Effects of Dietary Fructan on Cecal Enzyme Activities in Rats

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Abstract In this study, we have attempted to determine the effects of dietary fructose polymers (fructan), high molecular-weight β-(2,6)-linked levan, and low-molecular-weight β-(2,1)-linked inulin, on two intestinal enzymes (β-glucuronidase and β-glucosidase). As a preliminary experiment, when intestinal microflora were cultured in anaerobic media harboring levan or its oligosaccharides, bacterial cell growth was observed in the levanoligosaccharide-supplemented media, but not in the levan-supplemented media, indicating that levan's size is important for the utilization by intestinal bacteria of levan as an energy source. In our animal study, the intake of a levan-rich diet was determined to significantly attenuate the activity of the harmful enzyme β-glucuronidase, but did not affect the activity of β-glucosidase.

Keywords: levan, inulin, fructan, prebiotics, intestinal microflora

INTRODUCTION

The intestinal microbiota is a rich and complex ecosystem, which is inextricably intertwined with normal human functioning [1,2]. Lactic acid-producing bacteria (LAB), including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* species, are especially important factors in carcinogenesis, as they interact with intestinal bacteria within the digestive tract [3,4]. Harmful lumen bacteria can synthesize carcinogens or tumor promoters from a variety of substrates, obtained either from the host's diet, or from the bile. β-Glucuronidase and β-glucosidase have been isolated from intestinal bacteria [5]. The activity of bacterial β-glucuronidase is responsible for the hydrolysis of the hepatic conjugates of a host of foreign compounds, whereas the activity of β-glucosidase tends to involve the attack of the numerous plant glycosides encountered in the human diet, leading to the release of toxic aglycones, which are, in most cases, carcinogenic [6]. Bacterial species are capable of generating a staggering range of enzymes, but it has been reported that strict anaerobes (*Bacteroides*, *Eubacterium*, and *Clostridium*) generate high levels of β-glucuronidase, as compared to *Lactobacillus* and *Bifidobacterium* species [7]. Some dietary fibers appear to affect the composition and activity of the lumen microbiota. Therefore, the identification of bacterial enzymes with toxicological importance would provide a great deal of useful information regarding the influence of diet with regard to the modulation of the lumen microbiota.

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Fructans are widely recognized as prebiotics, and have been determined to modulate the intestinal microflora of both human adults and the elderly [8]. Two forms of fructans, which can be distinguished according to the type of linkage present, have been identified thus far: high-molecular weight (700~10,000 kDa) β-(2,6)-linked levan, and low-molecular weight (4~20 kDa) β-(2,1)-linked inulin [8,9]. Little information is currently available with regard to the activity of cecal enzymes in rats fed on a fructan-rich diet. In the present study, we have attempted to characterize the activities of β-glucuronidase and β-glucosidase in rats fed on a fructan-rich diet.

MATERIALS AND METHODS

Materials

Levan was prepared *via* enzyme reactions using levansucrase from *Zymomonas mobilis*, which was purchased from the RealBioTech Co. (Daejeon, Korea). Commercial chicory inulin was acquired from the Sigma Chemical Co. (St. Louis, MO, USA). The GAM media was obtained from Nissui Pharm. Co. Ltd (Tokyo, Japan) and contained 10 g of peptone, 10 g of proteose peptone, 13.5 g of horse blood, 5 g of yeast extract, 2.2 g of beef extract, 1.2 g of liver extract, 3 g of glucose, 2.5 g of KH_2PO_4, 3 g of NaCl, 5 g of soluble starch 5 g, 0.3 g of L-cystein hydrochloride hydrate, per liter, pH 7.1. Unless otherwise specified, all chemicals used in this study were purchased from Sigma.

Table 1. Composition of experimental diets (g/kg diet)

Constituents \ Group	Control diet	Levan diet	Inulin diet
Casein	360	360	360
Corn oil	70	70	70
Cellulose	70	70	70
Sucrose	448	378	378
Levan	0	70	0
Inulin	0	0	70
Choline chloride	6	6	6
Mineral mix[a]	36	36	36
Vitamin mix[b]	10	10	10

[a]AIN-76 Mineral mix was from Dyets, Pennsylvania (USA).
[b]AIN-76A Vitamin mix was from Dyets.

Preparation of Levanoligosaccharides

Levanoligosaccharides were constructed via acid hydrolysis. In brief, 5% levan solution was mixed with 0.4 M sulfuric acid for 3 min at 95°C, and the high-molecular weight levan was precipitated via the addition of 5 volumes of ethanol followed by a centrifugation step (7,500 × g, 10 min). The supernatants were extracted, concentrated with a rotary evaporator at 60°C, then applied to a glass column (3 × 50 cm) containing silica gel. The levanoligosaccharides were eluted using chloroform-ethanol-water (3/3/2, v/v/v). The samples (degree of polymerization 2~6) were then collected, freeze-dried, and stored at -70°C.

In vitro Fermentation of Levan by Lactic Acid-Producing Bacteria (LAB)

In order to assess the distribution of the *in vitro* fermentation of levan by LAB, 11 strains of LAB were incubated in sugar-free GAM agar, which had been supplemented with one of three carbohydrates (glucose, levanoligosaccharides, or levan) at a concentration of 0.5% (w/v). The strains of LAB used in this study included: *Lactobacillus plantanum* KCTC 3104, *Pediococcus pentosaceus* KCTC 3507, *Bifidobacterium bifidum* JCM 1254, *B. liberorum* JCM 1272, *B. infantis* JCM 7007, *B. longum* JCM 1217, *B. adolescentis* JCM 1275, *B. infantis* JCM 1210, *B. catenulatum* JCM 1194, *B. animalis* KCTC 3126, and *B. longum* KCTC 3215. Erlenmeyer flasks of 250 mL capacity containing 100 mL of appropriate media were inoculated, with an initial A_{600} of 0.1~0.2. Liquid cultures were grown for 16 h at 37°C and 200 rpm (no agitation for Bifodobacteria) in a shaking incubator. The samples (1 mL) were removed hourly for measurements of A_{600} and determination of specific growth rates (μ, h^{-1}). Doubling time was obtained during the exponential growth period from the slope of the curve obtained by plotting the logarithm of A_{600} against time.

Animals and Diets

24 male Sprague-Dawley rats (Korea Center for Experimental Animals, Seoul, Korea), all of the same age and weighing an average of 210 g, were assigned to three groups (n = 8) according to body weight. The animals were permitted free access to food and water throughout the adaptation period. The rats were fed on a control diet, a 7% levan diet, or a 7% inulin diet, for 3 weeks (Table 1). The animals were housed in individual metabolic cages, in a room which was maintained at a temperature of 22 ± 1°C, on a 12-h light/dark photocycle. Food was made available for 3 h, twice a day. Water was provided *ad libitum*. On the night of the final day of the experiment, the food was removed for a total of 18 h. Blood was then drawn from the hearts of the animals, under anesthetization with pentobarbital, and the cecum samples were obtained. The cecal contents were then transferred into two 1.5 mL tubes; one was used immediately for the pH measurement, and the other was used for the determination of enzyme (β-glucuronidase and β-glucosidase) activities.

Measurement of Enzyme Activities [10,11]

A 10% (w/w) suspension of cecal contents was then prepared in 50 mM potassium phosphate buffer (pH 7.0). The mixture was centrifuged (10 min at 7,500 × g, 4°C) and enzyme assays were conducted using the supernatant. In order to determine the quantity of β-glucuronidase, we used a reaction mixture containing 0.02 mL of 10 mM *p*-nitrophenyl-β-*D*-glucuronide (Sigma) as a substrate solution, and 0.01 mL of the supernatant from the cecal suspension. The samples were then incubated for 50 minutes at 37°C, after which the reaction was discontinued via the addition of 0.5 mL 0.5 N NaOH. After the addition of 0.5 mL of water, the samples were centrifuged and the *p*-nitrophenol concentration was determined according to the optical absorbance at a wavelength of 405 nm. In order to measure the quantity of β-glucosidase, we used a reaction mixture containing 0.02 mL of 10 mM *p*-nitrophenyl-β-*D*-glucopyranoside (Sigma) as a substrate solution.

Statistical Analysis

All data analyses were conducted using the Statistical Analysis System (SAS) program (SAS institute, Cary, NC, USA). All statistical analyses were performed using one-way analysis of variance (ANOVA), and the differences between means were assessed using Duncan's multiple range test. A *P* value of 0.05 was considered to be significant.

RESULTS AND DISCUSSION

The levan and inulin used in the present study had molecular weights of 6×10^7 and 5×10^3 daltons, respectively. Whereas the levan was extremely soluble in water at room temperature, the inulin was almost completely insoluble (<0.5%). The levanoligosaccharides represented a mixture of oligosaccharides (degree of polymerization of 3~6).

Table 2. Effect of supplement of 0.5% of levan or levanoligosaccharides to the media on the pH and growth of two lactic acid-producing bacteria

	L. plantanum KCTC 3140			*P. pentosaceus* KCTC 3507		
	Glucose	Levan	Levan-oligosaccharides	Glucose	Levan	Levan-oligosaccharides
pH, at 20 h in incubation	4.5	7.2	4.8	4.2	7.2	4.5
Specific growth rate, μ, h^{-1}	0.173	< 0.1	0.161	0.239	< 0.1	0.215

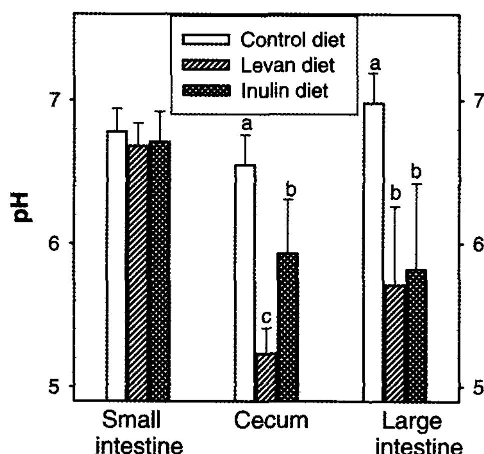

Fig. 1. Effects of levan and inulin on pH in experimental animals. Bars show means ± SD. Values with different superscript letters are significantly different among groups, at a significance level of 0.05.

In vitro Fermentation of Levan by LAB

In order to ascertain whether levan could be used as a substrate for LAB, 0.5% (w/v) levan was added to GAM broth, and used to foster the growth of LAB. None of the 11 tested strains induced any change in the pH of the culture broth. These results indicate that levan is not fermented by the LAB utilized in this study. The specific growth rates of the two strains (*L. plantanum* KCTC 3140 and *P. pentosaceus* KCTC 3507) of LAB in the sugar-free GAM agar supplemented with one of two carbohydrates (levan and levanoligosaccharides), at a concentration of 0.5% (w/v), were then compared, in order to identify the differences in sensitivity to the degree of polymerization (DP). In each set of experiments, the cultures were inoculated in sugar-free GAM agar, using 0.5% glucose as a control. When glucose was added to the growth medium, the specific growth rates and final pH in each strain, after 20 h of incubation, were as follows: 0.173 h^{-1} and pH 4.5 (*L. plantanum* KCTC 3140) and 0.239 h^{-1} and pH 4.2 (*P. pentosaceus* KCTC 3507) (Table 2). DP was demonstrated to affect both the growth rate and the pH. For *L. plantanum* KCTC 3140, the specific growth rate was shown to decrease to 0.161 h^{-1} when exposed to 0.5% levanoligosaccharides (DP 3-6), as the DP increased. Similar results were observed with *P. pentosaceus* KCTC 3507. In the levan medium, the growth of the *L. plantanum* and *P. pentosaceus* cells were completely inhibited. No or minimal changes in pH were observed in the culture fluids when the cells were grown for 20 h in the levan medium. Such low cell growth in the levan medium was probably attributable to the absence of levan-degrading enzymes in the two LAB strains employed. It has been also suggested that levan polymers of molecular mass greater than *ca.* 6,000 (DP 33) were not utilized by LAB, but that smaller polymers with a molecular mass of 3,200 (DP 18) were usable by the LAB [12]. Levanheptaose was, therefore, suggested as a candidate carbon source for LAB, whereas *Clostridium perfringens*, *Escherichia coli*, and *Staphylococcus aureus* were clearly unusable [13]. According to the results reported by others, as well as the results of the present study, it would appear that the difference in the molecular mass of the levan was quite relevant with regard to whether the levan could be utilized as a carbon source for bacterial growth.

In the case of inulin-type fructan, it has been suggested that the maximum specific growth rate of Bifidobacteria tended to be higher when using oligofructose (inulin oligosaccharide) as an energy source, followed by inulin, and then lowest when feeding on glucose [14]. This selectivity appears to be attributable to the specific β-(2,1) bond between the fructose units in the fructan chain [12].

Measurement of Enzyme Activities

We noted no significant differences in daily food consumption and weight gain between the rats fed on the control, levan, and inulin diets. The daily food intake of each group was as follows: 15.6 ± 1.9 in the control group, 15.4 ± 2.3 in the levan group, and 15.5 ± 2.1 in the inulin group. We also determined there to be no significant differences between the groups with regard to kidney and liver weight. In order to evaluate the effects of diet on the pH of the cecum and colon, the pHs of the small intestine, cecal, and colon contents were evaluated. However, we detected no differences between the three rat groups with regard to the pH of the small intestine contents (pH 6.6~6.8), thereby indicating that the small intestine may not be the location at which the fermentation of levan and inulin occurs (Fig. 1). The pH values of the cecum and colon samples from the rats grown on a levan diet were 5.23 and 5.71, whereas those fed on the control diet were 6.6 and 7.0, respectively. We noted significant differences between the control diet-fed rats and fructan-containing diets-fed rats with regard to the activity of cecal β-glucuronidase (Fig. 2). β-Glucuronidase activity was

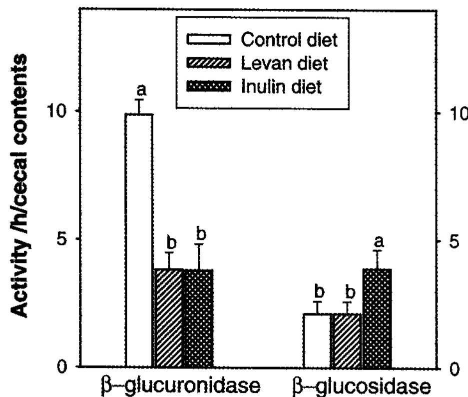

Fig. 2. Activities of β-glucuronidase and β-glucosidase in the cecal contents of rats fed on experimental diets. The bars show means±SD. Values with different superscript letters are significantly different among groups, at a significance level of 0.05.

found to be significantly lower in the rats fed on the levan diet and the inulin diet than in those fed on the control diet. β-Glucosidase activity was higher in the rats fed on the inulin diets than in those fed on the control diet. However, we determined there to be no or minimal differences were found between the levan-fed rats and the control rats with regard to β-glucosidase activity. Experimental studies in humans and animals evidenced an important variation, depending on the nature and amount of indigestible compounds [15]. Pectin in the diet can result in an increase in β-glucuronidase [16] activity, whereas the ingestion of bran, cabbage, carrots, cellulose, lactulose, and inulin tend to reduce these activities [15, 17-19]. Reports in the literature regarding the effects of inulooligosaccharide (FOS) on cecal activity are generally rather scarce. FOS tends to result in an increase in β-glucosidase and a decrease in β-glucuronidase [20]. The discrepancy between different experiments might be attributable to the relationship between fermentation pathways in the colon. One possible explanation for this might be related to the colonic area, in which the fermentation of both fructans and FOS takes place. Whereas FOS fermentation takes place in the proximal colon, fructans is fermented in both the proximal- and distal colon, and this clearly suggests different behavior conditions. In this study, we were unable to secure a sufficient quantity of levanoligosaccharides, and therefore we carried out no animal studies with levanoligosaccharides. No information is currently available regarding the effects of the levanoligosaccharides on cecal activity. Therefore, further experiments with levanoligosaccharides will clearly be required in order to characterize any real difference between levan and levanoligosaccharides in this regard.

CONCLUSION

In this study, we evaluated levan with regard to its potential as a functional food. Levan could not be fermented by LAB in our *in vitro* experiment, but was clearly utilized by lumen bacteria in our *in vivo* experiment. In our animal study, the intake of a levan-rich diet was determined to significantly attenuate the activity of the harmful enzyme β-glucuronidase. Therefore, we conclude that levan exhibits clear modulatory effects on the lumen microbiota.

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