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.
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 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 H2O 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 H2O.

*Animal 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.

**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% CO2/95% 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

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

* 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.
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 CaCl_2 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.

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

\[
\text{Inhibition of β-hexosaminidase release (%) = } \frac{(OD_{sample} - OD_{spontaneous})}{(OD_{control} - OD_{spontaneous})} \times 100
\]

**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.

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 H2O. ○; 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.

<table>
<thead>
<tr>
<th>Control</th>
<th>SDB</th>
<th>HP-20</th>
<th>Sephadex G-25</th>
<th>Bio-Gel P-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>×10⁸ CFU/mL</td>
<td>0.95</td>
<td>10.01</td>
<td>10.34</td>
<td>5.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-1</td>
<td>S-1</td>
<td>S-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-2</td>
<td>S-2</td>
<td>S-3</td>
</tr>
</tbody>
</table>

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.
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-
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).

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

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Oligosaccharide A</th>
<th>Oligosaccharide B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>730</td>
<td>530</td>
</tr>
</tbody>
</table>

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 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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