Note

Cloning and Expression of \( \beta \)-Fructofuranosidase Gene from

*Arthrobacter* sp. K-1

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A gene encoding an extracellular \( \beta \)-fructofuranosidase (\( \beta \)-FFase), designated *bff A*, was cloned from the chromosomal DNA of *Arthrobacter* sp. K-1, and expressed in *Escherichia coli*. The putative *bff A* consisted of 1734 bp encoding \( \beta \)-FFase of 578 amino acids. Comparison of the DNA sequence data with the N-terminal amino acid sequence of the purified enzyme indicated the presence of a precursor protein with a signal sequence of 36 residues. The deduced amino acid sequence of the \( \beta \)-FFase demonstrated a high degree of homology with that of levansucrase from *Acetobacter diazotrophicus*. The N-terminal amino acid sequence and some enzymatic properties of the recombinant enzyme were identical to those of the native enzyme.

\( \beta \)-D-fructofuranosidase (EC3.2.1.26; \( \beta \)-FFase) catalyzes the hydrolysis of sucrose as well as the transfructosylation from sucrose. This transfructosylation activity is useful for the production of fructose-containing oligosaccharides. The transfer ratio, defined as the amount of fructose transferred divided by the amount of allose moiety liberated, in addition to acceptor specificity and the structures of transfer products are dependent on the origin of the enzymes and on reaction conditions. Fungal \( \beta \)-FFase, one example being *Aspergillus niger*, catalyzes mostly hydrolysis of sucrose at a low concentration, whereas transfructosylation mostly occurs in high sucrose concentration. *A. niger* ATCC 20611 \( \beta \)-FFase selectively transfers the fructosyl residue of one sucrose molecule to the C-1 position of the fructose residue of another sucrose, and produces 1-kestose, nystose and fructosyl-nystose. On the other hand, *Arthrobacter* sp. K-1 \( \beta \)-FFase catalyzes mostly hydrolysis when incubated with sucrose alone even at high concentration. In the presence of suitable acceptors such as lactose, xylose, isomaltose and stevioside, the enzyme preferentially transfers the fructosyl residue of sucrose to the acceptor molecules, not including sucrose. In so doing, lactosucrose, fructosyl-xyloside, isomaltosylfructoside and fructosyl-stevioside are produced, respectively. The enzyme from *Arthrobacter* sp. K-1 has a wider range of acceptor specificities than other \( \beta \)-FFases, and is the most suitable agent for the production of heterooligosaccharides containing the fructose molecule. Information concerning the structure of this enzyme will be useful to help determine how \( \beta \)-FFase exhibits fructosyl transfer activity. In this paper, we describe the molecular cloning and the nucleotide sequences of \( \beta \)-FFase gene (*bff A*) derived from *Arthrobacter* sp. K-1.

We purified \( \beta \)-FFase from the cultured supernatant of *Arthrobacter* sp. K-1 according to a previously described procedure. The purified enzyme was digested with V8-protease by the method of Cleveland et al. to analyze the N-terminal and inner amino acid sequences of \( \beta \)-FFase using a Model 473A gas-phase protein sequencer (Perkin Elmer).
Genomic DNA of *Arthrobacter* sp. K-1 was used as a template for PCR with DNA polymerase (Ex-Taq, Takara). The following two oligonucleotides were used as primers for PCR: 5'-GGSCSTSCAGGACGGSCCSGA-3' corresponding to the amino acid sequence GLQDGPE in the N-terminal region of β-FFase, and 5'-TCCCASACC-CASACCTGTC-3' corresponding to EOVWVWD in a V8-protease cleaved fragment. PCR was performed using a DNA Thermal Cycler (Model 394, Perkin-Elmer) according to the following thermocycle program: 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. A major product of 0.2 kbp was purified from agarose gel after electrophoresis, and inserted into pT-Adv vector (CLONTECH) to generate pBFF200. The DNA fragment derived from pBFF200 was labeled using a rapid DNA labeling kit (Pharmacia Biotechnology), and then used as a probe for colony hybridization. Genomic DNA of *Arthrobacter* sp. K-1 was digested with BamHI because the probe hybridized with a BamHI fragment approximately 10 kbp in length according to Southern blot analysis. DNA fragments 9–11 kbp in length were obtained by agarose gel electrophoresis and ligated into the BamHI site of Charomid 9-36 (Toyobo). The ligated mixture was introduced into *E. coli* JM109 using an *in vitro* packaging kit (Gigapack III Gold Packaging Extract, Stratagene) to construct a limited genomic library of *Arthrobacter* sp. K-1. As a result of colony hybridization, 7 positive clones were obtained out of approximately 4000 transformants from the library. All 7 clones contained the same BamHI fragment. The nucleotide sequence of the region that hybridized with the probe was determined by a Model 373A DNA sequencer (Perkin-Elmer) using the Taq DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer).

The nucleotide sequence from the *Bal* I-*Fse*I region (2307 bp) of the cloned fragment is shown in Fig. 1. An open reading frame (ORF) sequence starting at position 258 and ending at position 1995 was located in this region. A Shine-Dalgarno-like sequence (5'-AGGAGA-3') was found 6–11 bp upstream from the initial codon of *bffA*, and the palindromic sequence (5'-CCTTGCCTCGG-ACGGCGATTCGCCGAGGCATCCGG-3') was found 5–46 bp downstream from the stop codon. The ORF of 1734 bp encodes a protein consisting of 578 amino acids with a molecular mass of 62,838 Da. The DNA sequence from position 373–593 in this ORF was completely identical to that of the probe amplified by PCR. From the N-terminal amino acid sequence of the purified protein, the first amino acid residue of a mature protein was identified as Gln37 in the deduced amino acid sequence, the ORF of which would encode a mature protein of 542 amino acids with a signal peptide of 36 amino acids. The amino acid sequences of peptides prepared from purified enzyme were found in the deduced amino acid sequence. The molecular mass of the deduced mature protein was 59,366 Da. This value is in good agreement with the 60,000 Da of the β-FFase purified from *Arthrobacter* sp. K-1. This ORF is therefore evidently the structural gene of β-FFase for *Arthrobacter* sp. K-1. The nucleotide sequence data reported in this paper are catalogued in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB062134.

An amino acid sequence homology search was performed using the FASTA program on the GenBank DNA database. The whole amino acid sequence of β-FFase from *Arthrobacter* sp. K-1 demonstrated homology to the sequence of levan- sucrase from *Acetobacter diazotrophicus* (51.0% identity), *Rahnella aquatilis* (31.1%), *Pseudomonas syringae* (30.3%) and *Zymomonas mobilis* (29.6%), according to the result of multiple alignment by the software CLUSTAL W (Fig. 2). Previously reported reaction analysis determined that this enzyme belongs to β-FFase rather than levan- sucrase. However, the sequence of β-FFase from *Arthrobacter* sp. K-1 was found to be similar to sequences of levan- sucrase produced by Gram-negative bacteria. Recently, enzymes with β-FFase activity are classified under two families, based on their amino acid sequences. One is β-FFases of family 32 of the glycosyl hydrolases, and the other is levan- sucrase type of enzymes. The deduced amino acid sequences of *Z. mobilis* extracellular **The molecular mass, 52,000 Da, reported previously by Fujita et al. was corrected to 60,000 Da in this report.**
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Fig. 1. Nucleotide and deduced amino acid sequences of the bff A.

The deduced amino acid sequence of the bff A is described below the nucleotide sequence in one-letter notation. The determined amino acid sequences from purified enzyme are underlined below the deduced sequence. The putative ribosome-binding site is underlined and labeled RBS. Convergent arrows indicate the palindrome sequence of the putative terminator.
Fig. 2. Comparison of deduced amino acid sequences of Arthrobacter sp. K-1 β-FFase with those of levansucrase.

Arthrobacter sp. K-1 β-FFase (AR), Acetobacter diazotrophicus levansucrase (AC), Rahnella aquatilis levansucrase (RA), Psedomonas syringae levansucrase (PS) and Zymomonas mobilis levansucrase (ZY) are aligned by the CLUSTAL W program. Asterisks indicates identical amino acids.
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invertase and Bacillus sp. V230 \( \beta \)-FFase have also been reported to be similar to those of levan-sucrase.

The pK1 plasmid was constructed to express the recombinant bffA gene in E. coli. The pK1 plasmid that contains the bffA gene for the mature protein region was prepared as follows. To introduce NdeI site at the translation initiation codon and BamHI site downstream of the stop codon of the bffA gene, primer 1 (5'-CATATGCAGTCA-GGGCTCCAG-3') and primer 2 (5'-GAAGGGA-T CCTCATGTCA GCCG) were synthesized. PCR amplification was performed using 25 cycles each at 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. After confirmation of the sequence between the NdeI and BamHI of the amplified fragment, the fragment was cloned to the NdeI-BamHI sites of the pET21a plasmid to construct pK1 plasmid. The transformant of E. coli BL21 (DE3) harboring the pK1 plasmid was grown at 30°C for 16h on Luria-Bertani broth containing 0.5 mm IPTG. Cells were washed using 20 mm potassium phosphate buffer (pH 6.0) and disrupted by sonication. Cell debris was removed by centrifugation, and the supernatant was used for purification of the recombinant \( \beta \)-FFase. SDS-PAGE and Western blot analysis of the recombinant enzyme revealed a single band corresponding exactly to those of the native \( \beta \)-FFase produced by Arthrobacter sp. K-1 (Fig. 3). In addition, the general properties of the recombinant enzyme corresponded well to the native enzyme (Table 1). The recombinant enzyme hydrolyzed \( \beta \)-2,1-fructofuranosidic linkage between fructose and glucose such as sucrose, raffinose, neokestose, and stachyose, but barely hydrolyzed the \( \beta \)-2,1-fructofuranosidic linkage between fructose and fructose such as 1-kestose and nystose. In the presence of acceptors such as lactose and xylose, the recombinant enzyme catalyzed transfructosylation from sucrose to acceptors, and produced lactosucrose and fructosyl-xyloside, respectively. These results indicate that the enzymatic properties of the recombinant enzyme were identical to those of the native enzyme.

In conclusion, the deduced amino acid sequence of the bffA gene product is similar to the sequence of levansucrase. Levansucrase transfers a fructosyl residue of sucrose toward 6-OH of fructose of sucrose, to synthesize a \( \beta \)-2,6-linked fructan (levan). However, the bffA gene product barely transfers a fructosyl residue of sucrose toward 1-OH of fructose of sucrose, and the resultant product is not levan but oligosaccharide as l-kestose. Of interest is

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**Fig. 3.** SDS-PAGE (A) and Western blot (B) analysis of recombinant \( \beta \)-FFase.

Lane 1: \( \beta \)-FFase from Arthrobacter sp. K-1. Lane 2: recombinant \( \beta \)-FFase from E. coli BL21(DE3)/pK1. The numbers on the left indicate the position of molecular weight markers (lane M).

**Table 1.** Some properties of recombinant \( \beta \)-FFase.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Recombinant</th>
<th>Native*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass</td>
<td>60 kDa</td>
<td>60 kDa</td>
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<tr>
<td>N-Terminal sequence</td>
<td>QSGLQDGPE</td>
<td>QSGLQDGPE</td>
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<tr>
<td>Optimum pH (40°C, 10min)</td>
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<td>6.5</td>
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<tr>
<td>pH stability (40°C, 2h)</td>
<td>5.5–10.0</td>
<td>5.5–10.0</td>
</tr>
<tr>
<td>Optimum temp. (pH6.5, 10min)</td>
<td>55°C</td>
<td>55°C</td>
</tr>
<tr>
<td>Temp. stability (pH6.5, 30min)</td>
<td>100%(45°C)</td>
<td>100%(45°C)</td>
</tr>
<tr>
<td>( K_m ) (Sucrose)</td>
<td>10.4 mM</td>
<td>9.1 mM</td>
</tr>
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*\( \beta \)-FFase purified from Arthrobacter sp. K-1
why the bffA gene product exhibits a different specificity to levansucrase. The bffA gene in this study will provide a clue as to the mechanisms by which the bffA gene product expresses fructosyl transfer activity different from levansucrase.

REFERENCES


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