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Transglycosylation of tagatose with maltotriose by *Bacillus stearothermophilus* maltogenic amylase (BSMA)

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Abstract—D-Tagatose was transglycosylated by *Bacillus stearothermophilus* maltogenic amylase (BSMA), and the physicochemical properties of the transfer products were analyzed. Maltosyl-tagatose was the main product of the transglycosylation reaction using maltotriose as the donor and D-tagatose as the acceptor. Glucosyl-tagatose was produced from maltosyl-tagatose by removal of a glucosyl moiety by glucoamylase. The ^{13}C NMR analysis of glucosyl-tagatose suggested that a linkage was formed between the C-1 carbon of the glucose unit and the C-1 carbon of the tagatose unit, thereby producing 1,1-α-glucosyl-tagatose from the transglycosylation reaction. Hygroscopicity measurements showed that glucosyl-tagatose had greater water sorption than did tagatose or sucrose. The glass transition temperature of glucosyl-tagatose was −29 °C, which is considerably higher than that of tagatose, −45 °C. Its structure and physicochemical properties suggest that glucosyl-tagatose has potential as a low-calorie sweetener and cryostabilizer.

1. Introduction

D-Tagatose, an isomer of D-galactose, is a rare natural ketose;[1] it was identified as a component of gum exudates of the cacao tree, *Sterculia setigera*[2] and was also detected as a component of an oligosaccharide in the lichens *Rocella linearis* and *Rocella fucoformis*.[3] One interesting property of D-tagatose is that, although it has sweetness similar to that of sucrose, it does not contribute calorie-wise to the diet.[4–6] In addition, D-tagatose lacks the laxative effect that is common with polyols used as sugar substitutes.[7,8]

Enzymatic transglycosylation has been widely used to modify sugars and sugar alcohols to improve their physicochemical properties as raw materials in foods.[9–13] The transfer products of sugars have a softer and milder taste, and give solutions of low viscosity and water activity, which makes them effective for controlling microbial contamination. The modified sugars also have particular physiological effects; they yield low energy upon metabolism and favor the production of beneficial intestinal microflora.[6] We previously reported that *Bacillus stearothermophilus* maltogenic amylase (BSMA) exhibited a high transglycosylation activity that we have applied to the modification of various food components.[14] Using different sugar moieties as acceptors, BSMA can transfer monosaccharides or disaccharides to acceptor molecules by forming α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkages. Sorbitol, a sugar alcohol, was modified by transglycosylation with BSMA to produce maltosyl-sorbitol, in which the maltose unit was attached to sorbitol by an α-(1,6)-glycosidic linkage.[12] The mechanism of transglycosylation was elucidated on the basis of the crystal structure of the enzyme.[15] It was reported that BSMA has broad acceptor specificity for glycosides.[11,16]

In this study, tagatose was transglycosylated by BSMA using maltotriose as a donor molecule, and the resulting transglycosylated product was analyzed by thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR). The water sorption characteristics, hygroscopicity, and glass transition temperature of the transfer product were also examined.

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2. Results and discussion

2.1. Transglycosylation of maltotriose with tagatose

Transglycosylation by BSMA and hydrolysis by glucoamylase were used to synthesize the glycosyl-tagatose product (Fig. 1). The transglycosylation activity of BSMA was used to form the transfer product using tagatose as the acceptor molecule. Maltotriose (G3) was used as the donor molecule at a concentration of 5% (w/v), and tagatose was added to the reaction mixture at a concentration of 20% (w/v). The enzyme was added at 0.2 U/mg maltotriose, and the mixture was incubated at 55 °C overnight. The mixture was reacted in 50 mM sodium citrate buffer (pH 6.0) with a reaction volume of 50 mL. The reaction was stopped by boiling for 5 min.

The transglycosylation reaction resulted in the production of both glucosyl- and maltosyl-tagatose transfer products. The glucosyl-tagatose and maltosyl-tagatose transfer products appeared as pink spots just below the glucose and maltose spots, respectively, on TLC. Glucoamylase was added to this mixture to hydrolyze the G2 or G3 compounds, producing the G1-tagatose transfer compound (Fig. 2). Transglycosylation by BSMA followed by hydrolysis with glucoamylase allowed the glucosyl-tagatose transfer product to be isolated as the major product.

2.2. Production and purification of glucosyl-tagatose

The reaction mixture was diluted 2-fold with distilled water and filtered through a 0.45-μm syringe filter. The transfer products were purified using recycling preparative HPLC. The fractions that were separated by recycling HPLC were analyzed using TLC developed with isopropyl alcohol/ethyl acetate/water (3/1/1). The glucosyl-tagatose fractions were pooled and freeze dried. Purified glucosyl-tagatose that was separated by recycling preparative HPLC was used for the NMR analyses.

2.3. Nuclear magnetic resonance (NMR) analyses of the transfer products

The structure of glucosyl-tagatose was analyzed using ^{13}C NMR spectrometry. The ^{13}C NMR spectra of glucose and tagatose were also measured. The ^{13}C NMR signals for these compounds are listed in Table 1.

Tagatose gave a C-1′ peak with a chemical shift of 65.0 ppm. In glucosyl-tagatose, this had shifted downfield to 70.5 ppm. The chemical shift for the glucosyl C-1 carbon that took part in the linkage showed a downfield change from 92.9 to 99.6 ppm, strongly suggesting that a linkage had formed between the C-1 carbon of glucose and the C-1′ carbon of tagatose (Fig. 1). This type of linkage between hexoses is not cleaved by human digestive enzymes or by intestinal microorganisms; thus, glucosyl-tagatose has high potential as a low-calorie sweetener.

2.4. Physicochemical properties of the transfer products

Glucosyl-tagatose, sucrose, maltose, tagatose, and glucose were freeze dried to investigate their sorption isotherms. Figure 3 shows sorption isotherms of

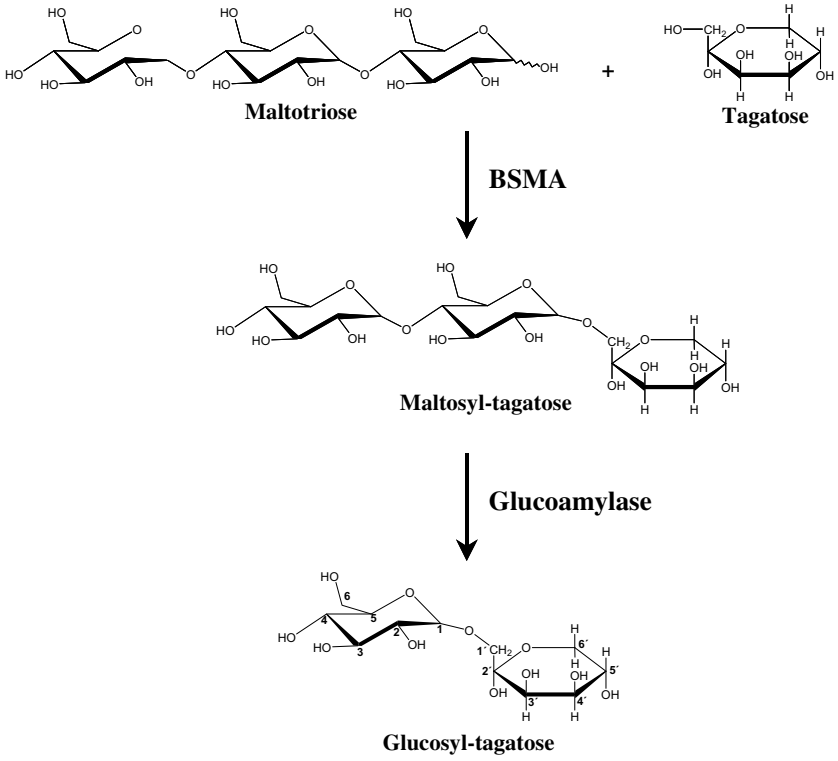

Figure 1. The synthesis of glucosyl-tagatose from maltotriose and tagatose by transglycosylation followed by hydrolysis.

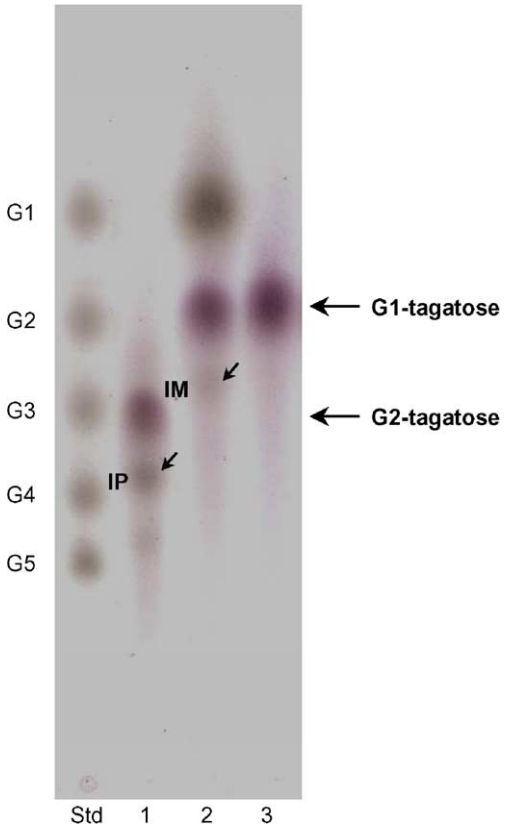

Figure 2. TLC analysis of glucosyl-tagatose produced by BSMA and glucoamylase. Std, G1–G5 standards; lane 1, after recycling preparative LC; lane 2, lane 1 treated with glucoamylase; lane 3, purified G1-tagatose. IM, isomaltose; IP, isopanose.

Table 1. ^{13}C NMR signals of glucose, tagatose, and glucosyl-tagatose

	Carbon atoms	Glucose[a]	Tagatose[b]	G1-tagatose[c]	Difference (ppm)
Glucose	C-1	92.9		99.6	**6.7**
	C-2	72.3		73.1	0.8
	C-3	73.6		74.2	0.6
	C-4	70.4		71.3	0.9
	C-5	72.2		72.6	0.4
	C-6	61.6		61.6	0.0
Tagatose	C-1′		65.0	70.5	**5.5**
	C-2′		99.2	99.1	−0.1
	C-3′		70.9	70.6	−0.3
	C-4′		72.0	72.0	0.0
	C-5′		67.4	67.4	0.0
	C-6′		63.3	63.5	0.2

[a] Glucose: α-form.
[b] Tagatose: α-form.
[c] G1-tagatose: glucosyl-tagatose.

glucosyl-tagatose, sucrose, maltose, tagatose, glucose, and fructose. Freeze-dried powders of glucosyl-tagatose and the various sugars produced typical isotherm curves. The sorption isotherm curve of tagatose was similar to that of sucrose. However, glucosyl-tagatose absorbed more water in the water activity (Aw) range of 0.3–0.9. When the moisture content of the sample was 0.2 g H_2O/g solid, the glucosyl-tagatose had an Aw of 0.7 compared with 0.83 for tagatose. For the

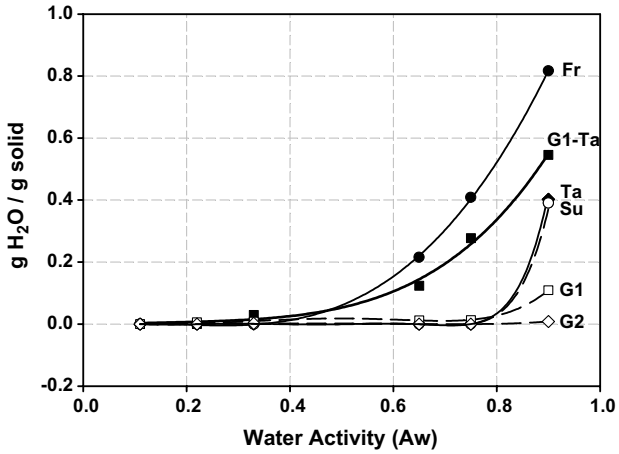

Figure 3. Water sorption isotherms of glucosyl-tagatose (G1-Ta), tagatose (Ta), sucrose (Su), fructose (crystal; Fr), glucose (G1), and maltose (G2).

water content range of 0.1–0.4 g H_2O/g solid sample, the Aw of tagatose and the related sugars were in the order: tagatose = sucrose > glucosyl-tagatose > fructose (crystal).

The hygroscopicities of glucosyl-tagatose and the various sugars were measured under a relative humidity of 90% at 25 °C (Fig. 4). As expected, maltose showed the maximum resistance to water sorption. Fructose had the highest hygroscopicity of the compounds tested. Glucosyl-tagatose also had high hygroscopicity, being higher than that of tagatose. Sucrose and tagatose showed less water sorption than did glucosyl-tagatose. These results confirmed that the transglycosylation of tagatose had improved its moisture absorptive powers.

The glass transition represents the phase transition of polymers, including many food materials, from a glassy state to a rubbery state and occurs at a specific

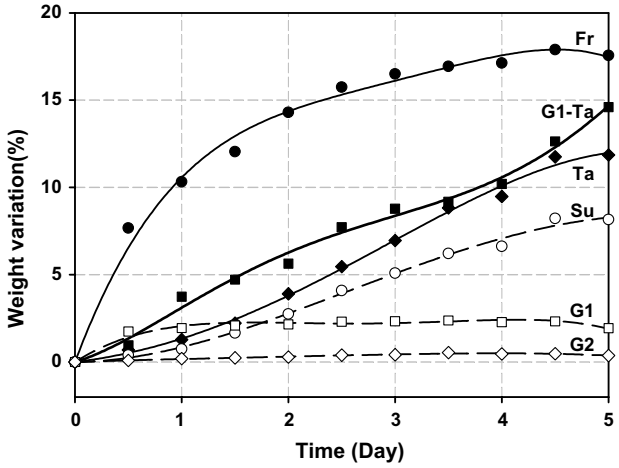

Figure 4. Hygroscopicities of glucosyl-tagatose and various sugars during storage at 25 °C under 90% relative humidity. G1-Ta, glucosyl-tagatose; Ta, tagatose; Su, sucrose; Fr, fructose (crystal); G1, glucose; G2, maltose.

Table 2. Glass transition temperatures (T'_g) and ice melting temperature (T'_m) of maximally freeze-concentrated glucosyl-tagatose, tagatose, and sucrose

Sugars	T'_g (°C)			T'_m (°C)
	Onset	Mid point	End point	
Glucosyl-tagatose	−29	−27	−26	−3
Tagatose	−45	−42	−40	−13
Sucrose[a]	−46	−41	−36	−34

[a] Data from Roos.[20]

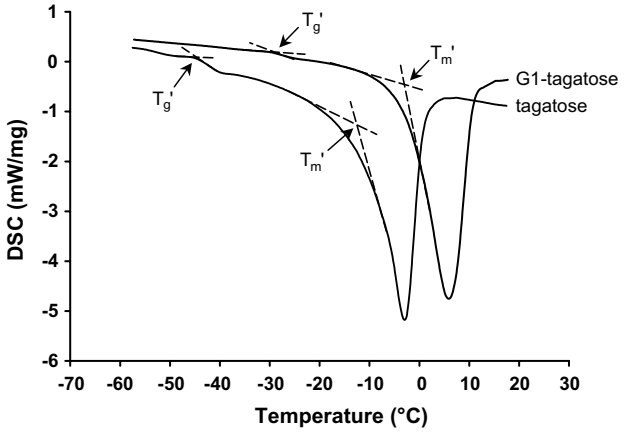

Figure 5. Differential scanning calorimetry (DSC) thermogram of 10% (w/w) solutions of glucosyl-tagatose and tagatose.

temperature, T_g. The glass transition temperature (T'_g) and ice melting temperature (T'_m) of maximally freeze-concentrated glucosyl-tagatose are listed in Table 2. A DSC thermogram of 10% (w/w) glucosyl-tagatose solution is shown in Figure 5. The glass transition (T'_g) and ice melting temperatures (T'_m) of glucosyl-tagatose were −29 and −3 °C, respectively. The results showed that the T'_g and T'_m values of glucosyl-tagatose were higher than the values of tagatose. These results for tagatose and glucosyl-tagatose were generally consistent with the findings by Levine and Slade,[17] that high-molecular-weight sugars had higher T'_g values than did low-molecular-weight sugars. Wang and Jane[18] demonstrated experimentally that the stability of frozen foods was positively dependent on the temperature difference between the freeze temperature (T_f) and the T'_g of the dissolved maltooligosaccharides ($\Delta T = T_f - T'_g$). The T'_g of glucosyl-tagatose was 16 °C, which is 17 °C higher than that of tagatose or sucrose, respectively. Therefore, these findings strongly suggest that glucosyl-tagatose has potential as a good cryostabilizer.

3. Conclusion

We have developed a new enzymatic method producing a glucosyl-tagatose and investigated its physiochemical properties. To produce the transferred product, first, 5% D-tagatose and 20% maltotriose mixture was incubated with BSMA at 55 °C for 5 min. Second, a series of transglycosylated products produced after the first reaction were incubated with glucoamylase to make glucosyl-tagatose. ^{13}C NMR analysis of the purified glucosyl-tagatose confirmed the formation of glucosidic linkage between C-1 carbon of the glucose and the C-1′ carbon of the tagatose. When the sorption isotherm was determined, this compound showed an enhanced moisture-containing ability compared with sucrose or tagatose itself. And hygroscopicity measurement confirmed that this product has a higher glass transition temperature than that of the tagatose. These properties support a potential application of glucosyl-tagatose as a cryostabilizer.

Tagatose has several unique physiological properties including strong sweetness, non-digestibility, non-laxative effect. And these properties make it possible to use glucosyl-tagatose as a sugar substitute that has a wide market in the food industry. On-going sensory evaluation project in our laboratory is testing the relative sweetness of glucosyl-tagatose compared with other sweetening compounds and also studying the effect of flavor-enhancing properties of glucosyl-tagatose for its application to the various food products such as confectionary and baking products. Recently, FAO strongly recommended lowering the sugar contents in food as part of an anti-obesity campaign. American Dentist Association also suggests the use of anti-cavity sweeteners especially for the children. Tagatose and its glucosylated products have a potential application as a sugar substitute in the food industry that satisfies the suggestions by these groups.

4. Experimental

4.1. Materials

D-Tagatose and maltotriose were purchased from Sigma Chemical Co., St. Louis, MO, USA. *B. stearothermophilus* maltogenic amylase (BSMA) was prepared as reported previously.[14] Glucoamylase was kindly provided by Novozyme.

4.2. Transglycosylation reaction

The following reaction was carried out for the production of maltosyl-tagatose and glucosyl-tagatose. Maltotriose (5%, w/v) and tagatose (20%, w/v) were mixed in 50 mM sodium citrate buffer (pH 6.0) and boiled until all solutes were dissolved. After pre-incubation at 55 °C for 10 min, 0.2 U of *B. stearothermophilus* maltogenic amylase (BSMA) per milligram of maltotriose was added to the mixture. The reaction was stopped by boiling for 5 min after the required incubation time at 55 °C.

4.3. Production of glucosyl-tagatose

The transglycosylation reaction resulted in the production of both glucosyl- and maltosyl-tagatose transfer products. Glucoamylase was added to this mixture to hydrolyze the G2 or G3 compounds, producing the G1-tagatose transfer product.

4.4. Purification using recycling preparative liquid chromatography (LC)

The reaction mixture that resulted from the transglycosylation by BSMA and hydrolysis by glucoamylase was diluted with one volume of filtered distilled water. The mixture of transfer products was separated by recycling preparative high-performance liquid chromatography (LC-918, JAI Co. Ltd, Japan) using a column (W-251) packed with a polymeric gel and elution with deionized water at a flow rate of 3 mL/min.

4.5. Thin-layer chromatography (TLC)

A silica gel TLC plate (K5F Whatman, Maidstone, UK) was activated by placing it in an oven at 110 °C for 1 h. Diluted samples of the reaction mixtures were spotted on the silica gel plate; the plate was placed in a TLC chamber containing a solvent mixture of isopropyl alcohol/ethyl acetate/water (3/1/1, v/v/v) or a mixture of acetonitrile/water (85/15, v/v) and developed once or several times at room temperature. The plate was dried thoroughly. To visualize the spots, the plate was dipped rapidly into a methanol solution containing 3 g of *N*-(1-naphthyl)-ethylenediamine and 50 mL of concentrated H_2SO_4 per liter, dried, and then placed in an oven at 110 °C for 10 min; purple-black spots developed on a white background.[19]

4.6. Nuclear magnetic resonance (NMR) analysis

The ^{13}C nuclear magnetic resonance spectrum was recorded with a JNMLA-400 FT-NMR spectrometer (JEOL, Japan) using the Distortionless Enhancement by Polarization Transfer (DEPT) mode.

4.7. Sorption isotherm measurements

Sorption isotherms of sugars, sugar alcohols, and transfer products were measured using a Novasina AW-Center (Pfaeffikon, Switzerland). The instrument was calibrated prior to use following the method in the instruction manual. Standard salt tablets provided with the instrument were used for both calibration and measurement. About 50 mg of a completely dried sample was placed on a net dish, and the total weight was measured and recorded. The net dish containing the sample was kept in contact with standard salt inside the Aw box for a defined time period at fixed temperature. Upon completion of the conditioning process, the sample weight was again measured. The procedure was repeated with standard salt at increasing relative humidity until the sample was fully saturated. The sorption isotherm of each sample was constructed by plotting the moisture content (gram H_2O/gram solid) versus water activity.

4.8. Hygroscopicity

About 0.5 g of each sample was kept at 53% relative humidity (RH) [saturated solution of $Mg(NO_3)_2{\cdot}6H_2O$] for 2 days. The samples were transferred to 90% RH [saturated solution of $BaCl_2{\cdot}2H_2O$] and kept at 25 °C. The time-dependent variation of weight was measured for each sample.

4.9. Glass transition temperatures

The glass transition temperature of the transfer product as freeze-dried glucosyl-tagatose was measured by differential scanning calorimetry (DSC) (DSC200, Netzsch, Selb, Germany). The glass transition temperature of the maximally freeze-concentrated solution (T'_g) was determined by the method of Roos[20] with modifications. Aliquots of the glucosyl-tagatose solution (15 °C; 20 mg; 60%, w/w) were placed into 20-μL aluminum DSC pans, initially cooled to −100 °C at the rate of 2.5 °C/min, and then heated to 25 °C to detect the T'_m, which is the onset temperature of ice melting in a maximally freeze-concentrated solution. Samples were then cooled to −100 °C at 5 °C/min, heated to the annealing temperature of −20 °C at 10 °C/min, and annealed for 30 min; after annealing, the samples were cooled to −100 °C at 5 °C/min and scanned from −100 °C to 25 °C at 10 °C/min to determine T'_g and T'_m, which are the onset temperatures of the glass transition and ice melting, respectively. In this experiment, an empty pan was used as a reference.

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