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Evaluation of Enhanced Hygroscopicity, Bifidogenicity, and Anticariogenicity of Enzymatically Synthesized β-Galactosyl-trehalose Oligosaccharides

SOO-IN RYU,† BONG-GWAN KIM,† MYUNG-SOO PARK,‡ YOON-BOK LEE,§ AND SOO-BOK LEE*,†

Department of Food and Nutrition, Brain Korea 21 Project, Yonsei University, Seoul 120-749, Research Center, Bifido Co., Ltd., Seoul 151-818, and Central Research Institute, Dr. Chung's Food Co., Ltd., Chungjoo 361-782, Korea

β-Galactosyl-trehalose oligosaccharides (β-GTOs) were enzymatically prepared as a mixture of 6-β-galactosyl-trehalose (**1**) and 4-β-galactosyl-trehalose (**2**) with a 9:1 ratio (w/w). The β-GTO mixture showed a highly enhanced hygroscopicity as compared to those of trehalose and other sugars used. At 72 h of incubation under 90% relative humidity and room temperature, it had a large increase in weight due to its moisture absorption, which was five times larger than that of trehalose, 1.9 times larger than that of sucrose, and 1.5 times larger than that of maltotriose. It was very effective in the growth promotion of *Bifidobacteria*, such as *Bifidobacterium longum* and *Bifidobacterium bifidum*, which was better than the growth promotion in the cases of trehalose and galactooligosaccharide. It also showed a highly anticariogenic property; it had only 10% cell proliferation of *Streptococcus sobrinus* for that of the sucrose control and 60% inhibition of insoluble glucan synthesis. Its effectiveness of inhibition was two and 1.5 times better than that of trehalose and one and two times than xylitol, respectively, against cell growth and glucan synthesis. Conclusively, the functionality of the β-GTO in terms of hygroscopicity, bifidogenicity, and anticariogenicity was considerably improved as compared to that of trehalose. It is thus suggested that the β-GTO might be applied as an effective humectant and prebiotic substitute with enhanced noncariogenicity in food applications.

KEYWORDS: β-Galactosyl-trehalose oligosaccharide; hygroscopicity; bifidogenicity; anticariogenicity; humectant

INTRODUCTION

Trehalose is a nonreducing disaccharide in which two glucose molecules are bonded in an α,α-(1↔1)-glucosidic linkage (*1*). This disaccharide is one of the most chemically stable sugars, in which the (1→1)-linkage makes it nonreducing and highly resistant to hydrolysis (*2*). This naturally occurring sugar is widely distributed in various organisms such as bacteria, yeast, fungi, insects, invertebrates, and lower and higher plants, where it may serve as a source of energy and a protectant of proteins and cellular membranes from a variety of environmental stress conditions, including desiccation, dehydration, heat, and freezing (*3*). Trehalose is known to hold the water of hydration tightly, forming a hydrogen-bonded tight-fitting complex. This complex allows it to penetrate more deeply than other sugars into certain membrane structures and functions to maintain the bioactivity (*4*). As compared to other sugars like sucrose, trehalose is not very hygroscopic (*4*, *5*). Up to a relative humidity (RH) of 92%, anhydrous and dihydrate forms of trehalose have only 10.5 and 9.5% moisture content, respectively. Thus, it appears that trehalose can be of benefit in dry-blending operations. In addition, trehalose is mildly sweet, more stable to wide ranges of pH and heat, and does not easily interact with proteinaceous molecules (*5*). It has also been suggested to be low cariogenic or anticariogenic, which makes it promising as a sugar substitute (*6*). Generally, ingested trehalose is hydrolyzed to glucose by trehalase and absorbed in the small intestine.

Meanwhile, oligosaccharides have been widely used as a food ingredient due to their favorable properties, such as high water holding, low calories with indigestibility, low sweetness, growth factors for *Bifidus*, and no dental caries (*7*). There have been several attempts to make glycosyl-trehalose oligosaccharides by the transglycosylation of cyclomaltodextrin glucanyltransferase or α-glucosidase, in which trehalose oligosaccharides were prepared by attaching only glucose residue in the α-(1→4)- or (1→6)-linkage (*8*). Naturally existing trehalose oligosaccharides have been reported in mycobacteria (*9*). They were tri- and tetrasaccharides all composed of trehalose as a basic structure

* To whom correspondence should be addressed. Tel: 82-2-2123-3124. Fax: 82-2-312-5229. E-mail: soobok@yonsei.ac.kr.
† Yonsei University.
‡ Bifido Co., Ltd..
§ Dr. Chung's Food Co., Ltd..

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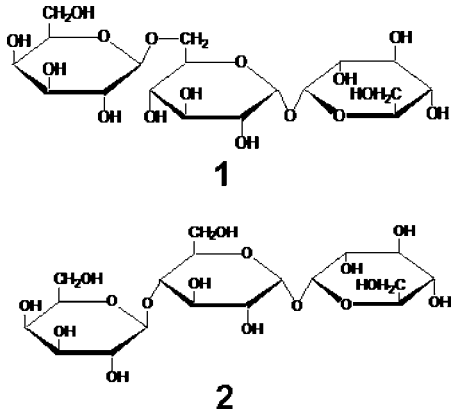

Figure 1. Chemical structures of the transgalactosylated products of trehalose. 6-*O*-β-D-Galactosyl-trehalose (**1**) and 4-*O*-β-D-galactosyl-trehalose (**2**).

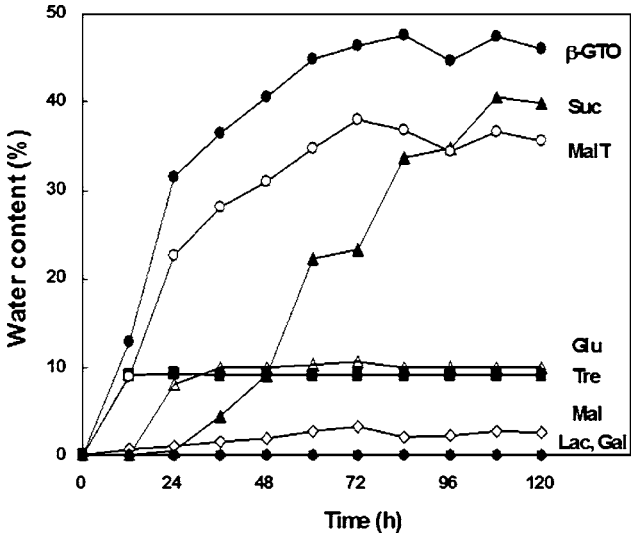

Figure 2. Hygroscopicity of the transgalactosylation products and several sugars. (●) β-GTO, mixture of oligosaccarides **1** and **2** in **Figure 1** (9:1 ratio); (▲) Suc, sucrose; (○) MalT, maltotriose; (△) Glu, glucose; (■) Tre, trehalose; (◇) Mal, maltose; (□) Gal, galactose; and (◆) Lac, lactose.

and additional glucose residues in the α-(1→4)- and β-(1→6)-linkages or a galactose residue in the α-(1→6)-linkage, respectively. These oligosaccharides were supposed to play a protective role in the microorganism. However, the physicochemical properties of trehalose-based oligosaccharides, except for glucosyl-trehalose, still have not been investigated. Recently, we enzymatically synthesized β-galactosyl-trehalose trisaccharides (*10*). It may be expected that the trehalose oligosaccharide prepared provides both benefits of trehalose and oligosaccharide as a food additive.

The objectives of this study were to evaluate β-galactosyl-trehalose oligosaccharides (β-GTOs) in terms of hygroscopicity, Maillard reactivity, bifidogenicity, and anticariogenicity and to compare the effectiveness with those of other sugars.

MATERIALS AND METHODS

Materials. Trehalose dihydrate was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Lactose and other sugars were obtained from Sigma Chemical Co. (St. Louis, MO). Commercial galactooligosaccharide [mainly composed of β-D-Gal*p*-(1→4)-α-D-Gal*p*, β-D-Gal*p*-(1→3)-α-D-Gal*p*, β-D-Gal*p*-(1→4)-α-D-Glc*p*, and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*] was obtained from Samyang Genex Co. (Seoul, Korea). Three *Bifidobacterium* strains (*Bifidobacterium longum* ATCC 15707, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium bifidum* ATCC 29521) were obtained from American Culture Collection (Manassas, VA). *B. longum* BORI was kindly provided from Bifido, Co. (Seoul, Korea), which was originally isolated from Korean healthy human feces. *Streptococcus sobrinus* and other bacteria used were purchased from Korean Culture Center of Microorganisms (Seoul, Korea). All other chemicals used were of reagent grade. β-GTO (a mixture with a 9:1 ratio of the transfer products **1** and **2** in **Figure 1**) was prepared by the transgalactosylation of *Escherichia coli* β-galactosidase in the presence of trehalose as an acceptor and lactose as a donor according to the previous method (*10*).

Evaluation of Hygroscopicity. Saturated salt solutions of $Mg(NO_3)_2 \cdot 6H_2O$ and $BaCl_2 \cdot 2H_2O$ were prepared in separate desiccators to give 53 and 90% RHs at 25 °C, respectively. Each lyophilized sample (about 1 g) of the transfer product mixture, trehalose, lactose, sucrose, and galactose was kept under 53% RH for 1 week to equilibrate them and then moved to a chamber of 90% RH and continuously kept for 1 week to investigate the absorption of water vapor (*11*). The weight gain for each sample was measured as a percentage of moisture absorbed on the basis of the initial sample weight.

Evaluation of Maillard Browning. Each 1 mL of McIlvaine (citrate/phosphate) buffer (pH 4−8 at intervals of 1 pH unit) containing 66.6 mM glycine and 292.1 mM β-GTO or other sugars was heated at 100 °C for 60 min. The absorbance values (A_{420}) for these solutions were respectively measured at 420 nm. The A_{420} value was used to show the Maillard reactivity in the model system of browning reaction (*12*). Each pH of McIlvaine buffer was prepared by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4 (*13*).

Effect on the Growth of *Bifidobacteria* and Other Bacteria. The effects of the β-GTO mixture on the growth of four *Bifidobacterium* strains (*B. longum*, *B. breve*, *B. bifidum*, and *B. longum* BORI) and three harmful bacteria (*E. coli*, *Clostridium botulinum*, and *Staphylococcus aureus*) were examined using modified MRS medium with phenol red as an indicator (*14*, *15*). The medium broth consisted of 10 g of proteose peptone no. 3, 5 g of casamino acid, 10 g of yeast extract (Difco Lab., MI), 1 g of beef extract, 1 mL of Tween 80, 2 g of ammonium acetate, 0.1 g of $MgSO_4$, 0.05 g of $MnSO_4 \cdot H_2O$, 2 g of Na_2SO_4, 1.92 g of $KHSO_4$, 0.2 g of Na_2CO_3, 0.1 g of $CaCl_2 \cdot H_2O$, 0.5 g of L-cystine $HCl \cdot H_2O$, and 0.18 g of phenol red in 1 L of deionized water. An equal volume of seed culture anaerobically precultured was inoculated to the MRS mediums containing 0.5% (w/v) β-GTO or other sugars, respectively, followed by incubation in an anaerobic chamber at 37 °C for 24 h. During the incubation, samples were collected and the cell density was determined by measuring the absorbance at 660 nm.

Effect on the Growth of *S. sobrinus* and the Synthesis of Insoluble Glucan. *S. sobrinus* NRRL 14555 was precultured in brain−heart infusion (BHI, Difco Lab.) broth (pH 7.0) at 37 °C with shaking (150 rpm) for 24 h. Then, an equal volume of the precultured solution (about 3×10^6 colony-forming units/mL) was inoculated into each 5 mL of BHI solution containing 1% of the respective sugar (sucrose, trehalose, galactooligosaccharide, and β-GTO) or sugar alcohol (sorbitol and xylitol), followed by incubation at 37 °C for 24 h (*6*). The cell density at the end of fermentation was determined using a spectrophotometer at 660 nm to investigate the effect of the β-GTO and other sugars on the microbial growth. The observation of growth was performed in triplicate. The inhibition effect of the β-GTO on water-insoluble glucan synthesis from sucrose by *S. sobrinus* was assayed and compared to those of other sugars (*16*). Precultured *S. sobrinus* was inoculated into 5 mL of BHI broth containing both sucrose (5%) and each sugar additive (5 and 10%). The bacterium was cultured at 37 °C for 24 h in a glass vial, and the supernatant of individual medium was discarded. The synthesized and precipitated glucan was washed with a buffer and solubilized with 0.5 M NaOH. The water-insoluble glucan was assayed by using the phenol−sulfuric method, and the absorbance was measured at 550 nm (*17*, *18*).

RESULTS AND DISCUSSION

Hygroscopicity and Maillard Browning of β-GTO Product. β-GTOs **1** and **2** were efficiently prepared after the enzymic reaction through baker's yeast fermentation and gel permeation

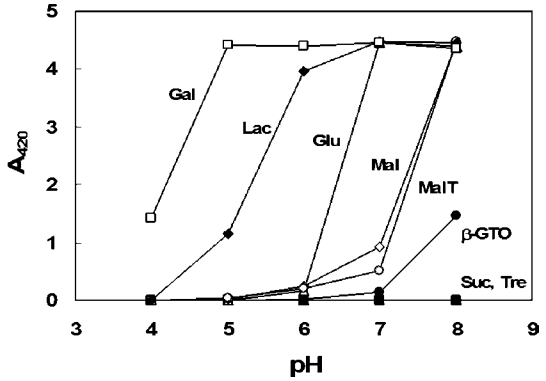

Figure 3. Browning development of the transgalactosylation products and several sugars with glycine at pH values ranging from 4.0 to 8.0. Abbreviations of sugars are shown in **Figure 2**. The absorbance was monitored at 420 nm.

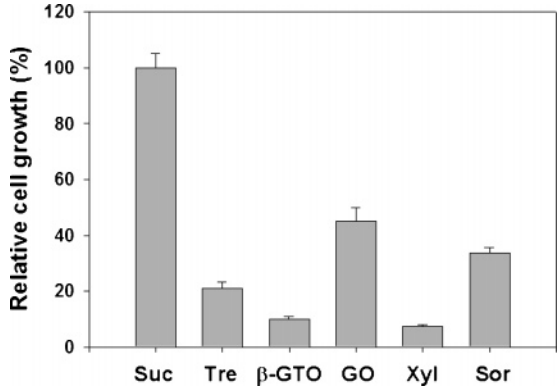

Figure 4. Relative percent of cell growth of *S. sobrinus* in the presence of several carbohydrates. Sucrose was a control (100%) and was substituted by other carbohydrates in the samples. Abbreviations of sugars are shown in **Figure 2**. Xyl, xylitol; Sor, sorbitol.

chromatography on W-251 (recycling high-performance liquid chromatography) polymeric columns with the yield of approximately 27−30% based on the amount of trehalose used (*10*). Because **1** was preferentially produced as compared to **2** and it was time-consuming for complete separation, the reaction product (β-GTO mixture) containing **1** and **2** with a 9:1 ratio was prepared and used for the evaluation followed.

Trehalose is usually found in the dihydrate form. The trehalose dihydate has a low hygroscopic property, the water content of which remains stable at 9.54% up to a RH of approximately 92% (*4*, *19*). In this study, lyophilized trehalose showed the same trend in the moisture absorption, remaining stable at 9.1% under 90% RH at 25 °C for 1 week (**Figure 2**). However, the lyophilized β-GTO product had an enormously enhanced ability to absorb moisture as compared to trehalose. The moisture absorption of β-GTO product was almost saturated (47−48%) at 3 days of equilibration, where its absorption rate was the highest among the mono- to trisaccharides employed and the water content gained was approximately five times higher than that of trehalose and 1.9 times higher than that of sucrose, respectively. After 5 days, the level of moisture absorption of β-GTO product was still 1.2 times higher than that of sucrose. The hygroscopic properties of the sugars compared were decreased in the order of β-GTO product, sucrose/maltotriose, glucose/trehalose, maltose, and galactose/lactose, which were evaluated in terms of the amount of moisture absorbed. The water content gained for maltotriose was largely increased as compared to that for maltose. Therefore, it is likely that the remarkable enhancement of moisture absorption for β-GTO product is basically due to the increase in the degree of polymerization via the galactose moiety attached and the glycosidic structures formed (*20*). This high ability of water absorption would make it applicable as a good humectant.

The β-GTO product was not strongly colored by a browning reaction in a model system, especially in the pH ranges less than 7.0; this was almost identical to the cases of trehalose and sucrose (**Figure 3**). At pH 8.0, the colorability of the β-GTO was significantly increased while those of trehalose and sucrose were nearly unchanged. The Maillard browning is generally caused by a chemical reaction between an aldehyde group of a reducing sugar and an amino group-containing compound (*21*). There is no reducing aldehyde group in the β-GTO product. Thus, the increase in the colorability of the nonreducing sugar β-GTO might come from its degradation, in which the glycosidic linkage between galactose and glucose residues could be labile in the alkaline pH with heating. However, the degree of browning of the β-GTO was still three times lower than those of glucose, galactose, maltose, lactose, and maltotriose at pH 8.0. These results suggested that the β-GTO could be a suitable sugar substitute to use when inhibition or control of the Maillard reaction is desired, especially in acidic conditions.

Table 1. Effect of β-GTOs on the Growth of Several *Bifidobacteria* and Harmful Bacteria in Comparison with Those of Trehalose and Galactooligosaccharide

microorganisms	trehalose	GO[a]	β-GTO[b]
B. longum ATCC 15707	++[c]	+[c]	+++[c]
B. bifidum ATCC 15700	+	+	+++
B. longum BORI	–[d]	+++	+++
B. breve ATCC 29521	+	–	+
E. coli	–	–	–
C. botulinum	–	–	–
S. aureus	–	–	–

[a] GO indicates galactooligosaccharide. [b] β-GTO indicates a product mixture (9:1 ratio) of 6-β- and 4-β-galactosyl-trehaloses. [c] The mark denotes a growth-promoting effect around 10% (+), more than 25% (++), and more than 40% (+++), respectively, as compared to the control (no sugar additive). [d] The mark denotes no effect on the growth as compared to the control.

Prebiotic and Anticariogenic Properties of β-GTO Product. Various oligosaccharides including galactooligosaccharide have been well-known to promote the growth of *Bifidobacteria* in the large gut of humans due to its indigestibility in the digestive tract (*22*, *23*). Trehalose disaccharide is hydrolyzed by trehalase in the small intestine, and the resulting glucose is uptaken (*5*). The trehalase is specific for the hydrolysis of α,α-(1↔1)-glucosidic linkage of trehalose. On the contrary, β-GTOs **1** and **2** in the present study were not hydrolyzed by trehalase in vitro (*10*). This result led us to investigate the possibility that the β-GTO product may play a role as a prebiotic compound for promoting the growth of *Bifidobacteria*. Four species of *Bifidobacteria* have been tested in vitro and have been compared for the growth with the addition of commercial galactooligosaccharide, trehalose, and the β-GTO; the same thing was also done for three kinds of harmful bacteria, as shown in **Table 1**. Galactooligosaccharide showed a positive promotion (10−40%) on the growth of *Bifidobacteria*, such as *B. bifidum* and *B. longum*, as compared to that for the control (no sugar additive). Interestingly, trehalose was also effective in promoting the growth (10−25%) of *Bifidobacteria*. The degree of growth enhancement was dependent on the species of *Bifidobacteria*. As expected, the β-GTO product also showed significant promotion (more than 40%) for the growth of *Bifidobacteria* such as *B. bifidum*, *B. longum*, and *B. longum* BORI, and no

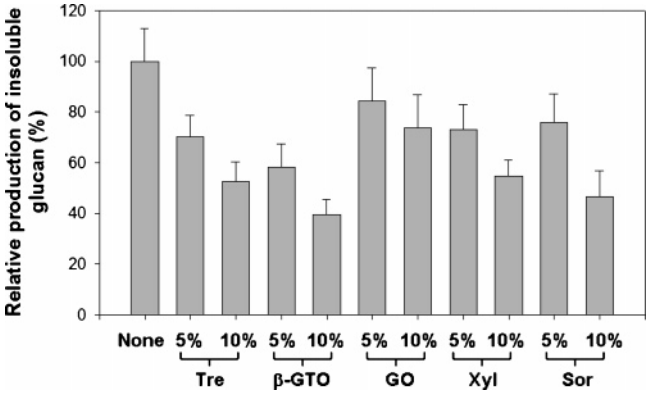

Figure 5. Relative percent of insoluble glucan synthesis by *S. sobrinus* in the presence of sucrose and other carbohydrates. Only sucrose was contained in the control (100%), and other carbohydrates (5 and 10%) were concomitantly added with sucrose in the samples. None, with only sucrose; Tre, trehalose; β-GTO, transfer product mixture; GO, galactooligosaccharide; Xyl, xylitol; and Sor, sorbitol.

growth-stimulating effect for the harmful bacteria used, which was very similar to the effect of galactooligosaccharide. Thus, β-GTO would have a potential as a prebiotic substance for promoting gut health.

In addition, the effects of the β-GTO product on the fermentability and the inhibition of glucan synthesis by *S. sobrinus* were investigated in comparison with those of sucrose, trehalose, galactooligosaccharide, xylitol, and sorbitol. The β-GTO was poorly utilized by the bacteria as was xylitol (**Figure 4**). It showed about 10 times lower cell proliferation than that of sucrose, two times lower than that for trehalose, and 3−4 times lower than that for sorbitol and galactooligosaccharide. The glucan synthesis by *S. sobrinus* was significantly inhibited by the sugar additives used, and the extent of the inhibition was increased with an increase in the concentration of sugar additives (**Figure 5**). The β-GTO was most effective in the inhibition of the glucan synthesis among the noncariogenic sugars compared, in which it was approximately 1.2−2.0 times better than xylitol, trehalose, and other sugars used for the inhibition. The β-GTO would be a good non- or anticariogenic carbohydrate to not significantly promote dental caries.

In conclusion, β-GTO showed significantly improved functionalities in such aspects as hygroscopicity, bifidogenicity, and anticariogenicity with low Maillard reactivity as compared to trehalose and other typical sugars employed. The enhancement in the effectiveness is supposed to be attributed to the combined nature of trehalose and galactosyl oligosaccharide preferentially in the major compound, 6-β-galactosyl-trehalose. The present study suggested that β-GTO could be an effective and multifunctional sugar substitute for humectant with anticariogenic and prebiotic efficacies in the food industry.

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