Food Sci. Biotechnol. Vol. 16, No. 1, pp. 127 ~ 132 (2007)

Food Science and Biotechnology

Enzymatic Synthesis and Characterization of Galactosyl Trehalose Trisaccharides

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Abstract α, α-Trehalose was efficiently modified by a transgalactosylation reaction of *Escherichia coli* β-galactosidase using lactose as a donor to yield two galactosyl trehalose trisaccharides. The reaction products of trehalose by the enzyme were observed by thin layer chromatography (TLC) and high performance anion exchange chromatography (HPAEC) and were purified by BioGel P2 gel permeation chromatography and recycling preparative HPLC. Liquid chromatography-mass spectrometry (LC-MS) and ^{13}C nuclear magnetic resonance (NMR) analyses revealed that the structures of the main products were 6^2-β-D-galactosyl trehalose (**1**) and 4^2-β-D-galactosyl trehalose (**2**). A reaction of 30%(w/v) trehalose and 15%(w/v) lactose at pH 7.5 and 45°C resulted in a total yield of approximately 27-30% based on the amount of trehalose used. The galactosyl trehalose products were not hydrolyzed by trehalase. In addition, the mixture of transfer products (9:1 ratio of **1** to **2**) showed higher thermal stability than glucose, lactose, and maltose, but less than trehalose, against heat treatment over 100 °C at pH 4 and 7. It also exhibited better thermal stability than sucrose at pH 4 alone.

Keywords: trehalose, β-galactosyl trehalose, transgalactosylation, *Escherichia coli*, β-galactosidase, thermal stability

Introduction

Trehalose is a nonreducing disaccharide to which two glucose molecules are bound in an α,α-(1→1)-glycosidic linkage (1). This disaccharide is one of the most chemically stable sugars, because the (1→1)-linkage makes it non-reducing, highly resistant to hydrolysis, and generally chemically inert (2). In comparison to other sugars, trehalose is mildly sweet, less hygroscopic, more stable to wide ranges of pH and heat, and does not easily interact with proteinaceous molecules (3). This naturally occurring sugar is widely distributed in various organisms such as bacteria, yeast, fungi, insects, invertebrates, and lower and higher plants (4), where it may serve as an energy source and a source of protection for proteins and cellular membranes from a variety of environmental stress conditions, including desiccation, dehydration, heat, freezing, and oxidation (5). The most widely distributed pathway involves trehalose-phosphate synthases (EC 2.4.1.15) that catalyze the transfer of glucose from glucose sugar nucleotides to glucose-6-phosphate to produce trehalose-6-phosphate and nucleoside diphosphates (6). The phosphate is removed by a trehalose-phosphate phosphatase leaving a free trehalose. A highly specific enzyme, trehalase, is responsible for the degradation of trehalose in many organisms.

Interestingly, the isolation of trehalose-based oligosaccharides has been reported in mycobacteria (7). They were tri- and tetrasaccharides all composed of trehalose as a basic structure and harbored an additional glucose residue in the α-(1→4)- and β-(1→6)-linkages, or a galactose residue in the α-(1→6)-linkage, respectively. These oligosaccharides were thought to play a protective role in the microorganism. In addition, attempts were made to synthesize glycosyl-trehaloses by the transglycosylation of cyclomaltodextrin glucanyltransferase or α-glucosidase, in which the trehalose oligosaccharides were prepared by attaching only glucose residues to the α-(1→4)- or (1→6)-linkage (8).

We have recently prepared β-galactosyl trehaloses using *Escherichia coli* β-galactosidase (EC 3.2.1.23). Generally, *E. coli* β-galactosidase has been known to hydrolyze lactose and also to transfer a galactosyl moiety to several acceptors, yielding β-galactooligosaccharides (9-11). In the present study, we report on the transgalactosylation reaction of trehalose and the thermal properties of the galactosyl trehalose products. The transfer compounds were purified and analyzed by thin layer chromatography (TLC) and high performance anion exchange chromatography (HPAEC), and their structures were determined by liquid chromatography-mass spectrometry (LC-MS) and ^{13}C nuclear magnetic resonance (NMR). The heat stability under high temperature was examined and compared to those of trehalose and other sugars.

Materials and Methods

Materials Trehalose dihydrate was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Lactose, other sugars, *p*-nitrophenyl-β-D-galactopyranoside (PNPβGal), baker's yeast, and trehalase (porcine kidney) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Wild-type strain *E. coli* K-12 ATCC 10798 was obtained from the American Type Culture Collection. All other chemicals used were of reagent grade.

Preparation and assay of enzyme *E. coli* β-galactosidase

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Received October 28, 2006; accepted November 8, 2006

(ECbG) was prepared by molecular cloning and over-expression into *E. coli* MC1061. The genetic manipulations and enzyme production were carried out according to the method previously reported (12, 13). The gene encoding β-galactosidase was amplified by polymerase chain reaction (PCR) with the genomic DNA of *E. coli* K-12 and was constructed into the HindIII site of the p6xHis119 expression vector. The *E. coli* MC 1061 transformant harboring the recombinant plasmid was able to constitutively produce a $(His)_6$-tagged recombinant enzyme. The recombinant β-galactosidase was purified to homogeneity using nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen, Hilden, Germany). The purified enzyme was concentrated by ultrafiltration (Millipore Co., Bedford, MA, USA) following dialysis against 50 mM Tris-HCl buffer (pH 7.5) and was used for further investigation.

The activity of ECbG was spectrophotometrically assayed under a standard condition at 37°C in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM PNPβGal as a substrate for the hydrolysis. The concentration of the liberated *p*-nitrophenol was determined by its absorbance at 420 nm (14). One unit (1 IU) of the enzyme activity was defined as the amount of the enzyme that releases 1 μmol of *p*-nitrophenol per min under the standard condition. The protein concentration was determined according to the Bradford method as previously described (15).

Transgalactosylation reaction The transgalactosylation reaction of trehalose by ECbG was performed in the presence of lactose as a donor. ECbG (800 IU) was added to a 100 mL mixture containing 15%(w/v) lactose and 30 %(w/v) trehalose in 50 mM Tris-HCl buffer (pH 7.5). The enzyme reaction was carried out at 45°C for 48 hr, and the reaction was terminated upon heating in boiling water for 5 min. The reaction products were analyzed by TLC on Whatman K5F silica gel plates (Whatman, Kent, UK). The plate was irrigated with isopropyl alcohol-ethyl acetate-water (3:1:1; v/v/v). The carbohydrates on the TLC plate were visualized by dipping the plate in a methanol solution containing 0.3%(w/v) *N*-(1-naphthyl) ethylenediamine and 5%(v/v) sulfuric acid, followed by heating at 110°C for 10 min (16, 17). The relative percentages of the carbohydrates were determined by TLC densitometry, using a Bio-Rad scanning densitomerter (GS-670; Bio-Rad Laboratories, Hercules, CA, USA).

Purification of the transgalactosylation products Glucose, unreacted trehalose and lactose were partially removed from the reaction mixture by gel filtration chromatography on a BioGel P2 (fine) column (1.5×100 cm) with the elution of deionized water at a flow rate of 1.0 mL/min. The partially fractionated reaction products were further purified using recycling preparative HPLC (LC-918; Jai Co., Ltd., Tokyo, Japan) equipped with a refractive index detector (RI-50) and a polymeric gel filtration column (W-251, 2×50 cm).

LC-MS analysis The molecular masses of the transfer products were determined by LC-MS using a Jeol LC mate instrument (Tokyo, Japan). The spectra were obtained in the positive-ion mode of atmospheric pressure chemical ionization (APCI). A 5 μL sample at a concentration of 1 mg/mL was directly injected into the instrument (18).

NMR analysis The ^{13}C NMR spectra of the transfer products were recorded with a Jeol JNM LA-400MHz NMR spectrometer (Tokyo, Japan). The samples were dissolved in DMSO-d_6 at 24°C using tetramethylsilane (TMS) as the chemical shift reference.

Evaluation of thermal stability Each 292.1 mM solution (1 mL) of the transfer products and other sugars at pH 4 and 7 McIlvaine buffers were heated to temperatures ranging from 100 to 145°C for 60 min in an air convection oven in order to investigate the heat stability and caramelization due to the heat-induced decomposition of the sugar (19). The samples were kept in screw-cap tubes during the heating process. The development of a brown color, caused by the caramelization, is generally assessed by monitoring the absorbance at 420 nm (A_{420}) (20). The browning intensity of the samples in the different conditions was determined by monitoring the absorbance at A_{420} with A_{720} as a base of absorption spectrum.

Results and Discussion

Transgalactosylation of trehalose by *E. coli* β-galactosidase The expression and purification of recombinant β-galactosidase from *E. coli* K-12 was efficiently performed (Fig. 1). The prepared β-galactosidase reacted with lactose as a donor and trehalose as an acceptor to prepare the β-galactosylated trehalose oligosaccharides. It catalyzed the transgalactosylation reaction to yield two reaction products (**1** and **2**), which were clearly separated by TLC (Fig. 2, lane 4). Reaction product **1** was preferentially produced at a higher concentration than **2**. The ratio of product **1** to **2** in the reaction (48 hr) with 15% lactose and 30% trehalose

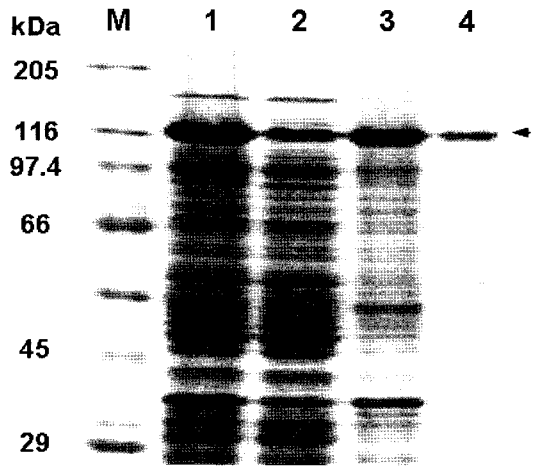

Fig. 1. SDS-PAGE of purified *E. coli* β-galactosidase. Lane M, standard proteins; myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa); lane 1, cell extract; lane 2, supernatant after centrifugation; lane 3, ammonium sulfate fractionation; lane 4, purified β-galactosidase (arrow) after Ni-NTA affinity chromatography.

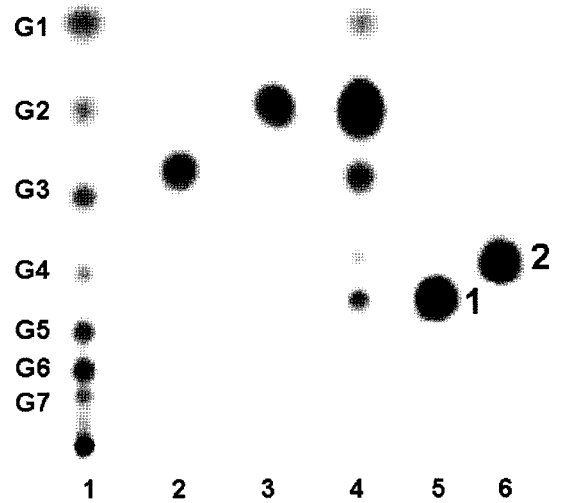

Fig. 2. Thin-layer chromatogram of the transgalactosylation reaction products by *E. coli* β-galactosidase. Lane 1, maltodextrin standards (G1-G7); lane 2, lactose; lane 3, α,α-trehalose; lane 4, transgalactosylation reaction products; lane 5, purified transfer product 1; lane 6, purified transfer product 2.

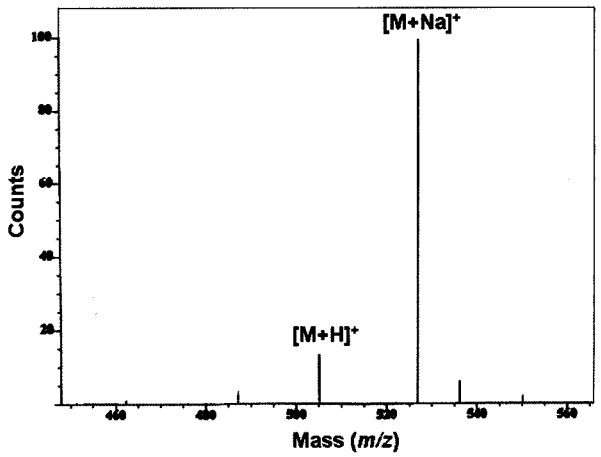

Fig. 3. Molecular mass determination of the purified transfer products using LC-MS. $[M+Na]^+$ and $[M+H]^+$ denote the sodium and proton adducts of the products respectively.

was about 9:1, as determined by TLC densitometry. In addition, the yields of products **1** and **2** were estimated to be approximately 25-27 and 2.5-3%, respectively, based on the concentration of trehalose. The additional spots produced by the transgalactosylation on lane 4 of the TLC chromatogram suggested that the transfer products were likely to be galactosyl adducts of trehalose.

Purification and structural identification of the reaction products The reaction mixture was followed with a treatment of baker's yeast for 24 hr to remove any free D-glucose produced (21). The yeast-treated reaction products were partially fractionated from the remaining substrates, such as trehalose and lactose, by BioGel P2 gel permeation column chromatography. They were further purified by recycling the preparative HPLC with 5 times of recycling to compounds **1** and **2** (Fig. 1, lane 5 and 6). The products were not hydrolyzed against 24 hr incubation when treated with trehalase and analyzed by TLC and HPAEC (data not shown). Trehalase is specific for the hydrolysis of the α,α-(1→1)-glycosidic linkage of trehalose. This result indicated that the addition of a galactosyl moiety into trehalose was effective at inhibiting the action of trehalase.

The molecular masses of the purified transfer products **1** and **2** were determined by LC-MS (Fig. 3). Both compounds were identically calculated to be 504 Da from the two peaks at *m/z* 505.1 ($[M + H]^+$) and *m/z* 527.1 ($[M + Na]^+$), which matched the expected molecular masses of hydrogen and the sodium adducts of hexose trisaccharide. This result implied that a single galactopyranosyl unit was attached to trehalose by *E. coli* β-galactosidase to yield galactosyl trehalose isomers, because both compounds had the same molecular mass but showed different mobility in the TLC analysis. The detailed glycosidic structures of the products were determined by ^{13}C NMR. The ^{13}C-chemical shifts of the galactosyl trehalose isomers **1** and **2** were compared with those of authentic trehalose and galactose, as shown in Table 1. Additional carbon signals were observed in both ^{13}C NMR spectra of compound **1** and **2**. Those signals undoubtedly resulted from the transfer of a galactosyl moiety to trehalose. In addition, there were large changes in the chemical shift of C-6 in one glucose moiety of trehalose from 61.365 to 68.988 ppm in **1** and in that of C-4 from 70.530 to 78.329 ppm in **2**. Chemical shifts were also observed in the C-1 of galactose from 97.289 to 104.163 ppm in **1** and 103.768 ppm in **2**. These results confirmed that the transferred galactosyl group was alternatively attached to C-6 and C-4 in one glucose moiety of trehalose. Therefore, the two transgalactosylation products, **1** and **2**, were defined as 6^2-β-galactosyl trehalose and 4^2-β-galactosyl trehalose, respectively.

In fact, the transgalactosylation activities of β-galactosidases from several microorganisms were employed to synthesize β-galactosylated compounds (11, 22-24). However, the resulting glycosidic linkages of the major products were diverse, including β-(1,6), (1,4), and (1,3), depending on the enzymes from the different sources. The β-galactosyl transfer by *E. coli* β-galactosidase in this study was preferentially performed onto C-6 in a glucose molecule of trehalose, which was consistent with the previous report using *N*-acetylglucosamine as an acceptor (23). A proposed scheme for the transgalactosylation of trehalose is described in Fig. 4.

Heat stability of β-galactosyl trehalose products The β-galactosyl trehalose products (9:1 ratio of **1** to **2**) showed reasonable stability in the heat treatment ranging from 100 to 145°C in comparison to other sugars in the acidic (pH 4.0) and neutral (pH 7.0) conditions (Fig. 5). At pH 4.0, the colorability of the products by heating at 145 °C was significantly higher than that of trehalose, but approximately four times lower than those of glucose and maltose, and 2.0-2.5 times lower than those of sucrose and lactose (Fig. 5A). When heated at pH 7.0 and 130°C, the colorability was 5 times higher than those of sucrose and trehalose, whereas it was approximately 4-6 times lower than those of glucose, maltose, and lactose (Fig. 5B).

Table 1. ^{13}C NMR chemical shifts[1] for trehalose, galactose, and β-galactosyl trehalose isomers 1 and 2 produced by the reaction of *E. coli* β-galactosidase with trehalose and lactose

	Position[2]	Trehalose	Galactose	1[3]	2[3]	Δδ (1)[4]	Δδ (2)[4]
Glc(I)[5]	C-1	94.064		94.204	93.945	0.140	0.119
	C-2	72.982		73.052	73.019	0.070	0.037
	C-3	73.356		73.307	73.365	0.049	0.009
	C-4	70.530		70.234	70.518	0.296	0.012
	C-5	71.871		71.830	71.797	0.041	0.074
	C-6	61.365		61.390	61.357	0.025	0.008
Glc(II)[5]	C-1	94.064		94.204	93.945	0.140	0.119
	C-2	72.982		73.052	73.019	0.070	0.037
	C-3	73.356		73.365	73.365	0.009	0.009
	C-4	70.530		70.535	78.329	0.005	7.799
	C-5	71.871		71.880	72.007	0.009	0.136
	C-6	61.365		68.988	60.707	7.623	0.658
Gal[6]	C-1		97.289	104.163	103.768	6.874	6.479
	C-2		72.715	72.842	72.007	0.127	0.708
	C-3		73.636	73.566	73.650	0.070	0.014
	C-4		69.580	69.543	69.403	0.037	0.177
	C-5		75.973	76.014	76.211	0.041	0.238
	C-6		61.810	61.871	61.871	0.061	0.061

[1]Chemical shifts were measured in DMSO-d_6 at 24°C using tetramethylsilane as a standard.
[2]The positions and chemical structures are represented in Fig. 4.
[3]**1** and **2** indicate the transfer products, 6^2-β-galactosyl trehalose (**1**) and 4^2-β-galactosyl trehalose (**2**).
[4]Differences in chemical shifts between the transfer products and trehalose or galactose.
[5]The glucose unit of trehalose.
[6]Free β-D-galactose and the galactose unit attached to trehalose.

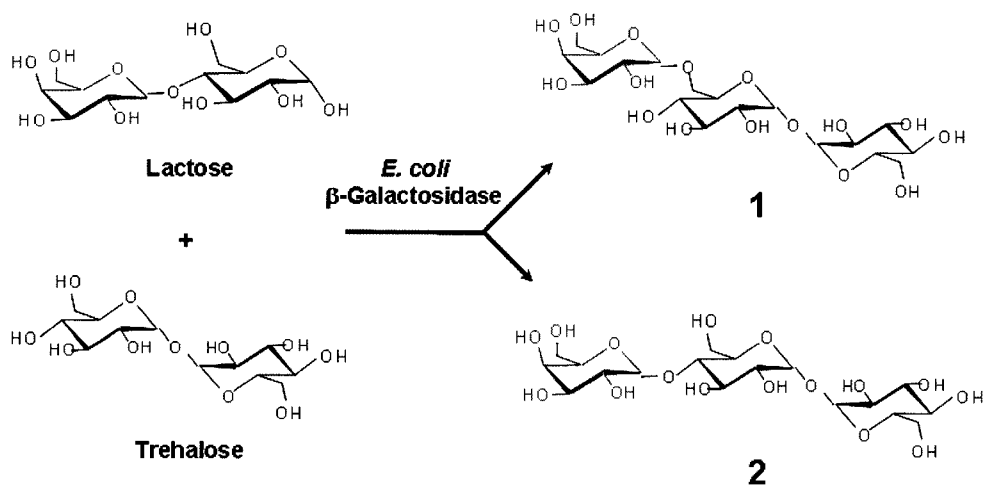

Fig. 4. A reaction scheme for the transgalactosylation reaction of trehalose by *E. coli* β-galactosidase using lactose as a donor.

Furthermore, the colorability of the transfer products by heating at pH 7.0 was higher than that at pH 4.0. The increase in absorbance for sugars in water by heating is generally related to the production of colors associated with a process of chemical degradation and polymerization called the caramelization process. In the color development process the fragmentation of sugars occurs to a significant extent at an acidic pH and considerably increases at alkaline pH. In addition, a pH increase from acidic to alkaline conditions strongly enhances the polymerization of the carbonyl compounds generated by the thermal degradation of saccharides (25, 26).

In conclusion, trehalose has been enzymatically modified by a transgalactosylation reaction primarily performed to

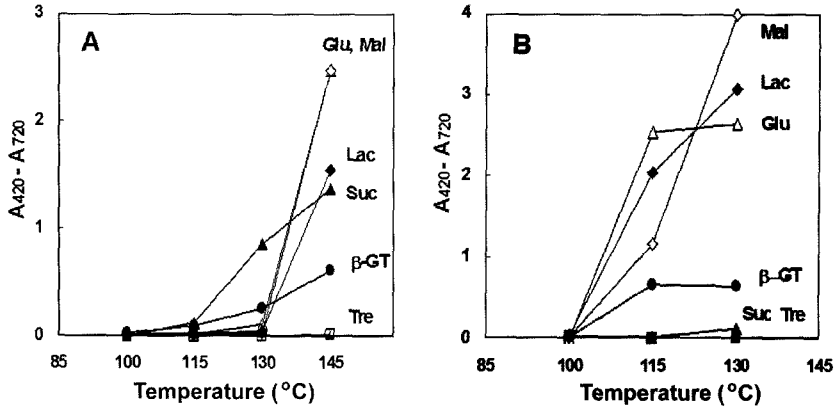

Fig. 5. Thermal stability of the transgalactosylation products and several sugars at pH 4 (A) and 7 (B). (●) β-GT, β-galactosyl trehaloses (9:1 ratio of 1 to 2); (▲) Suc, sucrose; (△) Glu, glucose; (■) Tre, trehalose; (◇) Mal, maltose; (◆) Lac, lactose.

produce two isomeric trisaccharides. The transfer products were determined to be 6^2-β-galactosyl trehalose, as the major compound, and 4^2-β-galactosyl trehalose as a minor product. The β-galactosyl trehaloses showed resistance to hydrolysis by trehalase and significant heat stability in comparison to the other sugars used. Additional physicochemical properties of the transfer products are under further investigation. It is presently assumed that the products might be used as an alternative non-digestible oligosaccharide containing the preferable features of trehalose.

Acknowledgments

This work was supported by a grant from the Korea Research Foundation Grant (KRF-2003-F00070) and in part by Yonsei University (2003). We acknowledge the financial support of the Brain Korea 21 Project, Yonsei University, in the form of scholarships to K.-J. Lee.

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