Cloning, Sequencing, and Characterization of the Intracellular Invertase Gene from Zymomonas mobilis

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The structural gene for the intracellular invertase E1 of Zymomonas mobilis strain Z6C was cloned in a 2.25-kb DNA fragment on pUSH11, and expressed in Escherichia coli HB101. The enzyme produced by the E. coli carrying pUSH11 was purified about 1,122 fold to homogeneity with a yield of 4%. The molecular weight and substrate specificity of the enzyme were identical with those of the intracellular invertase E1 from Z. mobilis. The nucleotides of the cloned DNA were sequenced; they included an open reading frame of 1,536bp, coding for a protein with a molecular weight of 58,728. The N-terminal amino acid sequence predicted was identical with the sequence of the first 20 N-terminal amino acid residues of the protein obtained by Edman degradation. Comparison of the predicted amino acid sequence of E1 protein with those of the four other known β-o-fructofuranosidases from Escherichia coli, Bacillus subtilis, and Saccharomyces cerevisiae indicated a stronger homology in the N-terminal portion than in the C-terminal portion.

Zymomonas mobilis is a Gram-negative bacterium capable of producing almost the theoretical yield of ethanol from glucose, and has higher productivity than the yeasts now used industrially. However, this bacterium can ferment only glucose, fructose, and sucrose. In addition, there is little information on the early metabolism of sucrose, although it has been known that sucrose is hydrolyzed to glucose and fructose by invertase that is produced inducibly,1) and the liberated fructose is converted to sorbitol2,3) and levane4,5) in part. Preliminary investigation suggested that there were three kinds of sucrose-hydrolyzing enzymes in Zymomonas, intracellular invertase (that was named E1), extracellular levansucrase (E2), and extracellular invertase (E3). Therefore, we have attempted to clone genes encoding for these enzymes and characterize them. This paper describes that the E1 gene was cloned from Zymomonas genomic library, and its nucleotide sequence was determined and characterized by comparing with genes encoding for invertase from E. coli,6) B. subtilis,7,8) and S. cerevisiae.9)

Materials and Methods

Plasmids and bacteria used. The bacteria used in this study were E. coli HB101 (F−, hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44, λ−), and Z. mobilis Z6C, a spontaneous mutant of Z. mobilis subsp. mobilis IFO13756 (=ATCC29191) capable of producing an extracellular levansucrase (E2) and invertase (E3), constitutively.1) The plasmids used were pZA22 (Cm−, Te−, 6.7kb), a shuttle vector between Z. mobilis and E. coli,10) and pZL7 (lacZ+, Y+, Cm−, 16.2kb), a lacY+ plasmid that can coexist with pUC118 or pUC119 in E. coli HB101.11)

Cultivation. Z. mobilis was cultivated statically at 30°C in RM medium (2% glucose, 1% yeast extract, and 0.2% KH2PO4, pH 6.0) and E. coli in the LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl,
The protein produced by the cloned El gene was purified from the E. coli HB101 carrying pUSH11 as follows. The transformant cells harvested from 7 l of the culture medium were washed with 20 mM phosphate buffer (pH 7.0), and suspended in 500 ml of the same buffer, then disrupted by sonication. The supernatant solution was fractionated with (NH₄)₂SO₄ precipitation between 30 to 50% saturation. The resulting precipitate was dissolved in the above buffer and dialyzed. The enzyme solution was put onto a DEAE-Toyopearl 650M column (2.3 × 30 cm) equilibrated with the buffer. The enzyme was recovered from unabsorbed fractions, and dialyzed against 20 mM acetate buffer, pH 5.0. The dialyzed solution was put onto a CM-Sephadex C-50 column (1.2 × 30 cm) equilibrated with the dialyzed buffer. After the column was washed thoroughly with the same buffer, the enzyme was eluted with a linear gradient from 0.1 to 1.0 M NaCl. Fractions showing the enzyme activity were pooled and concentrated in a CENTRICUT U-20 (Kurabo Biomedical P/J). The enzyme solution was fractionated with (NH₄)₂SO₄ of 0 to 10% saturation. The resultant precipitate was dissolved in 20 mM acetate buffer, pH 5.0.

The E1 enzyme from Z. mobilis Z6C cells was partially purified as follows. Cells harvested from 7 l of the culture medium were suspended in 700 ml of 20 mM phosphate buffer, pH 7.0, and incubated at 30°C for 10 min with shaking to release cell-bound levansucrase (E2) and invertase (E3) from cells. The washed cells were disrupted by sonication, and centrifuged to remove intact cells and debris. The supernatant solution was put onto a DEAE-Toyopearl 650M column (2.3 × 30 cm) equilibrated with 20 mM phosphate buffer, pH 7.0, and unabsorbed fractions were pooled. The enzyme was precipitated by adding (NH₄)₂SO₄ to 50% saturation, and the resultant precipitate was dissolved in a small volume of 20 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl. The enzyme solution was put onto a Sephadex G-200 column (2.3 × 100 cm) equilibrated with 20 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl, and chromatographed. Fractions having the enzyme activity were pooled and concentrated in a CENTRICUT U-20.

Molecular weight measurement. The molecular weight in a native form was estimated by gel filtration through a Sephadex G-200 column (1.0 × 50 cm) with 20 mM phosphate buffer, pH 7.0 containing 0.2 M NaCl. Cytochrome c, chymotrypsinogen A, ovalbumin, BSA, aldolase, and catalase were used as marker proteins. The molecular weight of the subunit was measured by the method of Laemmli. Marker proteins used were: α-lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, and phosphorylase b.

Analysis of N-terminal amino acid sequence. The E1 enzyme obtained in the final step of purification from E. coli HB101 carrying pUSH11 was blotted onto a polyvinylidene difluoride membrane (Millipore), and degraded sequentially with a protein analyzer, Applied
Biosystems model 4701A, equipped with an on-line HPLC apparatus model 120A.

**DNA sequencing.** The DNA sequence of the gene encoding for the *Zymomonas* E1 enzyme was analyzed by the dideoxy method of Sanger *et al.*[^20] The entire sequence of the insert contained in pUSH11 was analyzed in both directions using an overlapping set of Exonuclease III-generated deletions that had been cloned into derivatives of phage M13.

### Results and Discussion

**Cloning of the gene encoding a sucrose-hydrolyzing enzyme**

Plasmids containing our *BamHI* library of *Z. mobilis* chromosomal DNA in pZA22 were screened for the presence of a sucrose-hydrolyzing enzyme in *E. coli*, and seven positive clones were obtained. One of these clones, which contained a plasmid designated pZSH1, was chosen for further investigation. This plasmid contained a 7.85-kb *BamHI*-fragment inserted into pZA22, as shown in Fig. 1.

To identify the coding region of the enzyme on the 7.85-kb fragment, we did subcloning experiments using pUC118 or pUC119 as a vector, as shown in Fig. 2. First, the 7.85-kb fragment on pZSH1 was inserted into the *BamHI* site that is located in multi-cloning sites of pUC118 to construct pUSH1. A deletion mutant, pUSH3, was constructed by inserting the 5.4-kb *HindIII—BamHI* fragment of pUSH1 into pUC119 with the same transcriptional direction of *lac* promoter. Plasmid pUSH4 was

[^20]: Sanger *et al.*
constructed by inserting the 3.4-kb EcoRV–BamHI fragment of pUSH1 into the EcoRI(filled)–BamHI site of pUC119 with the opposite direction of lac promoter. Plasmid pUSH5 was constructed by inserting the 2.85-kb SacI–BamHI fragment of pUSH1 into pUC118, and the 2.1-kb Clal(filled)–BamHI fragment of pUSH1 inserted into pUC118 at the EcoRI(filled)–BamHI site to generate pUSH6. The deletion mutant pUSH5 retained the sucrose-hydrolyzing activity in E. coli. Next, the 2.85-kb of SacI–BamHI fragment on pUSH5 was further deleted approximately 500 bp from the BamHI site by Exonuclease III to produce pUSH11. The results suggested that the gene encoding for a sucrose-hydrolyzing enzyme and its own promoter were located on the 2.25-kb fragment in pUSH11. As the cell-free extracts of E. coli HB101 carrying pUSH11 showed about 25-fold higher activity than that of E. coli carrying pUSH1, an excess region that inhibited expression of the gene in E. coli seemed to be deleted by Exonuclease III. The restriction map of

Fig. 3. Chromatograms of the Sucrose-hydrolyzing Enzymes from Z. mobilis Z6C and E. coli HB101 Carrying pUSH11 on a DEAE-Toyopearl 650M Column.
A, Z. mobilis Z6C (——); B, E. coli HB101 with pUSH11 (——) or without (—). Cell-free extracts prepared from washed cells of E. coli or Z. mobilis from 100 ml of culture were put on the column (1.0 × 12 cm) equilibrated with 20 mM phosphate buffer, pH 7.0, washed with 10 ml of the same buffer, and then eluted with 50 ml of a linear gradient NaCl from 0 to 0.5 M (--.--.), collecting 1 ml per tube.

Fig. 4. Disc Gel Electrophoresis of the Cloned E1 Enzyme.
(A) Polyacrylamide gel electrophoresis. Purified enzyme was put on a 7.5% gel column and run at pH 4.3 for 40 min at 5 mA per gel. The direction of electrophoresis was from upper (anode) to lower (cathode).
(B) SDS-polyacrylamide gel electrophoresis. Purified enzyme treated with SDS at 100°C for 5 min was put on a 10% gel containing 0.1% SDS, are run at 7 mA per gel for 5 hr. The direction of electrophoresis was from upper (cathode) to lower (anode). Arrows indicate molecular weights (×10⁻³) of marker proteins.

Table 1. Purification of the Sucrose-hydrolyzing Enzyme from E. coli HB101 Carrying pUSH11

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>10,840</td>
<td>22,140</td>
<td>2.0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ ppt (30–50% saturated)</td>
<td>5,487</td>
<td>23,160</td>
<td>4.2</td>
<td>2.1</td>
<td>105</td>
</tr>
<tr>
<td>DEAE-Toyopearl 650M</td>
<td>258</td>
<td>17,750</td>
<td>68.4</td>
<td>33.5</td>
<td>80</td>
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<tr>
<td>CM-Sephadex C-50</td>
<td>15.8</td>
<td>5,080</td>
<td>322</td>
<td>158</td>
<td>23</td>
</tr>
<tr>
<td>1st (NH₄)₂SO₄ ppt</td>
<td>1.58</td>
<td>1,989</td>
<td>1,259</td>
<td>617</td>
<td>9</td>
</tr>
<tr>
<td>2nd (NH₄)₂SO₄ ppt</td>
<td>0.16</td>
<td>801</td>
<td>2,288</td>
<td>1,122</td>
<td>4</td>
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</table>
**Fig. 5.** Sequence and Translation of the *Z. mobilis El* Gene.

The -35 region, -10 region, and Shine-Dalgarno (S.D.) region deduced are underlined.
pUSH11 is shown in Fig. 1. The resultant 2.25-kb fragment in pUSH11 was sequenced.

Characterization of the gene product

A preliminary experiment suggested that Z. mobilis Z6C produced three different sucrose-hydrolyzing enzymes, E1, E2, and E3. To clarify which enzyme was coded for in the 2.25-kb fragment of pUSH11, the cell-free extracts prepared from E. coli HB101 carrying pUSH11 was chromatographed on a DEAE-Toyopearl 650M column. The chromatogram of the cell-free extract from Z6C cells (Fig. 3A) gave three distinct peaks of sucrose-hydrolyzing activity; the first peak was responsible for intracellular E1 enzyme, while the second and third peaks were responsible for extracellular E2 and E3 enzymes, respectively. As most of E2 and E3 enzymes were present in the washing solution, the amount of E2 and E3 enzymes found in this cell-free extract was equivalent to about 10% of the total of both enzymes. From the elution profile shown in Fig. 3B, it was clear that the E1 gene in Z. mobilis Z6C was cloned in the 2.25-kb fragment on pUSH11.

The E1 enzyme was purified from cells of E. coli carrying pUSH11 about 1,122-fold with a yield about 4%, as shown in Table I. The enzyme preparation was shown to be homogeneous by the criteria of polyacrylamide gel electrophoresis, as shown in Fig. 4. The molecular weight of the enzyme was 58,000 by Sephadex G-200 gel filtration, and 57,500 by SDS-polyacrylamide gel electrophoresis. It was a basic protein with an isoelectric point of pH 8.5. The first 20 N-terminal amino acids of the enzyme obtained by Edman degradation were Met-Glu-Ser-Pro-X-Tyr-Lys-Asn-Leu-Ile-Lys-Ala-Glu-Asp-Ala-Gln-Lys-Lys-Ala-Gly-. The enzyme catalyzed the hydrolysis of sucrose to liberate glucose and fructose with an equimolar ratio, and also raffinose to fructose and melibiose. In addition, it catalyzed the hydrolysis of inulin, but not maltose, meletzitose, or levan (data not shown). The relative activities for these substrates were 100 for sucrose, 54 for raffinose, and < 1 for inulin.

These results suggested that the enzyme was a β-D-fructofuranosidase, called invertase. The enzyme showed the maximum activity at pH 6.5 and was stable between pH 5.0 and 7.0. The highest reaction rate was observed at 37°C, and the enzyme was thermolabile. The enzyme activity was inhibited by 1 mM of mercuric compounds such as HgCl2, HgO, PCMB, mercuric acetate, and mersalyl acid.

To find whether E1 of the E. coli transformant was identical with the E1 enzyme of Z. mobilis Z6C or not, the E1 enzyme was partially purified from the cell-free extract of Z6C cells, and compared with the enzyme of the E. coli transformant. The molecular weight of the E1 enzyme was 59,000 by Sephadex G-200 gel filtration. The enzyme hydrolyzed sucrose, raffinose, and inulin with a relative ratio of 100 : 46 : < 1. The molecular weight and the substrate specificity of the cloned enzyme were identical with those of the E1 enzyme, suggesting that the 2.25-kb fragment coded for the intracellular invertase E1 of Z. mobilis Z6C.

Sequence of the intracellular invertase E1 gene

The sequence of the 2.25-kb DNA fragment on pUSH11 containing E1 enzyme was analyzed in both directions, as shown in Fig. 5. A single open reading frame was found starting with an ATG codon at position 371 from the HindIII end and ending with a TAA termination codon at position 1,907, including
Z. mobilis Intracellular Invertase Gene 1389

Fig. 7. Comparison of the Amino Acid Sequence of the Zymomonas Sucrose-hydrolyzing Enzyme El, Sucrose Hydrolase (rafD) from E. coli,6) Sucrase (sacA) from B. subtilis,7) Levanase (sacC) from B. subtilis,8) and Invertase (suc2) from S. cerevisiae.9) Identical residues in the first 278 amino acids are boxed. The arrow indicates a cystein residue in the putative active site.

1,536bp and coding for a protein with the molecular weight calculated to be 58,728. This was almost identical with the molecular weight of the El protein. The first 20 N-terminal amino acid residues predicted from the nucleotide sequence was also identical with those of the El enzyme obtained by Edman degradation.

Although we have not yet confirmed the initiation site of the El gene, we tried to find a candidate for the Shine-Dalgarino (S.D.) sequence and the promoter region, -35 region and -10 region, of the gene. Recently, pyruvate decarboxylase (pdc), alcohol dehydrogenase (adhB), glyceraldehyde-3-phosphate dehydrogenase (gap), phosphoglycerate kinase (pgk), and phosphatase (phoC) of Z. mobilis have been sequenced, and its S.D. sequence and promoter region were implied by pond et al.21) The consensus S.D. sequence was seemed to be *GGAG* 10 to 16 bases upstream from the translational start codon. Then by searching the analogous sequence upstream of the El gene, the sequence, AGGCAG, starting 11 bases upstream from the translational start codon was found, and deduced as the S.D. sequence. The consensus sequence of promoter region was also predicted as A/G****C/AT*G***---10 to 16 bases---TA*T/AG*A/T*T. In the El gene the sequence of AAAAGCCTTGCA---8 bases---TATAGAAAA starting at 120 bases upstream from the start codon seemed to be a promoter region, as shown in Fig. 5. The results of deletion analysis also implied that a promoter that could be recognized by E. coli RNA polymerase was upstream of the El gene. A sequence of TTGTCT---15 bases---TATAAT starting at 55 bases upstream from the start codon that seemed to adhere to the -35 and -10 consensus sequence of E. coli promoter was found.

Comparison of the El gene with other β-D-fructofuranosidase genes

As mentioned above, the El enzyme was an intracellular invertase, β-D-fructofuranosidase. The predicted amino acid sequence of the El gene was compared to that of other four β-D-fructofuranosidases, the intracellular sucrose hydrolase encoded by rafD on the plasmid-borne raf operon from E. coli,6) the intracellular sucrase encoded by sacA,7) extra-
cellular levanase encoded by sacC from Bacillus subtilis,\(^8\) and the extracellular invertase encoded by suc2 from Saccharomyces cerevisiae.\(^9\) The composition of a dot matrix analysis among these enzymes showed, in general, stronger homology in the N-terminal portion than in the C-terminal portion (data not shown). In particular, the E1 enzyme gave an extensive homology to the E. coli sucrose hydrolase, as shown in Fig. 6. Aligned sequences of the N-terminal portion of these enzymes are shown in Fig. 7. A comparison of the E1 enzyme with the E. coli sucrose hydrolase indicated that the score of identity was 50.7\% in the first 280 amino acid residues. The comparison with other enzymes showed the score was about 40\% in all cases. Several homologous sequences are shown in this figure with a boxed frame.

The amino acid sequence around a cysteine residue at aa position 230, which was suggested to be an active site of levanase by Martin et al.\(^8\), Trp(Tyr)-Glu-Cys-Pro-Asp(Gly)-Leu, was found in the E1 enzyme with a minor modification. Martin et al. have indicated a tempting speculation that sucrase, levanase, and invertase have evolved by fusion of two DNA segments corresponding to the N- and C-terminal parts of the protein and that the DNA sequence encoding the N-terminal part of these proteins derived from a common ancestor.\(^8\) There was no conflict between our data obtained from the E1 enzyme of Zymomonas and their speculation.

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References