Evidence for the Existence of a Soybean Resistant Protein That Captures Bile Acid and Stimulates Its Fecal Excretion

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Feeding HMF, an insoluble “high-molecular-weight fraction” from an industrial enzymatic digest of a soy protein isolate, increased the fecal excretion of bile acid concomitant with increased fecal nitrogen. An amino acid analysis revealed that this increased fecal nitrogen could be explained by an increase in the insoluble protein fraction. This suggests the existence of an indigestible protein or peptide that can be called a “resistant protein” in the feces. The presumed resistant protein was rich in hydrophobic amino acids and bound bile acid by hydrophobic interaction. The residual fraction of HMF obtained after in vitro pepsin and pancreatin digestion, showed higher in vitro bile acid-binding capacity and excreted more bile acid in vivo than HMF. Its amino acid composition was similar to that of the feces of rat fed with HMF. These results suggest that the fecal resistant protein with bile acid-binding ability could be derived from the indigestible fraction of HMF.

Key words: resistant protein; soybean; bile acid; enterohepatic circulation; feces

Soybean protein is widely known to have a cholesterol-lowering effect in human and animal models and may thus reduce the risk of atherosclerosis. Although the mechanism for this effect is not yet fully understood, there is evidence that either soy protein itself, its constituent amino acids, a non-protein constituent or a combination of these factors is involved in the cholesterol-lowering effect of soybean (see reviews1,2). Sugano and his coworkers digested a soy protein isolate in vitro by microbial proteases and prepared soluble and residual fractions, these being referred to as the low-molecular fraction (LMF) and high-molecular fraction (HMF), respectively. They examined their cholesterol-lowering effect on an animal model9–11 as well as on human volunteers12 and found that HMF caused higher fecal excretion of the steroids and consequently had a cholesterol-lowering effect in the serum than the intact soy protein isolate. They speculated from the results that specific peptide fragments rich in HMF would exert this cholesterol-lowering effect through interference with the steroid-absorption process.

On the other hand, we have found that feeding HMF suppressed the colon and liver carcinogenesis induced by deoxycholate.13–15) A nitrogen balance study indicated that nitrogen equivalent to about one fourth of HMF in the diet was excreted into the feces with an increased amount of fecal bile acids. On the basis of these observations, we hypothesized that a remnant of HMF which was no longer digestible remained in the intestines and thus could be called a “resistant protein.”13,16–19) This remnant might capture bile acids in the intestines and not only stimulate their fecal excretion, but also mask the promoter activity of deoxycholate in the colon. The increased fecal excretion of bile acids also reduced their serum concentration and sup-

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Abbreviations: CHAPS, 3-cyclohexylamino propane sulfonic acid; DTT, dithiothreitol; HMF, high-molecular-weight fraction; RF-HMF, residual fraction of HMF
pressed liver carcinogenesis. If such a “resistant protein” were to exist, it might be excreted into the feces with bile acids. However, there is no available data on the occurrence and characteristics of such a resistant protein in the feces of animals fed with HMF. The purpose of the present study is to confirm the existence and to characterize the resistant protein derived from soybean protein in the feces of rats fed with HMF.

**Experimental**

**Materials.** HMF was presented by Applied Research Institute of Fuji Oil Co. (Osaka, Japan). It has been prepared from SPI, Fujipro-R, in the same manner as that previously described and was supplied as freeze-dried powder at our request. The composition of HMF was 66.5% protein, 4.8% ash, 2.6% moisture and the rest was lipids and sugar. RF-HMF was prepared from HMF by in vitro digestion with pepsin and pancreatin as described later. The mineral and vitamin mixtures (Oriental formula, the compositions are described elsewhere) were products from Oriental Yeast Co. (Tokyo, Japan). Pepsin and pancreatin were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich Fine Chemicals (MO, USA), respectively. Total Bile Acid-Test Wako was a kit from Wako Pure Chemical Co. (Osaka, Japan).

**Preparation of RF-HMF by in vitro digestion of HMF with pepsin and pancreatin.** HMF was suspended in 20 volumes of 0.01 M HCl and digested by pepsin, which with pepsin and pancreatin. The mixture was centrifuged at 10,000 x g for 15 min at room temperature. The precipitate was washed once with water and extracted with ethanol, sonicated and then centrifuged as just described. The yield of indigested residue was about 30% in nitrogen content and is referred as to RF-HMF.

**Animals and feeding.** Male Fischer-344 rats (6 months old) were purchased from Nihon SLC (Hamamatsu, Japan). They were housed individually in metabolic cages in a temperature-controlled room (23 ± 1 °C) with a 12-hr light/dark cycle and allowed free access to the diet and water.

In experiment 1, 4 rats were given a casein diet for 1 week and then an HMF diet for the next 1 week. The compositions of these diets are given in Table 1. Feces were collected from each rat daily at 10:00 a.m. and stored at −80 °C until needed for analysis.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Casein</th>
<th>HMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>14.6</td>
<td>—</td>
</tr>
<tr>
<td>HMF</td>
<td>—</td>
<td>20.6</td>
</tr>
<tr>
<td>Pt-methionine</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>α-cornstarch</td>
<td>69.0</td>
<td>68.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>5.0</td>
<td>—</td>
</tr>
</tbody>
</table>

In experiment 2, 16 rats were used. They were each fed with the casein diet for 1 week, and then divided into 4 groups (n = 4). One group continued to receive the casein diet as a control, and the other 3 groups were fed the test diets shown in Table 2 for 1 week. Feces were collected as already described.

This experimental design was approved by the Animal Experiment Committee of Kyoto Prefectural University, and the animals were cared for according to the Guidelines for Animal Care and Use.

**Fractionation of peptide in the feces.** Feces from the rats fed on the experimental diets were dried under vacuum and milled. Fecal peptide was serially extracted as shown in Scheme 1. The extraction at each step was conducted twice, and the supernatants obtained from the respective steps were combined. Briefly, one gram of powdered feces was suspended in 25 ml of a 5 mM sodium phosphate buffer at pH 7.4, sonicated for 1 min and then centrifuged at 10,000 x g for 10 min at 20 °C. This supernatant is referred as to the water-soluble fraction. The precipitate was suspended in 25 ml of 50% ethanol, sonicated and then centrifuged as just described. The respective supernatants were collected and are referred to as the ethanol-soluble fraction. The precipitate was washed once with water and extracted with 25 ml of a lysis buffer consisting of 8 mM urea, 1% Triton X-100, 1% CHAPS, 65 mM DTT and 40 mM Tris-base as already described. The final residue was successively washed with water (twice) and methanol (twice), and then air-dried. The dried powder was defatted with ethyl
The precipitate was washed twice with the 5 mM sodium vapor by the method of Bidlingmeyer et al. for 3 min and then centrifuged at phosphate buffer containing sodium deoxycholate, stood hydrolyzed in a glass tube (6 mM used for an amino acid analysis. 0.1% TFA. The eluent and the internal solution were bound materials were eluted with 80% acetonitrile in methanol and equilibrated with 0.1% TFA, and the column (Waters) that had been pre-washed with 50% solution (50 ml) was loaded into a Sep-Pack C 18 3,000 (Spectrum Company, CA, USA). The external dialyzed against water with a Spectra/Por MW CO: insoluble fraction. The lysis buffer-soluble fraction was ether, using a Soxhlet extractor, and is referred to as the insoluble fraction. The lysis buffer-soluble fraction was dialyzed against water with a Spectra/Por MW CO: 3,000 (Spectrum Company, CA, USA). The external solution (50 ml) was loaded into a Sep-Pack C 18 column (Waters) that had been pre-washed with 50% methanol and equilibrated with 0.1% TFA, and the bound materials were eluted with 80% acetonitrile in 0.1% TFA. The eluent and the internal solution were used for an amino acid analysis.

Amino acid analysis. The soluble samples were hydrolyzed in a glass tube (6 mm x 5 cm) with HCl vapor by the method of Bidlingmeyer et al.20 The insoluble samples were hydrolyzed in 6 M HCl in a liquid phase at 150°C for 1 h. An amino acid analysis was performed according to the method of Bidlingmeyer et al.20 with a slight modification.21 The hydrophilicity of each fraction was calculated on the basis of the amino acid composition by the method of Parker et al.22

Determination of the nitrogen content in the feces and each diet. The nitrogen content in the feces and each diet were determined by the micro-Kjeldahl method. Feces were freeze-dried and ground to a powder, and a portion of 0.1 g was used.

Determination of fecal total bile acid. The fecal bile acid was extracted according to the method of Beher et al.23 and determined by the enzymatic procedure with commercially available enzymatic assay kits.13

Determination of the bile acid binding capacity of the defatted fecal insoluble fraction, HMF and RF-HMF. A powdered dried sample was suspended in a 5 mM phosphate buffer containing sodium deoxycholate, stood for 3 min and then centrifuged at 10,000 x g for 3 min. The precipitate was washed twice with the 5 mM sodium phosphate buffer at pH 7.4. The deoxycholic acid adsorbed to the precipitate was extracted with 1 ml of 75% ethanol. The resulting ethanol extract was dried in a 1.5 ml-centrifugation tube and then re-dissolved in the phosphate buffer. The amount of deoxycholic acid was determined by the enzymatic assay already described.

Statistical analysis. Each result is expressed as the mean ± SD, the statistical difference being evaluated either by a paired t-test or by Tukey’s test after one-way ANOVA. The difference is considered significant at p < 0.05.

Results

In experiment 1, four rats of 6 months old were fed the casein diet for the first one week. The feed was then changed to the HMF diet and the rats were kept for another one week. The body weight and daily food intake did not fluctuate significantly throughout the experiment whether they were given the casein or HMF diet (Table 3).

Figure 1 shows changes in the daily excretion of nitrogen, total amino acid and bile acid into the feces after the dietary change. The fecal nitrogen and total amount of amino acid that was released from the feces by acid hydrolysis began to increase markedly and reached a plateau on day 4. A similar change was observed for the fecal bile acid excretion. There was significant correlation between the fecal nitrogen and amino acid, and between the amino acid and bile acid (Fig. 2).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Food intake&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Casein</td>
<td>391 ± 9</td>
<td>400 ± 8</td>
</tr>
<tr>
<td>HMF</td>
<td>400 ± 8</td>
<td>402 ± 11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Statistical difference between initial and final body weight of rats fed with the casein or HMF diet was evaluated by a paired t-test.

<sup>b</sup>Statistical difference of food intake of rats fed with the casein or HMF diet was evaluated by Tukey’s test after one-way ANOVA.

Table 3. Body Weight Change and Daily Food Intake of Rats While Being Fed with the Casein and HMF Diets for 1 Week

![Scheme 1](image-url) Extraction of Resistant Proteins from Feces.
The apparent hydrophilicity of the presumed peptides in the respective fraction was based on their amino acid composition according to the equation proposed by Parker et al.\textsuperscript{22} Although the hydrophilicity parameters of both dietary proteins were no different (data not shown), the parameters of the feces and of the respective fraction from the HMF-fed rats were much lower than those from the casein-fed rats (Fig. 3). It is noteworthy that the parameter for the insoluble fraction of the rats fed with HMF became negative, indicating the highest hydrophobicity. The amino acid compositions of HMF itself, of the feces and of the insoluble

![Fig. 1](image1.png)

**Fig. 1.** Changes in the Fecal Excretion of Nitrogen, Amino Acid, and Bile Acid after Rats Had Been Fed with the HMF Diet.

Feces from each of four rats were collected at the day indicated, and the nitrogen content of each was determined. The rest of the daily feces were combined, and contents of amino acid and bile acid were determined as described in the experimental section. The nitrogen content is expressed as the mean ± SD, and different letters show significant difference at $P < 0.05$.

![Fig. 2](image2.png)

**Fig. 2.** Correlation between the Fecal Excretion of Nitrogen and Amino Acid (upper panel), and between Amino Acid and Bile Acid (lower panel).

Data were taken from Fig. 1.

![Fig. 3](image3.png)

**Fig. 3.** Apparent Hydrophilicity of Fecal Peptides in the Respective Fraction of the Fecal Extracts (see Scheme 1) from the Rats Fed with the Casein (unfilled bar) or HMF (filled bar) Diet.

The hydrophilicity parameter was determined from the amino acid composition as described in the experimental section.
fraction are shown in Fig. 4. The contents of such hydrophilic amino acids as Asx, Glx and Ser decreased, while such hydrophobic amino acids as Val, Leu and Ile increased in the order of HMF, feces and the insoluble fraction.

When fecal bile acids were sequentially extracted by a 5 mM phosphate buffer and then by 75% EtOH, more than 80% of bile acid in the feces of the casein-fed rats was extractable by the 5 mM phosphate buffer, while about 70% of bile acid in the feces of rats fed with HMF was recovered by EtOH extraction (Fig. 5A). On the other hand, the bile acid-binding capacity, which was estimated by a deoxycholic acid binding assay in vitro, of the defatted-insoluble fraction prepared from the feces of the HMF-fed rats was more than 3-fold higher than that of the casein-fed rats (Fig. 5B).

To simulate the formation of the indigestible peptides in the digestive tract, HMF was exclusively treated with pepsin and then by pancreatin to obtain the indigestive material as a residual fraction of HMF (RF-HMF). Figure 6 shows a comparison of the deoxycholate-binding ability of RF-HMF and HMF in vitro. The amount of RF-HMF required to precipitate 50% of deoxycholicacid was 20 mg (Fig. 6A), while that of HMF was 60 mg (Fig. 6B). Thus, the binding ability of RF-HMF was three times higher than that of HMF.

Fig. 4. Comparison of the Amino Acid Composition of HMF Itself (unfilled bar), Feces (hatched bar) and Insoluble Fraction of Feces (filled bar) from Rats Fed with the HMF Diet for 1 Week.

Fig. 5. Binding of the Insoluble Fraction of Feces from the HMF-Fed Rat with Bile Acid by Hydrophobic Interaction.
A, Bile acid was extracted from the feces of rats fed with the casein or HMF diet for 1 week with a 5 mM phosphate buffer (unfilled bar) and then by 75% EtOH (filled bar). B, 40 mg of the defatted insoluble fraction from the feces was suspended in 1 ml of a 5 mM phosphate buffer containing 2 mM sodium deoxycholate, and the bile acid-binding capacity was determined as described in the experimental section.
In experiment 2, we examined the effectiveness of RF-HMF for the fecal excretion of bile acids from the rats. Each group of four rats aged 6 months was fed with either the casein, HMF, 5% RF-HMF or 10% RF-HMF diet. The compositions of these experimental diets are shown in Table 2. The body weight and food intake of the respective groups did not change significantly during the experiment, and no significant difference was apparent among the four groups (Table 4).

As with the HMF diet, the fecal excretion of bile acids by the rats fed with RF-HMF increased as with increasing fecal nitrogen content. However, the 10% RF-HMF diet was enough to simulate the excretion of bile acids to the level by that of the 20% HMF diet (Fig. 7). In addition, the amino acid composition of RF-HMF was more like the fecal amino acids of the rats fed with the HMF or RF-HMF diet rather than with the original HMF (Fig. 8).

**Discussion**

We used 6-month-old rats in the present study to avoid the effects of growth and food intake during the experiment, since rats of this age have already matured and their growth has stopped. Moreover, the protein requirement of a mature rat is lower than that of a growing rat, and thus they can maintain a nitrogen balance as low as that provided by a 5% casein diet (unpublished observation). On the other hand, we have previously demonstrated by a nitrogen balance study that the nutritive value of HMF was less than that of casein, since about 1/4 of HMF could not be utilized by the rats and was excreted into the feces. We therefore considered this indigestible portion of HMF as dietary fiber, and the content of HMF in the diet was therefore increased by 5% compared to that of casein, and cellulose powder was omitted from the diet (Table 1). Since RF-HMF was the indigestible residue obtained after in vitro digestion of HMF by pepsin and pancreatin, the in vivo digestibility of RF-HMF was speculated to be less than that of HMF itself. We therefore fed RF-HMF to the rats with an appropriate amount of casein in the diet in which cellulose powder had also been omitted (Table 2). The results of experiments 1 and 2 show that the body weight as well as the food intake did not change significantly throughout each experimental period and were no different among the dietary groups (Tables 3 and 4).

In experiment 1, we confirmed for mature rats that feeding HMF stimulated the fecal excretion of bile acid as reported previously and demonstrated that the increase of fecal bile acid was accompanied by an increase in fecal nitrogen excretion (Fig. 1). There was good correlation between the amount of amino acid and bile acid in the feces (Fig. 2). The amino acid analysis strongly suggested that most of the fecal nitrogen was derived from a peptide that had higher hydrophobic capability than HMF itself (Fig. 3). In addition, most bile acid in the feces of the rats fed with HMF could be

![Fig. 6. Comparison of the Bile Acid-Binding Capacity of RF-HMF (A) and HMF (B) in Vitro.](image)
extracted by 75% EtOH (Fig. 5A) which suggests that the bile acid was adsorbed to the feces by hydrophobic interaction.

It has been demonstrated that the bile acid-binding capacity of a peptide was closely related to its hydrophobic nature and that a peptide showing higher bile acid-binding capacity exerted a stronger hypocholesterolemic action with the increase of fecal bile acid excretion.\(^3\)\(^-\)\(^5\),\(^10\) Such a bile acid-binding peptide has been identified in the soy glycinin subunit \(^2\) and in a beta-lactoglobulin tryptic hydrolysate.\(^8\) On the basis of these findings, we speculated the presence of a bile acid-binding peptide in the feces of rats fed with HMF. This peptide may resist intestinal digestion and bind bile acid by hydrophobic interaction, and then be excreted into the feces with bile acid.

To isolate and identify the hypothetical peptides, feces were fractionated by serial extraction. However, only a negligible amount of soluble peptides was present in the feces, and most of the increased fecal peptides by feeding HMF was suggested to be an insoluble form. Unexpectedly, we could not render soluble the peptide from the insoluble fraction, even in a sodium hydroxide, guanidine hydrochloride or SDS solution. On the other hand, the amino acid analysis (Figs. 3 and 4) and in vitro bile acid-binding assay (Fig. 5B) strongly suggested the presence in the insoluble fraction of a hydrophobic peptide that had bile acid-binding ability.

As shown in experiment 2, feeding RF-HMF increased the fecal nitrogen and bile acid excretion with a smaller dose than with HMF itself (Fig. 7). Furthermore, RF-HMF showed a similar amino acid composition to that of the insoluble fraction of the feces of the HMF-fed rats (Fig. 8) and had higher deoxycholate-binding capacity than HMF did (Fig. 6). It can be considered from the results that a portion of HMF, which resisted in vivo digestion and had bile acid-binding ability, was concentrated in RF-HMF by in vitro digestion with pepsin and pancreatin.

Taken together these observations, it is suggested that

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**Fig. 7.** Effect of the 10% and 5% RF-HMF Diets on the Excretion of Fecal Nitrogen (A) and Bile Acid (B).

Feces from four rats were collected at the day indicated. The feces were combined, and then the nitrogen content and bile acid were determined as described in the Experimental section.

**Fig. 8.** Amino Acid Composition of HMF (filled bar) and RF-HMF (unfilled bar), and of Feces from Rats Fed with the 20% HMF (slashed bar) or 10% RF-HMF (dotted bar) for 1 Week.

Feces were collected on day 6.
the peptide in the insoluble fraction could have been the hypothetical indigestible peptide derived from HMF. According to the recent proposals of Kato and Iwami\(^{18}\) and of Morita et al.,\(^{19}\) the hypothetical peptide can be called a soybean resistant protein.

Since secondary bile acid is an intrinsic promoter of colon carcinogenesis,\(^{20}\) it is possible that feeding HMF might increase colon cancer. In fact, it has been reported that an oral intake of cholestryamine, an anion-exchange resin used as a hypocholesterolemic drug, increased fecal bile acid excretion and the incidence of colon cancer.\(^{20,21}\) We, however, have previously reported that feeding HMF suppressed the colon carcinogenesis induced by azoxymethane and dietary deoxycholate in rats.\(^{13–15}\) Though the reason for this difference is not clear at present, it can be considered that the resistant protein not only interfered with the reabsorption of bile acid in the terminal ileum, but also captured bile acid in the intestines by hydrophobic binding and thus might have prevented the bile acid or its active site from contact with the colonic epithelium.

As already mentioned, several trials to render soluble the HMF-derived resistant protein in the feces failed. In addition, we also tried to obtain the peptide fragment of the resistant protein by digestion with several proteases. However, it was also resistant to bacterial, plant and mammalian proteases such as pepsin, pancreatin, pro-nase E, lysyl endopeptidase, leucine aminopeptidase, prolidase, carboxypeptidases A and W. On the other hand, a soy protein isolate can be rendered soluble and digested into soluble peptides and amino acid. Therefore, it is possible that the indigestibility of the resistant protein might have been induced enzymatically or chemically during the industrial preparation of the soy peptide.

A resistant protein is defined as a protein or peptide that is unsusceptible to mammalian digestive enzymes in the intestines. However, Morita et al. have demonstrated the possibility that resistant proteins provided the nitrogen source for an enterobacterium and altered the bacterial activity as well as the intestinal flora, before regulating the bacterial fermentation of dietary fibers in the colon.\(^{71}\) On the other hand, it seems likely that the indigestibility already mentioned protected the soybean resistant protein from not only intestinal digestion but also from bacterial degradation and endowed the resistant protein with its beneficial effect in the colon.

Considering the variety of chemical properties of amino acid residues, it can be expected that resistant proteins would play a role to eliminate undesired material from the digestive tract by the such actions as anti-oxidant, chelating, and ionic, hydrophilic and hydrophobic interaction if we could introduce indigestibility into the desired portion of a dietary protein. Further studies are therefore required to clarify the function-structure relationship of the soybean resistant protein.

### Acknowledgment

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