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Synthesis and Characterization of Fructooligosaccharides Using Levansucrase with a High Concentration of Sucrose

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Abstract A method for synthesizing branched fructo-oligosaccharides (BFOS) with a high concentration of sucrose (1~3 M) was developed using levansucrase prepared from *Leuconostoc mesenteroides* B-1355C. The degree of polymerization of oligosaccharides synthesized according to the present method ranged from 2 to over 15. The synthesized BFOS were stable at a pH ranges of 2 to 4 under 120°C. The percentage of BFOS in the reaction digest was 95.7% (excluding monosaccharides; 4.3% was levan). BFOS reduced the insoluble glucan formation by *Streptococcus sobrinus* on the surfaces of glass vials or stainless steel wires in the presence of sucrose. They also reduced the growth and acid productions of *S. sobrinus*. Oligosaccharides can be used as sweeteners for foods such as beverages requiring thermo- and acid-stable properties and as potential inhibitors of dental caries.

Keywords: levansucrase, fructooligosaccharides, inhibition, insoluble glucan

INTRODUCTION

Sucrose, [α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside], is the most common sugar. Large quantities of this disaccharide are accumulated in the edible parts of some plants, making it the most abundant natural sweetener in foods. The high available caloric content of sucrose and the ease by which it is metabolized by microbes that cause dental caries, has stimulated interest in other naturally occurring sweeteners. Oligosaccharides used in foods, feeds, pharmaceuticals, cosmetic industries as stabilizers, bulking agents, immunostimulating agents or prebiotic compounds are able to stimulate the growth of beneficial bacteria [1]. In general, oligosaccharides have a degree of polymerization (DP) of 2~10 monosaccharides (molecular weight of 300~2,000) that are dehydrated/condensed by a combination.

Presently, oligosaccharides that are commercially produced are fructo-oligosaccharides, isomalto-oligosaccharides, malto-oligosaccharides and galacto-oligosaccharides. The commercially produced malto-oligosaccharides consisting of glucose are resistant to acid and heat treatments. Yet, they are less sweet. On the contrary, the commercial fructo-oligosaccharides are sweet and are not resistant to acid and heat treatments. As a result, both of these oligosaccharides are restricted in their use as additives or sweeteners in foods that have a low pH and need a heat treatment during their processing. Many sweet foods are composed of mono- or disaccharides that are easily metabolized to acids by cariogenic bacteria and they also predispose enamel to dental caries. To satisfy the human preference for sweet substances without causing caries, the use of inert (non-metabolizable) dietary sweeteners has been proposed. A variety of different materials have been studied as potential inhibitors of various glucansucrases with the hope that an ideal compound that is safe, cheap, and effective in blocking the colonization of *Streptococcus* sp. on the teeth can be found [2].

Lactic acid bacteria (LAB) are a group of gram-positive, food-grade microorganisms consisting of many genera, *e.g. Lactococcus*, *Leuconostoc*, and *Lactobacillus*.

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Members of these genera, possessing the generally regarded as safe (GRAS) status, find applications in the production of food and feed [3]. Some LABs produce fructan which consists mainly of β-2,6 linked fructosyl residues and occasionally contains β-2,1 linked branches. Fructan is called levan and can reach a DP of more than 100,000 fructosyl units. Bacterial levan is produced extracellularly by the single enzyme, levansucrase (EC 2.4.1.9), which produces levan directly from sucrose. Fructan-producing bacteria can be found in a wide range of taxa, including plant pathogens and the bacteria present in oral and gut floras of animals and humans: the notable gram positive bacteria; *Bacillus, Streptococcus, Rothia, Lactobacillus, Arthrobater, Actinomyces,* and *Azotobacter,* and the gram negative bacteria; *Zymomonas, Glucobacter, Pseudomonas* and *Erwinia* [4-7]. The molds *Aspergillus, Auerobasidium, Fusarium, Penicillium,* and the yeast *Saccharomyces* also produce levan-type fructans. In addition to the fructan synthesizing activity, several of these bacterial levansucrases can transfer fructosyl units to other sugars that act as acceptors such as glucose, fructose, and raffinose [8]. *Leuconostoc mesenteroides* is a heterolactic acid bacterium and it elaborates dextransucrase, an enzyme that synthesizes dextran from sucrose. *L. mesenteroides* is also known to produce a levansucrase, yet a detailed study investigating the role of *L. mesenteroides* levansucrase with respects to the enzyme and sucrose acceptor reaction for fructo-oligosaccharide synthesis has not been reported.

In this paper, we report the synthesis of branched fructooligosaccharides (BFOS) by *L. mesenteroides* B-1355C levansucrase (LVSase) using a high concentration of sucrose, 1~3 M, as an acceptor and donor carbohydrate. The resulting BFOS were thermo-stable, acid-stable and sweet. These BFOS also inhibited the activity of mutansucrase from the growth of the oral pathogen, *Streptococcus sobrinus*.

MATERIALS AND METHODS

Materials

Carbohydrates were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Yeast extract, peptone and tryptone were purchased from Duchefa (Haarlem, Netherlands). All other chemicals were of reagent grade and commercially available.

Preparation of Levansucrase (LVSase)

A constitutive mutant of dextransucrase and levansucrase, *L. mesenteroides* B-1355C, was grown in LWG medium which consisted of 0.3% (w/v) yeast extract, peptone, 0.3% (w/v) K_2HPO_4, and a mineral solution (2% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, 0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot H_2O$, 0.13% $CaCl_2 \cdot 2H_2O$) containing 2% (w/v) glucose at 40°C for 12 to 16 h until the OD_{600} was approximately 3.0 [9]. LVSase was prepared from the culture supernatant. The LVSase activity was assayed by an incubation of the enzyme for different reaction periods at 28°C with 100 mM sucrose or raffinose in 20 mM sodium phosphate buffer (pH 6.4) as a substrate. Standard assay mixtures consisted of 200 μL of 200 mM sucrose and 200 μL of an enzyme solution (2 U/mL). Each enzyme reaction sample was spotted on a Whatman K5 TLC plate. The TLC plate was ascended twice on an acetonitrile: water (85:15, v/v) solvent system. Each carbohydrate was visualized by dipping the plates into 0.3% *N*-(1-naphthyl)-ethylenediamine and 5% sulfuric acid in methanol followed by a heat treatment for 10 min at 120°C [10]. The amount of separated glucose released from sucrose or of melibiose released from raffinose was analyzed with a NIH Image Program [9]. One unit of LVSase activity is defined as the amount of enzyme required to produce 1 μmol of glucose or melibiose per min under the assay conditions described above.

Partial Purification

Eight liters of an overnight culture of *L. mesenteroides* B-1355C grown on LWG medium was centrifuged for 15 min at 10,000 × *g*. The desired concentration of the culture supernatant was accomplished by passing the culture supernatant through a polysulfone ultrafiltration hollow fiber cartridge with a 63.8 cm length and a 0.45 m^2 surface area (H5P100-43 (100 kDa cut-off), Amicon, Inc., Beverly, MA, USA) at a flow rate of 4 mL/min at 22°C. As the volume of the concentrate reached approximately 1 L, 500 mL of 20 mM sodium phosphate buffer (pH 6.4) was added and a second concentrate until the volume reached approximately 1 L was followed. These steps were repeated three times, and the hollow fiber cartridge was washed with approximately 400 mL of the same buffer. The concentrate was equilibrated with the addition of 20 mM sodium phosphate buffer (pH 6.4), and 20 mg of this sample was loaded on a pre-equilibrated DEAE-Sepharose (2.8 × 35 cm) column with the same buffer. The column was washed with 200 mL of 20 mM sodium phosphate buffer (pH 6.4) to remove unbound proteins, and the bound protein was eluted with a linear gradient of NaCl (0~1 M) in the same buffer solution [11]. Fractions collected were screened for LVSase activity. Positive fractions were pooled and stored at -20°C for further studies.

Synthesis of Branched Fructo-oligosaccharides (BFOS)

In order to produce BFOS with B-1355C LVSase, a 4 M sucrose solution was mixed with the previously obtained enzyme. The final sucrose concentration of each enzyme reactor was 1~3 M, and this reacted between 28 and 55°C. The amount of the enzyme used was 0.1~10 U/mL. The reaction was performed until the sucrose of the reaction digest was consumed completely. After the reaction, a 1 μL aliquot was spotted on a Whatman K5F TLC plate (Whatman, Clifton, NJ, USA), then the plate was developed twice in acetonitrile/1-propanol/water (2/5/2.5, v/v/v). Each carbohydrate was visualized by dipping the plates into 0.3% *N*-(1-naphthyl)-ethylenedia-

mine and 5% sulfuric acid in methanol, and followed by a heat treatment for 10 min at 120°C [10]. The amounts of separated fructose were analyzed using the NIH Image Program [9].

Identification of Thermo-stable and Acid-stable Oligosaccharides

Fifty mL of each reaction digest was adjusted to different pHs of 20 mM imidazole-HCl buffer (pH 2, 3, and 4). Each preparation was divided into five portions and incubated at 25, 60, 80, 100, and 120°C for 15 min, and then quenched. For thermo stability test, the fifty mL of each reaction digest was adjusted to pH 5.2. Each preparation was divided into three portions and incubated at 100, 120, and 140°C for 1 h, and then quenched.

The composition change in BFOS was then identified by TLC as previously described [10].

The Effects of LVSase in *Streptococcus sobrinus* Growth and Acid Production

S. sobrinus was grown in BHI medium (0.5% yeast extract and 3.7% brain heart infusion, pH 7.0, 37°C) plus 0.5% oligosaccharides. The control culture was prepared in BHI medium containing 0.5% glucose. At the same time, the acid formation was monitored by the pH changes in the culture of *S. sobrinus* [12].

Sucrose-dependent, Glucan-mediated Adhesion

The adhesion of cells on the surface of a stainless steel wire or a glass vial was measured essentially as described by Ryu *et al.* [13]. *S. sobrinus* were grown in BHI medium. *S. sobrinus*, 2.5×10^6 cells/mL, were inoculated in BHI medium containing 10% (w/v) sucrose and 0.5% (w/v) oligosaccharides for 24 h at 37°C without shaking. Liquid and non-adherent cells were carefully removed by aspiration. Adherent cells were gently washed once with 1.0 mL of 20 mM phosphate buffer (pH 5.8), and dislodged with 1.0 mL of 0.5 M NaOH by vortex mixing. The adherent biomass was measured at the absorbance of 600 nm [13].

RESULTS AND DISCUSSION

Glycosyltransferases, glycosidases, and genetically engineered "glycosynthases" will play a role in this rapidly developing field. One of the glycosyltransferases is levansucrase (LVSase, EC 2.4.1.9), which produces levan (fructan consists mainly of β-2,6 linked fructosyl residues, and occasionally contains β-2,1 linked branches) directly from sucrose. In addition to the fructan synthesizing activity, several of these bacterial levansucrases can transfer fructosyl units to other sugars, which act as acceptors, such as glucose, fructose, and raffinose. The current study also found that sucrose at a high concentration was also used as an acceptor of LVSase, and that various sucrose acceptor products (BFOSs) were synthesized. Fig.

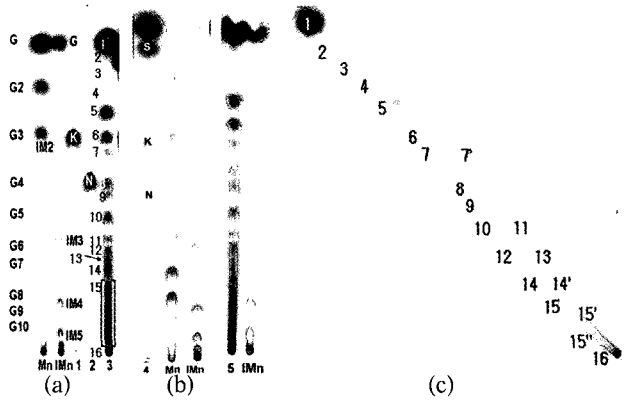

Fig. 1. Thin layer chromatogram of oligosaccharides synthesized using B-1355C levansucrase on 3 M sucrose. Mn: maltooligosaccharides; G, G2, G3 to G10 - Glucose, maltose, maltotriose to maltodecaose, IMn: isomaltooligosaccharides; IM2 to IM5 - isomaltose to isomaltopentaose, (a) lane 1: 1-kestose, lane 2: nystose, lane3: BFOS. The ascent developing solvent was 2:5:1.5 (v/v/v) nitromethane/1-propanol/water, (b) lane 4, commercial fructooligosaccharides; 1-monosaccharides, S-sucrose, K-kestose, N-nystose, the ascent developing solvent was 2:5:1.5 (v/v/v) nitromethane/1-propanol/water, (c) lane 5, two-dimensional TLC of oligosaccharides synthesized using levansucrase (GBOS) on 3 M sucrose. Composition of the first ascent developing solvent was 85:20:50:50 (v/v/v/v) acetonitrile/ethylacetate/1-propanol/water and then the second ascent developing solvent was 2:5:1.5 (v/v/v) nitromethane/1-propanol/water. Each component percent in the sucrose acceptor reaction digest (Fig. 1a, lane 3; Fig. 1c; % of total BFOS after excluding monosaccharides); 1-monosaccharides (glucose, fructose); 2- 3.4%; 3- 1.4%; 4-2.4%; 5-12.4%; 6-8.5% (kestose); 7+7'-5.2%; 8-5.7% (nystose); 9-4.3%; 10-6.6% (kestopentaose); 11-2.8%; 12-4.6% (kestohexaose); 13-5.8%; 14+14'-8.0%; 15+15'+15''-24.6%; 16-4.3% (levan).

1 illustrates the separation of oligosaccharides prepared with 3 M sucrose (as the final concentration) by two-dimensional thin layer chromatography (Fig. 1). The composition of the reaction digest was complex: Monosaccharides at 37.8%, BFOS at 59.5% and levan at 2.7% of the total saccharides. Excluding monosaccharides, the percentage of oligosaccharides was 95.7% and they were composed of over 15 different components including kestose (8.5% in BFOS), nystose (5.7% in BFOS), kestopentaose (6.6% in BFOS), kestohexaose (4.6% in BFOS) and their acceptor products of different linkages (73.6% in BFOS) (Fig. 1a, lane 3; Fig. 1c). The commercial fructo-oligosaccharides were generally composed of 59.5% monosaccharides, 29.3% sucrose, and 11.2% of some oligosaccharides (Fig. 1b, lane 4). They were also composed of fewer kinds of saccharides: kestose (47.7% of the total fructo-oligosaccharides), nystose (22.7% of the total fructo-oligosaccharides) and other fructo-oligosaccharides (29.6% of the total olgisaccharides) with different linkages. At present, each component is being purified using a P-2 column and paper chromatography, and detailed structure analyses are in progress.

The commercially produced malto-oligosaccharides,

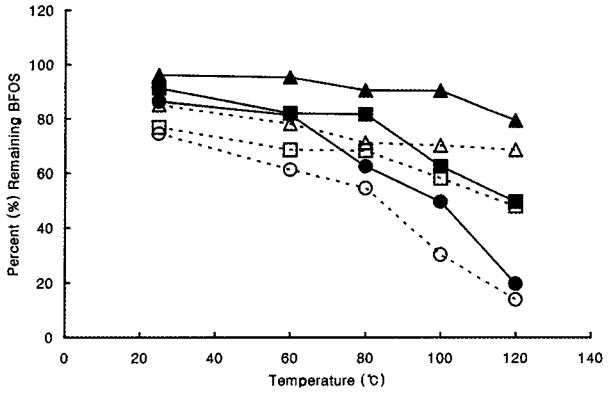

Fig. 2. The acid stability of BFOS at different temperatures (°C). Closed, with BFOS addition; Opened, Commercial Fructooligosaccharides; Circles (-●-, -○-), incubation at pH 2; Squares (-■-, -□-), incubation at pH 3; Triangles (-▲-, -△-), incubation at pH 4. Fifty mL of each reaction digest was adjusted to different pHs of 20 mM imidazole-HCl buffer (pH 2, 3, and 4). Each preparation was divided into five portions and incubated at 25, 60, 80, 100, and 120°C for 15 min, and then quenched.

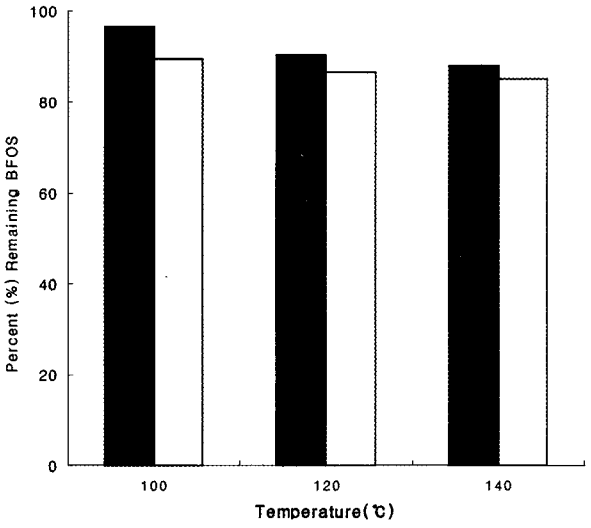

Fig. 3. The heat stability of oligosaccharides at different temperatures (°C). A closed bar, incubation for 30 min; Open bar, incubation for 60 min. Fifty mL of each reaction digest was adjusted to pH 5.2. Each preparation was divided into three portions and incubated at 100, 120, and 140°C for 1 h, and then quenched.

such as oligosaccharides consisting of glucose, are resistant to acid and heat treatments. Yet, they are less sweet. On the contrary, commercial fructo-oligosaccharides are sweet and are not resistant to acid and heat treatments. As a result, both of these oligosaccharides are restricted in their use as additives or sweeteners in foods that have a low pH and require a heat treatment during their processing. The stabilities of BFOS in acidic pHs and high temperatures were tested (Figs. 2 and 3). BFOS resisted the hydrolysis of glycosidic linkages at pH 5.2, 140°C for 1 h (Fig. 3). BFOS were also stable at pH 4, 120°C, at pH 3, 80°C and at pH 2, 60°C or lower temperatures: over 80% remained of the original amount of BFOS after 15 min (Fig. 2). This thermo-stability trait was higher when compared to that of commercial fructooligosaccharides. Since BFOS have thermo- and acid stable properties, they can be used as an additive for food processes that require relatively high temperatures and low pH conditions.

Dental plaque is a complex film of microorganisms on tooth surfaces that play an important role in the development of caries and periodental diseases [1]. Mutans *Streptococci* can colonize tooth surfaces thus initiating plaque formation by their ability to synthesize extracellular polysaccharides, mainly water-insoluble glucan, from sucrose [14]. This sucrose-dependent adherence trait and the accumulation of cariogenic *Streptococci* are critical steps in the development of pathogenic plaque [15].

Glucosyltransferases (GTFs including mutansucrase) are responsible for the synthesis of dextrans or insoluble glucans from sucrose [16-18]. These enzymes were suggested to have a two-site insertion mechanism in which glucose is added to the reducing end of the growing chain. In addition, GTFs catalyze the transfer of the D-glucopyranosyl group from sucrose to other carbohydrates that are present. If they are added to the enzyme digest, GTFs terminate the polymerization of dextran or glucan [19,20]. These added carbohydrates are called acceptors and the reaction is called an acceptor reaction. Mutansucrase, an insoluble glucansucrases from *S. sobrinus,* has been observed to utilize maltose and maltodextrins *via* acceptor reactions, thereby decreasing the production of glucan from sucrose in assays done in solution [20,21]. According to the acceptor reaction, an efficient acceptor can reduce the formation of glycans. The addition of BFOS effectively inhibited the formation of insoluble glucan by *S. sobrinus*. More specifically, the addition of BFOS (0.5% BFOS in the culture with 10% sucrose) inhibited the formation of insoluble glucan by about 50% when compared to that of the control (with the addition of 0.5% glucose with 10% sucrose instead of oligosaccharides). *S. sobrinus* plays a role in the aetiology of dental caries [14]. *S. sobrinus* makes extracellular soluble and insoluble polysaccharides from the sucrose that is associated with plaque formation and carcinogenicity. This organism can also synthesize intracellular polysaccharides which act as carbohydrate reserves, and can be converted to an acid when dietary carbohydrates are unavailable [2].

The growth of *S. sobrinus* was also inhibited by the addition of BFOS (Fig. 4). The pHs of the culture supernatants with a BFOS addition dropped slowly compared to the pH drop in the control without a BFOS addition (Fig. 4). The growth of *S. sobrinus* in the control culture reached an OD_{600} of 1.52 after 24 h. On the other hand, the growth of *S. sobrinus* in the culture containing BFOS decreased compared to that of the control: the growth value reached 1.15 (75.7% of control growth) for *S. so-*

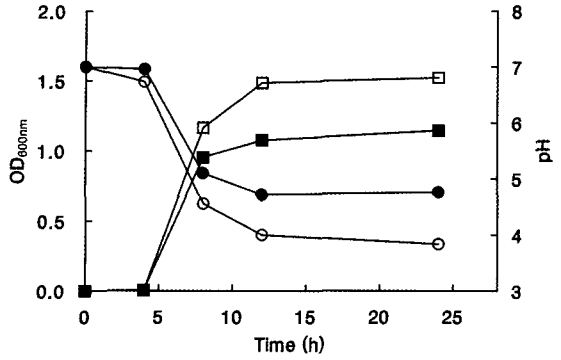

Fig. 4. The effect of BFOS on the growth of *S. mutans* and culture pH. The growth of *S. mutans* in a medium with (closed square) and without (open square) the addition of BFOS. The pH profile of the *S. mutans* culture with (closed circle) and without (open circle) the addition of BFOS. *S. sobrinus* was grown in BHI medium (0.5% yeast extract and 3.7% brain heart infusion, pH 7.0, 37°C) plus 0.5% oligosaccharides. The control culture was prepared in BHI medium containing 0.5% glucose. At the same time, the acid formation was monitored by the pH changes in the culture of *S. sobrinus*.

brinus. When glucose was the sole carbon source, the pH of the reaction mixture reached 4.0 in *S. sobrinus*, 7~8 h after inoculation. The pH in the culture with BFOS dropped more slowly than the controls; it bottomed out at a pH of 4.8 compared to a pH of 3.8 of the control.

BFOS also inhibited the formation of insoluble biomass by oral microorganisms. The amount of adherent biomass (both insoluble glucan and cells) was 40.1 mg on the surface of the control stainless steel wire and 11.5 mg on the surface of the stainless steel wire with the BFOS addition. The same pattern was seen on the surfaces of the glass vials; the inhibition of biofilm formation was 73% by absorbance. One reason for the decrease in the mutansucrase activity was due to the acceptor reaction; sucrose hydrolyzed by mutansucrase and most glucosyl residues was transferred to BFOS by acceptor reactions (data not shown). Since BFOS inhibit the formation of soluble and insoluble glucans by oral pathogens, they can be used as an active ingredient of oral care products to prevent dental caries and other oral diseases.

Studies of the physical properties and the roles of each purified BFOS as a prebiotic and/or an anti-cariogenic sucrose substitute, especially for various oral pathogens, are in progress.

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