Isolation and Characterization of Oligosaccharides Produced from Sucrose by Transglucosylation Action of *Serratia plymuthica*

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The oligosaccharides produced by the title reaction were dealt with. After removal of the most of palatinose the reaction mixture was used as the sample. Carbon-celite chromatography of the sample (40 g, 66° Brix) gave three saccharides (saccharide A, B and C), besides palatinose and sucrose. Saccharide A was obtained as syrup (2.0 g) and identified as 1-α-D-glucopyranosyl-D-fructofuranose by chemical reactions and some chromatographic analyses such as HPLC, GLC, TLC and PC, and by the fragmentation analysis of GC-MS. It tasted sweet. Saccharide B was obtained as syrup in a small amount with slight contamination. Hydrolysis and chromatographies suggested that it was identical with isomaltose. Saccharide C was isolated as white needles (2.3 g) and examined chemically and chromatographically. As the results, it was found that it had the same structure as isomelezitose. It was tasteless. Consequently, the sample sugar solution consisted of fructose (15.9%), glucose (9.7%), sucrose (21.8%), 1-α-D-glucopyranosyl-D-fructofuranose (16.6%), palatinose (21.8%), isomaltose (5.2%) and isomelezitose (8.2%). The transglucosylation reaction shows the remarkable specificity in that the newly synthesized oligosaccharides have a bond at which α-glucosyl residue attached to the primary alcoholic group of the sugars.

Various oligosaccharides having α-glucosidic linkage have been obtained by the application of transglucosylation action of enzymes from various origins. Of these sugars palatinose (isomaltulose, 6-α-D-glucopyranosyl-β-D-fructofuranose) has been produced from sucrose by the transglucosylation action of enzymes from some microorganisms, such as *Protaminobacter ruber*1), a haploid yeast2), and *Leuconostoc mesenteroides*3).

Recently, Shimizu et al.4) developed a method of mass production of this sugar, in which they treated sucrose solution with the fungal body of *Serratia plymuthica* NCIB 8285 to obtain abundant production of this sugar. Since this method has the great industrial possibility and the sugar is a non-cariogenic food sugar although its sweetness is as low as about 45% of that of sucrose, food manufacturers are taking much interest in this sugar. In the above production system, some oligosaccharides in addition to the main product palatinose are formed in small yield.

Now the authors have characterized these sugars.

Materials and Methods

**Standard preparations**: Turanose (Pfanstiehl Lab. Inc.) and isomaltose (Sigma Chemical Co.) were commercially available. 1-α-D-Glucopyranosyl-β-D-fructofuranose and maltulose were samples kindly donated by Dr. H. Schiweck, Leiter, Zentrallaboratorium der südzucker AG., and Mr. S. Hashimoto, Nikken Kagaku Co. Ltd., respectively. Palatinose was produced at Mitsui Sugar Co. Ltd.

**Immobilized Serratia plymuthica cells**: The immobilized preparation of *S. plymuthica* NCIB 8285 cells was produced according to the method described in a patent4) at Mitsui Sugar Co. Ltd.

**Sample sugar solution**: Sucrose solution was allowed to contact with the immobilized cells of *S. plymuthica*. The reaction mixture was purified and concentrated, and the crystallized palatinose was filtered off. The resulting filtrate (66° Brix) was used as the sample sugar solution.

**Carbon-celite column chromatography (CCC)**: The sample sugar solution (ca. 40 g)
was applied on a carbon-celite column (1:1 by wt., 10×60 cm) and eluted successively with deionized water (15 l), 2.5% ethanol (31 l), 5% ethanol (14 l), 7.5% ethanol (41 l) and 10% ethanol (16 l). Each 500 ml of effluent was collected. The total amount of sugars in each fraction was expressed as sucrose by measuring the absorbance at 490 nm by the phenol-sulfuric acid method.

**High performance liquid chromatography (HPLC):** A Hitachi model 635 A liquid chromatograph was used. The conditions used were as follows: column, µ-Bondapak Carbohydrate column (4 mm×30 cm, Waters Assoc., Inc.); solvent, acetonitrile-water azotrope; temperature, room temperature; flow rate, 1.5 ml/min; detector, Shodex RI model SE-11 differential refractive index monitor. The sample solution was filtered through a Sartorius membrane filter TM-80 (pore size, 0.45 µ) before injection.

**Gas liquid chromatography (GLC):** GLC analysis was carried out with a Shimazu model GC-6A gas chromatograph equipped with a FID detector. The following conditions were used for the analysis of TMS-derivatives: column, glass column packed with 5% OV-1 (Uniorp 60/80 µ 3 mm×2 m); column temperature, programmed from 200 °C to 310 °C at a rate of 3 °C/min; injection and detector temperature, 300 °C; carrier gas, N₂ (35 ml/min).

**Gas chromatography-mass spectrometry (GC-MS):** A Hitachi model RMU-6MG GC-MS spectrometer equipped with a glass column packed with 5% OV-1 on Chromosorb NAW DMCS (80/100 µ, 3 mm×1 m) was used. Operating parameters were as follows: column temperature, programmed from 220 °C to 310 °C at a rate of 3 °C/min; carrier gas, He, initial rate of 0.33 kg/cm²; ionizing potential, 20 eV; ion source temperature, 260 °C.

**Thin-layer chromatography (TLC):** TLC was carried out using a solvent system acetonitrile-water (85:15 by volume) with triple ascending. The spots were visualized with diphenylamine-aniline-phosphoric acid reagent⁴, urea-phosphoric acid reagent⁴ or naphthoresorcinol-phosphoric acid reagent⁷.

**Paper chromatography (PC):** Toyo Roshi No. 50 filter paper, n-butanol-pyridine-water (6:4:3 by volume) as solvent, and triple ascending technique were used. Diphenylamine-aniline-phosphoric acid reagent⁵ was used as spray reagent.

**Trimethylsilyl (TMS) derivative:** Preparation of TMS-derivative of sugars was performed by the method of SWEELY et al.⁸.

**Lead tetraacetate (LTA) oxidation:** Eight ml of glacial acetic acid containing 1 mg of LTA was added to each 2 ml solution of the saccharides (each 300 µg) to be tested in slightly moist glacial acetic acid. The reaction mixture was immediately transferred into a spectrophotometer cell. At appropriate time intervals the optical density at 260 nm was measured. The amount of LTA consumed by each sugar was calculated from the standard curve obtained in the range of 20~100 µg of LTA in glacial acetic acid.

**Results and Discussion**

**Sugar composition of sample sugar solution**

The result of HPLC of the sample sugar solution saccharides (Fig. 1, designated as Peak 1 to 7 in the increasing order of elution volume). Peaks 1 and 2 were of fructose and glucose, respectively, and the others were assumed to be of oligosaccharides. Then the sample sugar solution was subjected to CCC for the separation of the sugars showing Peaks 3 to 7. The elution curve obtained is shown in Fig. 2. After evaporation of each fraction, the sugars contained in each fraction was

![Fig. 1 High performance liquid chromatogram of sample sugar solution](image-url)
Fig. 2 Carbon-celite-column chromatographic elution pattern of sample sugar solution

detected by HPLC. The fractions Nos. 6-28 contained fructose and glucose; the fractions Nos. 31-48, one saccharide (Peak 5) referred to as saccharide A; the fractions Nos. 49-94, saccharide A, palatinose (Peak 4), one saccharide (Peak 6) referred to as saccharide B and sucrose (Peak 3); the fractions Nos. 95-122, sucrose; and the fractions Nos. 123-185, one saccharide (Peak 7) referred to as saccharide C.

The contents of these sugars in the sample sugar solution were determined by measurement of each peak area obtained in HPLC, viz., fructose, 15.9%; glucose, 9.7%; sucrose, 21.8%; saccharide A, 16.6%; palatinose, 21.8%; saccharide B, 5.2%; and saccharide C, 8.2%.

Characterization of saccharide A

The fractions Nos. 30-50 containing only saccharide A were collected and ultrafiltered through Amicon PM-10 membrane to remove any cloudy substances. Saccharide A was obtained as powder after drying in vacuo. The yield was 2.0 g. Attempt to crystallize saccharide A or its acetate has failed. The chromatographic results demonstrated that the powder consisted of a single substance. Anal. Found: C 41.98, H 6.62%; Calcd. for C12H22O11: C 42.10, H 6.48%; [α]D = +57.1 (c = 0.5, H2O).

From the results of HPLC and CCC, the sugar was inferred to be a disaccharide. The color reactions on TLC plate with the spray reagents used suggested the presence of ketose moiety in the molecule (Table 1). Acid hydrolysis (with N H2SO4, at 100°C, for 2h) of saccharide A gave glucose and fructose in a ratio of 1 to 0.81. Since A reduced FEHLING’s solution but gave negligible WILLSTÄTTER-SCHUDEL reaction, the reducing end assumed to be fructose moiety. The IR spectrum of saccharide A showed that the saccharide had α-glucosidic linkage (922, 828, 772 cm⁻¹). It is inferred from these that the saccharide is an α-glucosylfructose.

The result of LTA oxidation is shown in Fig. 3. One mole of saccharide A consumed about 2 moles of LTA. Theoretically, glucosylfructoses which consume 2 moles of LTA per mole are 1-, 5- and 6-glucosylfructose.

Then several chromatographic comparisons

Table 1 Chromatographic comparison of saccharide A with related α-D-glucosylfructoses

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Rv *</th>
<th>Color reaction on TLC</th>
<th>tRv *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLC</td>
<td>PC</td>
<td></td>
</tr>
<tr>
<td>Saccharide A</td>
<td>0.74 0.69</td>
<td>Red Blue Brown</td>
<td>1.50  1.46 1.59</td>
</tr>
<tr>
<td>1-α-D-Glucopyranosyl-β-fructose (1-1)</td>
<td>0.74 0.69</td>
<td>Red Blue Brown</td>
<td>1.50  1.46 1.59</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.00 1.00</td>
<td>Dark brown Blue Purple</td>
<td>1.00  1.00</td>
</tr>
<tr>
<td>Turanose</td>
<td>0.87 1.00</td>
<td>Maroon Blue Red purple</td>
<td>1.16  1.35</td>
</tr>
<tr>
<td>Maltulose</td>
<td>0.81 0.81</td>
<td>Red purple Blue Dark red purple</td>
<td>1.29  1.31</td>
</tr>
<tr>
<td>Palatinose</td>
<td>0.87 0.83</td>
<td>Faint brown Blue Dark red brown</td>
<td>1.18  1.42</td>
</tr>
</tbody>
</table>

* Index of migration or elution relative to sucrose.

** A, Diphenylamine-aniline-H3PO4 reagent; B, Urea-H3PO4 reagent; C, Naphthoresorcinol-H3PO4 reagent.

*** TMS-derivatives

Experimental conditions, see text.
with the related glucosylfructoses were carried out to determine the position where the fructose moiety combined with glucose. The data obtained (Table 1) in TLC and PC of saccharide A were identical with those of authentic 1-α-D-glucopyranosyl-D-fructose. The properties of A differed from those of leucrose. HPLC of saccharide A and the authentic one gave the same retention time (Table 1). GLC of TMS-derivative of saccharide A gave two peaks which were probably ascribed to α- and β-form of fructose moiety, and their retention times were the same as those of the authentic one.

By the way, the ring form of fructose moiety of 1-α-D-glucopyranosyl-D-fructose from Dr. SCHWECK has not been reported yet. According to KAMERLING and VLIEGENTHART, furanose ring gave the m/z 217/m/z 204 ratio above 1 while pyranose ring gave the ratio below 1. GC-MS was performed of the TMS-derivatives of saccharide A, and of the related saccharides for comparison. Table 2 shows the presence of furanose unit in saccharide A. Thus the sugar was identified with 1-α-D-glucopyranosyl-β-D-fructofuranose. The sugar had mild sweetness.

With the spray reagents, especially diphenylamine-aniline-phosphoric acid reagent, the authentic α-glucosylfructoses used gave respective unique color reactions on TLC plate although leucrose (1→5) which was not available could not be examined. It seems that the combination of the TLC and GC-MS technique used here is a very useful mean for identification of α-glucosylfructoses.

Identification of saccharide B

Saccharide B could not be obtained as single one. The Fractions Nos. 60–80 containing saccharide B were collected, evaporated to syrup and rechromatographed on a small column (5 cm × 25 cm) of carbon–celite using 2.5% ethanol as eluate. A fraction (2.5 l) containing saccharide B as major component was evaporated, yielding about 10 mg of syrup. The syrup was contaminated with a small amount of palatinose. Acid hydrolysis (with N H2SO4, at 100°C, for 1 h) gave only glucose. Under this condition, palatinose underwent negligible hydrolysis. Since saccharide B was assumed to be isomaltose from its retention time, the sugar was compared chromatographically with authentic samples. Table 3 shows that saccharide B is identical with isomaltose.

Table 2 Characteristic fragment peaks of 20 eV GC-MS spectra of TMS-saccharide A and related TMS–α–D-glucosyl–D–fructoses

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>m/z</th>
<th>Relative intensity (%)</th>
<th>217/204</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>204</td>
<td>217</td>
<td>271</td>
</tr>
<tr>
<td>Saccharide A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a *</td>
<td>75.8</td>
<td>100</td>
<td>4.4</td>
</tr>
<tr>
<td>b *</td>
<td>90.9</td>
<td>100</td>
<td>5.4</td>
</tr>
<tr>
<td>1-α-D-Glucopyranosyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-D-fructose</td>
<td>a *</td>
<td>82.6</td>
<td>100</td>
</tr>
<tr>
<td>b *</td>
<td>94.3</td>
<td>100</td>
<td>9.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.3</td>
<td>21.5</td>
<td>15.8</td>
</tr>
<tr>
<td>Turanose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.8</td>
<td>49.7</td>
<td>15.4</td>
</tr>
<tr>
<td>Maltulose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>43.1</td>
<td>19.4</td>
<td>15.5</td>
</tr>
<tr>
<td>Palatinose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>89.3</td>
<td>100</td>
<td>15.7</td>
</tr>
</tbody>
</table>

* a, tR = 1.46; b, tR = 1.59; described in Table 1.
Identification of saccharide C

The fractions Nos. 123-180 containing only saccharide C were collected, evaporated under reduced pressure to thick syrup and allowed to stand in a refrigerator. Crystallization occurred, yielding 2.3g of saccharide C. Recrystallization from water gave white needles. Mp 138-140°C. Anal. Found: C 41.48, H 6.47%. Calcd. for C_{13}H_{32}O_{10}·H_{2}O: C 41.37, H 6.51%. [α]_{D} = +103° (c = 0.5, H_{2}O). The acetate was derived from the saccharide with a usual method. Mp 143-145°C. [α]_{D} = 121° (c = 0.25, CHCl_{3}). Anal. Found: C 49.44, H 5.64%; Calcd. for C_{40}H_{54}O_{27}: C 49.67, H 5.59%. Saccharide C did not reduce Fehling's solution. From the results of HPLC and CCC, the saccharide was assumed to be a trisaccharide. Partial hydrolysis (with N/100 HCl, at 100°C, for 15 min) gave glucose and palatinose in a molar ratio of 1:1. Saccharide C was exclusively hydrolyzed with 2N sulfuric acid in a boiling water bath for 2h to give glucose and fructose in a ratio of 2:0.78. The IR spectrum of succharide C suggested that the sugar had α-glucosidic bond. These results showed that saccharide C to be 2α-β-D-glucosyl-palatinose or 6α-β-D-glucosylsucrose. Resistance to hydrolysis of the α-D-(1→2) bond was much lower than that of the α-D-(1→6) bond.

On the other hand, CHIBA et al. have synthesized a trisaccharide which had the same structure as that of the sugar mentioned above by the transglucosylation action of Brewer's yeast α-glucosidase and named it isomelezitose. The properties of this sugar and its acetate, as reported, are in good agreement with those of saccharide C. The sugar should be identified with isomelezitose. Nevertheless, the sugar had the structures of both sucrose and palatinose in its molecule the sugar was tasteless.

The three saccharides identified here, 1-α-D-glucopyranosyl-β-fructofuranose, isomaltose and isomelezitose, consisted of glucose and fructose.

All the newly formed α-glucosidic bonds in these oligosaccharides are produced in such a manner that α-glucosyl residue is transferred to primary hydroxyl group, viz., to C1- or C6-hydroxyl of fructose moiety, or to C4-hydroxyl of glucose moiety. This seems to show the specificity of the enzyme of S. plymuthica.

References

Serratia plymuthica の糖転位作用によりシュクロースから生産されるオリゴ糖類の単離と同定

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(* 神戸大学農学部, ** 三井製糖)

S. plymuthica NCIB 8285 の固定化菌体を シュクロースに作用させ生産するオリゴ糖類について検討を加え
た。試料はパラチノースを除去した反応母液 (Brix 66°)


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