Purification and Some Properties of a New Levanase from *Bacillus* sp. No. 71

Hiromi Murakami, Takashi Kuramoto,* Kenji Mizutani,* Hirofumi Nakano, and Sumio Kitahata

Osaka Municipal Technical Research Institute, 6-50 Morinomiya 1-cho, Jyotoku, Osaka 536, Japan

* Maruzen Kasei Co., Ltd., 14703-10 Mukaihigashi-cho, Onomichi-shi 722, Japan

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A levanase from *Bacillus* sp. was purified to a homogeneous state. The enzyme had a molecular weight of 135,000 and an isoelectric point of pH 4.7. The enzyme was most active at pH 6.0 and 40°C, stable from pH 6.0 to 10.0 for 20 hr of incubation at 4°C and up to 30°C for 30 min of incubation at pH 6.0. The enzyme activity was inhibited by Ag⁺, Hg²⁺, Cu²⁺, Fe²⁺, Pb²⁺, and p-chloromercuribenzoic acid. The enzyme hydrolyzed levan and phlein endowise to produce levanheptaose as a main product. The limit of hydrolysis of levan and phlein were 71% and 96%, respectively.

Levanases (2,6-β-d-fructan fructanohydrolase, EC 3.2.1.65) are enzymes that hydrolyze the β-2,6-linked main chain of levan.¹ There have been several reports about levanases and β-fructofuranosidasases (EC 3.2.1.26), which hydrolyze levan to produce d-fructose (FrU)²⁻⁴ or various levanoligosaccharides.⁵⁻⁶ A levanase from *Azotobacter chroococcus*⁶ produced a mixture of levanoligosaccharides the degrees of polymerization (DP) of which were more than 3. A levanase from *Arthrobacter sp.*⁵ acted randomly on levan to give a series of levanoligosaccharides with an average DP of 2.5. Those enzymes, however, have not been obtained in purified states. Furthermore, they did not accumulate levanoligosaccharides of particular DPs.

In our previous paper,⁶ we reported the isolation of several levanase-producing microorganisms. We also described the purification and characterization of a levanase from a *Streptomyces* sp., which hydrolyzed levan to produce levanbiose (F2) predominantly.

This paper deals with the purification and some properties of a levanase from a *Bacillus* sp. The new enzyme produced mainly levanheptaose (F7) from levan and phlein.

**Materials and Methods**

**Cultures.** The bacterial strain No. 71, which was one of those isolated from soil as levan-degrading enzyme producers, was used throughout this investigation. The microbial properties of strain No. 71 was investigated by the method and procedures of Smith *et al.* (Table I). Based on the results and Berge’s Manual,⁹ it was identified as a *Bacillus* sp. The medium for enzyme production was composed of 0.1% levan, 0.9% sucrose, 0.3% corn steep liquor, 1.0% polypropylene, 0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, and 0.02% MnCl₂·4H₂O, and the pH was adjusted to 7.0. Cultivation was done in 500-ml shaking flasks containing 50 ml of the medium at 28°C for 24 hr on a reciprocating shaker.

**Substrates.** Levan was synthesized from sucrose by immobilized levanaseurase from *Bacillus subtilis* var. *saccharolyticus* by the method described by Iizuka.⁹ Immobilized levanaseurase was incubated with 20% sucrose solution at 4°C for 7 days. After removal of the immobilized levanaseurase, the reaction mixture was dialyzed against running water to remove the remaining sucrose. The dialyzed enzyme solution was lyophilized. The DP of the enzyme was estimated to be about 360, which was calculated from the ratio of total sugars to reducing sugars. Phlein was a gift from Dr. M. Iizuka of Osaka City University. Phlein had been extracted from the bulbs of timothy (*Phleum pratense*), and purified by DEAE-cellulose column chromatography. The structure of phlein was reported to be without side chains on the basis of methylation analysis. Difructose anhydride IV (DFA IV) and F2 were prepared from levan by fructose-transferase from *Pseudomonas fluorescens* No. 949 (³) and levanase from *Streptomyces* sp. No. 7-3, respectively. Inulin was purchased from Isuzu Seiyaku Ltd.

**Enzyme assay.** Levanase activity was measured as follows: reaction mixture containing 0.125 ml of 2% levan in 20 mm phosphate buffer (pH 6.0) and 0.125 ml of the enzyme solution in the same buffer was incubated at 40°C for 10 min. The reaction was stopped by adding 0.25 ml of Somogyi’s reagent. The reducing sugars were measured by Somogyi and Nelson’s methods,¹² using FrU as a standard.

One unit of the enzyme activity was defined as the amount of enzyme liberating 1 µmol of reducing sugars per minute under the above conditions.

**Protein measurement.** Protein was measured by the absorbance at 280 nm with a Shimadzu spectrophotometer.

| Table 1. Microbial Characteristics of Strain No. 71 |
|-----------------------------------|-----|
| Cell shape | rod |
| Spore forming | + |
| Gram stain | + |
| Motile citium | - |
| Pigment production | - |
| Production of catalase | + |
| Production of oxidase | - |
| Production of lecinthinase | - |
| Nitrate reduction | - |
| Hydrolysis of gelatin | - |
| Hydrolysis of starch | + |
| Growth temperature | 10–30°C |
| Growth pH | 5.0–9.5 |
| Concentration of NaCl | 0–5% |
| Utilization of glucose | fructose |
| Utilization of sucrose | galactose |
| Utilization of xylose | inositol |
| Utilization of lactose | cellobiose |
| Utilization of mannitol | trehalose |
| Utilization of sorbitol | raffinose |

**Abbreviations:** FrU, fructose; F2, levanbiose; F3, levaniotriose; F4, levantetramose; F5, levampanotase; F7, levanheptaose; Fn, levanoligosaccharides; HPLC, High performance liquid chromatography; TLC, Thin layer chromatography; PCMB, p-chloromercuribenzoic acid; DFA IV, di-β-d-fructofuranose 2,6;2,6-dianhydride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FAB-MS, fast atom bombardment mass spectrum; DP, degree of polymerization.
Levanase from *Bacillus* sp.

Purification of the levanase.

**Step 1. Salting out.** The supernatant of a culture broth was used as the crude enzyme solution. Solid ammonium sulfate was added to the solution up to 40% saturation. After this was left overnight at 15°C, the precipitates that formed were removed by centrifugation. To the supernatant solution, ammonium sulfate was added to 70% saturation. After this was left overnight at 15°C, the precipitates that formed were collected, dissolved in 0.1 M phosphate buffer (pH 7.0), and dialyzed against the same buffer.

**Step 2. DEAE-Toyopearl column chromatography.** The enzyme solution was passed through a column of DEAE-Toyopearl 650 M (5 × 40 cm) previously equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 30% saturation of ammonium sulfate. The column was washed with the same buffer and the adsorbed enzyme was eluted with a linear gradient of ammonium sulfate from 30 to 0% saturation in the same buffer. The active fractions were combined and concentrated to 2.0 ml by polyethylene glycol.

**Step 4. Ultrogel AcA 44 gel filtration.** The concentrated solution was put on a column of Ultrogel AcA 44 (1.5 × 130 cm) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl. Elution was done with the same buffer. The active fractions were combined and dialyzed against 10 mM phosphate buffer (pH 8.0).

**Step 5. DEAE-Toyopearl column chromatography.** The dialyzed solution was put on a DEAE-Toyopearl column (2.5 × 80 cm) equilibrated with 10 mM phosphate buffer (pH 8.0). The column was washed with the same buffer and the adsorbed enzyme was eluted with a linear gradient of NaCl from 0 to 1.0 M in the same buffer. The active fractions were collected and used as the purified enzyme preparation.

**Fast atom bombardment mass spectrum.** The fast atom bombardment mass spectrum (FAB-MS) was taken on a JEOL JMS-SX-102 mass spectrometer.

**Electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) was done by the method of Davis using 7.5% gel and the pH 8.3 buffer system. SDS-PAGE was done by the method of Weber and Osborn using macroglublin (reduced form, molecular weight 170,000), phosphorylase b (97,400), glutamate dehydrogenase (55,400), lactate dehydrogenase (36,500), and trypsin inhibitor (20,100) as standard proteins. Isoelectric focusing was done by the method of Vesterberg and Svensson using carrier ampholites (pH 3.5–10.5) for 43 hr at 400 V and at 4°C.

**Thin layer chromatography.** Thin layer chromatography (TLC) of the reaction products was done on Kieselgel 60 plates (Merck Co., Ltd.), using chloroform–methanol–acetic acid–water (15:12:1.3:3, v/v) as a solvent. Carbohydrates were detected by heating the plates at 110–120°C after spraying with sulfuric acid–methanol.

**High performance liquid chromatography.** High performance liquid chromatography (HPLC) was done under the following three conditions. Condition A: column, Asahipak NH2P-50 (4.6 × 250 mm); solvent, 60:40 (v/v) acetonitrile–water; flow rate, 1.0 ml/min. Condition B: column, TSKgel G3000SW (7.5 mm × 60 cm × 2); solvent, water; flow rate, 1.0 ml/min. Condition C: column, TSKgel G2000PW (7.5 mm × 60 cm); solvent, water; flow rate, 0.5 ml/min. Detection was done by a Tosoh RI-8012 refractometer. Pullulan (Shodex standard P-82, Showa Denko Co.) and Fru were used as molecular weight standards. In analyzing under condition C, sample solutions were previously filtered through an ultrafiltration membrane (cut-off molecular weight, 10,000; UFP1 LGC24, Millipore Ltd.).

**Estimation of *K*ₚ.** The *K*ₚ of the levanase for levan and phlein was measured as follows. The enzyme (0.2 U/ml) was incubated with levan in various concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.25, 1.5, 2.5, 3.0, 3.2%) at 40°C, pH 6.0 for 10 min. The amount of reducing sugar produced was measured by Somogyi and Nelson’s method. The *K*ₚ was calculated from a Hanes–Woelf plot.

**Limits of hydrolysis of levan and phlein.**

Levan and phlein were estimated from the reducing power and the compositions of the reaction products. The levanase (0.9 U/ml) was incubated with 1% levan and phlein in 10 mM acetate buffer, pH 6.0, at 40°C. After 1, 3, 5, 10, 20, 30, 40, and 60 min, 2 hr, 5 hr, and 20 hr, samples of reaction mixtures were taken for the measurement of reducing power and HPLC analysis with condition A. The degradation ratios were calculated as follows:

\[
DR = 100 \times (RP - \sum_{i=1}^{n} DP_i C_i)/[S_0]
\]

for DR, degradation ratio; RP, reducing power of reaction mixture (mg/ml, as fructose); DPᵢ, degree of polymerization of products (i=1–9); Cᵢ, composition of products on the basis of HPLC analysis; [S₀], initial concentration of substrate (mg/ml).

**Results**

**Purification of the levanase.**

The results of the purification are summarized in Table II. The enzyme was purified about 55-fold over the starting culture broth, and had the specific activity of 21.7 U/A₂₈₀₅ₐₕ. The preparation showed a single protein band in PAGE and SDS-PAGE (Fig. 1).

**Molecular weight and isoelectric point**

The molecular weight of the levanase was estimated to be 135,000 by SDS-PAGE. The isoelectric point was measured to be pH 4.7 by isoelectric focusing method.

**Effects of pH on activity and stability**

The enzyme was most active at around pH 6.0, when the enzyme activity was measured under the standard assay conditions (Fig. 2A). The enzyme was stored at 4°C for 20 hr in buffers of various pHs, and the residual activity was measured at pH 6.0. The enzyme was stable at alkaline pHs from pH 6.0.

**Effects of temperature on activity and stability**

The enzyme activity was done at pH 6.0 and various temperatures for 10 min. The optimum temperature of the enzyme was 40°C (Fig. 2B). After 30 min of incubation at pH 6.0, the enzyme was stable at temperatures below 30°C.

**Table II. Purification Procedure of Levanase from *Bacillus* sp. No. 71**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (A₂₈₀₅ₐₕ)</th>
<th>Total activity (×10⁵U)</th>
<th>Specific activity (U/A₂₈₀₅ₐₕ)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture liquor</td>
<td>429</td>
<td>170</td>
<td>0.396</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (40–70%)</td>
<td>289</td>
<td>139</td>
<td>0.483</td>
<td>81.8</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650 M</td>
<td>106</td>
<td>133</td>
<td>1.25</td>
<td>78.2</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650 M</td>
<td>5.71</td>
<td>103</td>
<td>18.0</td>
<td>60.6</td>
</tr>
<tr>
<td>Ultrogel AcA 44</td>
<td>3.85</td>
<td>73.9</td>
<td>19.2</td>
<td>43.5</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650 M</td>
<td>2.22</td>
<td>48.3</td>
<td>21.7</td>
<td>28.4</td>
</tr>
</tbody>
</table>

**Fig. 1. Polyacrylamide Gel Electrophoresis of the Levanase.**

The purified enzyme protein, 24 µg (A, disc PAGE), and 19 µg (B, SDS-PAGE) were electrophoresed.
Fig. 2. Effects of pH (A) and Temperature (B) on Activity and Stability of the Levanase.

(A) Effects on activity (--; O, △--): The reaction mixtures consisting of levan solution (0.26 U, 250 μl), 300 μl of 2% levan, and 250 μl of 0.1 M McIlvain buffer (pH 2.2—8.2), 0.5 M borate-Na₂CO₃ buffer (pH 8.2—10.7), were incubated at 40°C for 10 min. Effects on stability (--; ○, △--): The enzyme solution (0.19 U, 200 μl) was kept in 10 mM McIlvain buffer (pH 3.0—7.8), and 10 mM borate-Na₂CO₃ buffer (pH 8.2—10.4) at 4°C for 20 hr. Then these solutions were diluted with 4 ml of 1 M acetate buffer (pH 6.0) and the residual activity was assayed.

(B) Effects on activity (--; ○--): The enzyme reaction was done at various temperatures for 10 min in 10 mM acetate buffer (pH 6.0). Effects on stability (--; ●--): The enzyme was incubated at various temperatures for 30 min in 0.9 M acetate buffer (pH 6.0), and the remaining activity was assayed under the standard conditions.

Table III. Effects of Various Chemicals and Metal Ions on the Activity of Levanase

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration of reagent (mm)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10.0</td>
<td>101</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0</td>
<td>100</td>
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<tr>
<td>MgCl₂</td>
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<td>10.0</td>
<td>93</td>
</tr>
<tr>
<td>SrCl₂</td>
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<td>84</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>10.0</td>
<td>78</td>
</tr>
<tr>
<td>CoCl₂</td>
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<td>95</td>
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<tr>
<td>NiCl₂</td>
<td>1.0</td>
<td>95</td>
</tr>
<tr>
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<tr>
<td>ZnCl₂</td>
<td>1.0</td>
<td>65</td>
</tr>
<tr>
<td>PbCl₂</td>
<td>1.0</td>
<td>14</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>1.0</td>
<td>5.2</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>EDTA-2Na</td>
<td>1.0</td>
<td>102</td>
</tr>
<tr>
<td>DTT</td>
<td>1.0</td>
<td>104</td>
</tr>
<tr>
<td>GSH</td>
<td>1.0</td>
<td>99</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.1</td>
<td>12</td>
</tr>
</tbody>
</table>

Effects of chemicals

The enzyme was incubated with various chemicals in 0.1 M acetate buffer (pH 6.0) at 30°C for 30 min, and the remaining activity was measured under the standard assay conditions. The enzyme activity was strongly inhibited by AgNO₃, HgCl₂, FeCl₃, PbCl₂, and PCMB. BaCl₂, CaCl₂, CoCl₂, MgCl₂, NaCl, NiCl₂, ethylenediaminetetraacetic acid disodium salt, dithiothreitol, and reduced form glutathione had no effects on the enzyme activity. The results are shown in Table III.

Fig. 3. High Performance Liquid Chromatograms of the Reaction Products from Levan and Phlein by the Levanase.

The levan (1.0 U/ml) were incubated with 1% levan (A) and phlein (B) in 10 mM of acetate buffer (pH 6.0) at 40°C. At intervals, 100 μl of the reaction mixtures were removed. Ten μl of 0.1 N HCl was added to the reaction mixture (100 μl) to stop the enzyme action, followed by addition of 0.1 N NaOH (10 μl) and ethanol (200 μl). The supernatant was used for HPLC analysis with condition A. The arrows show the elution points of each levanoligosaccharide in the partial acid hydrolyzates of phlein.
Fig. 4. Time Course of Hydrolysis of Levan and Phlein by the Levanase.

The levanase (0.9 U/ml) was incubated with 1% levan (○) and phlein (□) in 10 mM acetate buffer, pH 6.0, at 40°C. After 1, 3, 5, 10, 20, 30, 40, and 60 min, 2 hr, 5 hr, and 20 hr, samples of reaction mixtures were taken for the measurement of reducing power and HPLC analysis with condition A. The degradation ratios were estimated from the reducing power and the compositions of reaction mixtures. (see Materials and Methods)

**Substrate specificity**

The substrate specificity of the enzyme was studied by incubating the enzyme (4.4 U/ml) with phlein, inulin, sucrose, levanbiose, and DFA IV (1.0% of each) at 40°C for 20 hr followed by the measurement of reducing sugars and by HPLC analysis of the products. The three disaccharides (sucrose, levanbiose, and DFA IV) and inulin gave no products. Considerable increases of reducing sugars were detected during the reaction with levan and phlein, the latter of which was reported to be a β-2,6-fructan without side chains.10,11) The $K_m$ for levan was 0.37%.

**Hydrolysis products from levan and phlein**

Figure 3 shows the HPLC chromatograms of the enzymatic hydrolyzates of levan and phlein. From an early stage of reaction, the enzyme hydrolyzed levan and phlein to produce a saccharide A predominantly, the DP of which was estimated to be 7 from the retention time. The saccharide A was isolated from the hydrolyzates of levan by carbon column chromatography and HPLC, and its structure was examined.

The negative FAB-MS showed an [M-H]- ion peak at m/z 1151, indicating the molecular weight of the saccharide A to be 1152. Saccharide A (5 mg/ml) was completely hydrolyzed by F2-forming levanase from *Streptomyces* sp.6 (0.9 U/ml) for 5 hr incubation at pH 6.0, 40°C, and gave Fru and F2. Thus, it was confirmed that the main product (saccharide A) was linear F7.

After 20 hr of reaction, small amounts of various Fn and unknown saccharide X were accumulated in addition to F7. Similarly, the enzyme also produced F7 predominantly from phlein in the early stage of reaction. In a final stage of the reaction, small amounts of various Fn (n=2—9) were produced from phlein, but the product that corresponded to saccharide X from levan was not detected.

**Limit of hydrolysis of levan and phlein**

The degradation ratios calculated from the reducing power and the compositions of reaction products are shown in Fig. 4. The limit of hydrolysis of levan and phlein were 71% and 96%, respectively.
Fig. 6. High Performance Liquid Chromatograms of the Reaction Products from Levan by the Levanase.

The levan (0.653 U/ml) was incubated with 1% levan at 40°C. At intervals, 100 μl of the reaction mixture was removed, followed by addition of 0.1 N HCl (10 μl), then 0.1 N NaOH (10 μl). After ultrafiltration with a Millipore filter, the filtrate was used for HPLC analysis with condition C. Reaction time, (1) 3 min; (2) 10 min; (3) 30 min; (4) 60 min; (5), 120 min; (6) 20 hr.

Mode of action

To investigate whether the enzyme hydrolyzes levan exowise or endowise, the reaction products were analyzed by HPLC with a gel-permeation column. When the reaction mixture was directly analyzed by HPLC, a discernible shoulder attributable to the intermediate hydrolyzates with larger molecular weight was detected between the peaks of F7 and that of high molecular weight polysaccharides (Fig. 5). To detect the shoulder arising from the intermediate hydrolyzates more clearly, the reaction mixtures were pre-treated with an ultrafiltration membrane to remove the remaining substrates which were confused with the intermediates, and the filtrates were analyzed by another GPC column. As shown in Fig. 6, not only F7 but also larger molecular weight saccharides were detected even in the early stage of reaction. This result suggested that the mode of action of the levanase was endo-type.

Discussion

With respect to pH, effects of pH, temperature, and chemical reagents, the purified levanase of this paper had similar properties to those of the F2-forming levanase from Streptomyces sp.10 The pH of the levanase was estimated to be 4.7, which was almost the same as the latter enzyme’s (pH 4.7). The levanase was most active at pH 6.0 and 40°C, stable from pH 6.0 to 10.0 and up to 30°C, similarly to F2-forming enzyme (most active at pH 6.5 and 40°C, stable from pH 5.5 to 8.5, up to 40°C). The enzyme activity was inhibited by Ag⁺, Hg²⁺, Ca²⁺, Fe³⁺, P₆⁺, and PCMB. The F2-forming enzyme was also inhibited by Hg²⁺, Ag⁺, P₆⁺, and PCMB. The molecular weight of the levanase (135,000), however, was approximately twice as large as the latter (54,000).

The main product of the enzyme was F7, which did not contain β-2,1-branched points. The final hydrolyzates of levan consisted of 68.1% of F7, 17.5% of other levanoligosaccharides (Fru-F9), and 14.5% of unknown saccharide (X). The final hydrolyzates of phlein, which was reported to be linear β-2,6-fructan, contained 81.3% of F7 and 16.7% of other levanoligosaccharides (F2—F9). Products that corresponded to saccharide X from levan, however, was not detected in the hydrolyzates of phlein. Based on the structural differences in levan and phlein,9,10 together with the retention time of X on HPLC, the unknown saccharide X was considered to be a branched levanheptaose (Fig. 3), although the detailed structure remained to be confirmed.

The limits of hydrolysis was estimated from the amount of reducing sugars liberated and the composition of final reaction products. The limits of hydrolysis of levan and phlein were 71% and 96%, respectively, where F7 in the hydrolyzates amounted to 68.1% (levan) and 81.3% (phlein). In other words, about 48% of levan and 78% of phlein were finally converted into linear F7. No other enzymes except the F2-forming levanase (Streptomyces sp.)9,10 have been known to produce so much of a specific length of levanoligosaccharide. Therefore, the enzymes described in this and our previous paper are useful for the production of linear levanoligosaccharides.

At an early stage of hydrolysis of levan and phlein, only F7 was detected as a low molecular weight product (Fig. 3). The further examination on the reaction products from levan showed that higher molecular weight intermediates were produced, other than F7 (Fig. 5). The molecular weight of the intermediates produced in the early stage of reaction, calculated by GPC analysis were estimated to be 11,000—15,000. This result indicated that the levanase acted on levan endowise. A similar mode of action has been reported in an α-amylose from Bacillus circulans G-6 that hydrolyzed starch endowise to produce linear maltoolhexasose (G6) as a main product.119 Thus, it might not be unusual that the levanase accumulated large amounts of F7, while it acted endo-wise.

Acknowledgement. We wish to thank Dr. M. Iizuka of Osaka City University for his generous gift of immobilized levansucrase and phlein.

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