Maltosyl-erythritol, a Major Transglycosylation Product of Erythritol by Bacillus stearothermophilus Maltogenic Amylase

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This study was done to modify erythritol to change its physicochemical and sensory properties. Erythritol, a four-carbon sugar alcohol, was transglycosylated by Bacillus stearothermophilus maltogenic amylase with maltotriose as a donor molecule. The presence of various transglycosylation products of erythritol was confirmed by TLC and high performance ion exchange chromatography (HPIC). The major transfer product was purified by gel filtration chromatography on BioGel P-2. Examination by LC-MS, matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS), and 13C NMR showed that the major transfer product was maltosyl-erythritol. Results of 13C NMR of maltosyl-erythritol suggested that linkage was formed between the C1 carbon of glucose unit in maltose and either one of the two carbon atoms of the terminal hydroxyl groups of erythritol, so that a mixture of 1-O- and 4-O-α-maltosyl-erythritol was produced. The sweetness of maltosyl-erythritol was about 40% that of sucrose, and its negative sensory properties were less than those of erythritol.

Key words: maltosyl-erythritol; Bacillus stearothermophilus maltogenic amylase; transglycosylation; sweetness

Sugar alcohols are the reduction products of sugars and are referred to as polyols when all of their oxygen atoms are in hydroxyl groups. They are the primary alternative to sugar because of their low calorific value and other desirable characteristics. Sugar alcohols are less reactive than the corresponding sugars because of the absence of reducing carbonyl groups. As a result of this property, these alcohols cause little acid formation in dental plaque, and this difference helps to prevent dental caries. Sugar alcohols also are used in dietetic food formulations as humectants to control water activity in foods with intermediate levels of moisture and to improve the rehydration characteristics of dehydrated food. Erythritol is a linear carbohydrate with four carbons each carrying a hydroxyl group, and therefore is in the class of monosaccharide polyols like sorbitol, mannitol, xylitol, and glycerol. Erythritol is symmetrical and has only one form, the meso-form; it can exist as an anhydrous crystal with a moderately sweet taste without off-taste or odors. It is about 75% as sweet as sucrose and occurs in fruits such as pears, watermelons, and grapes, and in other foods including mushrooms and fermented foods (e.g., wine, soy sauce, and cheese). Oku found that more than 90% of the erythritol taken orally is excreted into the urine in its native form, suggesting that less than 10% of the erythritol is fermented, to contribute to its available energy (0.2 kcal/g). These properties make erythritol an ideal bulk sweetener for dietetic and diabetic foods.

Many glycosidases, primarily known for their hydrolysis of various polysaccharides and oligosaccharides, also catalyze transglycosylation reac-
In such reactions, the glycosyl moiety of the substrate is transferred to compounds containing hydroxyl groups other than water. The ability to catalyze the stereospecific formation of glycosidic bonds and to use simple carbohydrates as substrates make glycosidasases useful for these reactions. However, understanding of the regioselectivity of the enzymes is poor. Glycosidasases from different sources catalyze the synthesis of different linkages. Attempts have been made to establish a relationship between the substrate specificity of the hydrolytic reaction and the regioselectivity during transglycosylation. Recently, we found that Bacillus stearothermophilus maltogenic amylase (BSMA) has strong transglycosylation activity as well as hydrolytic activity. In the presence of maltotriose as a donor, BSMA transfers the maltose unit to the acceptor molecule by forming an α-(1-6)-glycosidic linkage. This property of BSMA has been used to improve the physicochemical characteristics of various natural compounds. The flavonoids neohesperidin and naringin have been modified to their glycosylated forms by transglycosylation with BSMA. The transfer products, 6-maltosyl-naringin and maltosyl-naringin, have properties different from those of the native compounds, such as increased solubility and sweetness.

Here, erythritol, a typical sugar alcohol, was modified by transglycosylation with BSMA. The transfer compounds were analyzed by HPIC and TLC, and the structure of the major transfer product, maltosyl-erythritol, was identified. The physicochemical and sensory properties of the maltosyl-erythritol were examined.

**Materials and Methods**

BSMA and assay of its activity. BSMA was purified from recombinant *Escherichia coli* DH5α [supE44, ΔlacU69 (φ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1], which harbors plasmid pSG12. Cultivation of the recombinant *E. coli* and purification of the enzyme was as described previously. Hydrolysis by BSMA was assayed with 1% (w/v) β-cyclodextrin as the substrate in 50 mM sodium citrate buffer (pH 6.0) at 55°C. The reducing sugar produced was identified by the 3,5-dinitrosalicylic acid method. One unit of enzyme activity was defined as the amount of enzyme that formed reducing sugar equivalents of glucose per minute. The reaction mixture was composed of 10% (w/v) maltotriose and 73% (w/v) erythritol in 50 mM sodium citrate buffer (pH 6.0). The reaction mixture was boiled until all of the solute dissolved. After incubation of the mixture at 55°C for 10 min, 0.5 unit of BSMA per milligram of maltotriose was added. After 12 h of incubation at 55°C, the reaction was stopped by boiling of the mixture for 5 min.

Detection of transglycosylation products. The transglycosylation reaction products were analyzed by TLC and HPIC. A silica-gel K5F TLC plate (Whatman, Maidstone, UK) with a solvent system of isopropyl alcohol/ethyl acetate/water (3:1:1 by volume) or a mixture of acetonitrile and water (85:15 by volume) was used. After irrigation twice, the plate was dried thoroughly and dipped in a mixture of 0.3% (w/v) N-(1-naphthyl)-ethylenediamine and 5% (v/v) H2SO4 in methanol, and the plate was heated at 110°C for 10 min. The amount of transfer products was estimated semiquantitatively from the spot on TLC by densitometry.

The reaction mixture was centrifuged at 12,000 × g for 10 min, and filtered through a 0.45-μm membrane filter before HPIC was done. Twenty microliters of sample was put onto a CarboPac PA1 column (0.4×25 cm, Dionex, Sunnyvale, CA) equipped with an electrochemical detector (ED40, Dionex). The sample was eluted with a linear gradient from 100% buffer A (150 mM NaOH in water) to 30% buffer B (600 mM sodium acetate in buffer A) during 30 min. The flow rate was 1.0 ml/min.

Purification of major transfer products. At the end of the transglycosylation reaction, the mixture was diluted by the addition of one volume of distilled water and put through a 0.45-μm filter. This reaction mixture was put onto a Bio-Gel P-2 column (2.6×95 cm) and gel filtration chromatography was done to purify ETP1 from the reaction mixture. Each fraction was assayed by TLC for the detection of transfer products. Although the chromatography removed much of the hydrolysis products in the reaction mixture, small amounts of panose were still in it. Hence, further separation by preparative TLC was done. The mixture of transfer products was spotted on a half-size Whatman K5F TLC plate, which was irrigated at room temperature with two ascents of a solvent mixture of isopropyl alcohol/ethyl acetate/water (3:1:1 by volume). The parts of the plate containing different transfer products were cut up, and the moistened silica powder was scratched off the plate and collected into a 50-ml Falcon tube. The transfer product was extracted from the silica powder with water by being shaken at room temperature for 4 h. The extract was centrifuged at 15,000 × g for 15 min and the supernatant was filtered through...
Cyano-4-hydroxy-cinnamic acid (Sigma Chemical each pH was prepared by the mixture of 0.1M citric bance values at 420 and 720 nm. McIlvaine buŠer of reactivity was found by comparison of the absor-

TLC was homogeneous. Analyses showed that ETP1 puriˆed by preparative TLC and HPIC contaminating silica compounds. TLC and HPIC speed concentrator under reduced pressure (Speed

Lapeer, MI). The ˆltrate was concentrated with a sample plate was placed in a Voyager-DE Biospectro-

metry workstation. The workstation was operated at the acceleration voltage of 20 kV.

MALDI-TOF-MS. The MALDI-TOF mass spectrum was obtained with a JEOL LC Mate (Tokyo, Japan) system in the mode of atmospheric pressure chemical ionization. Five microliters of the sample at the concentration of 100 µg/ml was injected into the instrument.

MALDI-TOF-MS. The MALDI-TOF mass spectrum was obtained with a Voyager-DE system (PerSeptive Biosystem, Framingham, MA). o-Cyano-4-hydroxy-cinnamic acid (Sigma Chemical Co., St. Louis, MO) was used as the matrix and des-Arg1-bradykinin was used for calibration. One microliter of the purified sample (1 µM) and o-cyano-

4-hydroxy-cinnamic acid (10 mg/ml) was dropped onto a sample plate and thoroughly dried. Then the sample plate was placed in a Voyager-DE Biospectrometry workstation. The workstation was operated at 25°C with tetramethylsilane as the internal reference.

Maillard reaction. McIlvaine buffers (pH 4–8) containing 0.5% (w/v) glycine and 10% (w/v) sugar or transfer product were put in Eppendorf tubes that were sealed and boiled for 1 h at 100°C. The Maillard reactivity was found by comparison of the absorbance values at 420 and 720 nm. McIlvaine buffer of each pH was prepared by the mixture of 0.1 M citric acid and 0.2 M Na2HPO4.

Measurement of flow behavior. Flow properties of sugar samples and transfer products were measured with a concentric cylindrical viscometer (Haake Rotovisco RV2O, Thermo Haake, Karlsruhe, Germany) at various temperatures and concentrations. Nine milliliters of sample solution was put in the cup and the shear stress was measured while the shear rate was increased from 0 to 1000 sec at a de-

defined temperature.

Sensory evaluation. The sensory characteristics of maltosyl-erythritol were compared with those of sucrose, maltose, and erythritol by quantitative description analysis and magnitude estimation. Samples were prepared fresh on the day of testing and served at room temperature (22 ± 2°C). Four milliliters of each sample solution was presented to nine trained panelists. Subjects were chosen on the basis of their performance in screening tests assessing their ability to identify accurately basic tastes, correctly rank solutions differing in concentration, and describe the taste properties of complex taste stimuli. All panelists had been exposed to a variety of sweeteners before the study. Quantitative descriptive analysis was used to evaluate the sensory attributes of maltosyl-erythritol. A sensory score sheet with a 12-cm unstructured line scale, each with anchored terms at both ends, was used for each descriptive term. The judges indicated the intensity of each attribute by placing a vertical line on the unstructured scale line. The five attributes profiled were aftertaste, bitterness, astringency, metallic taste, and mouth-

feel. Quantitative analysis involved measurement of the distance from zero to the vertical line. The relative sweetness of maltosyl-erythritol was found by magnitude estimation. Panelists were first asked to taste a reference solution containing 2% (w/w) sucrose. They then were given the test samples and asked to assign a sweetness score relative to the refer-

ence with a sip-and-spit procedure. Panelists were requested to assign numbers that reflected ratios of sensory intensity. Judges were free to choose any number, so the range selected by each judge was unique to him or her. Thus, for pooling of all judg-
mients, a normalization factor was calculated for each judge and used to adjust his or her daily magni-
itude estimates. The geometric means of the magnitude estimates given for various concentrations of su-
gars were multiplied by a number that made their grand mean equal to 10. Magnitude estimates were converted to logarithms and expressed as the geometric mean. Dose-response curves for each compound were then fitted to a power function (S = KC^n) where S and C are the sweetness and concentration of each compound, respectively, K is constant, and n is the characteristic exponent). Fisher’s least significant difference (LSD) method was used to evaluate the sig-

ificance of differences among treatment means.

Results and Discussion

Transglycosylation of erythritol by BSMA

BSMA hydrolyzes various carbohydrate substrates such as starch, pullulan, β-cyclodextrin, and maltooligosaccharides. In the presence of an acceptor, the enzyme catalyzes the transfer of the hydrolysis products glucose and maltose to many kinds of acceptor molecules. Transglycosylation with BSMA and with maltotriose as the donor and erythritol as the acceptor gave various transfer products in addition to the hydrolysis products glucose and maltose (Fig. 1). At the same molar ratio of the acceptor to
Fig. 1. TLC of Purified Maltosyl-erythritol.
Lane S1, maltodextrin standards from glucose (G1) to maltopentaose (G5); lane A, transglycosylation reaction mixture of erythritol with maltotriose; lane B, major fraction containing maltosyl-erythritol after Bio-Gel P-2 chromatography; and lane C, maltosyl-erythritol after preparative purification by TLC. The position of erythritol is not seen on TLC because the method in Materials and Methods for making products visible does not work for erythritol.

Fig. 2. TLC of Transglycosylation Reaction of Erythritol with Maltotriose at Various Molar Ratios of Acceptor and Donor.
Lane S1, maltodextrin standards from glucose (G1) to maltopentaose (G5); lane S2, glucose (G1), isomaltose (IM), and panose (Pan) standards; lane A, no acceptor; molar ratios of acceptor (erythritol) to donor (maltotriose; 0.2 M): lane B, 1:1; lane C, 2.5:1; lane D, 5:1; lane E, 10:1; lane F, 20:1; and lane G, 30:1. Spot 1 is maltosyl-erythritol (EPT1) and spot 2 is glucosyl-erythritol (EPT2).

Fig. 3. LC-MS of Maltosyl-erythritol.
The molecular weight of ETP1 was estimated by LC-MS and MALDI-TOF-MS. By LC-MS, the compound had three peaks corresponding to masses \((m/z)\) of 447.1, 469.2, and 485.1 Da (Fig. 3). The masses of those peaks matched the calculated molecular masses of proton, sodium, and potassium adducts of maltosyl-erythritol (447, 468, and 485 Da), respectively. MALDI-TOF-MS gave a single peak at the mass \((m/z)\) of 468.6, which was consistent with the molecular mass of maltosyl-erythritol combined with sodium ion \((\text{M} + \text{Na}^+)\) (Fig. 4). The molecular masses found by these methods were similar and the results confirmed that ETP1 was maltosyl-erythritol, in which a maltose molecule was transferred to the erythritol. In a similar way, we found that the minor erythritol-transfer product ETP2 was glucosyl-erythritol, in which a glucose was transferred to the erythritol instead of maltose (not shown).
**Fig. 4.** MALDI-TOF-MS of Maltosyl-erythritol.

**Fig. 5.** $^{13}$C NMR Spectra of Maltosyl-erythritol and Erythritol.

$^{13}$C NMR analyses of maltosyl-erythritol

Results of $^{13}$C NMR spectrometry are shown in Fig. 5 and Table 1, which lists the signals from maltose, erythritol, and maltosyl-erythritol. Erythritol, a meso compound, showed two chemical shifts at 63.5 and 72.8 ppm (Fig. 5). Four kinds of linkages might form between the anomeric carbon of maltose and one of the four carbon atoms of erythritol. The chemical shift for the anomeric carbon that took part in the linkage showed a downfield change from 92.7 to 98.8 and 99.3 ppm. The DEPT mode of $^{13}$C NMR gives a negative peak for the carbon of methylene groups (–CH$_2$–). We found a chemical shift from 63.5 to 69.3 and 69.9 ppm of the two methylene carbons (C-1 and C-4) of erythritol. This result suggested that the major erythritol transfer product of BSMA was maltosyl-erythritol, in which linkages formed between the anomeric carbon of maltose and either one of the two terminal methylene carbons of erythritol. Unfortunately, we could not identify the proportions of 1-0- and 4-0-α-maltosyl-erythritol because their mobility is identical in TLC and HPLC. However, erythritol is symmetrical, so the C-1 and C-4 of erythritol probably are involved equally in transglycosylation. Therefore, the major transfer product, ETP1, may be a mixture of equal amounts of 1-0- and 4-0-α-maltosyl-erythritol.

Cho et al. studied the transglycosylation of neohesperidin dihydrochalcone (NHDC) catalyzed by BSMA. The major transfer product of NHDC is maltosyl-α-(1,6)-NHDC, in which the transferred maltosyl group was attached to C-6 of the glucose moiety of NHDC. The major transglycosylation product of naringin by BSMA is maltosyl-α-(1,6)-naringin, in which the maltosyl unit is linked by an α-1,6 linkage to naringin. The transglycosylation products of naringin and neohesperidin by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species are glucosyl-α-(1,3)-glucopyranosyl naringin and glucosyl-α-(1,3)-glucopyranosyl neohesperidin, respectively, in which α-(1,3)-glycosidic linkages form between the acceptor and donor. It seems that the kind of glycosidic linkage that forms between the acceptor and donor depends on the enzyme that catalyzes the transglycosylation.

**Table 1.** $^{13}$C NMR Signals of Maltose, Erythritol, and Maltosyl-erythritol

<table>
<thead>
<tr>
<th>Carbon atoms</th>
<th>Maltose$^1$</th>
<th>Erythritol</th>
<th>Maltosyl-erythritol</th>
<th>Differences</th>
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<tr>
<td>Ring A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100.4</td>
<td>100.5</td>
<td>0.1</td>
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<tr>
<td>2</td>
<td>72.5, 72.6</td>
<td>72.5, 72.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>73.7</td>
<td>73.7</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70.2</td>
<td>70.2</td>
<td>0.0</td>
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</tr>
<tr>
<td>5</td>
<td>73.5</td>
<td>73.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>61.3</td>
<td>61.3</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Ring B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>92.7</td>
<td>98.8, 99.3</td>
<td>6.1, 6.6</td>
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</tr>
<tr>
<td>2</td>
<td>72.1</td>
<td>72.2, 72.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>74.1</td>
<td>74.3, 74.4</td>
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<tr>
<td>4</td>
<td>77.8</td>
<td>77.7, 77.6</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>70.8</td>
<td>70.9</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>61.3</td>
<td>61.3, 61.4</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Erythritol

| 1, 4         | 63.5       | 69.3, 69.9 | 5.8, 6.4            |             |
| 2, 3         | 72.8       | 71.1, 71.2 | 1.7                 |             |

$^1$ Maltose: α-form.

Physicochemical and sensory properties of maltosyl-erythritol

The formation of N-glycosides by the Maillard reaction generally increases at higher temperatures and pHs and with lower water activity. Glucose had the highest Maillard reactivity, followed by maltose (Fig. 6). The reactivities of the sugars were higher at higher pH. Maltosyl-erythritol had low reactivity in the pH range of 4 to 8, as did erythritol and sucrose. Takenaka and Uchiyama reported that another transglycosylation product, α-1,6-glucosylglycerol, has low Maillard reactivity. Unlike results for the Maillard reaction, a colorability test showed that the browning of maltosyl-erythritol at pH 7 was greater when the temperature was 120°C, although the color of sucrose changed the most (not shown). Maltosyl-erythritol was thermostable at various temperatures.
and pHs. Flow behavior of 20% (w/v) solution of maltosyl-erythritol is shown in Fig. 7. The linear mode of the solution gave the highest regression coefficient, suggesting that the maltosyl-erythritol solutions were Newtonian fluids. Comparison of 20% (w/v) solutions of maltosyl-erythritol, maltose, and erythritol showed maltosyl-erythritol had the highest shear stress, followed by maltose and erythritol, in that order.

Sensory evaluation with magnitude estimation showed that as the concentration increased, the sweetness of maltosyl-erythritol increased, and that its relative sweetness was about 40% of sucrose (Table 2). Most of the sensory scores of maltosyl-erythritol were less than those of erythritol (Fig. 8 and Table 3). Notably, the negative properties of erythritol (bitterness and astringency) were much less. Transglycosylation may change the sensory properties.

**Fig. 6.** Maillard Reactivity of Maltosyl-erythritol and Other Sugars at Various pHs.

**Fig. 7.** Flow Behavior of 20% (w/v) Solutions of Maltosyl-erythritol, Maltose, and Erythritol.

**Fig. 8.** Sensory Profiles of Sucrose, Maltose, Erythritol, and Maltosyl-erythritol.

<table>
<thead>
<tr>
<th>Sweetener</th>
<th>Slope (n)</th>
<th>Intercept (k)</th>
<th>$R^2$</th>
<th>$S_A/S_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>1.00</td>
<td>3.27</td>
<td>0.98</td>
<td>1.00</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.93</td>
<td>1.54</td>
<td>0.95</td>
<td>0.40</td>
</tr>
<tr>
<td>Erythritol</td>
<td>1.08</td>
<td>1.96</td>
<td>0.99</td>
<td>0.72</td>
</tr>
<tr>
<td>Maltosyl-erythritol</td>
<td>1.13</td>
<td>0.98</td>
<td>0.99</td>
<td>0.41</td>
</tr>
</tbody>
</table>

1 The equations were as follows:
\[ \log(\text{sweetness score}) = n \log C + \log k \]
where $n$ is the slope, $C$ is the concentration, and $k$ is the intercept.
Relative sweetness $= S_A/S_B = K_A C^n / K_B C^m$
where $S_A$ is the sweetness of sweetener $A$, $S_B$ is the sweetness of sweetener $B$, $K_A$, $K_B$, $m$, and $n$ are constants, and $m \neq n$.

**Table 2.** Relative Sweetness Equation Parameters and Relative Sweetness

**Table 3.** F-Value Calculated by Analysis of Variance and the Least Significant Difference of Scores from Descriptive Analysis for Sweetness of Sugars

<table>
<thead>
<tr>
<th>Sensory parameters</th>
<th>Means</th>
<th>F-value</th>
<th>Probability²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose</td>
<td>Maltose</td>
<td>Erythritol</td>
</tr>
<tr>
<td>Aftertaste</td>
<td>6.80a</td>
<td>4.00ab</td>
<td>4.94ab</td>
</tr>
<tr>
<td>Bitterness</td>
<td>1.76a</td>
<td>2.10a</td>
<td>6.69a</td>
</tr>
<tr>
<td>Astringency</td>
<td>0.77a</td>
<td>0.90a</td>
<td>3.29a</td>
</tr>
<tr>
<td>Metallic taste</td>
<td>1.31a</td>
<td>3.73a</td>
<td>3.63a</td>
</tr>
<tr>
<td>Mouth-feel</td>
<td>6.31a</td>
<td>4.46a</td>
<td>3.81a</td>
</tr>
</tbody>
</table>

1 Values with a different superscript in the same row are significantly different ($P<0.05$).
2 The level at which the observed result would just be statistically significant.
characteristics of the compound although its physicochemical properties are not greatly altered.

Acknowledgments

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