

Maltooligosaccharides have been used as reagents for research and clinical diagnostics, and have potential uses as nutrients in health care. However, the cost of production of pure maltooligosaccharide is surprisingly high, because of the difficulties of producing it in a pure state. Recently we reported a novel and efficient method that uses moranoline3,4 for the production of high purity maltose.5 The principle of this novel method can be applied to the preparation of another high purity maltooligosaccharide with the use of xeno-type maltooligosaccharide-forming amylases instead of β-amylase.

This paper describes the production of high purity maltotetraose (G₄) by our novel method.

Figure 1 is a diagram of the principle of the method for G₄ production, comprising a transglycosylation reaction by cyclodextrin glycosyltransferase [CGT-ase, 1,4-α-D-Glucan 4-α-(1,4-α-D-glucano)-transferase, cyclic endoglucanase, EC 2.4.1.19] and a hydrolysis reaction by maltotetraose-forming enzyme [G₄ amylase, 1,4-α-D-glucan maltotetrahydrolase, EC 3.2.1.60]. Glycosylmoranolines can be obtained by treating the reaction mixture of the transglycosylation reaction with a strong cation exchange resin. The components of the hydrolysate after a hydrolysis reaction are M₄, G₄M₃, G₄M₂, G₄M, G₄, M, G₄, G₄M₃, G₄M₂, G₄M, and G₄M are basic compounds and G₄ is a neutral compound, therefore, G₄ can be obtained easily from the reaction mixture by passage through a strong cation exchange resin in a column.

G₄ amylase was prepared as follows.4 Pseudomonas stutzeri IFO-3773 was cultivated on a rotary shaker for 1 day at 30°C in 500-ml flasks containing 100 ml of a medium consisting of 1% soluble starch, 1% Bacto casitone, 0.5% yeast extract, 0.28% KH₂PO₄, and 0.1% K₂HPO₄ (pH 7.0). This seed culture (200 ml) was transferred to a 30-liter jar fermentor (B. E. Marubishi Co., Ltd., MSJ-U-15) containing 15 liters of the same medium. The fermentation was done at 30°C at an agitation speed of 500 rpm and an aeration rate of 1vvm. The culture broth was centrifuged and the supernatant was precipitated with ammonium sulfate (30% saturation). This precipitate was collected by centrifugation and dissolved in a minimal volume of 0.01 M Tris-HCl buffer (pH 8.0) containing 5 mM CaCl₂, and dialyzed against the same buffer at 4°C. The dialyzed thus obtained was put on a Sephadex G-100 column (1.0 × 30 cm), which was used for affinity chromatography of G₄ amylase, as reported by Dellweg.5 The column was washed with the above dialyzing buffer and eluted with the same buffer containing 3% G₄.

The results of the purification procedures are shown in Table 1. The activity of G₄ amylase was measured by the method of Sakano et al.6 Reducing sugars in the reaction mixture were measured by the 3,5-dinitrosalicylic acid method,7 and protein was measured by a dye-binding assay (Bio-Rad protein assay) with bovine serum albumin as the standard. One unit of G₄ amylase is defined as the amount of enzyme which liberates 1 μmol of G₄ per min under these conditions.

Glycosylmoranolines (50 g) prepared by the method described previously5,6 were dissolved in 258 ml of 25 mM Tris-HCl buffer (pH 6.0) containing 5 mM CaCl₂. The G₄ amylase (25 ml, 30.4 units/ml) was added, and the hydrolysate reaction was done at 30°C for 46 h. The reaction mixture was ultrafiltered with a Pellicon cassette (MW 10,000; Millipore Ltd.), and the filtrate was adsorbed on 1.5 liters of Dowex 50W × 2 (H⁺). The resin was washed with distilled water (3.0 liters). The non-adsorbed fraction and washed solution were combined (4.1 liters), electrodialyzed with the micro acetyler G3 (Asahi Chemical Industry Co., Ltd.) with an ion-exchange membrane of Acrilox cartridge AC-110—400, and then lyophilized. High purity G₄ (11.6 g) was obtained. The HPLC is shown in Fig. 2. The substances adsorbed on the resin were eluted with 2.5 liters of 1 N NH₄OH and dried in vacuo. The recovered fraction containing moranolone was thus obtained as a powder (28.9 g). The HPLC is shown in Fig. 3. HPLC was measured as follows: 10 μl of an appropriate concentration of the sample solution was placed on a column (Asahipak NHZP-5, 4.2 mm i.d. × 25 cm) in an L-6000 HPLC apparatus (Hitachi Ltd.), developed with acetonitrile-water (68.32 v/v) at a flow rate of 1 ml/min in the column oven at 40°C, and monitored with a 655 A-30 RI detector (Hitachi Ltd.). The chromatogram was analyzed with a D-2000 chromato-integrator (Hitachi Ltd.).

The purity of G₄ obtained by this method was 98.7% with traces of contaminating maltose, maltotriose, and maltopentaose as shown in Fig. 2.

As shown in Fig. 3, the recovered fraction containing moranolone from the G₄ amylase reaction mixture consisted of moranolone, glucosylmoranolone, maltosylmoranolone, and maltotriosylmoranolone. The maltotetraose obtained was 1.1% as shown in Table 1.

**Table 1** Purification of Pseudomonas stutzeri G₄-Amylase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg/ml)</th>
<th>Activity (units/ml)</th>
<th>Specific activity (units/mg)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture sup.</td>
<td>0.061</td>
<td>0.992</td>
<td>16.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium</td>
<td>1.7</td>
<td>165.8</td>
<td>97.5</td>
<td>6.0</td>
</tr>
<tr>
<td>sulfate ppt</td>
<td>0.152</td>
<td>30.4</td>
<td>200</td>
<td>12.3</td>
</tr>
</tbody>
</table>

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Footnote:

1 This work was presented at the Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry, 1992, Tokyo.
tetraosylmoranoline was a trace. This means that G4 amylase hydrolyzes a α-1,4-glycosidic bond between glucose and terminal moranoline of the maltotetraosylmoranoline as shown in Fig. 1.

Nakakuki et al. reported that the purity of G4 produced from amylase by the action of G4 amylase was about 75% of the total sugar content at the late stage of the enzyme reaction.\(^{10}\) G4 amylase from Pseudomonas stutzeri is an exo-type amylase which hydrolyzes the accumulated G4 at a late stage of the reaction,\(^{11}\) resulting in the difficulty of manufacturing high purity G4. The courses of G4 production from glycosylmoranolines and G4 decomposition by G4 amylase from Pseudomonas stutzeri IFO-3773 were investigated under experimental conditions similar to those described in the preparation of G4 (G4: 3 w/v%, glycosylmoranolines; 15 w/v%, G4 amylase; 33.4 units/ml). The results are shown in Figs. 4 and 5.

In the case of G4 production from glycosylmoranolines (Fig. 4), G4 produced from glycosylmoranolines remained even after 100 h. In the case of G4 decomposition (Fig. 5), half of the G4 was hydrolyzed in 40 h incubation, and glucose, maltose, and maltotriose accumulated.

The IC\(_{50}\) the concentration needed for 50% inhibition, was measured by the activities of G4 amylase in the presence of inhibitors. The IC\(_{50}\) of the recovered fraction containing moranoline from a hydrolysis reaction by G4 amylase was 2300 μg/ml. All components of the recovered fraction containing moranoline were isolated, and their inhibitory activities against G4 amylase were investigated. Those of all the components except maltotriosylmoranoline were very weak (more than 5000 μg/ml), while maltotriosylmoranoline showed a potent inhibitory activity against G4 amylase (IC\(_{50}\): 30 μg/ml). These results show that the decomposition of G4 accumulated during a hydrolysis reaction was inhibited mainly by maltotriosylmoranoline.

Since the fractionation of the G4 syrup through a column of ion exchange resin is necessary to obtain a high purity G4 by the conventional method, this new method will be useful for the production of a high purity G4.

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References

High Purity Maltotetraose