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Transglycosylation properties of maltodextrin glucosidase (MalZ) from *Escherichia coli* and its application for synthesis of a nigerose-containing oligosaccharide

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ABSTRACT

The transglycosylation reaction of maltodextrin glucosidase (MalZ) cloned and purified from *Escherichia coli* K12 was characterized and applied to the synthesis of branched oligosaccharides. Purified MalZ preferentially catalyzed the hydrolysis of maltodextrin, γ-cyclodextrin (CD), and cycloamylose (CA). In addition, when the enzyme was incubated with 5% maltotriose (G3), a series of transfer products were produced. The resulting major transfer products, annotated as T1, T2, and T3, were purified and their structures were determined by TLC, MALDI-TOF/MS, ^{13}C NMR, and enzymatic analysis. T1 was identified as a novel compound, maltosyl α-1,3-maltose, whereas T2 and T3 were determined to be isopanose and maltosyl-α-1,6-maltose, respectively. These results indicated that MalZ transferred sugar moiety mainly to C-3 or C-6–OH of glucose of the acceptor molecule. To obtain highly concentrated transfer products, the enzyme was reacted with 10% liquefied cornstarch, and then glucose and maltose were removed by immobilized yeast. The T1 content of the resulting reaction mixture reached 9.0%. The mixture of T1 containing a nigerose moiety can have an immunopotentiating effect on the human body and may be a potential functional sugar stuff.

1. Introduction

α-Glucosidase is distributed widely in microorganisms, plants, and animals [1] and catalyzes the hydrolysis of α-1,4-glycosidic linkage in α-glucan, releasing α-D-glucose from the nonreducing end of the substrate. α-Glucosidase also has been known as transglycosidase because it catalyzes transglycosylation reactions [2,3]. In contrast, the maltodextrin glucosidase (MalZ) from *Escherichia coli* K12 is described as an enzyme that removes glucose residue from the reducing end of maltodextrin [4]. The cloning and sequencing of the gene encoding MalZ and the isolation and biochemical characterization of the encoded protein revealed an enzyme that hydrolyzed maltopentaose and the smaller maltodextrin to glucose and maltose, releasing glucose consecutively from the reducing end of the maltodextrin. The smallest substrate is maltotriose [4]. MalZ is involved in the maltose utilization system in *E. coli* whereby the physiological action is to degrade maltotriose to maltose, which is not the inducer of the maltose utilization system [5]. Notably, the MalZ isolated from the *malQ* knockout mutant exhibited a similar transferring activity to amylomaltase, MalQ [6]. The *malZ* gene has been found and characterized as a typical mal gene, but the biological function of the enzyme in cells remains unclear [6]. In addition, the formation of an unknown oligosaccharide compound whose chromatographic migration in thin layer chromatography was situated between maltotriose and maltotetraose was observed, indicating that MalZ exhibits transfer activity [4]. Many bacterial saccharifying amylases catalyze α-1,3- or α-1,6-transglycosylation in addition to the hydrolysis of α-1,4- or α-1,6-linkages [7,8]. The transglycosylation products used include all glucosyl saccharides with α-1,6- or α-1,3-glucosidic linkages such as isomaltose, panose, isopanose, branched products with a high degree of polymerization (DP; DP > 4), and nigerosyl oligosaccharides, when maltodextrins are incubated with bacterial enzymes. A transglycosylated mixture containing nigerose and nigerooligosaccharides was produced by using the enzyme from *Acremonium* sp. [9]. Physicochemical properties and physiological function of nigerose-containing oligosaccharides have been intensively investigated, and nigerosyl moiety is known to have an

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immunopotentiating effect on the human body [10–12]. In this study, we characterize MalZ from *E. coli* and report on the structural determination of the novel compound of the transfer product produced by the enzyme. We also discuss the potential bio-industrial applications of the transfer products.

2. Materials and methods

2.1. Cloning of the malZ gene

Based on the genome sequence of *E. coli* K12 (Accession No. NC 000,913), two primers, *MalZ-Nde*I (5′-AGGGGAATTCATATGTTAAATGCA-3′) and *MalZ-Pst*I (5′-AAACTGCAGGCCACGTTTTATCAA-3′), were synthesized to amplify the *malZ* gene. A 1.8-kb DNA fragment was PCR-amplified from the chromosomal DNA of *E. coli* K12 using two primers, and the nucleotide sequence of the resulting DNA fragment was confirmed using an ABI377 PRISM DNA sequencer (PerkinElmer, Carlsbad, CA, USA). The DNA fragment was digested with *Nde*I and *Pst*I, and ligated into expression vector p6×His119 [8] at the corresponding sites such that the open reading frame of the gene could be fused to six histamine residues in-frame at the C-terminus. The resulting plasmid, p6×H*malZ*, was transformed into *E. coli* strain MC1061 [F$^-$, *ara*D139, *rec*A13, (*ara*-ABC-*leu*)7696, *gal*U, *gal*K, *lac*X74, *rps*L, *thi*, *hsd*R2, *mcr*B] for expression of 6×His-tagged MalZ.

2.2. Purification of the enzyme

E. coli cells carrying the p6×H*malZ* gene were cultured in 1 L of LB medium [1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] with ampicillin (100 g/ml), and the cells were collected by centrifugation. The cells were resuspended in 100 ml of lysis buffer [50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10 mM imidazole] and sonicated over an ice bath (VC-600; Sonics & Materials Inc., Newtown, CT, USA; output 4, 5 min × 3 times). The crude cell extract was centrifuged (10,000*g*) at 4 °C for 15 min. After sonication, the cell lysates were subjected to Ni–NTA affinity chromatography (Qiagen, Valencia, CA, USA), as described previously [8].

2.3. Enzyme assay

The hydrolytic activity of MalZ was assayed at 37 °C in 50 mM sodium phosphate buffer (pH 7.0) with 0.5% (w/v) of substrates such as maltotriose, α-, β-, γ-cyclodextrins (CDs) and cycloamylose (CA) with DP25 by determining the amount of released glucose using the glucose oxidase/peroxidase method (Asan set glucose; Asan Pharmaceutical Co., Ltd., Seoul, Korea) [13]. One unit (U) of hydrolyzing activity was defined as the amount of enzyme producing 1 mM of glucose per minute. The protein concentration was determined using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard [14].

2.4. Purification of the transfer products

The reaction was carried out by incubation with MalZ (0.5 U/mg maltotriose) and 5% (w/v) maltotriose in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 2 h in a water bath. The major transfer products were purified by preparative thin layer chromatography (TLC) on Whatman K5F silica gel plates (Whatman, Maidstone, Kent, UK) with ethyl acetate/methanol/acetic acid/water (12:3:3:1, respectively, v/v/v/v) as the solvent system followed by paper chromatography to remove contaminating silica and binder compounds. Finally, impurities were removed by recycling preparative high-performance liquid chromatography (RP-HPLC; LC-918; JAI Co., Ltd., Tokyo, Japan) using a JAIGEL W251 column (20 × 50 cm; JAI Co., Ltd.). The products were analyzed by TLC and high-performance anion exchange chromatography (HPAEC). HPAEC was performed using a CarboPac PA1 column (0.4 × 25 cm; Dionex, Sunnyvale, CA, USA) and an electrochemical detector (ED40; Dionex). Buffers A (150 mM NaOH) and B (600 mM sodium acetate in buffer A) were used for the elution with a 0–30% (v/v) gradient of buffer B at a flow rate of 1.0 ml/min.

2.5. Mass spectrometry and NMR spectrometry

The MALDI-TOF mass spectrum was collected using a Voyager TM-DE system (Perceptive Biosystems, Framingham, MA, USA) with α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) as the matrix. Next, 1 μl of the purified sample and 1 μl of α-cyano-4-hydroxycinnamic acid were dropped on a sample applicator and dried thoroughly. The sample plate was operated with a 24-kV acceleration voltage. The ^{13}C nuclear magnetic resonance (NMR) spectrum was recorded using a JEOL LA-400 FTNMR spectrometer (JEOL, Tokyo, Japan). The sample was dissolved in H_2O/[D6] at 24.9 °C with tetramethyl silane as the internal reference.

2.6. Enzymatic analysis of the transfer products, T1, T2, and T3

Glucoamylase (AMG 300 L; Novozyme, Bagsværd, Denmark) was use to investigate the hydrolytic action pattern of transfer product T1. Glucoamylase (0.05 U/mg of substrate) was incubated with 0.25% (w/v) T1, T2, and T3 in 50 mM sodium acetate buffer (pH 4.5) at 55 °C for 4 h. The resulting hydrolysis products were analyzed by HPAEC.

2.7. Production and analysis of the transfer products

To produce transfer products, MalZ (1 U/mg of substrate) was added to 10% (w/v) liquefied cornstarch (Genedex; Samyang Genex, Seoul, Korea) in 50 mM phosphate buffer (pH 7.0) and incubated at 37 °C for 30 h. Glucose and maltose were removed by yeast fermentation at 30 °C for 24 h to produce a highly concentrated transfer mixture. Immobilized yeast was prepared using the procedure described by Yoo et al. [15]. The structures and purities of reaction products were analyzed by TLC, HPAEC, MALDI/TOF/MS, and NMR, as described previously [8,15,16].

3. Results and discussion

3.1. Overexpression and properties of MalZ

To overexpress MalZ using *E. coli*, the *malZ* gene was cloned into the p6×His119 vector [8]. The resulting recombinant was transformed into *E. coli*. The enzyme was harvested with a purification fold of 2.5 and yield of 58% via a one-step purification process using Ni–NTA affinity chromatography. Based on SDS–PAGE analysis, the estimated molecular mass was 69 kDa (data not shown), which was correlated with the molecular mass deduced from the predicted amino acid sequence of MalZ. The optimal reaction temperature of MalZ was 37 °C. Activity of the enzyme decreased drastically at temperatures of 40 °C and above, which is typical of most mesophilic enzymes. The optimal pH of MalZ was 7.0 when 50 mM sodium phosphate buffer was used.

3.2. Action pattern of MalZ on various substrates

To investigate the hydrolytic action pattern of MalZ, maltooligosaccharides (G3–G7), α-CD, β-CD, γ-CD, and CA with DP25 (0.5% each), were reacted with the enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 12 h, and the reaction products were

analyzed by TLC. MalZ hydrolyzed maltooligosaccharides, ranging from maltotriose (G3) to maltoheptaose (G7), mainly to glucose and maltose (Fig. 1A). MalZ did not hydrolyze either α- or β-CD, but efficiently hydrolyzed γ-CD, to glucose and maltose (Fig. 1C). Moreover, cycloamylose with DP25 was hydrolyzed to maltose and glucose by MalZ (Fig. 1). These results indicated that the enzyme preferred larger cyclized substrates to smaller ones. MalZ hydrolyzed G3 readily to maltose and glucose at a low concentration (0.5%; w/v) of substrate. However, several transfer products including T1, T2, T3 and other unidentified compounds with DP ⩾ 5 were produced when the enzyme was reacted at 5% (w/v) of G3 (Fig. 1B). In the TLC analysis of the MalZ reaction products, one spot of the unknown compound, whose migration distance was similar to that of β-CD [4], appeared on TLC developed in *n*-butanol/ethanol/water at a ratio of 5:3:2 (v/v/v), respectively. In contrast, as shown in Fig. 1B, the unknown compound was separated into three distinct spots, T1, T2, and T3, on TLC developed with ethyl acetate/methanol/acetic acid/water (12:3:3:1, respectively). These results indicated that MalZ transferred the sugar moiety to various positions at C–OH of the acceptor molecules.

3.3. Structural determination of the transfer products

To identify the major transfer products, T1, T2, and T3, they were purified and analyzed by TLC, HPAEC, and ^{13}C NMR and MALDI/TOF-MS (Table 1, Figs. 1–3). With a molecular mass of 527.17 Da, T2 was identified as a branched product with DP3, and with a molecular masses of 689.24 Da, T1 and T3 were identified as branched products with DP4 (Fig. 3A and C). When T1 was treated with glucoamylase (AMG 300 L; Novozyme) the T1 peak partially decreased, whereas peaks of glucose and the unknown compound appeared, indicating that glucoamylase hydrolyzed an

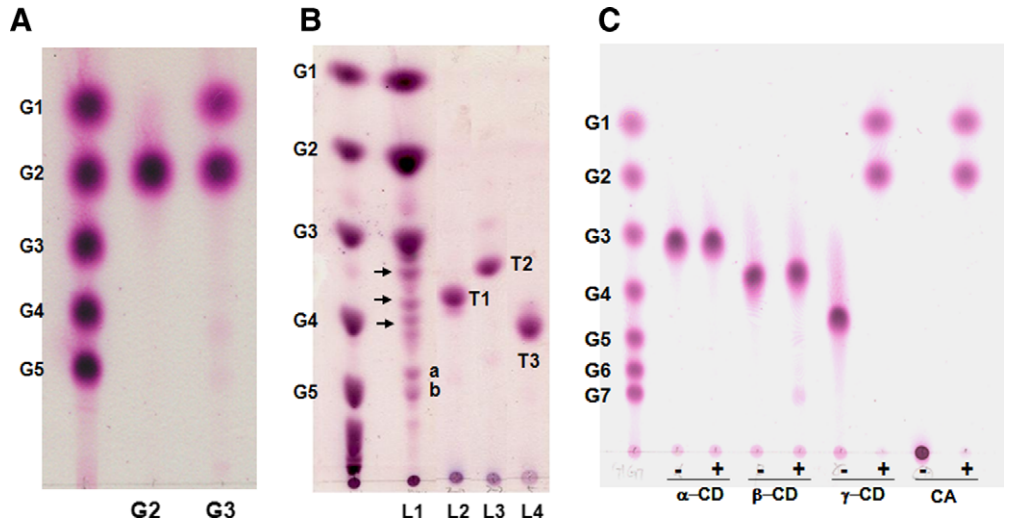

Fig. 1. Action patterns of MalZ on various substrates. (A) MalZ reaction with 0.5% (w/v) linear maltodextrins (G2 and G3); (B) MalZ reaction with 5% (w/v) G3. L1, reaction product of MalZ; a and b, unidentified compounds ⩾ DP5; L2, T1; L3, T2; L4, T3; (C) MalZ reaction with cyclodextrins (CD) and cycloamylose (CA; DP25).

Table 1
^{13}C NMR analysis of the compound T1 (units: ppm).

	Carbon atoms	Maltotetraose	T1	Differences
Ring A	1	92.17	92.19	0.02
	2	73.62	73.59	−0.03
	3	71.57	71.57	0.00
	4	77.19	77.23	0.04
	5	74.28	74.27	−0.01
	6	60.95	60.96	0.01
Ring B	1	100.00	100.14	0.14
	2	73.13	72.79	−0.34
	3	**71.48**	**79.65**	**8.17**
	4	77.04	70.60	−6.44
	5	74.81	74.88	0.07
	6	60.81	60.74	−0.07
Ring C	1	99.90	99.86	−0.04
	2	73.13	73.15	0.02
	3	71.83	71.75	−0.08
	4	76.95	77.03	0.08
	5	74.81	74.88	0.07
	6	60.74	60.74	0.00
Ring D	1	99.66	99.16	0.50
	2	72.97	72.92	−0.05
	3	72.00	72.01	0.01
	4	69.59	69.61	0.02
	5	76.46	76.42	−0.04
	6	60.69	60.54	−0.15

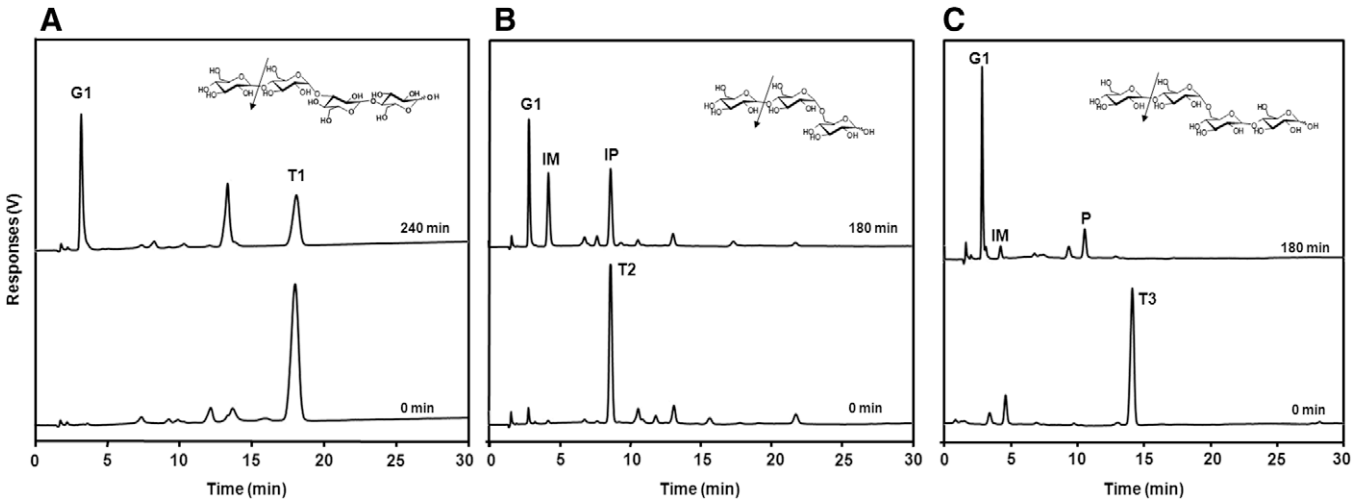

Fig. 2. Enzymatic analysis of transfer products. Each transfer product [(A), T1; (B), T2; (C), T3] was treated with glucoamylase (AMG, Novozyme) and the reaction products were analyzed using HPAEC. G1, glucose; IM, isomaltose; IP, isopanose; P, panose.

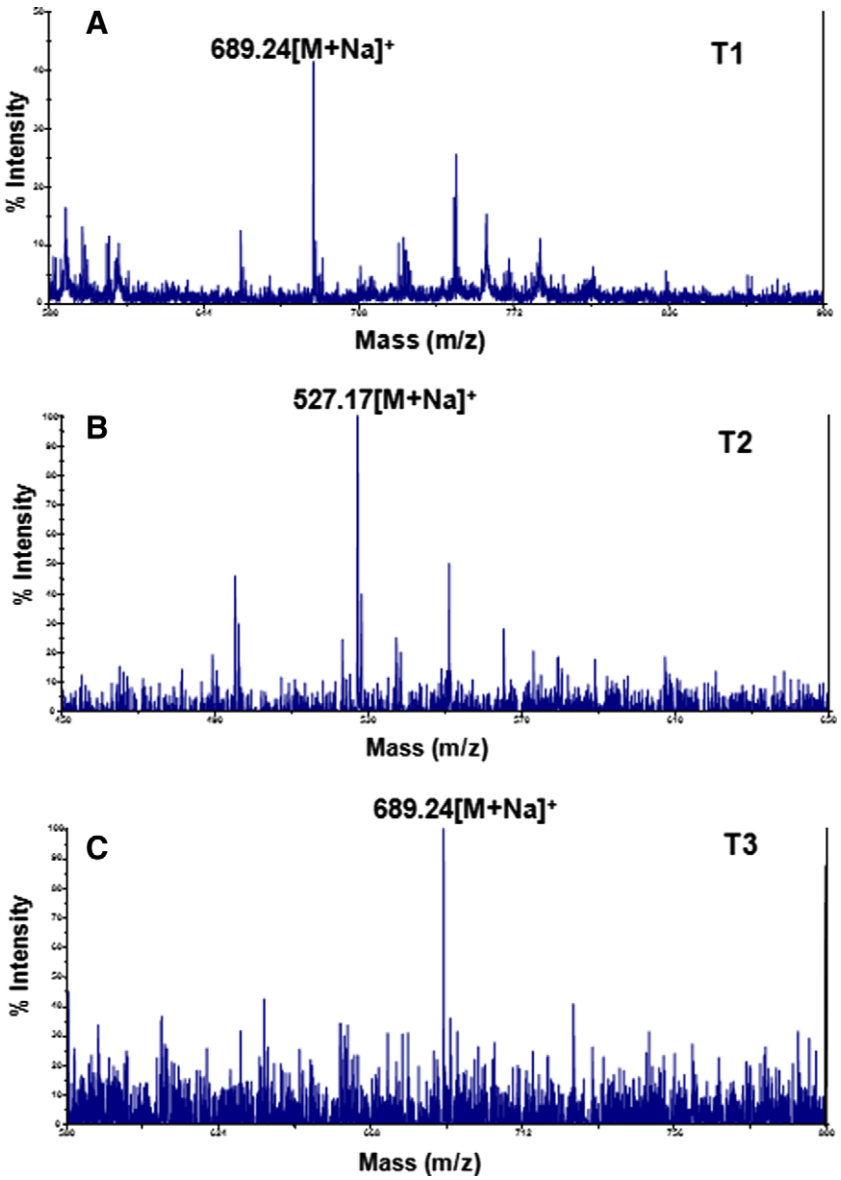

Fig. 3. MALDI-TOF/MS analyses of T1, T2, and T3.

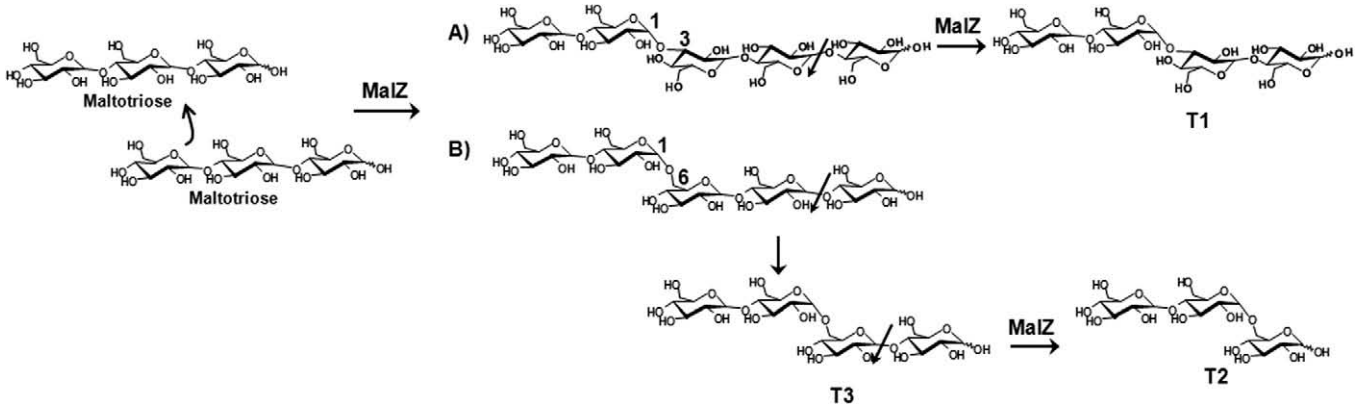

Fig. 4. Proposed mechanism of the MalZ transfer reaction.

α-1,4-linkage of T1 at the nonreducing end to glucose (G1), but the linkage of the resulting triglycoside could not be hydrolyzed (Fig. 2A). T2 had the same elution time in HPAEC as isopanose and was easily hydrolyzed, giving two peaks that corresponded to glucose and isomaltose. These results indicated that transfer product T2 is isopanose (Fig. 2B). Upon treatment with glucoamylase, the hydrolysis of T3 resulted in glucose and panose. Moreover, T3 had the same elution time in HPAEC as maltosyl-(α-1,6)-maltose [16]. Therefore, T3 was confirmed to be maltosyl-α-1,6-maltose (Fig. 2C). ^{13}C NMR analysis was performed to determine the structure of T1. Chemical shifts in the ^{13}C NMR spectrum were compared to those of maltotetraose (Table 1). The shifts occurred at position C-3 of the second glucose molecules from the reducing end, that is, from 71.48 to 79.65 ppm, implying that the maltosyl unit was attached to C-3 in the glucose of maltose. Therefore, the molecular structure of T1 was a novel compound named maltosyl-(α-1,3)-maltose. These results show that MalZ transfers sugar moiety mainly to C-3 or C-6–OH of glucose of the acceptor molecule. As proposed in Fig. 4, the maltosyl moiety of maltotriose (donor molecule) was transferred to either C-3–OH or C-6–OH of the nonreducing glucose of maltotriose (acceptor), thereby producing maltosyl-α-1,3-maltotriose or maltosyl-α-1,6-maltotriose, whose glucose residue was further hydrolyzed at the reducing end.

3.4. Composition of highly concentrated transfer product formed by MalZ

The highly concentrated transfer mixture was prepared by incubation of MalZ with liquefied cornstarch and sequential treatment of immobilized yeast [15]. The major components were determined by HPAEC. After removing glucose and maltose by yeast fermentation, the content of the transfer product was enhanced to 17.7% isopanose and 9.0% maltosyl-α-1,3-maltose.

3.5. Potential as a functional oligosaccharide

The mixture formed by the transglycosylation reaction of MalZ from *E. coli* contained significant amounts of maltosyl-α-1,3-maltose, isopanose, and maltosyl-α-1,6-maltose as the major transfer products. The compound T1, a tetrasaccharide containing an α-1,3-linkage within the molecule, can be converted first to nigerosyl glucose by α-glucosidase in the human intestine, removing the glucosyl residue on the nonreducing end. Nigerose and nigerooligosaccharide are known immunopotentiating oligosaccharides [17]. The hydrolyzing susceptibility of nigerose in the brush border membranes of rat jejunum is about 86% that of maltose [9]. Furthermore, a nigerose-containing oligosaccharide is hydrolyzed by isomaltase and glucoamylase from brush border membranes of rat jejunum, where only a small amount of nigerose is absorbed by the small intestine into the body [17]. The mixture contained 17.7% isopanose, a proportion higher than that in other commercial products. Isopanose is known to be hardly hydrolyzed by α-glucosidase. Therefore, this transfer mixture containing a high proportion of both isopanose and maltosyl α-1,3-maltose has a high potential as a substitute sugar for the prevention of diabetes, obesity, or cardiovascular disease.

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