J. Appl. Glycosci., **54**, 147−155 (2007)

Proceedings of the Symposium on Amylases and Related Enzymes, 2006

Synthesis of Thermo- and Acid-stable Novel Oligosaccharides by Using Dextransucrase with High Concentration of Sucrose

(Received November 14, 2006; Accepted December 28, 2006)

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Abstract: A method is presented for synthesizing thermo-, acid-stable glucooligosaccharides (TASOG) from sucrose (2.5−4 M) using a dextransucrase prepared from *Leuconostoc mesenteroides* B-512FMCM. The degree of polymerization (DP) of oligosaccharides synthesized was from 2 to 11. TASOG resisted hydrolysis of its glycosidic linkages at 140°C and pH 6.0 for 1 h. It was stable at pHs ranging from 2 to 4 at 120°C. A method for synthesizing fructo-oligosaccharides (TASOF) with high concentrations of sucrose (1−3 M) by using levansucrase prepared from *L. mesenteroides* B-1355C was also developed. The DP of oligosaccharides synthesized according to the present method ranged from 2 to over 15. The TASOF was also stable at pHs ranging from 2 to 4 under 120°C. The percentage of TASOF in the reaction digest was 95.7% (excluding monosaccharides; 4.3% was levan). Both oligosaccharides effectively inhibited the formation of insoluble glucan, and the growth and acid production of *Streptococcus sobrinus*. TASOG and TASOF potentially can be used as sweeteners for food and beverages where thermo- and acid-stable properties are required and as potential inhibitors of dental caries.

Key words: dextransucrase, levansucrase, thermo-stable, acid-stable, oligosaccharides

Oligosaccharides are used in foods, feeds, pharmaceuticals, or cosmetics as stabilizers, bulking agents, immunostimulating agents or prebiotic compounds.[1)] In general, effective oligosaccharides for various functions have a degree of polymerization (DP) from 2−10 monosaccharide units (molecular weight of 300−2000). Commercially produced oligosaccharides are fructooligosaccharides, isomaltooligosaccharides, maltooligosaccharides and galactooligosaccharides. It is well known that maltooligosaccharides and isomaltooligosaccharides are acid- and heat-stable carbohydrates. They are less sweet than fructooligosaccharides, which are susceptible to acid and heat treatment. As a result, both types have restricted use as additives or sweeteners in foods that require heat treatment or acidic pH during process. Many sweet foods contain mono- or disaccharides that are readily metabolized by cariogenic bacteria. To satisfy human craving 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 glucansucrases with the hope finding a compound that is safe, cheap and effective in blocking oral colonization of *Streptococcus* sp.[2)]

L. mesenteroides mutants from strains, B-512FM, B-742, B-1299 and B-1355 are constitutive for glucansucrase production.[3−7)] A mutant, *L. mesenteroides* B-512 FMCM, was obtained that produced 13 times more enzyme than the parent mutant strain, B-512FMC, and over a 100 fold more than the original B-512F commercial strain for dextran production.[8−10)]

Dextrans are a class of polysaccharides composed of D-glucans with contiguous α-(1→6) glycosidic linkages in the main chains and α-(1→2), α-(1→3) or α-(1→4) branch glycosidic linkages, depending on the specificity of the particular dextransucrase.[11)] The enzymes responsible for the synthesis of these dextrans from sucrose are dextransucrases (EC 2.4.1.5), glucansucrases and glucosyltransferases. In addition to catalyzing the synthesis of dextran from sucrose, dextransucrase also catalyzes the transfer of a D-glucopyranosyl group from sucrose to other carbohydrates.[12)] The added carbohydrates have been called acceptors. When the acceptor is D-glucose, maltose, isomaltose, or compounds having free hydroxyl group(s), a series of oligosaccharides and glycosides with isomaltodextrins of varying number of D-glucose units are at-

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tached by an α-(1→6) linkage to the nonreducing-end of the acceptor.[13–16)]

Lactic acid bacteria (LAB) are a group of gram-positive, food-grade microorganisms consisting of many genera, *e.g. Lactococcus, Leuconostoc* and *Lactobacillus*. Members of these genera possessing the generally regarded as safe (GRAS) status find applications in the production of food and feed.[17)] Some LABs produce fructan consisting mainly of β-(2→6) linked fructosyl residues, occasionally containing β-(2→1) linked branches. This fructan is called levan and can reach a DP of more than 100,000 fructosyl units. Bacterial levan is produced extracellularly by a single enzyme, levansucrase (EC 2.4.1.9), which produces levan directly from sucrose. In addition to fructan synthesizing activity, several of these bacterial levansucrases can transfer fructosyl units to other sugars such as glucose, fructose and raffinose as acceptors.[18)] *L. mesenteroides* is also known to produce a levansucrase, yet the detailed study for the *L. mesenteroides* levansucrase in the respect of enzyme and sucrose acceptor reaction for fructo-oligosaccharide synthesis is not reported.

Kim and Kim[10)] reported that reactions of *L. mesenteroides* B-512FMCM dextransucrase with increasing concentrations of sucrose, from 0.1 to 4.0 M, produced decreasing amounts of high-molecular weight dextran (HMWD) (>10^6 Da) with concomitant increases in low-molecular weight dextran (LMWD) (<10^5 Da). At 0.1 M sucrose, pH 5.5 and 28°C, 99.8% of the dextran had a Mw>10^6 Da and at 4.0 M sucrose, 69.9% had a Mw<10^5 Da and 30.1% had a Mw>10^6 Da. The degree of branching increased from 5% at 0.1 M sucrose to 16.6% for 4.0 M sucrose. Alsop[19)] reported that as the sucrose concentrations in a dextransucrase digest increased, from 0.06 to 0.6 M, the amount of low-molecular weight dextran (LMWD) increased. At 0.6 M sucrose, there was 43.9% LMWD, which is defined as dextrans with molecular weights below 5000 Da.

Along with these results, we report on the synthesis and characteristics of TASOG and TASOF synthesized by *L. mesenteroides* B-512FMCM dextransucrase and *L. mesenteroides* B-1355C levansucrase, respectively, at relatively high concentrations of sucrose, 0.5–4 M. The resulting oligosaccharides were thermo-, acid-stable and about 80% as sweet as sucrose. The oligosaccharides also inhibited the activity of glucosyltransferases from an oral pathogen, *Streptococcus sobrinus.*

MATERIALS AND METHODS

Materials. Carbohydrates were purchased from Sigma-Aldrich Inc. (Milwaukee, WI, USA). Media were purchased from Duchefa (Haarlem, Netherlands). All other chemicals were of reagent grade and commercially available.

Preparation of dextransucrase. Dextransucrase was prepared by culturing *L. mesenteroides* B-512FMCM in a glucose medium, as described by Kim and Robyt.[3–7)] The enzyme was purified as described by Kim and Kim.[10)] The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis that had a specific dextransucrase activity of approximately 250 U/mg, both indicative of a highly purified enzyme. The one unit of enzyme activity is defined as the amount of enzyme required to liberate one μmol of fructose from 150 mM sucrose in 20 mM sodium acetate buffer (pH 5.2) that contained 1 mM $CaCl_2$ and 1 mg/mL Tween 80 per min at 28°C. The amounts of fructose were analyzed using the NIH Image Program.[10)]

Preparation and purification of mutansucrase and levansucrase. *Streptococcus sobrinus* was grown in 4 L stirred batch cultures with the pH and temperature controlled at 7.0 and 37°C for 24 h, respectively.[20)] After fermentation, the culture was harvested, centrifuged and concentrated to 200 mL using hollow fiber membrane (30 K cut-off, Millipore, USA or 50 K cut-off, Pure Tech., Korea). One unit of mutansucrase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose per min at 37°C and pH 6.5 from 200 mM sucrose. The final enzyme activity for mutansucrase was 20 U/mL. A constitutive mutant for dextransucrase and levansucrase, *L. mesenteroides* B-1355C, was grown in LWG medium consisted of 0.3% (w/v) yeast extract, peptone, 0.3% (w/v) K_2HPO_4, and 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 OD_{600} was approximately 3.0[9)] and LVSase was prepared from culture supernatant. LVSase activity was assayed by 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 enzyme solution (2 U/mL). Each enzyme reaction sample was spotted on a Whatman K5 TLC plate. 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, and followed by heating 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.

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 concentration of culture supernatant was accomplished by passing the culture supernatant through a polysulfone ultrafiltration hollow fiber cartridge with 63.8 cm length and 0.45 m^2 surface area [H5P100-43 (100 kDa cut-off), Amicon, Inc., Beverly, MA] at a flow rate of 4 mL/min at 22°C. As the volume of the concentrate reached at approximately 1 L, 500 mL of 20 mM sodium phosphate buffer (pH 6.4) was added and followed by the second concentration to approximately 1 L. This step was repeated three times and the hollow fiber cartridge was washed with approximately 400 mL of the same buffer. The concentrate was equilibrated with 20 mM sodium phosphate buffer (pH 6.4) and 20 mL of sample was loaded on a DEAE-Sepharose (2.8×35 cm) column pre-equilibrated 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. Fractions collected were screened for LVSase activity. Positive fractions were pooled and stored at −20°C for further studies.

Synthesis of oligosaccharides. In order to produce oligosaccharides using B-512FMCM dextransucrase the enzyme was mixed with a 4.5 M sucrose solution. The final sucrose concentrations were from 0.5–4 M, and reaction conditions ranged from 28 to 65°C. The enzyme was used over a concentration range of 0.1–10 U/mL. The reaction was allowed to proceed until the sucrose was completely consumed. After reaction 1 μL aliquots were spotted on Whatman K5F TLC plates (Whatman, Clifton, NJ, USA), the plates were developed twice in the solvent mixture of acetonitrile/1-propanol/water (20/50/15, v/v/v). Components of the products were also identified by using HPLC with standard carbohydrates. Sugars were separated in a Macrosphere™ GPC 60 Å column (7.5×300 mm, Alltech Associates Inc., PA, USA), and were eluted at a flow rate of 0.5 mL/min with water.

Synthesis of Branched Fructo-oligosaccharides (TASOF). In order to produce TASOF with B-1355C LVSase, 4 M sucrose solution was mixed with enzyme obtained as above. The final sucrose concentration of each enzyme reactor was 1–3 M, and it was reacted at between 28 and 55°C. The enzyme used was 0.1–10 U/mL. The reaction was performed until sucrose of the reaction digest was consumed completely. After the reaction 1 μL aliquot was spotted on a Whatman K5F TLC plate (Whatman, Clifton, NJ), then the plate was developed twice in acetonitrile/1-propanol/water (2/5/2.5, v/v/v).

Isolation of oligosaccharides and analysis of its structure. Mixtures of oligosaccharides were spotted on preparative silica gel plates (Whatman, Clifton, NJ, USA). They were developed using the solvent mixture of acetonitrile/ethyl acetate/1-propanol/water (85/20/50/50, v/v/v/v), and then the lateral side was removed. The separated carbohydrate component from the TLC strip was visualized using dipping reagent as described above. The cut strip was compared with the undeveloped portion of the silica gel plates and silica gel containing each designated component was separately gathered. Each silica gel fraction was suspended in the developing solvent and centrifuged. The silica gel portion was re-suspended in water to remove the carbohydrate. Each component was identified by using TLC, then freeze dried and stored at 4°C. Identification of each oligosaccharide was through two-dimensional TLC with standard carbohydrates. The first developing solvent was the solvent mixture of acetonitrile/ethyl acetate/1-propanol/water (85/20/50/50, v/v/v/v). After two ascents, the TLC plates were dried and rotated 90°, then the plate was developed in the second solvent (nitromethane/1-propanol/water, 20/50/15, v/v/v). One μL of each compound (2 mg/mL) and/or the reaction products were transferred to the sample plate and mixed with 1.0 μL of 0.1 M 2,5-dihydroxybenzoic acid in 70% (v/v) acetonitrile/water solution. The solvent was evaporated under airflow. The molecular masses of the samples were analyzed by MALDI-TOF MS, using a Dynamo instrument (Thermo Bio-Analysis, Ltd., Paradise, UK) with a nitrogen laser (337 nm). Ions were detected in a positive mode at an acceleration voltage of 20 kV. Enzymes hydrolyzing different linkages (α-, β-, iso-amylase, α-, β-glucosidase, dextranase, α-amyloglucosidase, invertase; Sigma-Aldrich Inc., Milwaukee, WI, USA) were used to treat each purified oligosaccharide at 37°C with optimum pH for each enzyme. Then one microlitter of enzyme digest was spotted onto the silica gel plate and then developed twice in the nitromethane/1-propanol/water (20/50/15, v/v/v) to identify the hydrolysis products. The structure was predicted based on the results above.

To identify each component of oligosaccharide, each purified fraction was mixed with an equal volume of 1 M hydrochloric acid. After hydrolysis at 100°C for 30 min, the hydrolyzates were vacuum dried and re-dissolved in water. One microlitter of each reaction solution was spotted on silica gel plates and developed twice in the solvent mixture of acetonitrile/water (85/15, v/v). In order to identify the acceptor reaction product from the oligosaccharide mixture, each fraction was mixed with dextransucrase (3 U/mL) and 100 mM sucrose in 20 mM sodium acetate buffer (pH 5.2) at the proportion of 1/1/1 (v/v/v). Then it was reacted at 28°C for 12 h. Each acceptor reaction product obtained from the above reaction was identified by using TLC and compared to the original oligosaccharide mixture.

Thermo- and acid-stable oligosaccharides. Fifty mL each of reaction digests were incubated at different temperatures (60°C to 140°C) for 30 and 60 min. Fifty mL each of reaction digests were adjusted to different pHs with 20 mM imidazol-HCl buffer (pH 2, 3, 4 or 5). Each preparation was divided into 5 equal portions and incubated at 40, 60, 80, 100 or 120°C for 15 min. The composition change was identified using TLC as described previously.[21]

The effects of oligosaccharides in cell growth and acid production. *Bifidobacterium infantis* (KCCM 11207) and *B. adolescentis* (KCCM 11206) were grown in reinforced clostridium medium (0.3% yeast extract, 1% beef extract, 1% peptone, 0.1% soluble starch, 0.5% glucose, 0.05% cysteine-HCl, 0.5% NaCl, 0.3% sodium acetate, pH 6.8, 37°C) plus 0.5% oligosaccharides. *B. bifidum* (KCCM 12096) was grown in TPY medium (1% tryptone, 0.5% pancreatic digest of soybean meal, 0.5% glucose, 0.25% yeast extract, 0.05% cysteine, 0.2% K_2HPO_4, 0.05% cysteine-HCl, 0.05% $MgCl_2$, 0.025% $ZnSO_4 \cdot 7H_2O$, 0.015% $CaCl_2$, 0.001% $FeCl_3$, 0.001% Tween 80, pH 6.8, 37°C) plus 0.5% oligosaccharides. *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% glucose with or without the addition of 0.5% oligosaccharides with the pH and temperature at 7.0 and 37°C, respectively. At the same time, the acid formation was monitored by the pH changes in cultures of *S. sobrinus*.[22]

Sucrose-dependent, glucan-mediated adhesion. Adhesion of cells on surfaces of stainless steel wire or glass vials was measured essentially as described by Ryu *et al.*[23] *S. sobrinus* was grown in BHI medium. *S. sobrinus*, 2.5 $\times 10^6$ cells mL^{-1}, were inoculated in BHI medium containing 10% (w/v) sucrose, 0.5% (w/v) or 4% (w/v) oligosaccharides for 24 h at 37°C without shaking. Liquid

and non-adherent cells were carefully removed by aspiration. Adherent cells were washed once gently 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 by the absorbance at 550 nm.[23,24]

RESULTS AND DISCUSSION

Synthesis and structure analysis of oligosaccharides.

Sucrose [α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] has five structural isomers including palatinose (isomaltulose), trehalulose, turanose, maltulose and leucrose. Two of the most promising compounds for reducing both caloric uptake and dental caries are isomers of sucrose, palatinose [α-D-glucopyranosyl-(1→6)-D-fructofuranose] and leucrose [α-D-glucopyranosyl-(1→5)-D-fructofuranose].[11] The relative sweetness of TASOG (containing 13.5% of fructose) was about 80% that of sucrose. The sweetness of palatinose or leucrose is about 45% or 60% that of sucrose (in a 10% solution), respectively. The structure and relative quantity of each oligosaccharide was determined (Table 1). TASOG contains no glucose or sucrose but fructose (13.5%). In addition, the bulk (37.5%) of the total carbohydrates was oligosaccharides containing fructose. Analyses of each purified component by acidic hydrolyses for monomer components, MALDI-TOF for molecular size determination, various enzyme hydrolyses for linkages determination, and acceptor reactions of each oligosaccharide as an acceptor were used to identify designated acceptor reaction products.

More than 14.3% was palatinose and its acceptor reaction products. These are non-cariogenic sugar substitutes (Designated Nos. 2, 6, 10, 14). Various palatinose-producing microorganisms have been used industrially due to their α-glucosyltransferase production (sucrose isomerase; EC 5.4.99.11), catalyzing of conversion of sucrose into isomaltulose or other isomers.[25] More than 23.2% was leucrose and its acceptor reaction products (Designated Nos. 3, 7, 11, 15). Essentially no acid formation was observed after incubation of leucrose with suspensions of human dental plaque, *S. mutans* NCTC 10449, *Lactobacillus casei* LSB 132 and *Actinomyces viscosus* Ny 1 No. 30.[26–28] Leucrose is a competitive inhibitor of the acid formation from sucrose by *S. mutans* NCTC 10449 at neutral pH. Furthermore, leucrose inhibits uptake of sucrose by *S. mutans* NCTC 10449 at neutral pH.

Both palatinose and leucrose can be synthesized by acceptor reactions with fructose, although both disaccharides show no or low acceptor reaction efficiencies with dextransucrase under standard reaction conditions (pH 5.2, 28°C, 300 mM or lower concentration of sucrose).[29] In high sucrose concentrations, however, various acceptor reaction products, containing different degrees of glucosyl residue(s) at the non-reducing end of palatinose (Designated Nos. 6, 10, 14) or of leucrose (Designated Nos. 7, 11, 15), were synthesized. Those oligosaccharides are synergistic non-cariogenic sucrose substitutes with palatinose and leucrose. Inhibition studies of insoluble glucan formation and pH drop in cultures of various oral pathogens with each palatinose or leucrose acceptor reaction product are in progress.

We also found that sucrose in high concentration was used as an acceptor of LVSase and various sucrose acceptor reaction products (TASOF) were synthesized. Figure 1

Table 1. Structure and quantities of TASOG produced by dextransucrase.

Structure of (Oligo) saccharide	Name of (Oligo) saccharide	Relative Amt (%)
Fru	D-fructose	13.5
α-Glc(1→6)Fru	Isomaltulose (Palatinose) (6-α-D-glucopyranosyl-D-fructofuranose)	4.6
α-Glc(1→5)Fru	Leucrose (5-*O*-α-D-glucopyranosyl-D-fructopyranose)	12.4
α-Glc(1→6)Glc	Isomaltose	7
α-Glc(1→6)Glc ↗ α-Glc(1→3)	3^2-*O*-α-D-glucosyl-isomaltose	5.2
α-Glc(1→6)α-Glc(1→6)Fru	6-*O*-α-isomaltosyl-D-fructose	3.7
α-Glc(1→6)α-Glc(1→5)Fru	5-*O*-α-isomaltosyl-D-fructopyranose	4.3
α-Glc(1→6)α-Glc(1→6)Glc	Isomaltotriose	6.3
α-Glc(1→6)α-Glc(1→6)Glc ↗ α-Glc(1→3)	3^2-*O*-α-D-glucosyl-isomaltotriose	2.9
[α-Glc(1→6)]$_2$α-Glc(1→6)Fru	6-α-isomaltotriosyl-D-fructose	3.3
[α-Glc(1→6)]$_2$α-Glc(1→5)Fru	5-*O*-α-isomaltotriosyl-D-fructopyranose	3.8
[α-Glc(1→6)]$_3$Glc	Isomaltotetraose	6.2
[α-Glc(1→6)]$_3$αGlc(1→6)Glc ↗ α-Glc(1→3)	3^2-*O*-α-D-glucosyl-isomaltotetraose	2.8
[α-Glc(1→6)]$_3$α-Glc(1→6)Fru	6-*O*-α-isomaltotetraosyl-D-fructose	2.7
[α-Glc(1→6)]$_3$α-Glc(1→5)Fru	5-*O*-α-isomaltotetraosyl-D-fructopyranose	2.7
[α-Glc(1→6)]$_4$Glc	Isomaltopentaose	5.6
	OligosaccharidesHaving DP of over 5	8.4
	Dextran	4.6

Reaction condition: B-512 FMCM dextransucrase (3 U/mL), 4 M sucrose for 30 h.

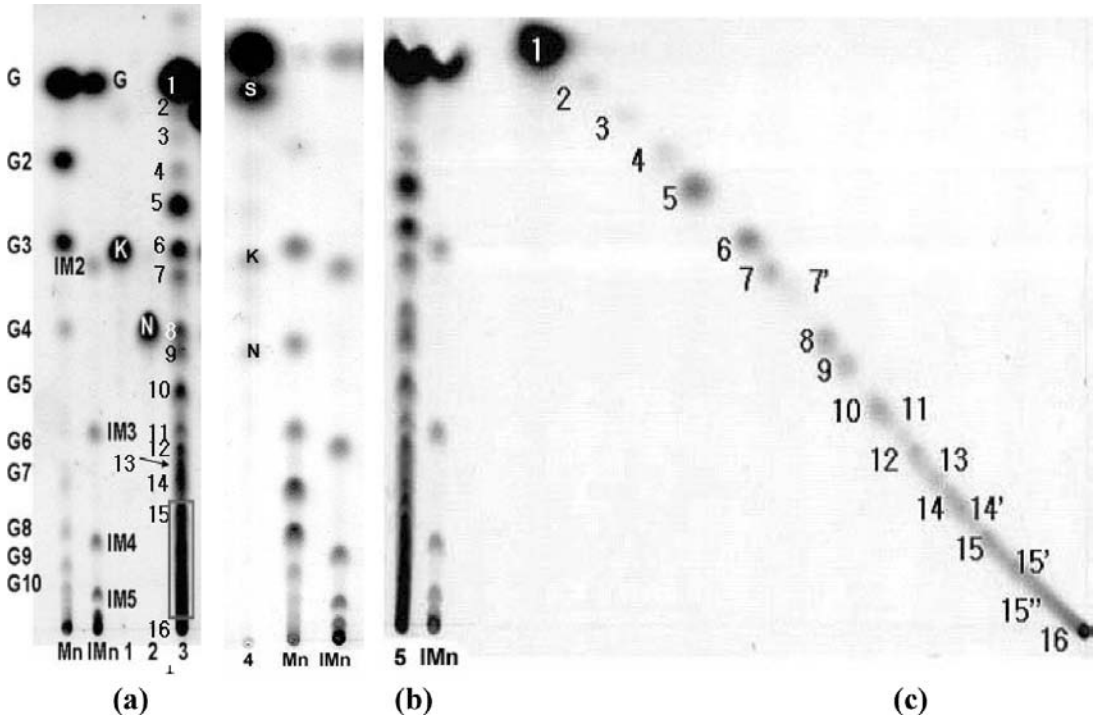

Fig. 1. Thin layer chromatogram of oligosaccharides synthesized by using B-1355C levansucrase on 3 M sucrose.

Mn: maltooligosaccharides; G, G2, G3 to G10 : Glucose, maltose, maltotriose to maltodecaose; IMn : isomaltooligosaccharides; IM_2 to IM_5 : isomaltose to isomaltopentaose; (a) lane 1, 1-kestose; lane 2, nystose; lane 3, TASOF. 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 by using levansucrase (TASOF) 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 % in sucrose acceptor reaction digest (Fig. 1a, lane 3; Fig. 1c; % of total TASOF 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)

illustrates the separation of oligosaccharides prepared with 3 M sucrose (as final concentration) by using two-dimensional thin layer chromatography (Fig. 1). The composition of reaction digest was complex: Monosaccharides compromised 37.8%, TASOF 59.5% and levan 2.7% of 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 TASOF), nystose (5.7% in TASOF), kestopentaose (6.6% in TASOF), kestohexaose (4.6% in TASOF) and their acceptor reaction products of different linkages (73.6% in TASOF) (Fig. 1a, lane 3; Fig. 1c). The commercial fructo-oligosaccharides were mostly composed of monosaccharides and sucrose at 59.5 and 29.3%, respectively, and some oligosaccharides at 11.2% (Fig. 1b, lane 4). Also it was composed of fewer kinds of saccharides; kestose (47.7% of total fructo-oligosaccharides), nystose (22.7% of total fructo-oligosaccharides) and other fructo-oligosaccharides (29.6% of total oligosaccharides) with different linkages. Each component is being purified by using P-2 column and paper chromatography, and detailed structure analyses are in progress.

The effect of various reaction conditions on the synthesis of oligosaccharides.

Table 2 shows the effects of sucrose concentration, reaction temperature, and final dextransucrase activity on synthesis of oligosaccharides by *L. mesenteroides* B-512 FMCM dextransucrase. The acceptor reaction was enhanced with higher sucrose concentrations since oligosaccharide content was increased from 27.2% at 0.5 M sucrose to 63.4% at 4 M sucrose. However, polymer and monosaccharide (glucose and fructose) contents were decreased by the increased sucrose concentration (Table 2). This indicates more acceptor reaction occurred with carbohydrates than water since there were higher concentrations of available carbohydrates. This result is consistent with a study of Kim *et al*.[21] in that the small saccharides (Mw-10^{3-5} Da) were increased but large saccharides (Mw-10^{4-6} Da) were decreased the most by increasing of sucrose content from 1 M to 4 M.

The effect of temperature on acceptor was measured at 28, 37, 45, 55 and 65°C. At below 28°C or above 65°C, the un-consumed sucrose was increased and the yield of oligosaccharides was decreased (data not shown). Optimum temperature for acceptor reactions was 45°C with the highest oligosaccharides yield of 59.1%. Dextransucrase produced the highest oligosaccharides yields at 28–30°C with below 300 mM sucrose but at 45°C with above 300 mM sucrose. The effect of increasing the temperature to give increased yields of the oligosaccharide products might have been expected from the mechanism of acceptor reactions of dextransucrase.[29] At the high concentration of sucrose, sucrose binds to the third, low-affinity binding site on dextransucrase, allosterically changing the conformation of the active site so that the dextran is not preferentially formed; rather acceptor reaction products are formed.[29] Kim *et al*.[21] reported an increase in the de-

Table 2. The effect of various reaction conditions on the synthesis of TASOG.

Reaction conditions		Yield[a] (%)	
		Fructose and glucose (Sucrose left unreacted)	Oligosaccharides/polymer
Sucrose concentrations	0.5 M	49.9(0)	27.2/22.9
-28°C,	1.0 M	49.0(0)	35.3/15.7
-2.5 U/mL dextransucrase,	2.0 M	47.1(0)	40.0/12.9
-36 h reaction	3.0 M	40.0(0)	50.0/10.0
	4.0 M	28.2(0)	63.4/ 8.4
Temperatures	28°C	20.0(21.4)	50.8/ 7.8
-3 M sucrose,	37°C	23.2(13.1)	53.9/ 9.8
-2.5 U/mL dextransucrase,	45°C	28.4(0)	59.1/12.5
-24 h reaction	55°C	20.6(28.8)	42.3/ 8.3
	65°C	18.2(42.7)	34.2/ 4.9
Dextransucrase activity			
-28°C,	1 U/mL	12.5(33.5)	46.7/ 7.3
-3 M sucrose,	5 U/mL	26.9(0)	63.1/10.0
-24 h reaction	10 U/mL	26.2(0)	61.9/11.9
-37°C,	1 U/mL	18.3(24.4)	51.6/ 5.7
-3 M sucrose,	5 U/mL	26.6(0)	61.0/12.4
-24 h reaction	10 U/mL	25.9(0)	61.3/12.8
-45°C,	1 U/mL	20.0(19.4)	51.4/ 9.8
-3 M sucrose,	5 U/mL	27.0(0)	60.0/13.0
-24 h reaction	10 U/mL	27.7(0)	59.6/12.7

[a] Yield was determined as relative percentage on total carbohydrate.

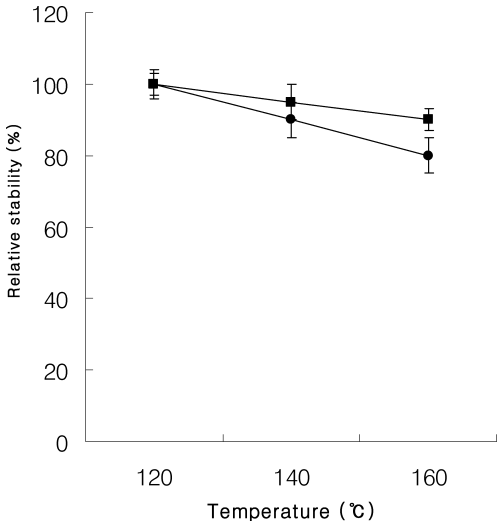

Fig. 2. The heat stability of oligosaccharides at different temperatures (°C).

■, 30 min; ●, 60 min. Activity was measured after incubation of TASOG (thermo-, acid-stable oligosaccharides) at the designated temperature for 30 or 60 min.

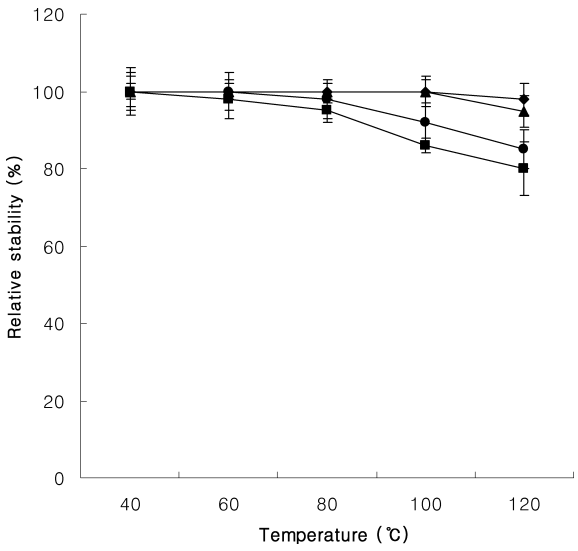

Fig. 3. The acid stability of oligosaccharides at different temperatures (°C).

■, pH 2 ; ●, pH 3 ; ▲, pH 4 ; ◆, pH 5. Activity was measured after incubation of TASOG (thermo-, acid-stable oligosaccharides) at the designated temperature for 15 min.

gree of branching as reaction temperature increased. The binding of dextran to the acceptor-site is controlled by the diffusion of the dextran molecules, and the diffusion is temperature dependent. Thus, the higher the rate of diffusion that is dependent on the temperature, the higher the degree of branching due to greater interaction between dextransucrase, sucrose and acceptor dextran.[21,27]

However, increased dextransucrase activity (5.0 U mL^{-1} or higher) did not influence final oligosaccharides yields (Table 2). Overall, the optimum conditions for the synthesis of oligosaccharides would be obtained at 3–4 M sucrose concentrations, 2.5–5.0 U mL^{-1} dextransucrase activities at 28–45°C until all of the sucrose was consumed (around 24 h).

Thermo- and acid-stable oligosaccharides.

The stabilities of oligosaccharides at acid pHs and high temperatures were tested. Oligosaccharides resisted the hydrolysis of glycosidic linkage at 140°C for 1 h at pH 6.0 (Fig. 2: less than 10% oligosaccharides were hydrolyzed). They were stable at pH ranges from 2 to 4 at 120 °C or lower (Fig. 3). Therefore, our TASOG having thermo- and acid stable properties can be used as an additive in food processes requiring high temperature and low pH conditions. Growth of *S. sobrinus* was inhibited by

Table 3. Growth and pH comparisons of bacteria with or without thermo-, acid-stable oligosaccharides (TASOG).

	Streptococcus sorbinus	*Bifidobacterium adolescentis*	*Bifidobacterium infantis*	*Bifidobacterium bifidium*
	pH	OD_{600nm}	OD_{600nm}	OD_{600nm}	OD_{600nm}
Without TASOG[a]	4.73	1.31	0.89	1.02	1.25
With TASOG[b]	6.32	0.83	1.14	1.13	1.39

The data shown are the mean of three replicates. [a] Without TASOG, the addition of 0.5% glucose in culture medium alone. [b] With TASOG, 0.34% TASOG was added with 0.5% glucose

the addition of TASOG (containing 13.5% fructose), but growth of *Bifidobacterium* sp. was activated by the addition of TASOG (Table 3). The pHs of the culture supernatants dropped slowly compared to the pH drop in the control, without TASOG addition (Table 3). When glucose was the sole carbon source, the pH of the reaction mixture reached pH 4.73 in *S. sobrinus* 24 h after inoculation. The pH of cultures with TASOG dropped more slowly than the controls and bottomed out around pH 6.32 (Table 3). The stabilities of TASOF at acid pHs and high temperatures were tested. TASOF resisted the hydrolysis of glycosidic linkage at pH 5.2, 140°C for 1 h. TASOF was also stable at pH 4, 120°C, at pH 3, 80°C and at pH 2, 60°C or lower temperatures: there remained over 80% of original amount of TASOF after 15 min (data not shown). This thermostability was higher compared to that of commercial fructooligosaccharides. Since TASOF has thermo- and acid stable properties, it can be used as an additive in food processes requiring relatively high temperature and low pH conditions.

Sucrose-dependent, glucan-mediated adhesion.

The mixture of TASOG effectively inhibited the formation of insoluble glucan by *S. sobrinus* mutansucrase: the addition of 0.5% oligosaccharides in culture containing 10% sucrose inhibited the formation of insoluble glucan about 60% compared to that of control without the addition of oligosaccharides (Fig. 4a). The mixture of 4% glucooligosaccharides in culture containing 10% sucrose significantly inhibited the formation of insoluble glucan by *S. sobrinus* (Fig. 4b). *S. sobrinus* plays a role in the aetiology of dental caries.[2] *S. sobrinus* produces extracellular soluble and insoluble polysaccharides from sucrose that becomes dental plaque on teeth, resulting in tooth decay. They also synthesize intracellular polysaccharides which act as carbohydrate reserves, and can be converted to acid when dietary carbohydrates are unavailable.[2] As oligosaccharides inhibit the formation of both soluble and insoluble glucans by oral pathogens, they can be used as an active ingredient in oral care products to prevent dental caries and other oral disease. The addition of TASOF also effectively inhibited the formation of insoluble glucan by *S. sobrinus*: by the addition of TASOF (0.5% TASOF in culture with 10% sucrose) the formation of insoluble glucan was inhibited about 50% compared to that of the control (without the addition of oligosaccharides but 0.5% glucose with 10% sucrose). Growth of *S. sobrinus* was also inhibited by the addition of TASOF (Fig. 5). The pHs of the culture supernatants with TASOF addition dropped slowly compared to the pH drop in the control, without TASOF addition (Fig. 5). The growth of *S. sobrinus* in the control culture reached OD_{600} of 1.52 after

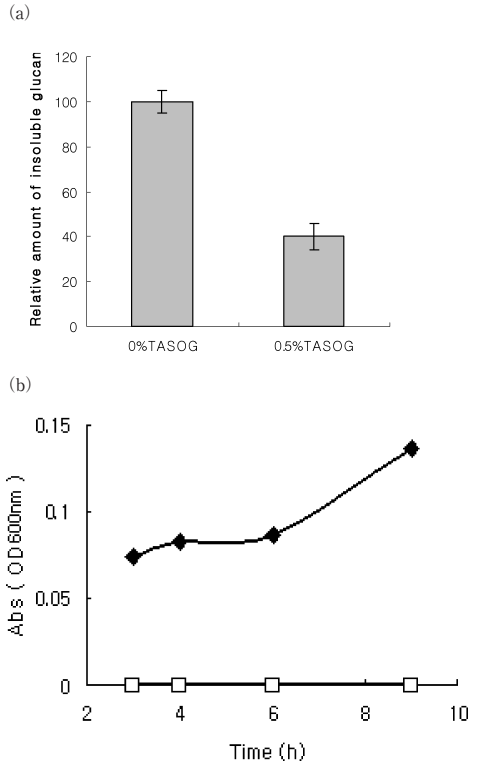

Fig. 4. The inhibition effect of TASOG addition in the insoluble glucan formation by *S. sobrinus.*

(a) Relative amount of insoluble glucan (left bar) and its adhesion on glass vial (right bar) by addition of final 0% TASOG or 0.5% TASOG in 10% sucrose culture medium. (b) Cell density detection by addition of 0% (closed diamond) or 4% (open square) TASOG in 10% sucrose culture medium.

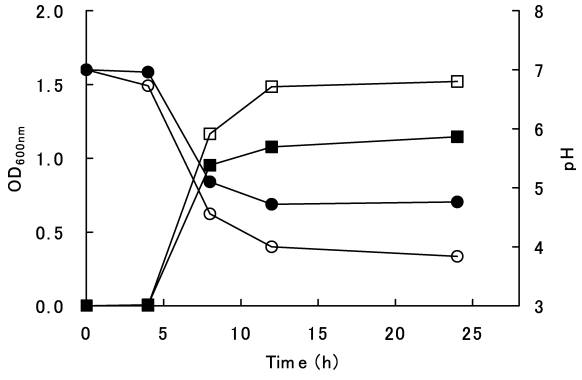

Fig. 5. The effect of TASOF for the growth of *S. mutans* and culture pH.

The growth of *S. mutans* in the medium with (closed square) and without (open square) the addition of TASOF. The pH profile of *S. mutans* culture with (closed circle) and without (open circle) the addition of TASOF.

24 h. On the other hand, the growth of *S. sobrinus* in culture containing TASOF decreased compared to that of the control: the growth reached 1.15 (75.7% of control growth) in *S. sobrinus.* When glucose was the sole carbon source, the pH of the reaction mixture reached pH 4.0 in *S. sobrinus* 7−8 h after inoculation. The pH in culture with BFOS dropped more slowly than the controls and bottomed out at pH 4.8 compared to pH 3.8 of control. The TASOF 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 stainless steel wire with TASOF addition. The same pattern was seen on the surfaces of glass vials: inhibition of biofilm formation was 73% by absorbance. One reason for decreasing mutansucrase activity was the acceptor reaction: sucrose hydrolyzed by mutansucrase and most glucosyl residues was transferred to the TASOF by acceptor reactions (data not shown). Since TASOF inhibited the formation of soluble and insoluble glucans by oral pathogens, it 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 specific role of each purified oligosaccharide as a prebiotic for the inhibition of type II diabetes and/or an anticariogenic sucrose substitute, especially for various oral pathogens, are in progress.

CONCLUSION

A method for synthesizing glucooligosaccharides (TASOG) and fructooligosaccharides (TASOF) with high concentration of sucrose (1−4 M) by using dextransucrase and levansucrase, respectively, prepared from *Leuconostoc mesenteroides* was developed. The DP of oligosaccharides synthesized according to the present method ranged from 2 to 15. Both oligosaccharides were stable at low pHs ranges under 100°C. TASOG and TASOF reduced the insoluble glucan formation 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.* The oligosaccharides can be used as the sweeteners for the food such as beverages requiring thermo- and acid-stable properties and as potential inhibitors of dental caries.

This work was underwritten by 21C Frontier Microbial Genomics and the Applications Center Program, Ministry of Science and Technology (Grant MG05-0301-1-0) and supported in part by Korean Ministry of Education under the second stage of BK21 project. Experimentation at PLS was supported by MOST and POSCO.

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〔Questions〕

Motomitsu Kitaoka, National Food Research Institute

1. Is your oligosaccharide produced by co-culture of two strains going to be commercialized?

2. How about the estimation of the cost?

3. Will the cost be competitive for food usage?

〔Answers〕

1. We are planning to do so. Yet, it is not in the market.

2. For the synthesis of glucooligosaccharides using glycansucrases, it is about $ 2.5–3/kg for enzyme and substrate.

3. If it can be optimized for large scale production, depending on the application target, I think it is cost effective.

〔Questions〕 Sumio Kitahata

1. In your case, isomaltooligosaccharides are synthesized by dextransucrase and dextranase from sucrose. How much % of isomaltooligosaccharides is produced from sucrose?

2. If the enzyme is incubated with 10–20% sucrose, how many isomaltooligosaccharides are produced?

〔Answers〕

1. About 50% is isomaltooligosaccharides including some % of glucose (depending on the reaction condition). The rests are fructose and lecurose.

2. By using hybrid enzyme, about 5–10% is isomaltooligosaccharides. By using glucansucrase, the products are not just isomaltooligosaccharides but oligosaccharides containing sucrose at the reducing end and connected by α-1,6 linked glucose at the non-reducing end with different degree of polymerization.

〔Question〕 Atsuo Kimura, Hokkaido University

DXAMase exhibited the endo-hydrolytic reaction to α-1,4-, α-1,6- and α-1,3-glucosidic linkages. Are these reactions catalyzed by a single catalytic site or multiple catalytic sites?

〔Answer〕

We confirmed the α-1,4-, and α-1,6-glucosidic linkages are hydrolyzed by different sites of DXAMase. Yet, we do not have a confirmation, yet, for the α-1,3-glucosidic linkage hydrolysis.