effect of purified phospholipid also has been reported (Imaizumi et al. 1989, O’Mullane and Hawthorne 1982). Sirtori et al. (1985) studied the effects of textured soy protein2 containing 6% lecithin in type-II hyperlipidemic patients, among whom they noted an increase in HDL cholesterol concentration. However, there is little information about textured soy protein containing 6% lecithin, and no studies have yet been reported on the effects of proteins that bind phospholipids in large quantities and, in particular, their hydrolysates.

A soy protein peptic hydrolysate (SPH)3 has been reported to have a stronger lowering effect on serum cholesterol than that of intact soy protein (Sugano et al. 1990). These authors showed that SPH both decreased the blood cholesterol level and promoted fecal excretion of steroids, compared with casein. From the fecal steroid excretion data, they suggested that SPH might have inhibited cholesterol absorption. Although the soy protein effect on cholesterol absorption has been examined previously in comparison with that by casein (Nagaoka et al. 1982), the in vivo experimental system did not allow determination of its direct effect on cholesterol absorption. Saeki et al. (1987) suggested that the inhibition of cholesterol absorption was not the major factor involved in the differential effects of dietary proteins on serum cholesterol. Moreover, Lovati et al. (1992) suggested that the activation of LDL receptor activity in liver cells induced by soybean globulin may be related to the serum cholesterol-lowering action of soy protein. Therefore, to elucidate the molecular mechanism of the inhibitory effect of soy protein on cholesterol absorption, we evaluated this effect with the use of a cell strain cultured in vitro. In an earlier paper (Nagaoka et al. 1997), we used cultured Caco-2 cells and found that SPH directly inhibited the absorption of micellar cholesterol. However, no direct in vivo or in vitro studies of the effects of SPHP on cholesterol absorption from the intestine have been reported. Interestingly, no work has been reported to date evaluating the effects of proteins or their hydrolysates on the micellar solubility of cholesterol.

We postulate that SPHP-induced hypocholesterolemia may

1 To whom correspondence and reprint requests should be addressed.
2 Textured soy protein is defined as soy protein treated with extruder (Sirtori et al. 1985).
3 Abbreviations used: CTH, casein tryptic hydrolysate; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; SP, soy protein with bound phospholipids; SPH, soy protein peptic hydrolysate; SPHP, soy protein peptic hydrolysate with bound phospholipids.


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TABLE 1

Chemical composition of casein, soy protein, SP, SPH and SPHP

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Casein</th>
<th>Soy protein</th>
<th>SP</th>
<th>SPH</th>
<th>SPHP</th>
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<tbody>
<tr>
<td>Protein</td>
<td>843.8</td>
<td>863.0</td>
<td>719.0</td>
<td>819.0</td>
<td>619.0</td>
</tr>
<tr>
<td>Lipids</td>
<td>0</td>
<td>0</td>
<td>201.0</td>
<td>0</td>
<td>237.0</td>
</tr>
<tr>
<td>Sugar</td>
<td>0</td>
<td>54.0</td>
<td>66.0</td>
<td>62.0</td>
<td>48.0</td>
</tr>
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<td>141.2</td>
<td>37.0</td>
<td>9.0</td>
<td>19.0</td>
<td>79.0</td>
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<tr>
<td>Ash</td>
<td>15.0</td>
<td>46.0</td>
<td>5.0</td>
<td>100.0</td>
<td>17.0</td>
</tr>
</tbody>
</table>

1 Abbreviations used: SP, soy protein with bound phospholipids; SPH, soy protein peptic hydrolysate; SPHP, soy protein peptice hydrolysate with bound phospholipids.

have resulted in the inhibition of both cholesterol absorption in the intestinal epithelial cells (accompanying the lowering of micellar cholesterol solubility) and ideal reabsorption of bile acids. Thus, we used Caco-2 cells, rats or in vitro assays related to both the micellar solubility of cholesterol and the binding capacity of taurocholate to investigate the mechanisms of the serum cholesterol-lowering action of SPHP.

MATERIALS AND METHODS

Preparation of soy protein peptic hydrolysate (SPH). Soy protein (New Fujipro-E; Fuji Oil, Osaka, Japan) was hydrolyzed by porcine pepsin (activity: 1:10,000, Nacalai Tesque, Kyoto, Japan) at pH 2.0 and 37°C for 24 h. Porcine pepsin (1 g/100 g) was added to the protein. The digest was heated at 90°C for 30 min and neutralized with 2 mol/L NaOH. The digest was centrifuged at 4500 × g for 20 min. The sediment was washed with water three times and centrifuged at 4500 × g for 20 min. The sediment was freeze-dried and identified as SPH.

Preparation of soy protein with bound phospholipids (SP). Enzyme-modified soy phospholipids (Elmizer AC; T&K Lecithin, Mie, Japan) were used as a phospholipid source. Enzyme-modified soy phospholipids were prepared from soy phospholipids (SLP; True Lecithin Co., Tokyo, Japan) hydrolysis as described previously (Pryde 1985). Fecal neutral steroids were assayed with trimethylsilyl ether by using 1.5% OV-17 with a GC-14A instrument (Shimadzu, Kyoto, Japan) and 5α-cholesterol as the internal standard (Miettinen et al. 1965).

Animals and diets. Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) were used in two experiments. Room temperature was maintained at 22 ± 2°C with a 12-h light:dark cycle (lights on 0800–2000 h). The approval of Gifu University Animal Care and Use Committee was given for our animal experiments. All of the rats were housed individually in metal cages and were allowed free access to food and water. After acclimation to a commercial nonpurified MF diet (Oriental Yeast, Osaka, Japan) for 3 d, rats were divided into groups on the basis of body weight in Experiments 4 and 5. The basic diet was that recommended by the AIN (1977). All diet compositions for Experiment 5 are given in Table 3.

TABLE 2

Amino acid compositions of casein, soy protein, SPH and SPHP

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Casein</th>
<th>Soy protein</th>
<th>SP</th>
<th>SPH</th>
<th>SPHP</th>
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<tr>
<td>Asp</td>
<td>89.5</td>
<td>110.4</td>
<td>90.3</td>
<td>94.3</td>
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<tr>
<td>Thr</td>
<td>56.2</td>
<td>35.5</td>
<td>36.8</td>
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<tr>
<td>Ser</td>
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<td>52.2</td>
<td>51.2</td>
<td>52.8</td>
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<tr>
<td>Glu</td>
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<td>193.8</td>
<td>141.1</td>
<td>141.6</td>
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</tr>
<tr>
<td>Pro</td>
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<td>56.3</td>
<td>70.4</td>
<td>72.1</td>
<td></td>
</tr>
<tr>
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<td>41.9</td>
<td>45.9</td>
<td>45.8</td>
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</tr>
<tr>
<td>Ala</td>
<td>38.8</td>
<td>42.2</td>
<td>46.9</td>
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<tr>
<td>Cys</td>
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<tr>
<td>Val</td>
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<td>47.4</td>
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<td>Met</td>
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<tr>
<td>Ile</td>
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<tr>
<td>Leu</td>
<td>94.3</td>
<td>80.2</td>
<td>96.4</td>
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</tr>
<tr>
<td>Tyr</td>
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<td>38.7</td>
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<td>Phe</td>
<td>38.3</td>
<td>56.8</td>
<td>69.0</td>
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<tr>
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<td>21.6</td>
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<tr>
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<td>19.0</td>
<td>12.5</td>
<td>14.3</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>

1 See Table 1 for abbreviations.
2 Asp: Asp reflects aspartate derived from the hydrolysis of both aspartate and asparagine from proteins.
3 Glu: Glu reflects glutamate derived from the hydrolysis of both glutamate and glutamine from proteins.
monolayers became confluent 3–4 d after seeding at between 7 and 10 
incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The 
ettented with 10% fetal calf serum (FCS), 4 mmol/L glutamine, 
maintained in Dulbecco’s modified Eagle’s medium (DMEM) supple-
Caco-2 cells were generously provided by the Central Research 
Institute of Meiji Milk Products, (Tokyo, Japan). The cells were 

were measured by the method of Kritchevsky and Story (1974) with 
some modifications as described previously (Hirose et al. 1991). The 
mixtures containing 1.85 kBq of tauro [carbonyl-14C] cholic acid (sodium salt) (1.89 Gbq/mmol, Amersham International, Bucking-
hamshire, UK), 0.1 mol/L sodium taurocholate in 5 mL of 0.1 mol/L 
Tris-HCl buffer (pH 7.4), and 1–500 mg binding substances (cho-
lesteramine, CTH, SPH or SPHP) were prepared by sonication. Then the mixture was 

were divided into five groups of six rats each on the basis of body weight. 
Experiment 3: Cholesterol absorption in Caco-2 cells in vitro. 
Caco-2 cells were generously provided by the Central Research 
Institute of Meiji Milk Products, (Tokyo, Japan). The cells were 

were measured by the method of Ikeda et al. (1988) with some 
modifications. Micellar solutions (1 mL) containing 6.6 mmol/L 
sodium taurocholate, 0.5 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 
mmol/L monolein, 0.6 mmol/L phosphatidylcholine, 132 
mmol/L NaCl, 15 mmol/L sodium phosphate (pH 7.4), CTH, SPH or 
SPHP (5.0 g/L) were prepared by sonication. Then the mixture was 

were measured by liquid scintillation counting.

Experiment 2: Micellar solubility of cholesterol and tauro-
cholate. Micellar solubility of cholesterol with various proteins in 
vitro was measured by the method of Ikeda et al. (1988) with some 
modifications. Micellar solutions (1 mL) containing 6.6 mmol/L 
sodium taurocholate, 0.5 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 
mmol/L monolein, 0.6 mmol/L phosphatidylcholine, 132 
mmol/L NaCl, 15 mmol/L sodium phosphate (pH 7.4), CTH, SPH or 
SPHP (5.0 g/L) were prepared by sonication. Then the mixture was 

were measured by the method described previously (Nagaoka et al. 1997).

The final concentration of each [14C]-labeled micellar solution (0.5 mL) was as follows: 3.7 kBq [4,14C]-cholesterol (2.1 Gbq/mmol, 
LEN, Boston, MA), 0.1 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 
mmol/L monolein, 6.6 mmol/L sodium taurocholate, 0.6 mmol/L 
phosphatidylcholine, and CTH, SPH or SPHP (2.5 mg/0.5 mL). The 
micellar solution was mixed by ultrasonic vibration.

After 14 d, the cells were rinsed two times with 1 mL of PBS. 
[14C]-labeled micellar solution (0.5 mL) containing CTH, SPH or 
SPHP was then added to the dishes, which were incubated at 37°C 
for 20 min in a CO2 incubator. After this incubation, the cells were 
rinsed two times with 1 mL of PBS. The cells were finally lysed in 
0.1% SDS solution; then 7.5 mL of Aquasol-2 (NEN) was added, and 
the radioactivity in the cellular debris was counted to determine the 
amount of cholesterol associated with the cells.

Experiment 4: Cholesterol absorption in vivo. After acclimation 
to a commercial nonpurified diet (MF, Oriental Yeast, Osaka, Japan), 
for 3 d, 8-wk-old rats weighing 176–205 g were deprived of food 
for 48 h with free access to water. Casein tryptic hydrolysate (CTH) was 
provided by Meiji Milk Products. The chemical composition of CTH 
was as follows (g/kg): protein, 862; ash, 47; moisture, 91; lipid, 0; 
sugar, 0. Rats received the test solutions by intragastric intubation 
with the use of a polyethylene catheter. Rats were anesthetized with 
diethyl ether and killed 1 h after the administration of the test 
solutions. Blood was collected by cardiac puncture for the separation of 
serum. The liver and intestine were excised quickly. The liver was 
rinsed with ice-cold saline, and the luminal contents of the small 
intestine were removed by flushing with ice-cold saline. The test 
solutions consisted of 1 mmol/L monolein (Sigma, St. Louis, MO), 
5 mmol/L taurocholic acid (Sigma), 37 kBq [1,2-3H]-cholesterol 
(1972.1 Gbq/mmol, NEN) and CTH, soy protein, SPH or SPHP 
(62.5 mg) in 1 mL of 15 mmol/L phosphate buffer (pH 7.4). These 
solutions were emulsified by sonication (Ultrasonic Homogenizer, 
Model VP-5, Taitec, Saitama, Japan). [3H]-Cholesterol incorporated 
into the serum, liver and intestine was extracted with hexane after 
saponification of KOH-ethanol as described previously (Borel et al. 1990). Aliquots of the organic extract were used for scintillation 
counting.

Experiment 5: Lipid metabolism in rats fed casein, soy protein, 
SP, SPH or SPHP. After acclimation to a commercial nonpurified 
diet (MF, Oriental Yeast), 5-wk-old rats weighing 115–130 g were 
divided into five groups of six rats each on the basis of body weight. 
Each group had free access to one of the test diets (Table 3) con-
taining casein, soy protein, SP, SPH or SPHP as the protein source 
for 10 d. After 24 h without food, the rats were anesthetized with diethyl

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Casein</th>
<th>Soy protein</th>
<th>SP</th>
<th>SPH</th>
<th>SPHP</th>
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<td>g/kg diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>237.02</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Soy protein</td>
<td>231.75</td>
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<td>—</td>
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<tr>
<td>SP</td>
<td></td>
<td>—</td>
<td>278.17</td>
<td>—</td>
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<tr>
<td>SPH</td>
<td></td>
<td>—</td>
<td></td>
<td>244.20</td>
<td>—</td>
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<td>—</td>
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<td>—</td>
<td>323.10</td>
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<tr>
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<td>5</td>
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<tr>
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</tbody>
</table>

1 See Table 1 for abbreviations.
2 AIN-76 diet (AIN 1977).
FIGURE 1 Binding of taurocholate to cholestyramine, casein tryptic hydrolysate (CTH), soy protein peptic hydrolysate (SPH) or soy protein peptic hydrolysate with bound phospholipids (SPHP) in vitro. Individual values represent means of assays performed in duplicate. Error bars (SEM) are too small to show. From 25 to 300 mg, SPHP was significantly greater than SPH and CTH, \( P < 0.05 \).

RESULTS

Experiment 1: Taurocholate binding capacities of CTH, SPH and SPHP measured in vitro. From 25 to 300 mg, the bile acid–binding capacity of SPHP was significantly higher than that of CTH or SPH, indicating that SPHP has the highest bile acid–binding capacity among the protein hydrolysates tested (Fig. 1).

Experiment 2: Micellar solubility of cholesterol and taurocholate. We used cholestyramine as a standard for the micellar solubility of cholesterol in preliminary test (in mmol/L: cholestyramine, 0.02 ± 0.001; SPH, 0.18 ± 0.02). The micellar solubility of cholesterol was significantly less in the presence of cholestyramine compared with SPH. As shown in Table 4, the micellar solubility of cholesterol was significantly lower in the presence of SPH or CTH than CTH or soy protein. In contrast, no significant effects of protein hydrolysates on bile acid solubility were observed.

Experiment 3: Cholesterol uptake in Caco-2 cells in vitro. Cholesterol uptake from micelles containing SPH (5.70 ± 0.34 pmol/well) or SPHP (4.87 ± 0.21 pmol/well) was significantly lower than that from cholesterol micelles containing casein, CTH or soy protein (9.85 ± 0.97, 9.50 ± 0.87, 8.12 ± 0.82 pmol/well, respectively).

Experiment 4: Cholesterol absorption in rats infused with SPHP, SPH, soy protein or CTH. Final body weights (CTH, 170.0 ± 2.0 g; soy protein, 170.7 ± 2.1 g; SPH, 170.5 ± 2.5 g; SPHP, 170.3 ± 2.0 g) and relative liver weights (in g/100 g body weight: CTH, 2.88 ± 0.03; soy protein, 2.85 ± 0.05; SPH, 2.87 ± 0.02; SPHP, 2.85 ± 0.02) were unaffected by treatments. The incorporation of \(^{3}\)H-cholesterol into the serum, liver and intestine was significantly lower in the SPH and SPHP groups than in the CTH and soy protein groups (Table 5).

Experiment 5: Lipid metabolism in rats fed SPHP, SPH, SP, soy protein or casein. Food intake and body weight gains were unaffected by dietary treatment, but the relative liver weight was significantly greater in the casein-fed group than in all other groups (Table 6). Serum total cholesterol levels in the SP, SPH and SPHP groups were significantly lower than in the casein group. Serum HDL cholesterol concentration and the ratio of HDL cholesterol to total cholesterol in groups fed soy protein or its hydrolysates were significantly higher than in the casein-fed group. Serum triglyceride and phospholipid concentrations were unaffected by dietary treatments. Liver total lipid and cholesterol concentrations were significantly lower in all soy protein–fed groups than in the casein-fed group. Fecal dry weight was significantly higher in the SPHP and SPHP-fed groups than in the casein-fed group. The fecal outputs of acidic and total steroids were significantly higher in the SPHP group than in all other groups.

DISCUSSION

By binding phospholipid to soy protein or SPH, its cholesterol-lowering activity was promoted prominently as shown in the effects of SP or SPHP. Sirtori et al. (1985) reported that textured soybean protein containing 6% lecithin increased the effects of SP or SPHP. Sirtori et al. (1985) reported that textured soybean protein containing 6% lecithin increased HDL cholesterol levels in type-II hyperlipidemic patients. We
found that SP and SPHP had higher phospholipid concentrations (~20%) than those reported by Sirtori et al. (1985). In particular, SPHP clearly demonstrated serum cholesterol-lowering effects. In these studies, soy protein and soy phospholipids were added in the ratio of 4:1 (wt/wt). By increasing the content of soy phospholipids in SPHP, the level of unbound phospholipids was increased. SPHP, rich in unbound phospholipids, is oxidized more readily than SPHP that are rich in bound phospholipids. Thus, this ratio (4:1) is appropriate for identifying the active components affecting the cholesterol absorption. Also, the extent of the effect of casein on serum cholesterol level (Nagaoka et al. 1997) or on cholesterol uptake in Caco-2 cells in this study is almost the same as that of CTH. Thus, we used the CTH group instead of the casein group as a control group in Experiments 1, 2 and 4.

In recent studies of lipid metabolism, monolayers of Caco-2 cell cultures have been used as a model system (Field et al. 1987, Hughes et al. 1987, Ranheim et al. 1992). For example, Field et al. (1987) reported that Caco-2 cells, like the small intestine, have the ability to absorb micellar cholesterol and to express marker enzymes like alkaline phosphatase, as in small intestinal epithelial cells. However, there have been few experimental studies to date to evaluate any effects of peptides on cholesterol uptake by using cultured intestinal cells. In this study, we found that SPHP also directly inhibited the uptake of micellar cholesterol in Caco-2 cells in vitro. These results suggest that the suppression of cholesterol absorption by direct interaction between cholesterol-mixed micelles and SPHP in the intestinal epithelia is part of the mechanism of the hypcholesterolemic action of SPHP.

Cholesterol is rendered soluble in bile salt–mixed micelles and then absorbed (Wilson and Rudel 1994). We have found for the first time that micellar solubility of cholesterol in the presence of SPH or SPHP was significantly lower than with CTH. Sitosterol (Ikeda et al. 1988), sesamin (Hirose et al. 1991) or catechin (Ikeda et al. 1992) also lowered the micellar solubility of cholesterol, in conjunction with the serum cholesterol-lowering effects in rats. These findings suggest that suppression of the 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 SPH and SHPH, some other dietary proteins or peptides may also affect such solubility. Although the micellar solubility of cholesterol in the presence of SPH and SHPH was almost the same, fecal excretion of acidic steroids was significantly greater in rats fed the SHPH diet compared with those fed the SPH diet. Iwami et al. (1986) showed a correlation between the hydrophobicity of a protein hydrolysate and its binding capacity to bile acids and suggested that a peptide with a high bile acid–binding capacity could inhibit the reabsorption of bile acids in the ileum and decrease the blood cholesterol level. In our feeding study, SHPH had a significantly greater bile acid–binding capacity than did SPH, concomitant with a greater increase in fecal bile acid excretion. Thus, the difference in the degree of hypocholesterolemic action between SPH and SHPH may result from the differences in their bile acid–binding capacities.

In summary, there have been many studies on the hypocholesterolemic effects of proteins, most of which emphasized 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. These possibilities may be applicable to the case of SHPH on the basis of the evidence of fecal bile acid excretion and bile acid–binding capacity in this study. However, our earlier study (Nagaoka et al. 1997) and this study clearly suggest that the inhibition of micellar solubility of cholesterol, which causes the suppression of cholesterol absorption by direct interaction between cholesterol–mixed micelles and SPH in the jejunal epithelia, is part of the mechanism of hypocholesterolemic action induced by SHPH. Thus, the hypocholesterolemic action of SPH may involve both jejunal and ileal effects, as shown in this study.

Our experimental system to evaluate cholesterol uptake in Caco-2 cells is useful for clarifying both the molecular mechanism and active components underlying the inhibitory effect of soy protein or other proteins on cholesterol absorption from the small intestine; it can also be expected to facilitate greatly an elucidation of the effects of various food constituents on cholesterol absorption in the future.

LITERATURE CITED


