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Allergy-suppressing Activity of Oligosaccharides in Sweet Potato-*Shochu* Distillery By-product

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We obtained anti-allergic oligosaccharides from a sweet potato-*shochu* distillery by-product (SDB). Oral administration of oligosaccharides A and B in SDB to ovalbumin/aluminum-sensitized mice for 4 weeks resulted in a significant decrease in serum IgE concentration. The release of β-hexosaminidase from rat basophilic leukemia cell line RBL-2H3 was also suppressed by the addition of oligosaccharides A and B to the cell culture. The molecular weights of oligosaccharides A and B were about 730 and 530, respectively, determined from the retention time on Bio-Gel P-2. Oligosaccharide A was composed of galactose, glucose and uronic acid in a molar ratio of 2:1:1. Oligosaccharide B was composed of galactose, glucose and arabinose (1:1:1 molar ratio) or galactose, glucose and uronic acid (1:1:1 molar ratio), showing that oligosaccharide B is a heterogeneous mixture of other oligosaccharides. Oligosaccharides A and B exhibited growth-stimulating activity with *Bifidobacterium lactis* BB-12. The present results indicate that the oligosaccharides in SDB are promising ingredients for the effective control of allergic diseases via suppression of IgE production and inhibition of basophilic leukemia cells degranulation.

Keywords: anti-allergy, *shochu* by-product, oligosaccharide, suppression of IgE production, suppression of β-hexosaminidase release, *Bifidobacterium* growth stimulation

Introduction

Shochu is a traditional alcohol spirit in the south of Japan. After alcoholic fermentation of sweet potato with rice *Koji* and yeast, the alcohol and other volatile components were distilled. Sweet potato-*shochu* distillery by-product (SDB) contains numerous components originating from its raw materials, rice malt and yeast, and is rich in nutrients, including oligosaccharides, peptides, dietary fiber, polyphenols and vitamins. There are many reports on the functions of SDB, such as in the prevention of hypertension, anti-cancer (Matsumoto *et al.*, 2005) and antioxidant activities (Yoshimoto *et al.*, 2004; Ye *et al.*, 2004), and anti-allergic immunomodulation (Iguchi *et al.*, 2009; Kobayashi *et al.*, 2004; Nagura *et al.*, 2002). In recent years, Furuta *et al.* (1998) and Nakano *et al.* (2010) reported that the low molecular weight fraction of SDB, in which tetrasaccharides were the main component, had a stimulatory effect on bifidobacterial growth. We investigated the effects of SDB on allergy in mice and on the intestinal bacterial flora in humans (Yonemoto *et al.*, 2010). In these reports, it is suggested that SDB has potential utility as a prebiotic.

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Prebiotics are food ingredients that selectively stimulate the growth and activity of specific bacterial species in the gut, usually bifidobacteria and lactic acid bacteria, with resultant health benefits. The prebiotics are usually oligosaccharides, are typically short-chain carbohydrates that are nondigestible by human digestive enzymes. It has been suggested that intestinal microflora play an important role in the prevention of allergic diseases (Fuller, 1989). Serum IgE was suppressed by oral administration of *Bifidobacterium* in human infants (Sudo *et al.*, 1997). In mice, elimination of indigenous microflora by kanamycin in infancy resulted in increased serum IgE levels (Watanabe *et al.*, 2010). It was reported that glucomannan from alimentary yam paste prevented the development of allergic symptoms and IgE response (Onishi *et al.*, 2007) Therefore, the administration of prebiotics that stimulate the growth of bifidobacteria and lactic acid bacteria in the intestine are thought to enhance immunity and reduce allergic symptoms.

In the present study, we characterized the factors in SDB that suppress allergy, and described relations between the anti-allergic functions and the stimulatory effect on *Bifidobacterium* growth.

Materials and Methods

Materials Sweet potato-*shochu* post-distillation slurry was obtained from a sweet potato-*shochu* manufacturing company (Satsuma Shuzo Co., Kagoshima, Japan) and kept at −20°C until use. The slurry was centrifuged at 12,000 × *g* for 10 min, and the supernatant was used as sweet potato-*shochu* distillery by-product (SDB).

Fractionation of SDB SDB was fractionated by HP-20, followed by Sephadex G-25 and Bio-Gel P-2 column gel chromatography. Elution in HP-20 column chromatography (3 × 13 cm, Mitsubishi Chemical Co., Tokyo, Japan) was carried out with H_2O and 50% ethanol. Saccharides in the eluate were determined with the phenol-sulfuric acid method, and protein and polyphenol contents were assessed by absorbance at 280 nm. An animal experiment and a β-hexosaminidase release assay were employed to assay the allergy-suppressing activity of the collected fractions.

The unabsorbed fraction from HP-20 chromatography was concentrated under vacuum using a rotary evaporator, and was then subjected to G-25 gel filtration (7.3 × 48 cm, GE Healthcare UK Ltd., UK). All fractions from the gel filtration were monitored for allergy-suppressing activity in an animal experiment and with a β-hexosaminidase release assay. After concentration under vacuum, the S-2 fraction from Sephadex G-25 gel filtration was subjected to Bio-Gel P-2 gel filtration (1.2 × 85 cm, Bio-Rad Lab., CA, USA) in H_2O.

Animals and diets Five-week-old female BALB/c mice (6 mice per experimental group, Japan SLC, Shizuoka, Japan) were housed at 23 ± 2°C under a 12-h light/dark cycle. All animal protocols used in this study were approved by the Committee for Animal Experiments of Kagoshima University. The mice were provided with the experimental diets and water *ad libitum*. All experimental diets were prepared according to the AIN-93M formula (Table 1). The SDB and fractionated SDB diets were prepared by adding SDB and fractionated SDB to the control diet to give concentrations of 10% and 2% (w/w), respectively.

Table 1. Components of experimental diets.

Ingredients	Control diet (%)	SDB diet (%)	SDB fraction diet (%)
α-Corn starch	47.0	37.0	45.0
Milk casein	25.0	25.0	25.0
Cellulose	7.0	7.0	7.0
Corn oil	6.0	6.0	6.0
Sugar	10.0	10.0	10.0
Vitamin mix*	3.5	3.5	3.5
Mineral mix**	1.0	1.0	1.0
Choline Chloride	0.2	0.2	0.2
DL-Methionine	0.3	0.3	0.3
SDB	—	10.0	—
SDB-Fractions	—	—	2.0

* The vitamin contents were according to the AIN-93 formula and supplied by Nihon Nosan Co., Ltd.

** The mineral contents were according to the AIN-93 formula and supplied by Nihon Nosan Co., Ltd.

Ovalbumin-induced allergy model The mice were fed the experimental diets for 4 weeks. The mice were injected intraperitoneally with 20 μg of ovalbumin (OVA) (Grade V, Sigma, MO, USA) and 2 mg of $Al(OH)_3$ (Pierce, IL, USA) in a total volume of 0.2 mL PBS. Sensitization was repeated two times at 7-day intervals. Blood was collected from the tail every week, and the serum was kept at −20°C until use.

Serum IgE concentration was measured with a mouse total IgE ELISA kit (Morinaga, Tokyo, Japan).

Cells The rat basophilic leukemia cell line RBL-2H3 was obtained from Cell Resource Center, Tohoku University (Miyagi, Japan). The cells were maintained in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker Co., MD, USA), 2 mM L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Beta-hexosaminidase release assay To evaluate IgE-mediated degranulation of rat mast cells, a histamine release assay was employed. Because high histamine content is found in fermented products like SDB, a β-hexosaminidase

release assay was employed to evaluate IgE-mediated degranulation in this study (Kobayashi and Tanabe, 2006; Shinomiya *et al.*, 2009). RBL-2H3 cells (2×10^5 cells/well) were seeded onto 48-well plates and cultured for 24 h. The cells were treated with 0.5 mg/mL anti-DNP IgE (Sigma, MO, USA) and incubated for 12 h. After washing three times with PIPES-BSA buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, 1 mM CaCl2 and 0.1% BSA, pH 7.2), 200 µL of PIPES-BSA buffer containing from 5 to 20 µL of SDB or fractionated SDB was added to the wells and incubated for 30 min. Then, 1 µg/mL DNP-BSA was added to the culture medium. Thirty minutes later, cells were put on ice for 10 min to end the reaction. Next, supernatants were harvested by centrifugation at 300 × *g* at 4°C for 10 min. The supernatants (50 µL) were transferred to 96-well plates and reacted with 50 µL of 0.1 M citrate buffer (pH 4.5) including 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (PNAG) at 37°C. One hour later, stop buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0) was added to terminate the reaction. Absorbance was measured at 405 nm using a microplate reader (Mutiskan FC, Thermo Scientific, Finland).

The increase in OD reflects β-hexosaminidase release. Calculations were performed using the following equations. To obtain valid values, it was necessary to exclude factors not typically induced by samples. In "spontaneous", neither DNP-BSA nor sample was added to cells to determine spontaneous β-hexosaminidase release from cells. In "control", DNP-BSA but not samples was added to cells to determine β-hexosaminidase release from cells in the absence of sample. In "total", cells were sonicated to determine the total amount of β-hexosaminidase contained in the cells. In "sample", both DNP-BSA and samples were added to cells to determine β-hexosaminidase release from cells under these conditions.

$$\text{Ratio of β-hexosaminidase release (\%)} = (OD_{control} \text{ or } OD_{sample} - OD_{spontaneous})/(OD_{total} - OD_{spontaneous}) \times 100$$

$$\text{Inhibition of β-hexosaminidase release (\%)} = (OD_{sample} - OD_{sponteneous})/(OD_{control} - OD_{spontaneous}) \times 100$$

Determination of Bifidobacterium lactis BB-12 growth stimulation In order to examine the effects on *Bifidobacterium lactis* BB-12 growth, SDB fractions obtained by chromatography were monitored as described by Nakano *et al.* (2010) with some modifications.

Determination of saccharide composition Samples were hydrolyzed with 1 M HCl for 2 h at 100°C, and the remaining HCl was then repeatedly removed with a rotary evaporator under vacuum. The sugar composition was determined by anion-exchange-HPLC with a Carbo Pac PA-1 column (Dionex, CA, USA) as previously described (Ando *et al.*, 2000), with modifications pertaining to the elution (Table 2). The uronic acid content was determined by the method of Galambos (1967).

Table 2. Gradient Program for HPAEC.

Time (min)	Water (% vol)	0.1 M NaOH (% vol)
0.0	90	10
2.9	90	10
3.0	100	0
35.0	100	0
40.0	0	100

Saccharides were identified using high-performance anion-exchange chromatography (HPAEC), equipped with a pulsed amperometric detector (PAD) and a CarboPac PA-1 column (Dionex, CA, USA). HPLC was operated at 30°C with 1.0 mL/min flow rate of a mixture of water and 0.1 M sodium hydroxide with 0.3 M sodium hydroxide, according to the modified method of Ando *et al.* (2000). At this time, 0.3 M sodium hydroxide was mixed with the eluent (water and 0.1 M sodium) at the exit of the column and was supplied to the PAD using the high purity nitrogen gas cylinder at 45 psi. Mixing conditions of water and 0.1 M sodium hydroxide are shown in Table 2.

Determination of mean molecular weights Mean molecular weights of the anti-allergic fraction were determined using Bio-Gel P-2 gel filtration as mentioned above. Maltotriose (Mw 504.44), maltotetraose (Mw 666.58) and maltohexaose (Mw 990.87) were used as standards.

Statistical analysis The statistical significance of differences was assessed by Dunnett's multiple comparison test.

Results

Fractionation of SDB As shown in Fig. 1, SDB was divided into the flow-through fraction and the 50% EtOH eluate using HP-20 chromatography. Most of the sugar was not adsorbed on the column while most of the polyphenols or proteins were eluted in the 2nd fraction. The anti-allergic activity of each purification step fraction was monitored by both *in vitro* and *in vivo* methods, as described in the methods.

The baseline level of total IgE in the control mice (n = 5) was 167 ± 36 (mean ± SE) ng/mL serum, and increased to 1240 ± 132 ng/mL serum in the OVA-sensitized mice at 28 days. Administration of 10% SDB and 2% fractionated SDB resulted in significantly suppressed total IgE levels in mice, 956 ± 134 ng/mL serum and 903 ± 68 ng/mL serum, respectively ($p < 0.05$).

We also monitored allergy-suppressing activity using the cell culture method described in the methods, and found that the chromatographed fractions with IgE inhibitory activity in mice also showed strong suppressive activity in the β-hexosaminidase release assay (Fig. 6 and Fig. 7).

In addition, the stimulatory activity toward bifidobacterial

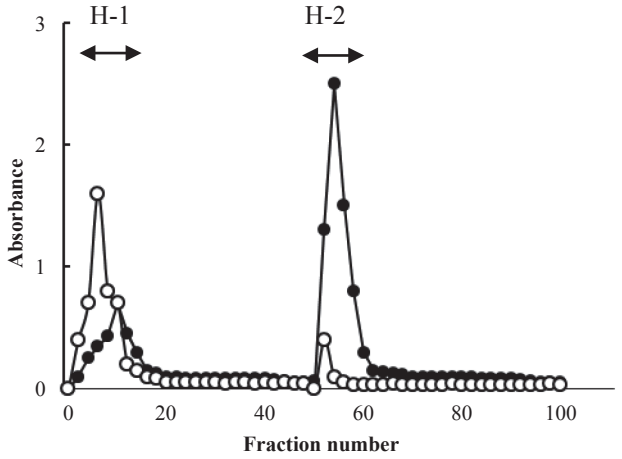

Fig. 1. HP-20 column chromatography of SDB.

After SDB was applied to the HP-20 column, the unabsorbed fraction (H-1) was removed with water, and the polyphenolic fraction (H-2) was recovered by washing with 50% ethanol.

Column size: 4 × 25 cm. Fraction vol: 18 mL/tube. ○; absorbance at 490 nm (sugar determination); ●; absorbance at 280 nm (protein and polyphenol determinations).

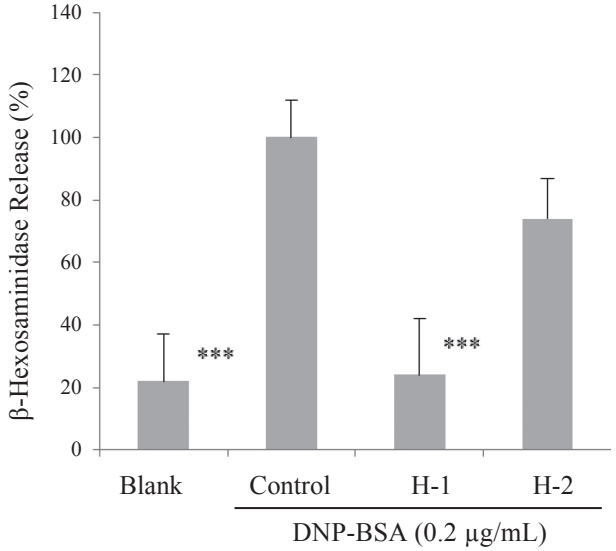

Fig. 2. Suppression of β-hexosaminidase release from RBL-2H3 cells by fractions H-1 and H-2.

IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA in the presence or absence of each fraction. Each column represents mean ± SE of five experiments. Asterisks denote significant differences from the control at ***, $p < 0.001$.

growth was monitored for all purification step fractions. We found that fractions exhibiting allergy-suppressing activity also showed bifidobacterial growth stimulatory activity (Table 3).

SDB and the SDB fractions did not suppress β-hexosaminidase activity.

Strong allergy-suppressing activity, assayed by both the animal experiment and cell culture method, was found in the flow-through fraction of HP-20 chromatography (Fig. 2). The flow-through fraction was concentrated under vacuum and subjected to Sephadex G-25 gel filtration (Fig. 3).

The allergy-suppressing activity detected by the animal experiment, as well as cell culture method, was detected in the low molecular weight 2nd sugar fraction (Fig. 4); therefore, the 2nd sugar fraction was concentrated under vacuum and applied to a Bio-Gel P-2 column. Two sugar-containing fractions were obtained using Bio-Gel P-2 gel ration (Fig. 5), and IgE inhibitory activity was found in the mice fed either oligosaccharide fraction (Fig. 6). Inhibition of β-hexosaminidase release from RBL-2H3 cells was also

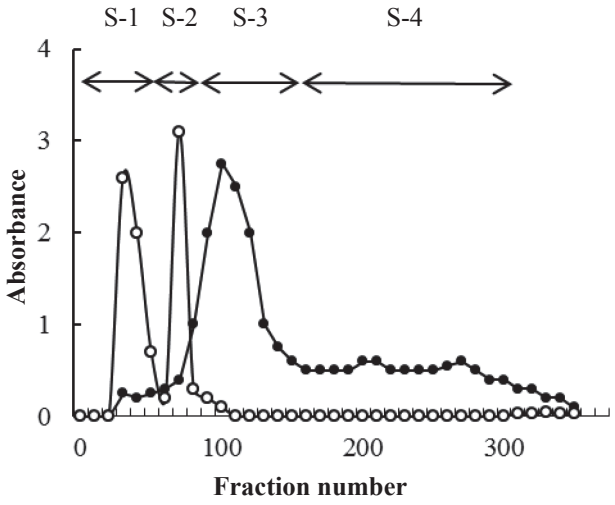

Fig. 3. Sephadex G-25 gel filtration of H-1.

After concentration under vacuum, H-1 was subjected to Sephadex G-25 gel filtration in H_2O.

○; absorbance at 490 nm (sugar determination), ●; absorbance at 280 nm (protein or polyphenol determinations). Column size; 4 × 40 cm. Fraction vol; 12 mL/tube. Flow rate; 350 mL/h.

Table 3. The effect of HP-20, Sephadex G-25 and Bio-Gel P-2 chromatography fractions on *Bifidobacterium lactis* BB-12 growth.

	Control	SDB	HP-20		Sephadex G-25				Bio-Gel P-20		
			H-1	H-2	S-1	S-2	S-3	S-4	A	B	C
$\times 10^8$ CFU/mL	0.95	10.01	10.34	5.32	4.97	13.7	5.97	5.97	13.76	9.37	4.76

Examination of *Bifidobacterium lactis* BB-12 growth was conducted using a modified method of Nakano *et al.* (2010). Each freeze-dried sample was diluted to the original concentration. Skim milk (10 g) and sample (90 mL) were mixed and adjusted to pH 6.7 with 30% KOH, then sterilized at 90°C for 10 min. After cooling to 40°C, 10 mL of *Bifidobacterium lactis* BB-12 culture was added. After cultivation at 37°C for 48 h, the growth of bacteria was evaluated. The number of microorganisms was expressed as Colony forming units (CFU /mL). CFU were determined anaerobically by plating on TOS propionate agar (Eiken Chemical Co., Japan). The sugar content in each fraction used for assay of *Bifidobacterium lactis* BB-12 growth was about 0.4 mg.

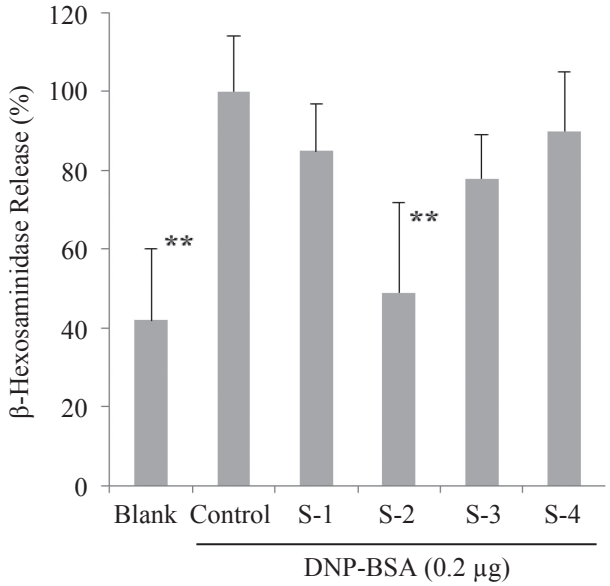

Fig. 4. Suppression of β-hexosaminidase release from RBL-2H3 culture by S-1~4 fractions from Sephadex G-25 gel filtration.

IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA in the presence or absence of each fraction. Each column represents mean ± SE of five experiments. Asterisks denote significant differences from the control at **, $p < 0.01$, respectively.

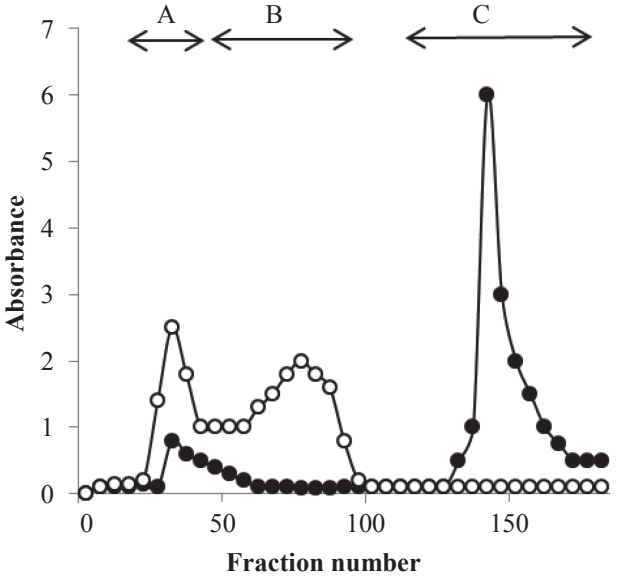

Fig. 5. Bio-Gel P-2 column chromatography of fraction S-2.

After concentration under vacuum, S-2 was subjected to Bio-Gel P-2 column chromatography.

○; absorbance at 490 nm (sugar determination), ●; absorbance at 280 nm (protein and polyphenol determination). Column size; 3 × 70 cm. Fraction vol.; 3.5 mL/tube. Flow rate; 80 mL/h.

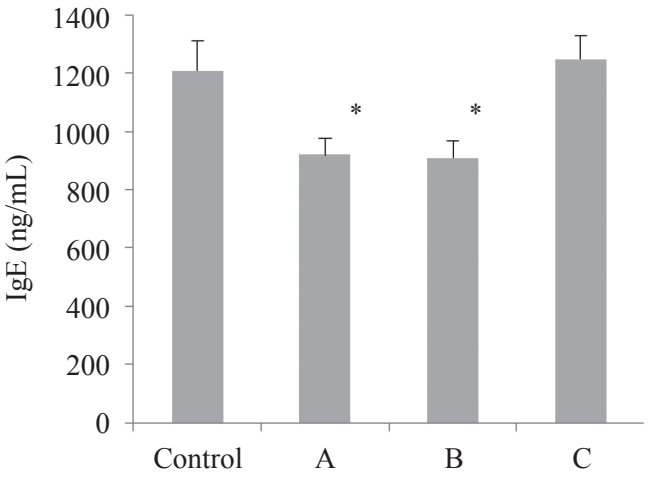

Fig. 6. Suppressive effect of oligosaccharides A and B on IgE levels in mice.

After the Bio-Gel P-2 gel filtration fractions (A, B and C) were lyophilized, each fraction, at a concentration of 2% in the experimental diet (Table 1), was fed to mice for 4 weeks. Each column represents mean ± SE of five experiments. Asterisks denote significant differences from the control at *, $p < 0.05$ in comparison with control.

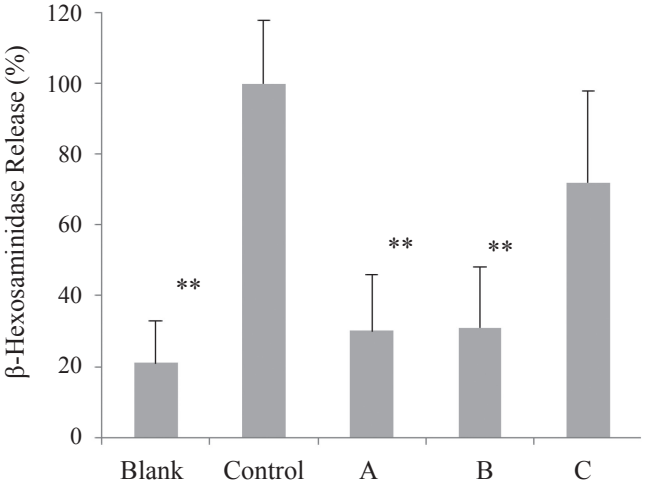

Fig. 7. Suppression of β-hexosaminidase release from RBL-2H3 culture by oligosaccharides A and B obtained after Bio-Gel P-2 chromatography.

IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA in the presence or absence of each fraction. Each column represents mean ± SE of five experiments. Asterisks denote significant differences from the control at **, $p < 0.01$, respectively.

found in both oligosaccharide fractions (Fig. 7). In addition, a stimulatory growth effect on *Bifidobacterium lactis* BB12 was found in both oligosaccharides A and B (Table 3).

Oligosaccharide properties Mean molecular weights were calculated from peak retention times by comparison with standard oligosaccharides (see methods). The molecular weights of the oligosaccharides were determined by Bio-Gel P-2 gel filtration; molecular weights of 730 for oligosaccharide A and 530 for oligosaccharide B were obtained from the gel filtration profiles.

The sugar composition of the two oligosaccharides was assayed after acid hydrolysis as described in the methods. Based on the sugar content and molecular weight of the two oligosaccharides, oligosaccharide A was composed of ga-

Table 4. Molar ratios of sugars in oligosaccharides A and B and molecular weights.

	Oligosaccharide A	Oligosaccharide B
Arabinose		1
Galactose	2	2
Glucose	1	2
Uronic acid	1	1
Molecular weight	730	530

lactose, glucose and uronic acid (2:1:1 molar ratio), while oligosaccharide B was composed of galactose, glucose and arabinose (1:1:1 molar ratio) or galactose, glucose and uronic acid (1:1:1 molar ratio), showing that oligosaccharide B is a heterogeneous mixture with other oligosaccharides (Table 4).

Discussion

In this study, we showed that two oligosaccharides in SDB suppressed the IgE response induced by intraperitoneal immunization with OVA/Alum in a mouse model. In addition, these oligosaccharides suppressed β-hexosaminidase release from the rat basophilic leukemia cell line RBL-2H3. The molecular weight of oligosaccharide A was about 730 and that of oligosaccharide B was about 530 from the elution profiles from Bio-Gel P-2 column. The molecular weights and sugar composition indicated that oligosaccharide A was composed of galactose, glucose and uronic acid (2:1:1 molar ratio), while oligosaccharide B consisted of galactose, glucose and arabinose (1:1:1 molar ratio) or galactose, glucose and uronic acid (1:1:1 molar ratio), indicating the possibility that oligosaccharide B was contaminated with impurities. These oligosaccharides are likely derived from hemicelluloses of rice or sweet potato during *shochu* fermentation.

Furuta *et al.* (1998, 2007) and Nakano *et al.* (2010) previously reported that the oligosaccharides in SDB accelerated the growth of *Bifidobacterium lactis* BB-12, the sugar components were similar to those of this paper, and the molecular weights were < 1000, indicating that the oligosaccharides reported in this paper were very similar to those previously reported. We monitored the growth-stimulating function against *Bifidobacterium lactis* BB-12 in SDB using the first purification step in this study, and found that both oligosaccharides A and B stimulated *Bifidobacterium lactis* BB-12 growth, indicating that the oligosaccharides A and B obtained in this study are identical to the oligosaccharides reported by Nakano *et al.* (2010). From these findings, oligosaccharides A and B suppressed allergy symptoms *in vivo* and *in vitro*, and also stimulated the growth of *Bifidobacterium lactis* BB-12.

We previously reported that stool samples of volunteers administered fermented milk with SDB and lactic acid bacteria for 2 weeks indicated that *Bifidobacterium* in the intestine was increased, and oral administration of SDB to mice for 28 days decreased IgE serum concentration significantly (Yonemoto *et al.*, 2010).

The intake of oligosaccharides digestible by bifidobacteria should result in an increase in intestinal bifidobacteria, which plays an important role in imparting health benefits, including immune function as generally understood and widely reported (Fuller, 1989; Gilliland, 1989; Fernandes and Shahani, 1990; Sudo *et al.*, 1997; Nagura *et al.*, 2002; Sonoyama *et al.*, 2005; Matsushita *et al.*, 2006; Onishi *et al.*, 2007; Ukawa *et al.*, 2007).

The intestinal bacterial flora plays an important role in the maintenance of host health. It is generally recognized that bifidobacteria and lactic acid bacteria are beneficial species, and are associated with various health-promoting functions, such as the production of short-chain fatty acids, immune stimulation, and inhibitory effects on the growth of harmful bacteria.

During fermentation for *shochu* production, the digestible polysaccharides are easily utilized by *koji* and yeast; therefore, the remaining oligosaccharides in SDB remain as nondigestible components for the microorganisms in *shochu* fermentation. Some nondigestible polysaccharides are selectively assimilated by intestinal bacteria and thought to exert a prebiotic function. It was reported that the administration of probiotics and prebiotics suppressed Th2 type allergic reaction, as a result of enhanced intestinal bacterial flora (Gilliland, 1989; Fernandes *et al.*, 1990; Sudo *et al.*, 1997; Sonoyama *et al.*, 2005).

We previously demonstrated that SDB improved intestinal bacterial flora in humans (Yonemoto *et al.*, 2010). The results of this study support the view that enhanced intestinal bacterial flora contributes to the suppression of IgE production.

An immediate allergic response is caused by chemical mediators released from basophils and mast cells via cellular degranulation, as a result of the interaction between IgE antibodies and antigen. Further studies of the mechanism regulating inhibition of degranulation by the oligosaccharides in SDB are needed.

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