Identification of Neokestose Produced from Sucrose by an Enzyme of *Penicillium oxalicum*

Hideyuki Yasuda, Takeo Shitoh, Toshiyuki Yamano, Yoshio Itoh and Susumu Shimura

Central Laboratory, Lotte Co., Ltd., Numakage, Urawa-shi, Saitama 336, Japan

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Several oligosaccharides prepared from sucrose–enzyme mixtures have been extensively reported. The chemical structures of several oligosaccharides, synthesized from sucrose by an enzyme of *Penicillium oxalicum*, were investigated. In this paper we report that one of these oligosaccharides was identified as O-β-D-fructofuranosyl-(2→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside, namely neokestose, which is present in Liliaceae and Amaryllidaceae plants.

To isolate the oligosaccharides, the reaction solution was treated with Amberlite IR-120B and IRA-45, followed by chromatography on a column of charcoal-celite (1:1) with successive solutions with H2O, 5% EtOH and 10% EtOH. The fractions eluted with 10% EtOH, containing mainly an oligosaccharide, which is abbreviated herein as "S-A", and small amounts of other oligosaccharides, were collected, concentrated in vacuo at 40°C and then lyophilized. The white powder thus obtained was chromatographed on a column of Sephadex G-15 with H2O to afford purified S-A, which did not show any reducing power with the method of Fehling. After S-A had been completely hydrolyzed with 0.1 N HCl at 100°C for 1 hr, reducing sugars were quantitatively determined by the method of Somogyi and Nelson. Glucose was determined with a commercial Glucostat reagent (Worthington Biochemical Corp.). The ratio of fructose to glucose was determined by comparison of the areas under the peaks obtained on gas-liquid chromatography of the oximated and trimethylsilylated products by the usual methods. Gas-chromatographic analyses were carried out with a Hitachi Model 073 Gas-chromatograph equipped with a glass column (3 mm x 2 m) packed with 3% Silicon SE-30 on Chromosorb W (column temp.: 180~250°C/5°C/min). The flow rate of carrier nitrogen gas was 40 ml/min.

The degree of polymerization of S-A was calculated to be 3 from i) the relative *Rf*-value of S-A (0.72, *Rf* of sucrose = 1) on thin layer chromatography (Silica gel 60, Merck) with the solvent system of *n*-BuOH–MeOH–H2O = 5:4:1, and ii) the molar ratios of reducing sugars to glucose (3.06), and fructose to glucose (2.02), in acid-hydrolyzates of S-A. Thus, S-A was found to be a non-reducing trisaccharide consisting of glucose (1 mol) and fructose (2 mol).

S-A was methylated by the method of Hakomori, and then the permethylated S-A was methanolyzed with 3% methanolic hydrochloric acid at 100°C for 30 min. The reaction mixture was treated with Amberlite IR-120B and IRA-410 to remove HCl and then evaporated in *vacuo* to yield methanolyzates. The methanolyzates were dissolved in a small amount of MeOH and then subjected to gas-chromatography with a Shimadzu Model 4CM Gas-chromatograph equipped with a glass column (3 mm x 1 m) packed with 15% butane-1,4-diol succinate polyester on Chromosorb W AW DMCS (column temp.: 170°C). The flow rate of carrier nitrogen gas was 40 ml/min. The relative retention times of the methyl glycosides found in the methanolyzates are shown in Table I. The methanolyzate from S-A gave four peaks corresponding to methyl-1,3,4,6-tetra-O-methyl-β-D-fructoside (relative retention times, 0.82 and 1.27) and methyl-2,3,4-tri-O-methyl-β-D-glucoside (2.56 and 3.67). Also, it was found that S-A possessed the β-fructofuranosyl configuration because it was hydrolyzed by yeast β-fructofuranosidase (Sigma VII) to β-fructose and glucose. The structure of S-A was determined to be 6-O-β-D-fructofuranosyl sucrose by gas-chromatographic analyses of methanolyzates of S-A and enzymatic hydrolysis of S-A.

In addition to the above methods, S-A was also analyzed by mass spectrometry and 13C-NMR spectrometry. The trimethylsilyl derivative of S-A was prepared with anhydrous pyridine, hexamethyl-disilazane.
Table II. $^{13}$C-NMR Chemical Shifts$^a$ of S-A Measured in Deuterium Oxide

<table>
<thead>
<tr>
<th>Assignment$^b$</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>65.21</td>
</tr>
<tr>
<td>C-1'</td>
<td>65.21</td>
</tr>
<tr>
<td>C-2</td>
<td>106.58</td>
</tr>
<tr>
<td>C-2'</td>
<td>106.51</td>
</tr>
<tr>
<td>C-3</td>
<td>84.18</td>
</tr>
<tr>
<td>C-3'</td>
<td>83.98</td>
</tr>
<tr>
<td>C-4</td>
<td>79.94</td>
</tr>
<tr>
<td>C-4'</td>
<td>79.47</td>
</tr>
<tr>
<td>C-5</td>
<td>77.47</td>
</tr>
<tr>
<td>C-5'</td>
<td>76.99</td>
</tr>
<tr>
<td>C-6</td>
<td>63.36</td>
</tr>
<tr>
<td>C-6'</td>
<td>63.36</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>94.86</td>
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<td>C-2</td>
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<td>C-5</td>
<td>74.40</td>
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<tr>
<td>C-6</td>
<td>64.60</td>
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</table>

$^a$ In ppm.

$^b$ Primed numbers denote that a $\alpha$-fructofuranosyl unit is attached to sucrose.

and trimethylchlorosilane by the usual method. The GC-MS (EI) spectra were recorded with a Hitachi M-80A at 70eV connected to a Hewlett Packard Model 5790 Gas-chromatograph: column, glass (3 mm x 30 cm); column packing, Diasolid ZT (column temp.: 100~360°C/10°C/min); and flow rate of carrier helium gas, 30 ml/min. The mass spectrum of TMS-S-A showed ions at m/z (rel. int.): 217 (100), 231 (8), 244 (7), 272 (13), 319 (6), 361 (60), 435 (18), 451 (21), 671 (3) and 813 (2), which proved to be identical with in the case of TMS-neokestose isolated from onion bulbs.

$^{13}$C-NMR spectra were recorded with a JEOL JNM-FX 90Q, with complete proton-decoupling. Chemical shifts were measured, relative to external tetramethylsilane. The chemical shift data for S-A on $^{13}$C-NMR are shown in Table II. The general assignment of resonances of S-A in D$_2$O was made on comparison of the chemical shifts with the data for 1-kestose isolated from onion bulbs. The chemical shifts at C-1, C-5 and C-6 of the $\delta$-d-glucopyranosyl group were considerably different from those of 1-kestose. The resonances at $\delta$ 65.21 and $\delta$ 63.36 were assigned to C-1, C-1' and C-6, C-6' of $\alpha$-fructosyl groups, which were assumed to be in the same magnetic environments, respectively. From the above $^{13}$C-NMR results, S-A was assumed to be neokestose and the spectrum of S-A was found to be identical with that of neokestose isolated from onion bulbs.

On the basis of all the experimental results mentioned above, it seemed most reasonable to conclude that S-A is neokestose, that is, $O$-$\beta$-$D$-Fruc-$\beta$(2→6)-$O$-$\alpha$-$D$-Glc-$\alpha$(1→2)-$\beta$-$D$-Fruf.

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REFERENCES

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