Note

Purification and Properties of Inulin Fructotransferase (Depolymerizing) from Arthrobacter globiformis C11-1

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Inulin is a linear β2,1-linked fructose polymer terminated by a sucrose residue. In studies of inulin decomposing enzymes, hydrolases from yeast11 and mold21 were mainly reported. Uchiyama et al.31 found a new type of inulin decomposing enzyme. The enzyme converted inulin into di-D-fructofuranosyl 1,2:2,3 dianhydride (DFA III) and a small amount of oligosaccharides. The enzyme was named inulin fructotransferase (depolymerizing) (EC 2.4.1.93).

One of the authors isolated a bacterium which produced this kind of enzyme with high activity. The microorganism was identified as Arthrobacter globiformis C11-1. The details of the identification will be presented in another article.

The microorganism was cultivated aerobically at 30°C for 24 hr in a 500 ml shaking flask containing 100 ml of a medium composed of 0.4% Na2HPO4·12H2O, 0.1% KH2PO4, 0.05% MgSO4·7H2O, 0.1% NH4NO3, 0.001% FeSO4·7H2O, 0.003% CaCl2·2H2O, 0.05% yeast extract (Difco) and 0.3% inulin, pH 7.0. The culture was inoculated into a 5 l Erlenmeyer flask containing 1 l of the same medium. The microorganism was cultivated aerobi-

ically at 30°C for 10 hr. The cells were removed by cen-
trifugation (8,000 × g, 20 min) and the supernatant was used as the crude enzyme solution.

For measurement of the enzyme activity, the enzyme solution (0.05 ml), 0.1 M citrate buffer, pH 5.0 (0.2 ml), deionized water (2.25 ml) and 1% inulin (2.5 ml) were mixed. The mixture was incubated at 30°C for 30 min. The enzyme reaction was stopped by heating at 100°C for 7 min. The DFA III produced was determined by HPLC (column: Zorbax ODS, 4.6 mm × 25 cm; liquid phase: water; detector: ERC 7510 RI detector (Erma Optical Works Co., Ltd.)). One unit of the enzyme was defined as the amount of the enzyme which produced 1.0 μmol of DFA III per min at 30°C and pH 5.0. Protein concentrations were determined by the method of Lowry et al.41

The crude enzyme was dialyzed against 0.02 M Tris-HCl buffer, pH 8.0. The enzyme solution was applied on a column of DEAE-Toyopearl 650M (1st, 2.5 cm × 20 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0. The enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer. The fractions showing the enzyme activity were pooled and then dialyzed against 0.02 M Tris-HCl buffer, pH 8.0. The enzyme solution was applied on a column of DEAE-Toyopearl 650M (2nd, 1.5 cm × 14 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0. The enzyme was eluted with a linear gradient of 0 to 0.2 M NaCl in the same buffer. The fractions showing the enzyme activity were pooled and then dialyzed against 0.005 M phosphate buffer, pH 7.0. The enzyme solution was applied on a column of DEAE-Toyopearl 650M (3rd, 1.5 cm × 10 cm) equilibrated with 0.005 M phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient of 0 to 0.2 M NaCl in the same buffer. The purification procedure is shown in Table I. The fraction obtained on DEAE-Toyopearl chromatography (3rd) was used as the purified inulin fructotransferase (depolymerizing) solution. It gave a single band by Coomassie Brilliant Blue R250 staining after polyacrylamide gel electrophoresis51. The molecular weight of the enzyme was estimated in two ways. By SDS-PAGE,61 it was estimated to be 45,000. By gel filtration (TSK-gel G3000SW), it was estimated to be 50,000. From these results, the enzyme was considered to be a monomeric enzyme. The isoelectric point of the enzyme was found to be pH 5.1 by isoelectric focusing on a polyacrylamide gel.71

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>138</td>
<td>1900</td>
<td>13.8</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>5.50</td>
<td>1430</td>
<td>260</td>
<td>75.2</td>
</tr>
<tr>
<td>(lst)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>2.64</td>
<td>762</td>
<td>289</td>
<td>40.1</td>
</tr>
<tr>
<td>(2nd)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>0.94</td>
<td>276</td>
<td>294</td>
<td>14.5</td>
</tr>
<tr>
<td>(3rd)</td>
<td></td>
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</tr>
</tbody>
</table>

Purification of the enzyme was started from 1.921 of culture broth.
The enzyme activity was measured in the pH range of 3.0 to 8.0 in 0.004 M citrate buffer or 0.004 M phosphate buffer at 30°C. The enzyme showed maximal activity at pH 5.0. The optimal pH of the enzyme reaction was lower than that of Uchiyama's enzyme (pH 6.0). The effect of temperature on the enzyme activity was assayed at pH 5.0. The enzyme showed maximal activity at 55°C. The optimal temperature was slightly higher than that of Uchiyama's enzyme (50°C). To investigate its pH stability, the enzyme was incubated at various pHs at 25°C for 24 hr. The enzyme was stable in the pH range of 4.0 to 9.0. The thermostability of the enzyme after 20 min incubation was assayed at pH 5.0. The enzyme was stable up to 75°C, but was rapidly inactivated over 80°C.

For preparation of the main reaction product, the enzyme solution (1.0 ml, 12 units), 0.1 M citrate buffer, pH 5.0 (1.0 ml) and 5% inulin (20 ml) were mixed. The mixture was incubated at 37°C for 6 hr. The enzyme reaction was stopped by heating at 100°C for 7 min. Paper chromatography was carried out with Toyo filter paper No. 50, with a solvent system of n-butyl alcohol–pyridine–water (3:2:2, by volume) at 60°C. The spots of the products were revealed with resorcinol–HCl reagent. Figure 1 shows the paper chromatogram of the reaction products from inulin. Spots of DFA III and oligosaccharides were detected. To remove oligosaccharides by alcohol fermentation, baker's yeast (1.0 g wet weight; Sankyo Co., Ltd.) was added to the reaction mixture. The suspension was incubated at 37°C for 8 hr. The yeast cells were removed by centrifugation (9,000 x g, 15 min). The supernatant was filtered with a Millipore GV filter (Millipore Co., Ltd.) and then passed through an Amberlite MB-3 column (7 mm x 5 cm). The solution was condensed with a rotary evaporator. The condensate was applied on a column of Toyopearl HW40S (2.5 cm x 32 cm) equilibrated with water. Elution was performed with water. The fraction containing the main reaction product was collected. Crystallization of the main reaction product was performed by freeze drying (ca. 0.5 g). The material was not fermented by baker's yeast and was a non-reducing sugar; mp 164°C (DFA III authentic9); 162°C, [x]D +135° (c = 1.0, H2O) (DFA III authentic9): +136°). The properties of the material agreed very closely with those of DFA III.

REFERENCES