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In vitro digestibility and fermentability of levan and its hypocholesterolemic effects in rats

Yukiko Yamamoto,* Yuko Takahashi,* Mitsuyoshi Kawano,* Masaru Iizuka,† Takashi Matsumoto,† Shigeru Saeki,* and Hidemasa Yamaguchi*

**Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, Osaka, Japan; and †Department of Biology, Faculty of Science, Osaka City University, Osaka, Japan*

This study describes the in vitro digestibility and fermentability of high molecular weight (ca. 2,000,000) levan and its effect on the metabolism of lipids in growing rats fed cholesterol-free diets. Levan was synthesized from sucrose using bacterial levansucrase immobilized on a honeycomb-shaped ceramic support. Although body weight gain, weight of visceral organs, morphologic changes in the digestive tract, and the serum triacylglycerol and glucose concentrations were not affected by feeding levan diets for 4 weeks, a significant hypocholesterolemic effect was observed. Serum cholesterol level was decreased to 83% or 59% by feeding a 1% or 5% levan diet, respectively. The hypocholesterolemic effect was accompanied by a significant increase in fecal excretion of sterols and lipids. High molecular weight levan, though not hydrolyzed by the salivary amylases, was hydrolyzed by artificial gastric juice and was changed to a low molecular weight (ca. 4,000) levan with a small amount of fructose, but did not produce any fructooligosaccharides. Low molecular weight (ca. 6,000) levan was not hydrolyzed by either pancreatic juice or small intestinal enzymes. This suggests that, in vivo, low molecular weight levan derived from the high molecular weight material is not further digested and reaches the colon intact. The fermentation of low molecular weight levan (ca. 6,000) by several strains of bifidobacteria was not observed. These results showed that the hypocholesterolemic effect of levan may result from the prevention of intestinal sterol absorption, and not from the action of the fermentation products of levan. (J. Nutr. Biochem. 10:13–18, 1999)

Keywords: levan; serum cholesterol; dietary fiber; digestibility; hypocholesterolemic effect; bifidobacteria

Introduction

There has been a great deal of interest in the ability of dietary fibers to reduce the serum cholesterol and triacylglycerol levels in humans and animals. Nondigestible oligomers of fructose and other saccarides are types of dietary fiber that are known to be effective physiologically and biochemically. Enzymatically synthesized short-chain β-(2-1) fructooligosaccharides are interesting because they have many kinds of nutritional and biological properties.[1–10] Fructooligosaccharide stimulates the growth of bifidobacteria[1–5] and improves the intestinal microflora production of short-chain fatty acids (SCFA), which are expected to have physiologic effects on human health.[11] The cholesterol- and triacylglycerol-lowering effects of fructooligosaccharides in rats are also well known.[1,7,8]

Another source of β-(2-1)fructan, inulin, is a longer chain length molecule extracted from the root of plants such as chicory and Jerusalem artichoke. Inulin and its partial hydrolysate fructooligosaccharides are fermented by bifidobacteria and human fecal bacteria,[12,13] and their fermentation products are thought to contribute to human health.[2,14,15] The hypocholesterolemic effects of inulin have been reported for rats[8,16] and for hyperlipidemic and diabetic humans.[17,18]

In contrast to the extensive studies on the biochemical and physiologic effects of β-(2-1)fructan such as fructooligosaccharides and inulin, few investigations of levan, which

Address correspondence to Dr. Hidemasa Yamaguchi, Division of Environmental Food Science, Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, Sugimoto 3-3-138, Sumiyoshi, Osaka 558-8585, Japan.
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655 Avenue of the Americas, New York, NY 10010
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is composed of β-(2-6) linked fructose, have been carried out, with the exception of the work of Cho et al.,[19] who failed to show the hypocholesterolemic effect of levan produced by *Bacillus natto*. Although fructooligosaccharides and inulin are available as natural oligomers and polymers of the sugar fructose found in many plants and microbial products, it has not been possible to obtain sufficient quantities of β-(2-6)fructan levan from natural sources.

We have successfully synthesized high molecular weight (ca. 2,000,000) levan from sucrose by using bacterial levansucrase immobilized on a honeycomb-shaped ceramic support.[20] In the present study, the digestibility and fermentability of levan and its hypocholesterolemic effect on rats fed cholesterol-free diets were investigated using the high molecular weight levan synthesized by our methods.

Materials and methods

Materials

All of the digestive enzymes, chemicals, and diagnostic kits used in this experiment were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A honeycomb-shaped ceramic support (SM-10) was supplied by NKG Insulators, Ltd. (Aichi, Japan). Columns for high performance liquid chromatography (HPLC)—Asahipak GS-710H [7.6 mm inner diameter (ID) × 250 mm long], Tosoh TSKgel G3000 (or 6000) PW (7.5 mm ID, 30 mm long) and Shimpack SCR 101N (7.9 mm ID, 300 mm long)—were obtained commercially from Showadenko (Tokyo, Japan), Tosoh Corporation (Tokyo, Japan), and Shimadzu Corporation (Kyoto, Japan), respectively. Bio Gel P-2 column (4.5 cm ID, 61 cm long) for gel permeation chromatography was purchased from Bio Rad (Hercules, CA USA). Thin layer chromatography (TLC) plates (Silicagel 60) were supplied from Merck KGaA (Darmstadt, Germany).

Preparation of high molecular weight levan

High molecular weight (ca. 2,000,000) leven was prepared by bacterial levansucrase immobilized on a honeycomb-shaped ceramic support. The preparation method was reported previously.[20] Judging from H- and C-nuclear magnetic resonance spectroscopy, the resulting levan consists of β-(2-6) fructosyl units with small amounts of branch β-(2-1) linkages. The molecular weight of the levan measured by HPLC (Asahipak GS-710H) was estimated to be approximately 2,000,000, which is equivalent to more than 10,000 monosaccharide moieties. From methylation analysis of the levan, it was concluded that the average number of fructose residues in the linear part was approximately 9.

Preparation of low molecular weight levan

Low molecular weight levan (ca. 6,000) was synthesized by incubating a mixture of sucrose and *Bacillus natto* levansucrase in the presence of 6 M NaCl. Isolation of levan from the reaction mixture was done by gel permeation chromatography with Bio Gel P-2 after heating the reaction mixture at 100°C for 10 minutes to inactivate the levansucrase. The molecular weight of the levan measured by HPLC (Asahipak GS-710H) was estimated to be approximately 6,000.

Animals and diets

Male 3-week-old Sprague-Dawley rats were obtained from Clea Japan, Inc. (Tokyo, Japan). They were housed individually in cages with wire mesh bottoms in a room kept at 24 ± 1°C and with a 12:12 hour light:dark cycle. The animals were given free access to distilled water and diets. Body weight and food intake were determined every day. The composition of the experimental diets is given in *Table 1*. The animals were randomly divided into test groups of six animals each. One group received a diet with 5% cellulose as a control group, and the other two groups had diets containing 1% or 5% (wt/wt) levan. The rats were fed the high molecular weight levan. The animals were given ad libitum access to food for 4 weeks.

Table 1 Composition of experimental diets

Ingredients	Experimental diets (%)		
	Control	1% Levan	5% Levan
Cellulose	5	–	–
Levan*	–	1	5
Casein	20	20	20
Mineral mix†	5	5	5
Vitamin mix‡	2	2	2
Corn oil	5	5	5
Corn starch	63	67	63

*High molecular weight levan (ca. 2,000,000) was used.
†Mineral mixture (%): $CaHPO_4 \cdot 2H_2O$, 14.56; KH_2PO_4, 25.72; NaH_2PO_4, 9.35; NaCl, 4.66; Ca-lactate, 35.09; Fe-citrate, 3.18; $MgSO_4$, 7.17; $ZnCO_3$, 0.11; $MnSO_4 \cdot 4H_2O$, 0.12; $CuSO_4 \cdot 5H_2O$, 0.03; KI, 0.01.
‡Vitamin mixture (in 100 g): vitamin A acetate, 100 mg; vitamin D_3, 0.25 mg; vitamin $B_1 \cdot HCl$, 120 mg; vitamin B_2, 400 mg; vitamin $B_6 \cdot HCl$, 80 mg; vitamin B_{12}, 0.05 mg; vitamin C, 3,000 mg; vitamin E, 500 mg; vitamin K_3, 520 mg; biotin, 2 mg; folic acid, 20 mg; Ca pantothenate, 500 mg; p-aminobenzoic acid, 500 mg; nicotinic acid, 600 mg; inositol, 600 mg; choline chloride, 20 g.

Fecal and tissue collection

During the final 2 days, feces were collected for determination of fecal sterol and total lipids excretion. Feces were lyophilized, weighed, ground in a mill, and stored at –20°C until analyzed. At the end of the experimental period, food was removed 24 hours before the animals were euthanized. Water was provided ad libitum after food removal. The animals were anesthetized with pentobarbital. The peritoneal cavity was opened rapidly, and blood was collected from trunk blood into a centrifuge tube and kept on ice. The serum was then separated by centrifugation (3,000 rpm, 0°C, 15 minutes) and stored at –20°C while awaiting biochemical determinations. After blood sampling, the kidneys, liver, spleen, thymus, and testis were removed. Adherent fat and mesentery were detached, and the organs were washed, blotted dry with paper tissue, and weighed.

Lipids and glucose analysis

Concentrations of total cholesterol, triacylglycerol, and glucose in serum were determined using cholesterol CII-test (Wako Pure Chemical Industries Ltd.), triglyceride G-test (Wako Pure Chemical Industries Ltd.), glucose CII-test (Wako Pure Chemical Industries Ltd.), respectively. Total lipids in feces were extracted by a toluene and petroleum ether extraction procedure[21] and measured gravimetrically. Total sterol in lipids extracted from feces was determined using the diagnostic kit cholesterol CII-test.

In vitro digestibility of levan

Hydrolysis of levan in the digestive tract was simulated by in vitro digestion methods. Digestion of levan was carried out at 37°C by

Table 2 Effect of dietary levan* on body weight gain, relative weight of visceral organs, and the length of small intestine and colon

Diets	Body weight gain (g/4w)	Relative weight† (%)					Length (cm)	
		Kidneys	Liver	Spleen	Thymus	Testis	Small intestine	Colon
Control	211.0 ± 6.4	0.83 ± 0.03[a]	3.15 ± 0.05	0.26 ± 0.02	0.28 ± 0.02	1.11 ± 0.08	90.3 ± 3.5	10.8 ± 0.7
1% Levan	196.6 ± 6.1	0.76 ± 0.01[b]	3.16 ± 0.07	0.26 ± 0.02	0.27 ± 0.03	1.08 ± 0.02	92.5 ± 3.7	11.2 ± 0.3
5% Levan	203.7 ± 6.3	0.78 ± 0.01[ab]	3.21 ± 0.08	0.24 ± 0.01	0.23 ± 0.02	1.07 ± 0.01	82.2 ± 5.6	10.8 ± 0.3

*High molecular weight levan (ca. 2,000,000) was used.
†Relative weight of visceral organ was expressed as wet g × 100/g body weight.
Values are means ± SEM (N = 6). Values within a column with different superscript letters are significantly different from each other ($P < 0.05$).

incubating a levan solution with artificial digestives. For digestion by the artificial saliva, 3 mL of 1% aqueous solution of high molecular weight levan was incubated with 3 mL of artificial saliva. The contents of 100 mL of artificial saliva were as follows: sodium CM-cellulose, 1 g; sorbitol, 3 g; KCl, 0.12 g; NaCl, 0.084 g; $CaCl_2$, 0.015 g; $MgCl_2$ $6H_2O$, 0.005 g; K_2HPO_4, 0.034 g; α-amylase from human saliva, 2,200 U; lysozyme from human milk, 3,950 U; acid phosphatase from milk, 1.2 U; alkaline phosphatase from porcine, 3.3 U; and lypase from porcine, 6.65 U.

For the digestion by artificial gastric juice, 5 mL of 4% aqueous solution of high molecular weight levan was incubated with 5 mL of artificial gastric juice. The gastric juice consisted of 320 mg of pepsin, 200 mg of NaCl, and 2.4 mL of 0.1 M HCl in 100 mL solution (pH 1.2).

For the digestion by pancreatic juice or small intestinal juice, 5 mL of 4% aqueous solution of low molecular weight levan (ca. 6,000) was incubated with 5 mL of artificial pancreatic juice or small intestinal juice, respectively. Artificial pancreatic juice contained 2.5 g of pancreatin from porcine and 100 mL of 50 mM K-phosphate buffer (pH 8.0). Artificial small intestinal juice contained 500 mg of a powdered acetone extract from rat small intestine and 100 mL of 50 mM maleate buffer, which was prepared by mixing 25 mL of 0.2 M sodium maleate and 20.8 mL of 0.2 M NaOH and water to 100 mL (pH 6.6).

HPLC and TLC analysis of hydrolyzed products of levan

The changes in molecular weight distribution of levan during the in vitro hydrolysis were measured using Tosoh TSKgel G6000PW and TSKgel G3000PW columns. Results were expressed in terms of the retention times of the main products of hydrolysis. The qualitative analysis of hydrolyzed compounds of levan (oligolevans, fructose, and glucose) was carried out by HPLC using a Shimpack SCR 101N column and TLC.

Fermentation of levan by bifidobacteria

In vitro fermentation of low molecular weight levan (ca. 6,000) and levanbiose was investigated using different bacterial species, including eight strains of bifidobacteria in anaerobic batch culture fermenters. The decrease in pH of the culture medium, due to formation of acids, was taken as a measure of utilization of these substrates by the bacteria.

Statistical analysis

Data were expressed as mean ± SEM for six rats. All statistical analyses were performed by one-way analysis of variance, and the differences between means were tested using Duncan's multiple range test[22] when the F value was significant. A *P*-value of 0.05 was considered significant.

Results

Growth and visceral organs

Growth of rats fed a diet containing 1% or 5% levan is shown in *Table 2*. No significant differences in the body weight gain were found between rats fed levan and the control rats over the experimental period. In addition, there was no difference in the relative weight of visceral organs, except kidneys to body weight, in all groups ($P < 0.05$). The change in the kidney weight may be not a meaningful result, because the change was not a dose response. In addition, levan did not affect the length of small intestine and colon for all groups.

Scanning electron microscopy showed no damage to the surface of the small intestine, colon, or cecum in levan-fed rats (data are not shown).

Hypolipidemic effect

As shown in *Table 3*, the serum cholesterol level of rats fed 1% and 5% levan diets was significantly lower than that of the control rats ($P < 0.05$). The cholesterol level decreased with increase in the dietary levan content, and the differences between 1% and 5% levan were significant ($P < 0.05$). On the other hand, neither serum triacylglycerol nor glucose level were affected by the dietary levan.

The effects of levan on the fecal excretions of total sterol and total lipids are shown in *Table 4*. The total sterol excretion in 1% and 5% levan was significantly higher than the control ($P < 0.05$). Total lipid excretion was also significantly higher in levan groups than the control ($P < 0.05$).

Table 3 Effects of levan* on the serum cholesterol, triacylglycerol, and glucose

Diets	Cholesterol	Triacylglycerol (mmol/L)	Glucose
Control	2.93 ± 0.04[a]	1.13 ± 0.07	7.16 ± 0.69
1% Levan	2.43 ± 0.09[b]	1.20 ± 0.08	6.45 ± 0.21
5% Levan	1.72 ± 0.05[c]	1.08 ± 0.06	6.82 ± 0.19

*High molecular weight levan (ca. 2,000,000) was used.
Values are means ± SEM (N = 6). Values within a column with different superscript letters are significantly different from each other ($P < 0.05$).

Table 4 Effects of levan* on excreted total sterol and lipids in feces

Diets	Total sterol	Total lipid (mg/g dry feces)
Control	10.09 ± 0.83[a]	59.21 ± 3.88[a]
1% Levan	21.19 ± 1.42[b]	104.25 ± 7.96[b]
5% Levan	19.64 ± 1.52[b]	105.15 ± 3.52[b]

*High molecular weight levan (ca. 2,000,000) was used.
Values are means ± SEM (N = 6). Values within a column with different superscript letters are significantly different from each other ($P < 0.05$).

Digestibility of levan

The products of digestion were measured by their HPLC retention times. The main products of hydrolysis were then expressed as a function of the reaction period (*Figure 1*). High molecular weight (ca. 2,000,000) levan was not hydrolyzed after 60 minutes of exposure to amylase-containing saliva. It was hydrolyzed by artificial gastric juice after 60 minutes of reaction time and reduced to a low molecular weight levan (ca. 4,000) with a small amount of fructose (5.6% of levan) and a trace amount of glucose. No fructooligosaccharides were detected. Hydrolysis of levan by pancreatic juice and small intestinal enzymes was carried out with the low molecular weight levan (ca. 6,000) as a substrate, because high molecular weight (ca. 2,000,000) levan was hydrolyzed to low molecular weight (ca. 4,000) levan by the artificial gastric juice. Pancreatic juice and small intestinal enzymes did not hydrolyze the low molecular weight (ca. 6,000) levan.

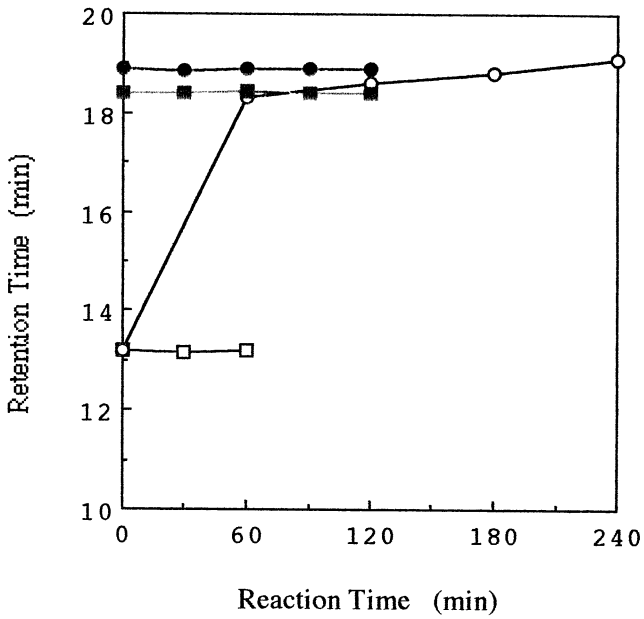

Figure 1 Hydrolysis products of levan by digestive enzymes. The hydrolysis products of levan were monitored by high performance liquid chromatography and are shown in terms of their retention times. –●– Low molecular weight levan (ca. 6,000) was hydrolyzed by small intestinal juice. –■– Low molecular weight levan (ca. 6,000) was hydrolyzed by pancreatic juice. –○– High molecular weight levan (ca. 2,000,000) was hydrolyzed by gastric juice. –□– High molecular weight levan (ca. 2,000,000) was hydrolyzed by salivary juice.

These results suggest that, in vivo, the low molecular weight levan is derived from the high molecular weight levan by the action of gastric juice, but is not further hydrolyzed by digestive enzymes, and reaches the colon intact.

Fermentation of levan by bifidobacteria

It is possible that levan may be a substrate for potentially beneficial bacteria (e.g., bifidobacteria and lactobacilli). We used the changes in pH of the culture medium to judge the utilization of levan by the bifidobacteria. The low molecular weight (ca. 6,000) levan was used as substrate. The pH of the culture medium containing levan was not significantly different ($P < 0.05$) from that of the control at the end of fermentation (*Table 5*). In cases of levanbiose, fructose, and glucose, the pH of the culture medium dropped significantly compared with that of the control, although a few exceptions were observed ($P < 0.05$). The above results suggested that levan was not fermented by these bacteria.

Discussion

In vitro digestibility experiments showed that high molecular weight (ca. 2,000,000) levan was hydrolyzed to low molecular weight (ca. 4,000) levan by gastric juice. Because the artificial gastric juice does not contain β-fructosidase, the hydrolysis of levan might be carried out under the acidic conditions in gastric juice. β-Fructosidase has never been reported to be present in the digestive fluids of the stomach, small intestine, or colon. Therefore, in vivo levan must be hydrolyzed by the acidic conditions in the stomach. Low molecular weight levan produced in the stomach should not be further hydrolyzed and should reach the colon intact, because the levan (ca. 6,000) was not digested in vitro by the pancreatic and small intestinal juices.

In this experiment, we showed a cholesterol-lowering, but not triacylglycerol- or glucose-lowering, effect of levan in rats fed a cholesterol-free diet. We have never seen any other papers reporting this effect of levan. Cho et al.[19] failed to show a hypocholesterolemic effect of levan on rats fed cholesterol-containing diets. They used levan, which is produced by *Bacillus natto* and composed of from 10 to 12 fructose residues. The molecular size of their levan was much smaller than that of our levan. We used high molecular weight (ca. 2,000,000) levan and cholesterol-free diets in this experiment. The discrepancy in the effects of levan might result from the differences in experimental conditions, and the high molecular weight will be important for the hypocholesterolemic effect of levan.

Although levan used in this study was not fermented by bifidobacteria, it is proposed that the fermentation products, especially the SCFA-like propionate, may modulate cholesterol metabolism. The production of SCFA causes a drop in colonic pH and may depress the reabsorption of bile acids.[23] In addition, the propionate may reach the liver and affect liver cholesterol synthesis.[24–26] On the other hand, some authors express doubt about these actions of SCFA.[27,28] Thus, we have no conclusive evidence of the role of SCFA,

Table 5 Utilization test* of sugars by bifidobacteria

Bacteria	Value of pH				
	Glucose	Fructose	Levan†	Levanbiose	Control‡
Mitsuokella multiacidus	4.64 ± 0.04[a]	4.61 ± 0.06[a]	6.71 ± 0.05[b]	4.59 ± 0.05[a]	6.46 ± 0.04[b]
Clostridium sordellii	5.95 ± 0.03[a]	6.10 ± 0.02[a]	6.69 ± 0.06[b]	6.62 ± 0.02[b]	6.50 ± 0.02[b]
Eubacterium aerofaciens	4.65 ± 0.05[a]	4.65 ± 0.05[a]	6.47 ± 0.04[b]	4.67 ± 0.05[a]	6.45 ± 0.02[b]
Bacterioides vulgatus	5.47 ± 0.04[a]	5.84 ± 0.03[b]	6.61 ± 0.02[c]	5.90 ± 0.03[b]	6.51 ± 0.03[c]
Bifidobacterium infantis	4.65 ± 0.03[a]	4.67 ± 0.04[a]	6.43 ± 0.04[b]	4.77 ± 0.03[a]	6.50 ± 0.02[b]
Bifidobacterium liberorum	4.63 ± 0.06[a]	4.67 ± 0.03[a]	6.47 ± 0.03[b]	4.67 ± 0.04[a]	6.56 ± 0.01[b]
Bifidobacterium lactentis	4.63 ± 0.05[a]	4.65 ± 0.05[a]	6.49 ± 0.03[b]	4.69 ± 0.04[a]	6.53 ± 0.02[b]
Bifidobacterium animalis	4.63 ± 0.03[a]	6.51 ± 0.03[b]	6.54 ± 0.04[b]	4.76 ± 0.03[a]	6.51 ± 0.03[b]
Bifidobacterium breve	4.55 ± 0.04[a]	4.60 ± 0.05[a]	6.40 ± 0.02[b]	4.66 ± 0.04[a]	6.54 ± 0.02[b]
Bifidobacterium longum	4.66 ± 0.07[a]	4.66 ± 0.04[a]	6.46 ± 0.03[b]	4.66 ± 0.05[a]	6.53 ± 0.02[b]
Bifidobacterium bifidum	4.90 ± 0.04[a]	4.66 ± 0.03[b]	6.51 ± 0.05[c]	6.52 ± 0.03[c]	6.56 ± 0.03[c]
Bifidobacterium adolescentis	4.65 ± 0.05[a]	4.65 ± 0.04[a]	6.45 ± 0.04[b]	4.97 ± 0.06[c]	6.55 ± 0.02[b]
Lactobacillus acidophilus	4.70 ± 0.04[a]	5.10 ± 0.04[b]	6.42 ± 0.03[c]	6.54 ± 0.02[c]	6.55 ± 0.02[c]

*The initial pH of all cultures was 7.0.
†Low molecular weight levan (ca. 6,000) was used.
‡Control was fermented without test carbohydrate.
Values are means ± SEM (N = 3). Values within a row with different superscript letters are significantly different from each other ($P < 0.05$).

including propionate on lipid metabolism. β-(2-1)Fructans such as fructooligosuccharides and inulin, which show the hypocholesterolemic effect, are known to be highly fermented in the cecum and the colon.[12,13] However, the present study showed that β-(2-6)fructan, levan, is not fermented by the bifidobacteria. Therefore, the hypocholesterolemic effect of levan should be produced independently of the fermentation products.

The other possible mechanism is that reduced intestinal absorption of steroids could lead to a lowering of the serum cholesterol concentration. It is well known that soluble fibers such as pectin and psyllium hydrocolloid increase steroid excretion and lower serum cholesterol in rats.[29–31] The reduced steroid reabsorption by dietary fibers is produced by binding and entrapping properties.[32–34] In this experiment, the fecal excretions of total sterols and lipids were significantly higher in levan-fed rats than in controls (*Table 4*). These results suggest that levan may bind or entrap the steroids in the intestine and disturb their reabsorption. The finding that levan binds taurocholate in vitro[35] supports this hypothesis. Therefore, the hypocholesterolemic effects of levan may result from the disturbed enterohepatic circulation of steroids. However, further studies should be carried out to elucidate the exact mechanisms of the hypocholesterolemic effects of levan.

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