Polysaccharide Produced by the Genus *Pullularia*

II. Trans-α-Glucosidation by Acetone Cells of *Pullularia*

SEINOSUKE UEDA and HARUYOSHI KONO

Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University, Fukuoka, Japan

Received for publication 25 May 1965

**Abstract**

UEDA, SEINOSUKE (Kyushu University, Fukuoka, Japan), and HARUYOSHI KONO. Polysaccharide produced by the genus *Pullularia*. II. Trans-α-glucosidation by acetone cells of *Pullularia*. Appl. Microbiol. 13:882-885. 1965.—Acetone cells of *Pullularia* sp. were incubated with maltose, and the saccharides produced were fractionated on a charcoal column. The fractions were subjected to paper-chromatographic analysis and enzymatic assay. By these methods, the saccharides have been shown to include glucose, maltose, maltotriose, panose, and the lower members of glucose polymers containing the 1,4- and 1,6-α-glucosidic linkages, but neither isomaltose nor dextrantrirose. The use of glucose oxidase results in the formation of the higher members of glucose polymer.

Bender, Lehmann, and Wallenfels (1959) reported that a strain of *Pullularia*, when incubated in a glucose or sucrose medium with aeration, synthesized a dextran-type polysaccharide, "Pullulan." More recently, we isolated a strain of *Pullularia* which was capable of utilizing maltose effectively for polysaccharide production, in contrast to Bender's strain. (Ueda et al., 1963).

The ability of maltase preparations from a variety of biological sources to catalyze the synthesis of oligosaccharides from maltose has been reported by many workers (Saroja, Venkataraman, and Giri, 1955; Barker and Carrington, 1953; Faizur and French, 1952; Giri et al., 1955). On the other hand, Monod and Torriani (1950) prepared a cell-free enzyme from a special strain of *Escherichia coli* which converts maltose into a starchlike polysaccharide by use of glucose oxidase.

In continuation of our studies on polysaccharide production by *Pullularia*, we now describe an investigation of the metabolism of maltose by acetone cells of *Pullularia*.

**Materials and Methods**

**Chromatography.** Paper chromatography of free sugars was carried out as previously described with n-butanol-pyridine-water (8:4:3, v/v) as solvent (Ueda et al., 1963). A column containing a 1:1 (w/w) mixture of activated charcoal (Takeda Pharmaceutical Co. Ltd.) and Celite (Hyflo Super-Cel; Johns-Manville, New York, N.Y.) was prepared by the general method of Whistler and Durso (1950). After glucose and oligosaccharides were adsorbed on charcoal, sugars were eluted with an increasing concentration of aqueous ethyl alcohol solution, and fractions of 700 ml were collected from the column (30 by 3 cm).

**Estimation of degree of polymerization.** The eluted solutions from the charcoal column were concentrated to 50 ml in vacuo, and the reducing power of samples was estimated with the micro Bertrand method. The reducing power was calculated as equivalents of glucose. Samples also were hydrolyzed with sulfuric acid (2 N, equal volume) at 100 °C for 3 hr, neutralized with sodium hydroxide (phenolphthalein as indicator), and diluted; the glucose content was then determined. From the ratio of the total reducing value to the direct reducing value, the degree of polymerization was calculated.

**Determination of glucose, maltose, and oligosaccharide.** These sugars were estimated separately by the method of Stark and Somogyi (1942).

**Partial acid hydrolysis.** The sugar [1% (w/v) solution, 1 ml] and sulfuric acid (0.5 N, 0.6 ml) were heated at 100 °C for 1 hr, neutralized, concentrated, and analyzed by paper chromatography.

**Preparation of acetone cells.** As previously reported (Ueda et al., 1963), *Pullularia* sp. S-1 was grown by shaking at 24 °C for 48 hr in 100 ml of a synthetic medium containing 5% sucrose, 0.5% K$_2$HPO$_4$, 0.1% NaCl, 0.02% MgSO$_4$·7H$_2$O, 0.06% (NH$_4$)$_2$SO$_4$, and 0.04% yeast extract (as nitrogen). The contents of seven such flasks were combined, and the cells were collected by centrifugation. The cells were washed twice with 250 ml of 0.1 N NaCl and were then dispersed in 100 ml of 0.1 N NaCl. The cell suspension was poured into the cold acetone (1 liter) at 0 °C and was centrifuged at 650 X g for 2 min. The precipitated acetone cells were washed by cold acetone twice and by ether once, and were dried over CaCl$_2$ in vacuo. A 7-g amount of yellow powder was obtained as acetone-dried cells.

**Results**

**Preparation and isolation of oligosaccharides.** A digest was prepared containing: recrystallized
maltose, 3 g; 0.2 M acetate buffer (pH 6.0), 5 ml; acetone, 1 g; distilled water, 25 ml; and toluene. After incubation at 40°C for 24, 48, 72, and 96 hr, the digest (0.1 ml) was withdrawn and heated to inactivate the enzyme, and was diluted to 50 ml with distilled water and centrifuged. The supernatant solution was subjected to determination of glucose, maltose, and oligosaccharides by the method of Stark and Somogyi (1942). Time course of the production of oligosaccharides is shown in Fig. 1.

To facilitate separation of the oligosaccharides, the remaining portion of 96-hr digest was concentrated and fractionated on a charcoal-Celite column (30 by 3 cm) by the method of Whistler and Durso (1950). The glucose and maltose were eluted with water and 5% (v/v) aqueous ethyl alcohol, respectively. The oligosaccharides were then eluted with 8% ethyl alcohol and with increasing concentrations of ethyl alcohol. Fractions (700 ml) were collected, concentrated, and examined by paper chromatography. None of the fractions became acid on concentration (Table 1).

The oligosaccharides will be referred to as M₀, M₁, M₂, etc., in order of elution from the column. Fraction III was rechromatographed on a second column (30 by 1.5 cm), yielding pure M₂ (60 mg). Fraction V also was applied to a third column (30 by 1.5 cm). Elution with 12 and 18% aqueous ethyl alcohol gave chromatographically pure M₃ (trace) and M₄ (34 mg), respectively.

A control digest containing maltose (3 g) and 0.03 M acetate buffer (pH 6.0, 30 ml) without acetone cells was incubated at 40°C for a similar period. After heating, it was concentrated and applied to a charcoal-Celite column (30 by 3 cm) and was developed with increasing concentrations of aqueous ethyl alcohol. No sugar other than maltose could be detected. The observed synthesis of oligosaccharides in the main digest is not, therefore, due to microbial contamination of the buffer and sugar solution. Further, the purity of the maltose used as substrate was confirmed.

Preliminary characterization of the oligosaccharides. The purified oligosaccharides, which were homogeneous by paper chromatography, were reducing sugars which contained only glucose as the constituent sugar. The results in Table 2 indicate that M₂ and M₃ are trisaccharides and that M₄ is tetrasaccharide.

The four main oligosaccharides were partially hydrolyzed with acid, and the products were tentatively identified by paper chromatography (Table 2). Further evidence of the structures of M₁, M₃, and M₄ was obtained by enzymatic assay (Table 2).

Characterization of M₂ as panose. M₂ had [α]D

+147° in water (c, 1.0). Pazur and French (1952) reported [α]D + 150°. M₂ had the same R₀ on the paper chromatogram as an authentic sample of panose, and was not attacked by β-amylase, but M₃ was completely hydrolyzed to glucose by amylglucosidase. (R₀ values are relative to D-glucose.) The data reported in Table 2 and the enzymatic studies identified M₂ as panose.

Characterization of M₃ as maltotriose. M₃ had [α]D + 162° in water (c, 1.0), compared with [α]D + 160° reported by Whelan, Bailey, and Roberts (1953) for maltotriose. M₃ was slowly hydrolyzed by bacterial α-amylase and by β-amylase, giving glucose and maltose, as shown by paper chromatography and reducing power measurements. The data reported in Table 1 and 2 and the enzymatic studies identified M₃ as maltotriose.
TABLE 2. Identification of sugars

<table>
<thead>
<tr>
<th>Determination</th>
<th>Designation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₁</td>
<td>M₂</td>
<td>M₃</td>
<td>M₄</td>
<td>M₅</td>
<td></td>
</tr>
<tr>
<td>α-Amylase</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Glucose</td>
<td>Maltose</td>
<td></td>
</tr>
<tr>
<td>β-Amylase</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Glucose</td>
<td>Maltose</td>
<td></td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>—</td>
<td>Glucose</td>
<td>Glucose</td>
<td>Glucose</td>
<td>Maltose</td>
<td></td>
</tr>
<tr>
<td>Partial hydrolysis</td>
<td>—</td>
<td>Glucose</td>
<td>Maltose</td>
<td>Glucose</td>
<td>Maltotriose</td>
<td></td>
</tr>
<tr>
<td>(α)ᵩ</td>
<td>+52.5°</td>
<td>+140°</td>
<td>+147°</td>
<td>+162°</td>
<td>+175°</td>
<td></td>
</tr>
<tr>
<td>Suggested name</td>
<td>Glucose</td>
<td>Maltose</td>
<td>Panose</td>
<td>Maltotriose</td>
<td>Malto-pentaose</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 2.** Paper chromatogram showing the formation of oligosaccharides from maltose by transglucosidation with and without added glucose oxidase. *I*, with glucose oxidase; *II*, without glucose oxidase. *G*, glucose; *M*, maltose; *T*, maltotriose; *P*, panose. Sake contains glucose, nigerose, isomaltose, dextrantrirose and other dextrins.

Characterization of M₄ as maltotetraose. M₄ had [α]ᵩ + 175° in water (c, 0.7), compared with [α]ᵩ + 176° and +177° reported by Whistler and Hickson (1955) and Whelan et al. (1953), respectively, for maltotetraose. M₄ was rapidly hydrolyzed by bacterial α-amylase and β-amylase from sweet potato to give maltose. The enzymatic studies and the data in Tables 1 and 2 characterize M₄ as maltotetraose.

**Effect of glucose oxidase on the degree of polymerization of oligosaccharides.** The average degree of polymerization of a mixture of glucose polymers resulting from the new enzyme—a kind of transglucosidase—action is normally quite small, but products of a little higher molecular weight are produced when the glucose formed in the synthesis is continuously removed by means of glucose oxidation with glucose oxidase.
TABLE 3. Effect of glucose oxidase on degree of polymerization of oligosaccharide produced*

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Degree of polymerization</th>
<th>Without added glucose oxidase</th>
<th>With added glucose oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>5.2</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>6.1</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>6.2</td>
<td>9.0</td>
<td></td>
</tr>
</tbody>
</table>

*Glucose oxidase (50 mg, Nagase Industries Co., Ltd.) was added to the reaction mixture. Gluconic acid produced was frequently neutralized to pH 6.0 by sodium bicarbonate, and the reaction mixture was kept at pH 6.0.

Chromatograms I and II in Fig. 2 and Table 3 show the comparison of the presence and absence of glucose oxidase.

Discussion

The present study shows that acetone cells of *Pullularia* sp. S-1 catalyze the synthesis of oligosaccharides from maltose when the concentration of maltose is sufficiently high. The main products of enzymatic action, which have been characterized by chemical and enzymatic methods, are panose, maltotriose, and maltotetrose. In addition, small amounts of other oligosaccharides are also formed.

Although the yields of the oligosaccharides recorded in Table 1 are not quantitative, they are a measure of the relative proportions of the various sugars present in the synthetic reaction. They show that the main transfer of α-glucosyl residues from maltose is to the hydroxyl group at C₄ or C₆ of the nonreducing end group in maltose or maltotriose. The hydroxyl group at C₆ of the nonreducing end group in glucose or isomaltose may not be carbohydrate acceptor for α-glucosyl radicals transferred from maltose, because isomaltose and dextranotriose do not appear in the reaction mixture.

These observations differ from many reports that mold enzymes readily transfer glucosyl radicals to the primary alcoholic group in glucosaccharides, including glucose and isomaltose, whereas the animal and bacterial enzyme systems can transfer only to the hydroxyl group at C₄ of the nonreducing end group of the acceptor (Pazur and French, 1952; Barker and Bourne, 1952; Giri et al., 1955).

Glucose oxidase increased the degree of polymerization of oligosaccharides which were produced by acetone cells of *Pullularia*; thus, this transglucosidase activity may act in polysaccharide production by *Pullularia*.

Acknowledgments

We express our appreciation to Motoyoshi Hongo, Kyushu University, for his thoughtful suggestions throughout this work. Some amylases and glucose oxidase preparations were placed at our disposal by generous colleagues to whom sincere thanks are hereby tendered: crystalline α-amylase from bacteria and glucose oxidase preparation by A. Komaki (Nagase Industries Co., Ltd., Amagasaki, Japan); crystalline β-amylase from sweet potato by I. Igaue (Niigata University, Niigata, Japan); crystalline amyloglucosidase from *Aspergillus awamori* by S. Hayashida (Kyushu University, Fukuoka, Japan).

Literature Cited


