Feeding Soybean Resistant Protein to Rats Raises Fecal Bile Acid Excretion but Counteracts a Deoxycholate-Caused Decrease in Colonic Aberrant Crypt Foci

Naoyuki AZUMA, Masahiro KANAYA, Ryuhei KANAMOTO and Kimikazu IWAMI*

Department of Biological Resource Chemistry, Kyoto Prefectural University, Kyoto 606-8522, Japan

(Received September 25, 1998)

Summary A high-molecular-weight fraction after removal of water-soluble peptides from proteinase-treated soybean protein isolate (referred to as HMF) was examined for its effect on preneoplastic lesions in the rat colon. For this purpose, male Fisher-344 rats 7 wk old were divided into 8 groups (n=5), of which 6 groups received 3 injections of azoxymethane (AOM, 15mg/kg of body weight) for 3 wk once a week, while all were fed HMF or casein diets supplemented with or without deoxycholic acid (DCA) over a period of 4 wk. Two groups of AOM-treated rats were allowed free access to HMF or casein diets without supplemental DCA, respectively, while the others were pair-fed so as to be well matched in their food intake. There were no significant differences in growth parameters among the pair-fed groups. Feeding HMF diets raised fecal lipid and acidic steroid excretions to a greater extent than feeding casein diets, secondary bile acids being conspicuous among acidic steroids in the excreta irrespective of the presence or absence of DCA supplementation. As a result of observation for colonic aberrant crypt foci (ACF), the intake of HMF proved to reverse the reduction of ACF appearance by DCA. This result implies that secondary bile acids are caught and brought out by HMF, or rather its derivative "resistant protein," so as not to keep contact with colonic mucosae.

Key Words high-molecular-weight fraction, deoxycholic acid, azoxymethane, aberrant crypt foci, resistant protein

*To whom correspondence should be addressed.

Abbreviations: SPI, soybean protein isolate; HMF, an insoluble high-molecular-weight fraction from digestive products of SPI with microbial proteinases; AOM, azoxymethane; DCA, deoxycholic acid; ACF, aberrant crypt foci; AC, aberrant crypts.
Dietary soybean protein is hypocholesterolemic compared with milk casein (1). There are conflicting explanations for the mode of action. The more acceptable explanation is that intraluminal soybean protein, as such or in fragments, disturbs the intestinal absorption of neutral and acidic steroids, and elevates their excretion into the feces. The "high-molecular-weight fraction (HMF)" remaining insoluble after the digestion of soybean protein isolate (SPI) by the use of microbial exo-type proteinases was more effective in suppressing a rise in the serum cholesterol level when used as the sole nitrogen source instead of SPI for rat growth (2, 3). The daily amount of fecal steroid excretion was larger in rats fed HMF than in rats fed SPI. HMF surpassed soybean protein itself in bile acid-binding capacity in vitro, although being far inferior to cholestyramine, which had been previously used as a remedy for cholesterolemia (4). Cholestyramine capable of binding many bile acids changed not only the total amount of bile acids excreted but also their individual content ratio in the feces of rats provided with it (5).

Experimentally induced colonic tumors grew more in rats receiving cholestyramine at a 2% level of the diet than in ones not receiving it (6). Taking into account the risk that secondary bile acids may promote carcinogenesis in the colon (7–9), further investigation is required for the application of HMF products as hypocholesterolemic provisions. The purpose of this investigation was to assess the pro- or anti-tumorigenic effect of HMF given to azoxymethane (AOM)-treated rats with the intervention of dietary deoxycholic acid (DCA).

**MATERIALS AND METHODS**

**Materials.** HMF was a gift from Applied Research Institute, Fuji Oil Co., Osaka, Japan. It was prepared from SPI, Fujipro-R, in the same manner as previously described (4) and supplied as freeze-dried powder upon our request. Mineral and vitamin mixtures (AIN-76 likeness, but the vitamin premix fortified with choline chloride) were products of Oriental Yeast, Tokyo, Japan. Other chemicals used were commercially available, of analytical grade and used without further purification.

**Animals and feeding.** This experimental design was approved by the Animal Experiment Committee of Kyoto Prefectural University in line with the Guidelines Concerning the Care and Use of Laboratory Animals. Young male Fischer-344 rats were purchased from Japan Clea, Osaka, Japan and housed in our animal care facility (air-conditioned at 25°C with a 12 h light/dark cycle).

Forty Fischer-344 rats weighing 150–160 g were divided into 8 groups \((n = 5)\) as shown in Table 1 of which A–F groups were pair-fed for 4 wk by adjusting the daily average food intake to the minimum intake on the preceding day (usually for group C or D) because AOM and DCA caused a decrease in appetite. Thirty rats of groups C–H were intraperitoneally injected with AOM (15 mg per kg of body weight) at 0, 1 and 2 wk, while the 10 rats of groups A and B were treated with saline in a similar fashion. The feces were collected for 4 d before the end of
Table 1. Composition of experimental diets.

| Dietary composition for each group (g/100g diet) |
|-----------------------------------------------|
| A    | B    | C    | D    | E    | F    | G    | H    |
| Casein\(^1\) (+4.0\% L-Arg)\(^3\) | 20   | –    | 20   | –    | 20   | –    | –    |
| HMF\(^2\) (+0.7\% DL-Met)\(^3\)   | –    | 26   | –    | 26   | –    | 26   | –    |
| α-Corn starch\(^4\)                     | 64.8 | 58.8 | 64.8 | 58.8 | 65   | 59   | 65   |
| Soybean oil\(^2\)                        | 5    | 5    | 5    | 5    | 5    | 5    |
| Mineral mixture\(^1\)                    | 5    | 5    | 5    | 5    | 5    | 5    |
| Vitamin mixture\(^1\)                    | 1    | 1    | 1    | 1    | 1    | 1    |
| Cellulose powder\(^1\)                   | 4    | 4    | 4    | 4    | 4    | 4    |
| Sodium deoxycholate\(^5\)                | 0.2  | 0.2  | 0.2  | 0.2  | –    | –    |
| Azoxymethane\(^5\)                       | –    | –    | +    | +    | +    | +    |

\(^1\) Products of Oriental Yeast, Tokyo.
\(^2\) Supplied from Fuji Oil, Osaka.
\(^3\) Casein and HMF were supplemented with 4\% L-arginine and 0.7\% DL-methionine, respectively, so as to resemble the Arg/Lys ratio and sulfur amino acid content.
\(^4\) Purchased from Kansai Denpun, Kyoto.
\(^5\) Purchased from Nacalai Tesque, Kyoto.

the 4th week. In 4 wk, all 8 groups were food-deprived overnight and then anesthetized with ethylether. Immediately, blood was taken from the abdominal aorta, and then the colon was excised for preneoplastic observation.

**Fecal lipid and steroid assays.** The daily feces collected for each rat were crushed to pieces and freeze-dried. Part of the powdered feces was extracted with 10 volumes of chloroform–methanol (2 : 1). The residue after complete evaporation was weighed to estimate the daily average amount of total lipids excreted into the feces. Another part was saponified for 60 min at 70°C with 1 mol/L NaOH in 90\% ethanol, and its aqueous suspension was washed with petroleum ether to remove neutral steroids. The aqueous layer was strongly alkaliified with 10 mol/L NaOH and autoclaved for 120 min, and after acidification below pH 2, extracted with chloroform–methanol (2 : 1) to collect acidic steroids. The residue after complete evaporation, was again dissolved in methanol; acidic steroids were colorimetrically determined by the enzymatic procedure (10) using a commercially available assay kit (Wako Pure Chemical, Osaka, Japan). The individual bile acids were separated on a Silica Gel-60 sheet (E. Merck AG, Darmstadt, Germany) by thin-layer chromatography using isooctane/ethylacetate/acetic acid (5 : 5 : 1) as a developing solvent, and quantified by UV measurement at 385 nm of 65\% sulfuric acid-treated extracts from their corresponding spots using authentic bile acids at various concentrations as reference standards.

**Plasma protein and albumin concentrations.** Arterial blood samples taken using
heparin-coated syringes were immediately separated into plasma by centrifugation, and stored at \(-80^\circ\text{C}\) until use. Then the individual plasma samples were thawed and analyzed for their total protein and albumin concentrations according to the Lowry-Folin (11) and bromocresol-green (12) methods, respectively. The A/G ratio was calculated by regarding globulin as the difference between total protein and albumin.

**Inspection of preneoplastic lesions and tumors.** The colons excised at 4 wk were cut open lengthwise, rinsed in cold saline, and fixed in 10% formalin. Their extended tissues were put between two pieces of filter paper. Then the mucosal surface of each colon was stained with 0.2% methylene blue and the number of preneoplastic lesions (i.e., aberrant crypt foci (ACF)) was counted concurrently with the interior aberrant crypts (AC) under a stereoscopic microscope at 40\(\times\) magnification (13, 14).

**Statistical analysis.** Data were expressed as means\(\pm\)SE, and for multiple comparisons, were analyzed using ANOVA followed by the Student-Newman-Keuls test (15); differences among these means were considered significant at \(p<0.05\). Statistical calculation was carried out with the aid of SPSS software for the Macintosh-Power Macintosh (version 6.1J, SPSS Japan, Tokyo, Japan).

**RESULTS**

Table 2 summarizes the results of measuring growth parameters through the feeding period of 4 wk. The rats of groups A–F were pair-fed because their appetite was diminished not only by intraperitoneal injection of AOM but also by the bitterness of the DCA added to their diets. For this reason, there were no significant differences in daily food intake and body weight gain among the six groups, which weighed lighter by about one-seventh as compared to groups G and H, which were given free access to their respective diets. There were nevertheless no significant differences in tissue weight (liver, kidney and spleen) or plasma protein (total protein, albumin and A/G ratio) among all the experimental groups.

Figure 1 compares fecal lipid and acidic steroid excretion in the HMF groups with those in the casein groups. Both total lipid and acidic steroids were excreted in larger amounts into the feces of HMF-fed rats irrespective of DCA supplementation or AOM treatment, on account of the bile acid-binding capacity of HMF rather than bulky feces. The excretory amounts of the main bile acids, which were roughly estimated by thin-layer chromatography, are illustrated in Fig. 2. A comparison between pairs of groups having the presence or absence of AOM and/or DCA revealed that the HMF groups excreted much more secondary bile acids than their twin casein groups did.

The colons excised at 4 wk were fixed with formalin, stained with methylene blue, and observed for preneoplastic lesions with a stereoscopic microscope. Neither ACF nor AC were found in the A (casein) and B (HMF) groups that did not undergo previous AOM injection. The results of inspection of preneoplastic

*J Nutr Sci Vitaminol*
Table 2. Growth parameters for each experimental group.

|                     | A          | B          | C          | D          | E          | F          | G          | H          |
|---------------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Daily food intake (g/d during 4 wk) | 9.62 ± 0.15 | 9.68 ± 0.12 | 9.78 ± 0.16 | 9.65 ± 0.15 | 9.72 ± 0.03 | 9.78 ± 0.10 | 12.60 ± 0.20* | 13.11 ± 0.18* |
| Body weight gain (g over a period of feeding) | 15.6 ± 2.0 | 12.9 ± 3.3 | 11.7 ± 0.9 | 12.8 ± 1.7 | 13.5 ± 1.0 | 11.3 ± 0.7 | 55.2 ± 3.5** | 46.2 ± 2.4** |
| Tissue weight (g/100 g body weight) |            |            |            |            |            |            |            |            |
| Liver               | 3.25 ± 0.09 | 3.18 ± 0.07 | 3.26 ± 0.11 | 3.09 ± 0.03 | 3.05 ± 0.03 | 3.15 ± 0.02 | 3.04 ± 0.04 | 3.11 ± 0.04 |
| Kidney              | 0.77 ± 0.02 | 0.78 ± 0.02 | 0.78 ± 0.03 | 0.76 ± 0.01 | 0.77 ± 0.03 | 0.73 ± 0.02 | 0.74 ± 0.01 |         |
| Spleen              | 0.36 ± 0.03 | 0.34 ± 0.01 | 0.36 ± 0.02 | 0.35 ± 0.01 | 0.32 ± 0.02 | 0.32 ± 0.01 | 0.32 ± 0.01 | 0.30 ± 0.02 |
| Plasma protein (g/L) |            |            |            |            |            |            |            |            |
| Total               | 66.7 ± 1.1  | 66.3 ± 1.3  | 65.1 ± 0.8  | 63.7 ± 1.5  | 62.8 ± 0.5  | 64.5 ± 1.5  | 63.0 ± 1.2  | 63.9 ± 1.0  |
| Albumin             | 37.5 ± 1.0  | 36.5 ± 0.9  | 37.3 ± 0.6  | 35.1 ± 0.8  | 34.9 ± 0.2  | 35.9 ± 0.5  | 35.4 ± 0.3  | 34.9 ± 0.4  |
| A/G ratio           | 1.25 ± 0.04 | 1.20 ± 0.02 | 1.28 ± 0.08 | 1.23 ± 0.05 | 1.27 ± 0.02 | 1.26 ± 0.04 | 1.29 ± 0.06 | 1.24 ± 0.02 |

Values are the means ± SE (n=5) for each group provided with the corresponding diet shown in Table 1. Although either A vs. B, C vs. D or E vs. F makes a pair on conditions of treatment with AOM and/or DCA, pair-feeding was carried out among these 6 groups by adjusting the daily average food intake to the minimum intake on the preceding day of group C or D in most cases.

* Significantly different from pair-fed groups at p < 0.05.
** Significantly different from pair-fed groups at p < 0.01.
Fig. 1. Fecal lipid and acidic steroid excretions in rats fed casein and HMF diets. The dietary composition and the presence or absence of treatment with AOM and/or DCA for each group (A–H) are the same as shown in Table 1. The feces gathered from every rat for 4 d before the finish of feeding were assayed for total lipids and acidic steroids. The lengths of bar graphs represent means±SE (n=5); there are significant differences (p<0.01) between casein and HMF groups irrespective of AOM and/or DCA treatment(s).

![Bar graph showing fecal excretion of total lipids and acidic steroids](image)

Fig. 2. Amounts of individual bile acids excreted into the feces. These bile acids were separated by preparative thin-layer chromatography on the basis of Rf-values corresponding to authentic cholic, chenodeoxycholic, deoxycholic and lithocholic acids (muricholic acid were unobtainable), and were individually extracted with 60% sulfuric acid. Their concentrations were estimated by UV measurement at 385 nm. Values are the means with standard error of several % on the whole (at most 12%).

![Bar graph showing individual bile acids excreted](image)

lesions are graphically depicted in Fig. 3. When a comparison was made between DCA-fed casein or HMF groups and DCA-unfed casein or HMF groups, the number of ACF was significantly smaller in the former than in the latter. A significant difference was also observed between DCA-fed casein and HMF groups but not between DCA-unfed casein and HMF groups. Taken altogether, it is reasonable to consider that DCA intake caused a significant decrease in AOM-
Fig. 3. Effects of casein and HMF diets with and without supplementation of deoxycholate on azoxymethane-induced aberrant crypt foci in rat colon. The feeding conditions are the same as in Fig. 1. All the rats were sacrificed by blood-letting to excise the colons, in which ACF and AC were enumerated under a stereoscopic microscope after staining the mucosal flats with methylene blue. The heights of columns represent means ± SE (n=5); those not sharing common superscript letters are significantly different at p<0.05.

 induced ACF but that HMF feeding was more effective in counteracting such a decrease by DCA.

DISCUSSION

A “soluble peptide fraction” obtained from SPI as a result of its treatment with exo-type microbial proteinases is almost equal to the SPI in amino acid composition (16); that is to say, HMF has an amino acid composition similar to SPI. A main difference between HMF and SPI consists in resistance to animal digestive enzymes. In a preliminary experiment, we had found that about a quarter of HMF was undigested and excreted into the feces of rats fed 26% HMF (data not shown). The undigested HMF fraction was estimated as dietary fiber in a broad sense including an indigestible “resistant protein” (the gap in protein content between casein and HMF diets in Table 1). This resistant protein is characterized by binding bile acids and bringing them out (Figs. 1 and 2), which is faithfully reflected in a bile acid-like tendency for fecal lipid excretion among dietary groups. Above all, secondary bile acids are in larger amount in the feces of HMF
groups (Fig. 2), implying that the colonic mucosa may have been exposed to the risk of direct contact with such injurious bile acids.

When AOM, dimethylhydrazine or methylazoxymethanol is subcutaneously or intraperitoneally administered to rats a few times at weekly intervals, ACF (or AC) taken for preneoplastic lesions appear on the surface of the colonic mucosa, of which the number and multiplicity are used as a convenient means to roughly evaluate carcinogenic modulators at early stages before tumor development (14, 17, 18). A causal relationship between preneoplastic and carcinomatous lesions, however, has not been elucidated yet. DCA actually occurs in the intestine and serves as a carcinogenic promoter, of which the use is necessary to translate experimental carcinogenesis into practice by shortening the duration of feeding. The daily food intake in young-adult Fischer-344 rats was 10 g/d among groups A–F (Table 2), while the pool size of bile acids in Wistar rats weighing 300 g is 40 mg/rat, of which one-fourth is excreted into the feces daily (19). Although the Fischer-344 strain is not necessarily equal in bile acid circulation to the Wistar strain, the amount of DCA intake from 0.2% DCA-supplemented diets lies within the gut bile acid pool size. This is the reason for us to consider that the DCA supplementation of our experimental diets at their 0.2% level will be allowable. In practice, we have never observed any inflammatory lesions arising from detergent-like action on the mucosal surface of both small and large intestines until now.

In regards to primary bile acids, it has been demonstrated that the addition of 0.2% cholic acid to a formulated diet exerts a hyperproliferative response in the colon (17), and thereby enhances tumor incidence (20) in spite of a significant decrease in ACF as compared with no addition (14, 21, 22). Cholic acid is easily converted to DCA by the action of microflora. Accordingly, it may safely be said that DCA plays a more direct role in the rise or fall of ACF than its primary bile acid. Regardless of the presence or absence of DCA in the diets, no tumor was macroscopically observed in the colon of rats given three injections of AOM periodically when excised at 4 wk. At that time, the total number of ACF appearance was significantly less in the DCA-fed casein group than in the DCA-unfed casein and HMF groups as well as the DCA-fed HMF group (Fig. 3). The crypt multiplicity of ACF (i.e., plural crypts in focus) was significantly higher in group C than in its twin group D, to which DCA-unfed groups E and F were inferior in rank (the inset in Fig. 3). A long-term experiment in oncogenesis is needed to determine whether or not this difference carries substantial implications for the inspection of neoplastic lesions.

In any case, it is premature to assess either the risk of colonic tumorigenesis or the possibility of anti-tumorigenicity from the standpoint of the total number or crypt multiplicity of ACF alone at this stage (within a month of feeding). The view that there is little correlation between ACF count and actual tumor development will be described in more detail in a subsequent paper.
This study was supported by research funds from the Soy Protein Research Committee of Japan, and by a Grant-in-Aid for Scientific Research (No. 09660142) from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1) Caroll KK, Kurouska EM. 1995. Soy consumption and cholesterol reduction: review of animal and human studies. *J Nutr* **125**: 594S–597S.

2) Sugano M, Yamada Y, Yoshida K, Hashimoto Y, Matsuo T, Kimoto M. 1988. The hypocholesterolemic action of the undigested fraction of soybean protein in rats. *Atherosclerosis* **72**: 115–122.

3) Ogawa T, Galchalain-Yee M, Sugano M, Kimoto M, Matsuo T, Hashimoto Y. 1992. Hypocholesterolemic effect of undigested fraction of soybean protein in rats fed no cholesterol. *Biosci Biotechnol Biochem* **56**: 1845–1848.

4) Sugano M, Goto S, Yamada Y, Yoshida K, Hashimoto Y, Matsuo T, Kimoto M. 1990. Cholesterol-lowering activity of various undigested fractions of soybean protein in rats. *J Nutr* **120**: 977–985.

5) Imai Y, Kawata S, Inada M, Miyoshi S, Minami Y, Matsuzawa Y, Uchida K, Tarui S. 1987. Effect of cholestyramine on bile acid metabolism in conventional rats. *Lipids* **22**: 513–516.

6) Nigro ND, Bhadrachari N, Chomchai C. 1973. A rat model for studying colonic cancer; effect of cholestyramine on induced tumors. *Dis Colon Rectum* **16**: 438–443.

7) Reddy BS, Narisawa T, Weisburger JH, Wynder EL. 1976. Promoting effect of sodium deoxycholate on adeno-carcinoma in germ-free rats. *J Natl Cancer Inst* **56**: 441–442.

8) Wilpart M, Mainguet P, Masken A, Roberfroid M. 1983. Structure-activity relationship amongst biliary acids showing co-mutagenic activity toward 1,2-dimethylhydrazine. *Carcinogenesis* **4**: 1239–1241.

9) McSherry CK, Cohen BI, Bokkenheuser VD, Mosbach EH, Winter J, Matoba N, Scholes J. 1989. Effects of calcium and bile acid feeding on colon tumors in the rat. *Cancer Res* **49**: 6039–6043.

10) Mashige F, Tanaka N, Maki A, Kamei S, Yamanaka M. 1981. Direct spectrophotometry of total bile acids in serum. *Clin Chem* **27**: 1352–1356.

11) Oliver HL, Nira J, Rosebrough A, Lewis F, Rose JR. 1951. Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275.

12) Doumas BT, Watson WA, Biggs HG. 1971. Albumin standards and the measurement of serum albumin with bromocresol green. *Clin Chim Acta* **31**: 87–96.

13) Bird RP. 1987. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* **37**: 147–151.

14) Bird RP. 1995. Further investigation of the effect of cholic acid on the induction, growth characteristics and stability of aberrant crypt foci in rat colon. *Cancer Lett* **88**: 201–209.

15) Granz SA. 1992. Primer of Biostatistics, 3rd ed. McGraw-Hill, New York.

16) Hasida W. 1989. Development and application of soybean peptides (in Japanese). *Shokuhin Kakou Gijutsu (Techniques in Food Processing)* **9**: 89–96.

17) Bird RP. 1986. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* **7**: 1657–1661.

18) McLellan EA, Medline A, Bird RP. 1991. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. *Carcinogenesis* **12**: 2019–2023.
19) Uchida K, Okuno I, Takase H, Nomura Y, Kadowaki M. 1978. Distribution of bile acids in rats. *Lipids* **13**: 42–48.

20) Magnuson BA, Carr I, Bird RP. 1993. Ability of aberrant crypt foci characteristic to predict colonic tumor incidence in rats fed cholic acid. *Cancer Res* **53**: 4499–4504.

21) Magnuson BA, Bird RP. 1993. Reduction of aberrant crypt foci induced in rat colon with azoxymethane or methylnitrosourea by feeding cholic acid. *Cancer Lett* **68**: 15–23.

22) Magnuson BA, Shirrtliff N, Bird RP. 1994. Resistance of aberrant crypt foci to apoptosis induced by azoxymethane in rats chronically fed cholic acid. *Carcinogenesis* **12**: 969–972.