Note

Purification, Crystallization, and Properties of the Extracellular Levansucrase from Zymomonas mobilis

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Levansucrase (sucrose: 2,6:fructan 6:fructosyl-transferase; EC 2.4.1.10) is an enzyme that catalyzes the following reaction:

Sucrose + Acceptor=Glucose + Fructosyl-acceptor

Various molecules may act as an acceptor, but levans, hexoses and water are highly reactive. Most studies on levansucrases have been done in Bacillus subtilis. Chamber et al.1,2 have investigated the mechanism of the transfructosylation process. Petit-Glatron et al.3,4 have characterized a macromolecular form of levansucrase. LeBrun et al.5 have presented the tertiary structure of the enzyme. The gene sacB, which codes for the exoenzyme levansucrase of B. subtilis, has been cloned by Gay et al.6,7 and its DNA has been sequenced by Steimmet al.8,9

On the other hand, Zymomonas mobilis is known as a facultative anaerobic bacterium capable of producing ethanol from glucose efficiently. However, ethanol yields from sucrose are always low on account of the production of levans and sorbitol by by-products, as indicated by Viikari.10 The first report of the existence of levansucrase in Z. mobilis comes from Dawes et al.9 who described the formation of levansacrose during sucrose hydrolysis. Lyness and Doelle11 have partially purified levansucrase from cell-free extracts of Z. mobilis NCIB 11199 (ATCC29191). In a preliminary experiment, we found that there were three kinds of sucrose-hydrolyzing enzyme, E1 (invertase), E2 levansucrase, and E3 (invertase), in Z. mobilis subsp. mobilis IFO13756 (Z6), and that approximately 80% of the total activities of E2 and E3 was released into the supernatant of a washing suspension of cells, and almost of the E1 activity was associated with the cell cytoplasm, indicating that both E2 and E3 are extracellular enzymes and E1 is an intracellular enzyme. Recently, we have cloned the gene encoding for the intracellular invertase (E1) and sequenced its DNA.12 This note describes the purification and characterization of the E2 enzyme.

The levansucrase activity was measured by estimating reducing sugars formed from sucrose, as follows. A reaction mixture, containing 10% sucrose and enzyme solution in a total 1.0 ml of 0.1 m acetate buffer, pH 5.0, was incubated at 30°C. The reducing sugars produced was measured by the method of Somogyi-Nelson.13,14 One unit was defined as the amount of enzyme that produces reducing sugars equivalent to 1 µmol of glucose per min. The glucose and fructose liberated from sucrose were also measured separately by using an F-kit for Glucose-Fructose (Boehringer Mannheim, GmbH). Z. mobilis Z6C, a spontaneous mutant of the Z6 strain capable of producing an extracellular levansucrase (E2) and extracellular invertase (E3), constitutively, was used as the source of the enzyme. Z6C strain was cultured statically in T medium containing 10% glucose, 1% yeast extract, 1% KH₂PO₄, 0.1% (NH₄)₂SO₄, 0.05% MgSO₄·7H₂O, and 1% sucrose, pH 5.6, at 30°C for 24 hr. Z6C cells harvested from 6 liters of the culture medium were suspended in 600 ml of 20 mm potassium phosphate buffer, pH 7.0, incubated at 30°C for 15 min with shaking to release the enzyme from the cell surface, centrifuged, and the supernatant was pooled. After washing twice the supernatant was collected, as a crude enzyme solution. The solution was fractionated with (NH₄)₂SO₄ precipitation between 0 to 50% saturation. The resultant precipitate was dissolved in the above buffer and dialyzed. The enzyme solution was put onto a column of DEAE-Tosyoparl 650M (4.0×10 cm) equilibrated with the same buffer. The enzyme was eluted with 500 ml of a linear gradient

![Fig. 1. Photomicrograph of Crystals of Levansucrase E2](https://example.com/image1)

The bar represents 20 µm.

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Fig. 2. HPLC Chromatograms of Products from Sucrose.

(A) The reaction products obtained by incubation at 15°C for 24 hr, indicating product I, II, sucrose, glucose, and fructose. Ethanol is an internal standard.

(B) Product I isolated, and its hydrolyzate.

(C) Product II isolated, and its hydrolyzate.

HPLC conditions: column, Shodex Ionpack KS-802 (at 80°C); effluent, water (0.5 ml/min; monitor, Shodex R1 Model SE-11). Symbols: Suc, sucrose; Glc, glucose; Fru, fructose; EtOH, ethanol.
of NaCl (0 to 0.5 M). Fractions showing the enzyme activity were pooled, concentrated, and put onto a column of Toyopearl 55W (2.0 x 90 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.0, supplemented with 0.2 M NaCl, and chromatographed with the same buffer. The enzyme eluted was precipitated by (NH₄)₂SO₄ with 20% saturation and dissolved in a small volume of the phosphate buffer. To this solution (about 4 mg protein/ml) solid (NH₄)₂SO₄ was gradually added until a faint turbidity appeared. On standing at 4°C needle-shaped crystals were formed (Fig. 1). Finally, 11 mg of E2 enzyme was isolated (7.8-fold purification with 4% activity yield). The homogeneity of the enzyme preparation was proved by native PAGE and SDS-PAGE.

The molecular weight of the enzyme was estimated to be 94,000 by Sephadex G-200 gel filtration, and 56,000, by SDS-PAGE. The pI was pH 2.6 by Ampholine-PAGE. The first 19 N-terminal amino acids of the enzyme identified by Edman degradation were Met-Leu-Asn-Lys-Ala-Oly-Ile-Ala-Glu-Pro-Ser-Leu-Trp-Thr-Arg-Ala-Asp-Ala-Met-

The optimum pH for the enzyme activity was around pH 5.0. The temperature optimum was at 50°C. The enzyme was stable at pH 3.5-6.0 in 24 hr at 30°C, and stable up to 37°C in a 30-min incubation at pH 6.0.

The purified enzyme catalyzed the liberation of reducing sugars from substrates having 2-β-D-fructofuranose residues on a terminal such as 150 mM sucrose, 150 mM raffinose, 2% levan from Aerobacter leguanicum (Sigma Chemical Co., St. Louis, Mo.), and 2% inulin, with a relative activity of 100:104:1:0.01. However, it could not cleave maltose or melezitose. The apparent Km for sucrose was 122 mM. The enzyme was inhibited by some metal ions, such as Ag⁺, Hg²⁺, and Cu²⁺, and mercuric acid. However, no inhibition was observed with EDTA, suggesting that metals are not essential for the reaction.

The reaction products from sucrose were analyzed by HPLC, using a Shodex Ionpack KS-802 column (Showa Denko Co., Ltd., Tokyo). As shown in Fig. 2, a highly polymerized saccharide (peak I in Fig. 2A) and oligosaccharides (peak II in Fig. 2A) were found by HPLC, besides the residual sucrose, and liberated glucose and fructose. To identify these products, the polysaccharide and the oligosaccharide were isolated by preparative HPLC, and hydrolyzed with 0.1 M HCl at 100°C for 30 min. Fructose was found in the hydrolysate of peak I by HPLC analysis (Fig. 2B), indicating highly polymerized fructan. The hydrolysate of peak II gave glucose and fructose in a molar ratio of 1:2 (Fig. 2C), indicating a fructosyl sucrose. The results suggested that the E2 enzyme appeared to be levansucrase that catalyzes transfructosylation of fructose residues liberated from sucrose to form fructan and fructosyl sucrose.

The effects of temperature on the transfructosylation were investigated to measure the amount of glucose and fructose produced from 10% sucrose at pH 5.0. A ratio of the amount of difference between glucose and fructose liberated to the amount of glucose liberated was defined as the transfructosylation activity. Besides, poly- and oligo-saccharides produced were also identified by HPLC (Table I). It was found that at lower temperatures such as 5°C and 15°C, the transfructosylation was preferentially catalyzed rather than the hydrolysis of sucrose, but inversely at higher temperature such as 30°C and 40°C the hydrolysis was preferentially catalyzed.

The effects of pH on transfructosylation were investigated (Table II). When the enzyme was incubated with 10% sucrose at 15°C and at acidic pH such as pH 4 to 6, the transfructosylation preferentially occurred, and oligo- and polysaccharides were produced. However, when incubated at pH 7 to 8, the hydrolysis of sucrose was enhanced, and oligosaccharides were produced rather than polysaccharides as transfructosyla-

### Table I. Effects of Temperature on the Transfructosylation Reaction from Sucrose by Levansucrase E2 in Z. mobilis Z6C

The reaction mixture contained 10% sucrose, 100 mM acetate buffer, pH 5.0, and 3.7 unit/ml of E2 enzyme in a total volume of 1.0 ml. Incubation was done at the indicated temperature for 24 hr.

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Sucrose consumed (%)</th>
<th>Glucose liberated (%)</th>
<th>Fructose liberated (%)</th>
<th>G-FaG (%)</th>
<th>Oligosaccharideb formed (%)</th>
<th>Polysaccharidesc formed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td>8.6</td>
<td>4.3</td>
<td>0.5</td>
<td>88.0</td>
<td>0.4</td>
<td>1.4</td>
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<tr>
<td>15°C</td>
<td>9.6</td>
<td>4.8</td>
<td>1.0</td>
<td>78.9</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>30°C</td>
<td>9.3</td>
<td>4.9</td>
<td>3.5</td>
<td>28.5</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>40°C</td>
<td>9.5</td>
<td>5.0</td>
<td>4.1</td>
<td>17.3</td>
<td>0.2</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a Transfructosylating activity: the ratio of the amount of difference between glucose (G) and fructose (F) liberated to the amount of glucose liberated.

b Converted into raffinose.

c Converted into the total sugar content measured by the phenol-sulfuric acid method using fructose as the standard.

### Table II. Effects of pH on the Transfructosylation Reaction from Sucrose by Levansucrase E2 in Z. mobilis Z6C

The reaction condition was same as described in Table I, except the 100 mM buffer indicated and incubation temperature at 15°C.

<table>
<thead>
<tr>
<th>Buffer and pH</th>
<th>Sucrose consumed (%)</th>
<th>Glucose liberated (%)</th>
<th>Fructose liberated (%)</th>
<th>G-FaG (%)</th>
<th>Oligosaccharideb formed (%)</th>
<th>Polysaccharidesc formed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McIlvain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>5.1</td>
<td>2.0</td>
<td>0.8</td>
<td>60</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>9.6</td>
<td>4.3</td>
<td>0.8</td>
<td>81</td>
<td>0.4</td>
<td>2.6</td>
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<tr>
<td>Acetate</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9.7</td>
<td>4.5</td>
<td>1.0</td>
<td>78</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>5</td>
<td>9.6</td>
<td>4.8</td>
<td>1.0</td>
<td>84</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>6</td>
<td>9.6</td>
<td>4.2</td>
<td>0.9</td>
<td>76</td>
<td>0.7</td>
<td>2.5</td>
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<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
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<td>6</td>
<td>9.7</td>
<td>4.1</td>
<td>0.9</td>
<td>78</td>
<td>0.6</td>
<td>2.1</td>
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<td>7</td>
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<td>1.8</td>
<td>1.1</td>
<td>39</td>
<td>1.9</td>
<td>&lt;0.1</td>
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<tr>
<td>8</td>
<td>3.8</td>
<td>1.0</td>
<td>0.6</td>
<td>40</td>
<td>1.2</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

a Transfructosylating activity.
b Converted into raffinose.
c Converted into the total sugar content as fructose.
tion products.

The \textit{Z. mobilis} enzyme transferred a fructose unit from sucrose to another sucrose molecule to form a high molecular weight of fructan as well as oligosaccharides. Tanaka \textit{et al.}\textsuperscript{1,2} have reported that synthesis of levan by \textit{B. subtilis} levansucrase occurs far more effectively at lower temperatures than at room temperature. Similar effects were observed on transfructosylation by the \textit{Z. mobilis} enzyme. Furthermore, we found that the pH of reaction affected the transfructosylation and polymerization of fructan. To clarify the molecular alteration of levansucrase between transfructosylation and hydrolysis of sucrose, we have cloned and sequenced the gene of the E2 enzyme. We are now trying to construct chimeric enzymes by site-directed mutagenesis.

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\textbf{References}


