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High-Level Production of Low-Branched Levan from *Pseudomonas aurantiaca* S-4380 for the Production of di-β-D-Fructofuranose Dianhydride IV

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Abstract The *lsc*A gene, encoding a levansucrase of 424 amino acids (aa) residues, was cloned from the genomic DNA of *Pseudomonas aurantiaca* S-4380, and overexpressed in *Escherichia coli*. The recombinant levansucrase overexpressed in *E. coli* was then used to produce levan from sucrose. Levan crystals with 98% purity could be obtained from the reaction mixture with 62% yield using an alcohol precipitation method. The molecular weight of the levan was 7×10^5 daltons. Methylation studies showed that the levan was branched: main linkage C-2,6; branched linkage C-2,1; and degree of branching 6%. Three bacterial levans from different strains were incubated with levan fructotransferase (LFTase) from *Arthrobacter ureafaciens* K2032, which produced di-β-D-fructofuranose dianhydride IV (DFA IV); final conversion yields from the levans to DFA IV were 39% in *Zymomonas mobilis*, 53% in *Serratia levanicum*, and 59% in *P. aurantiaca* S-4380 levansucrase. The levan from *P. aurantiaca* S-4380 levansucrase gave the highest conversion yield of levan to DFAIV so far reported.

Key words: di-β-D-Fructofuranose dianhydride IV, *Pseudomonas aurantiaca* S-4380, levan

Fructan is a homopolysaccharide composed of D-fructofuranosyl residues linked as either β-(2,6) or β-(2,1) [9, 12, 14]. Two types of fructan, which are distinguishable by their type of linkage, are inulin and levan. Chemically, levan consists of β-D-fructofuranosyl residues linked predominantly through β-(2,6) linkages, with extensive branching through β-(2,1) linkages. In contrast, inulin is composed of β-D-fructofuranose attached by β-(2,1) linkages [5]. Levan is found in plants and especially in bioproducts of microorganisms. Microbial levans, whose molecular weights are up to several million daltons with multiple branches, are produced by levansucrase (E.C. 2.4.1.10) from a wide range of taxa such as bacteria, yeasts, and fungi [5, 8, 17]. As an industrial gum, levan can be utilized in making levan oligosaccharides including difructose anhydrides (DFAs) [3]. DFAs are cyclic disaccharides consisting of two fructose units linked at their reducing carbons, and there are structural varieties according to their linkage type [21]. So far, four kinds of DFAs, including DFA I, III, IV, and V, have been reported as the results of degradation of inulin or levan by microbial enzymes [21]. Unlike other DFAs, β-D-fructofuranose-β-D-fructofuranose-2',6:6,2'-dianhydride (DFA IV) is the only DFA produced from levan by levan fructotransferase (LFTase). DFAs play roles in inhibiting tooth decay and stimulating growth of lactic acid-producing bacteria, and also function as an absorption factor for minerals in the body [1, 20]. The molecular weight and structure of levan have significant effects on the yield of its conversion to DFA IV by LFTase [28]. In particular, low branching at the C-1 position in levan might be favorable for the high-yield production of DFA IV [19]. Of bacterial levans, the properties of levan have been studied in most detail in *Bacillus* species and *Zymomonas mobilis*, and the degree of branching of two bacterial levans were the same; 12% [5, 16]. Only the levansucrase from *Serratia levanicum* produces low-branched levan with 6% degree of branch,

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but is available in a very small quantity [15]. Recently, we have screened a large collection of soil bacteria for strains producing levan from sucrose, and found that one of these strains, *Pseudomonas aurantiaca* S-4380, synthesized large amounts of low-branched levan. The present study reports the biochemical and structural characterization of the low-branched levan produced by *P. aurantiaca* S-4380 and a recombinant *E. coli* harboring the levansucrase gene from *P. aurantiaca* S-4380.

MATERIALS AND METHODS

Materials

Z. mobilis levan was prepared on a laboratory scale as described previously [26]. *S. levanicum* levan, kestrose, and nystose were purchased from Wako Pure Chemical Co. (Osaka, Japan). DFA IV and LFTase were prepared from *Arthrobacter ureafaciens* K2032 using previously reported methods [25]. A silica gel 60 F_{254} thin-layer chromatography (TLC) plate used for sugar analysis was purchased from Merck (Darmstadt, Germany), and chemicals for gel electrophoresis were from Bio-Rad. Unless otherwise specified, the rest of the chemicals were purchased from Sigma (St. Louis, MO, U.S.A.), Boehringer Mannheim (Mannheim, Germany), or Takara (Tokyo, Japan).

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strain used in this study as the source of gene coding for levansucrase was *P. aurantiaca* S-4380 (KCTC 0943BP), previously isolated and grown aerobically in YPS broth (yeast extract 1%, peptone 2%, sucrose 5%) at 30°C. *E. coli* BL21(DE3) [27] was used for expression of *lscA* of *P. aurantiaca* S-4380 (GenBank Accession No. AF306513). Recombinant *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium containing (g/l): 5 yeast extract, 10 tryptone, and 10 NaCl. When appropriate, ampicillin was added to a final concentration of 100 μg/ml. For pLK1, a 2.4-kb fragment containing the *lscA* gene from *P. aurantiaca* S-4380 was cloned into pBluescript II KS(+). For pELK1, a 2.5-kb BamHI-EcoRI fragment containing the *lscA* gene from *P. aurantiaca* S-4380 was cloned into pET-21a(+). The gene in this plasmid had the same orientation as *lacZα*.

Isolation, Maintenance, and Identification of Microorganism

Isolation procedures of *P. aurantiaca* S-4380 were described previously [6]. Briefly, microorganisms showing levansucrase activity were isolated from sucrose-disclosed soils in Korea. Cells were cultivated on nutrient agar plate at 30°C for 2–3 days. The developed colonies were transferred to YPS agar plates, and then incubated at 30°C for 24–48 h. Bacterial colonies with sticky polymer were selected and incubated in the liquid media. After 48 h of cultivation, the cells were separated by centrifugation at 8,000 ×*g* for 10 min. The product analysis and determination of sucrose-hydrolyzing activity were carried out using the cell-free culture broth. One of the strains producing low-branched levan was selected and used for this study. Identification of the genus and species was confirmed through analysis of the 16S rRNA sequence. For nucleotide sequence determination of the 16S rRNA gene, the genomic DNA was isolated by the modified Rochelle method [18, 23], and polymerase chain reaction was performed to amplify the 16S rRNA coding region, using two primers: 5'-AGAGTTTGATCMTGGC-TCAG-3' and 5'-AAGGAGGTGWTCCARCC-3'. After purification of the 16S rRNA product, direct sequencing was performed with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) as specified by the manufacturer [24]. The DNA fragments were analyzed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The homology search and construction of a phylogenetic tree were performed using DNADIST program

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmids	Relevant characteristics or genotype	Reference or source
P. aurantiaca S-4380	Wild-type, Lev[*a]	This study
E. coli		
BL21	DE3, F⁻ *ompT* r^-_B m^-_B	Takara[b]
Plasmids		
pBluescript II KS(+)	Ap[r]	Stratagene[c]
pET-21a(+)	Ap[r], a 5.4-kb vector containing an N-terminal T7 promoter sequence plus a C-terminal His-tag sequence	Novagen[d]
pLK1	Ap[r]; 2.4-kb fragment containing the *lscA* gene from *P. aurantiaca* S-4830 inserted into pBluescript II KS(+).	[6]
pELK1	Ap[r]; 2.5-kb BamH1-EcoRI fragment from pLK1 inserted into pET-21a(+).	[6]

[a]Producing levansucrase.
[b]Stratagene Cloning Systems, La Jolla, CA, U.S.A.
[c]Novagen, Inc., Madison, WI, U.S.A.
[d]Takara Korea Biomedical Inc., Seoul, Korea.

(Mac-molly, Mackintosh) and NEIGHBOR program of PHYLIP 3.5 package [18, 23].

Isolation and Purification of Levan

For the preparation of levan from *P. aurantiaca* S-4380, cells were grown in the YPS, and the culture fluids were collected by centrifugation. Three volumes of cold ethanol were added to culture supernatant, and the mixtures were mixed by vortexing for 1 min and then centrifuged (8,000 ×*g* for 10 min at 4°C). Precipitated materials were resuspended in water and mixed again with three volumes of cold ethanol. Samples were collected by centrifugation, and the procedure was repeated. The amount of levan was determined by measuring the final dry weight of the pellets after centrifugation. For the preparation of levan from the recombinant *E. coli* strain, the purified levansucrase (see next section for the purification procedures) was used for the production of levan, and levan was then purified from the mixtures as mentioned above.

Purification of Modified Levansucrase from the Recombinant *E. coli*

Plasmid pELK1 was constructed by PCR amplification using pLK1 as a template and two primers: forward primer, 5'-TAGGATCCATGAAAAGCAACACTGAA-3'; reverse primer, 5'-GGAATTCCTTGAGCGTTACATC-3'. PCR-amplified 2.5-kb DNA fragments encoding the *lsc*A were EcoRI-BamHI digested and inserted into the same enzyme-digested pET-21a(+) vector. This replacement removed the stop codon from the amplified *lsc*A region and provided the *lsc*A with a 6× His tag. After IPTG induction, the harvested *E. coli* BL21(DE 3) harboring the plasmid pELK1 was resuspended in 50 mM phosphate buffer (pH 6.0) and then disrupted by ultrasonication. The cell debris was removed by centrifugation (4°C, 12,000 rpm, 10 min), and the supernatant was used as a cell-free extract. Levansucrase with His-affinity tag was purified from the supernatant using a chelate adsorbent, Ni-NTA resin, which was purchased from Qiagen (Valencia, CA, U.S.A.).

Determination of Molecular Mass

The degree of polymerization of levan was determined by HPLC equipped with two successive columns, $GMPW_{XL}$ (Viscotek Co. Houston, TX, U.S.A.) and a refractive index detector (conditions: 0.1 M $NaNO_3$; 0.8 ml/min; temperature, 30°C; injection volume, 0.02 ml). Levan was dissolved in water, filtered through a 0.45-μl filter (Millipore, Bedford, MA, U.S.A.), and analyzed by HPLC [7]. Polyethylene oxides (9.7×10^5, 7.6×10^5, 2.8×10^5, 7.9×10^4, 7.9×10^4) from Polymer Laboratories (U.S.A.) were used as standard compounds.

Analysis of Monosaccharide

After acid hydrolysis of levan (20 min in 0.2 N HCl at 100°C), the pH of the mixture was neutralized by the addition of NaOH. The hydrolyzates were analyzed by HPLC equipped with a refractive index detector and Asahipk NH2P-50 (Asahi Chemical, Inc., Japan) column (conditions: deionized water:acetonitrile=25:75; flow rate, 1.0 ml/min; injection volume, 0.02 ml) [13].

Methylation Analysis

For methylation and acid hydrolysis of levan, 1 mg of the levan was dissolved in dimethyl sulfoxide (0.1 ml) by stirring until a clear solution was obtained. Hakomori's reagent (0.07 ml) and methyl iodide (0.06 ml) were added to the solution [11]. The reaction was allowed to proceed for 12 h, and then 0.4 ml of water was added. This mixture was extracted three times with $CHCl_3$ (0.2 ml). The $CHCl_3$ layer was rotary-evaporated in vacuum at 30°C. After hydrolysis with 0.1 ml of 4 M trifluoroacetic acid (16 h, 30°C), the sample was made into syrup by rotary evaporation in vacuum at 30°C, and the syrup mixture was extracted 3 times with methanol (0.1 ml). Aliquots (1 μl) were applied on a silica gel 60 F_{254} TLC plate. The methylation products were separated by two ascents on plate using CH_3CN/$CHCl_3$/CH_3OH, 3:9:1 (vol/vol/vol) at room temperature, followed by development of the plate by dipping it into 0.3% *N*-(1-naphthyl)-ethylenediamine and 5% sulfuric acid in methanol, and heating at 120°C [11]. Quantitative determination of the *O*-methylated sugars on the TLC plate was carried out by scanning the plate with a Bio-Rad Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The degree of branching was determined by dividing the area of 3,4-di-*O*-methyl-D-fructose by the sum of the area from 1,3,4,6-tetra-*O*-methyl-D-fructose, 1,3,4-tri-*O*-methyl-D-fructose, and 3,4-di-*O*-methyl-D-fructose [16].

Assay of Levansucrase Activity

Levansucrase activity was determined by measuring sucrose-hydrolyzing activity. One unit of levansucrase activity was defined as the amount of the enzyme to release one mmole of glucose per minute. Enzyme reactions were initiated by adding levansucrase solution (1 U/ml) to 50 mM sodium-acetate buffer (for pH 4.6–5.6) or sodium-phosphate buffer (for pH 6.0–8.0), containing 10% (wt/vol) sucrose, and 100 μl of samples was collected at regular time intervals to check the levan formation. The enzyme reaction was terminated by the addition of 100 μl of 50 mM NaOH. The quantitative determination of glucose, fructose, sucrose, and levan was carried out by HPLC (Beckman) with a refractive index detector and Shodex Ionpack KS-802 (Showa Denko Co., Japan) column (conditions: water 100%; flow rate, 0.4 ml/min; temperature, 50°C; injection volume, 0.02 ml).

Assay of LFTase Activity

LFTase activity was assayed as described previously [25]. Enzyme reactions were carried out using 50 ml of sodium-phosphate buffer (pH 5.8), containing 5% (wt/vol) levan

and 10 U of LFTase, at 37°C for 60 h with gentle shaking. One unit of enzyme activity was defined as the amount of enzyme to release one mmole of reducing sugar equivalent to glucose per minute. The quantitative determination of DFA IV and levan was conducted by HPLC equipped with a KS-802 column. Protein concentration was estimated by the method described by Bradford, and a BioRad protein assay kit was used [2].

RESULTS AND DISCUSSION

Screening of Microorganism Producing Low-Branched Levan

About 250 isolates showed sucrose-hydrolyzing activity on sucrose-containing agar medium. Among them, 8 colonies were isolated as levan producers from sucrose, based on the migration rate and sugar compositions of the products on TLC and HPLC. One of the colonies, which showed relatively high activity and produced the enzyme in a stable manner, was selected for further experiments.

Identification of Bacteria

Phylogenetic analysis with the 16S rRNA sequence showed that the microorganism shared 99.7% sequence homology with *P. aurantiaca* ATCC 33663T (Fig. 1). Analysis of the secondary structure with 16S rRNA of other bacteria also revealed the highest homology with that of *P. aurantiaca*. Therefore, this strain was named as *P. aurantiaca* S-4380 and registered at the Korean Collection for Type Cultures (KCTC) (Reference number 0943BP).

Fructan Analysis

The HPLC elution patterns and size distribution analysis of the fructan produced by *P. aurantiaca* S-4380 were studied.

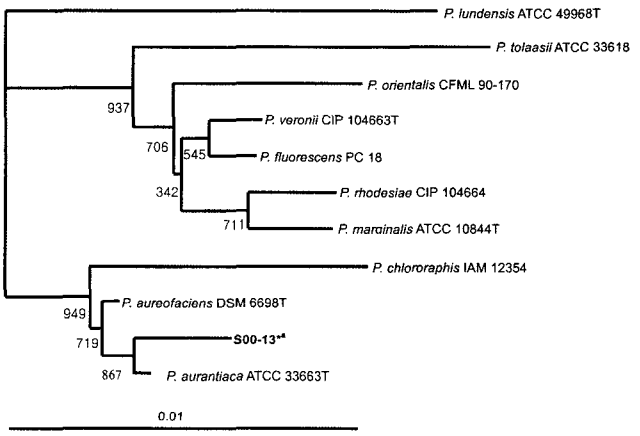

Fig. 1. Phylogenetic tree of the isolated strain (*a: *P. aurantiaca* S-4380) showing levansucrase activity.
Indicated number is the boostrap value.

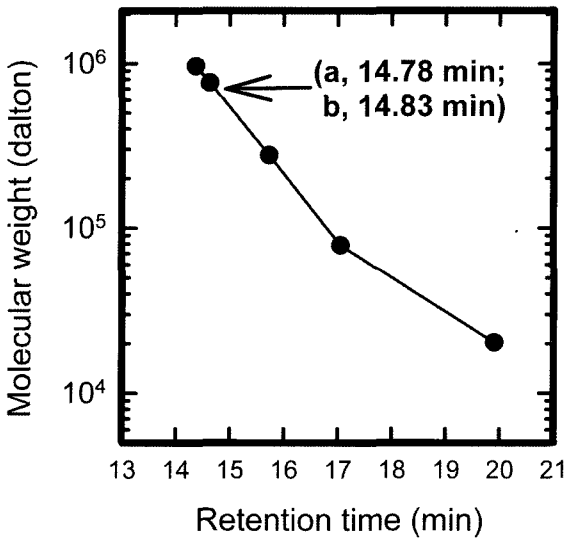

Fig. 2. Molecular weight determination of levan formed by *P. aurantiaca* S-4830 (a), and levansucrase purified from *E. coli* BL21(DE3) containing pELK1 (b) incubated with 100 g sucrose/ml in 1 ml of 50 mM sodium-phosphate buffer (pH 5.6) at 0°C. HPLC analyses were performed using two successive GPC columns with 0.1 M $NaCO_3$ as a mobile phase at 0.8 ml/min.
Polyethylene oxides (9.7×10^5, 7.6×10^5, 2.8×10^5, 7.9×10^4, 7.9×10^3) from Polymer Laboratories (U.S.A.) were used as standard compounds.

With two successive GPC columns, *P. aurantiaca* S-4380 was found to produce fructan with a peak at 14.8 min (Fig. 2). The estimated molecular size of the fructan (at 14.8 min) was about 7.0×10^5 M.W. The product was readily soluble in water and insoluble in 70% ethanol at room temperature. The sugar components in the acid hydrolyzates of the fructan were analyzed by HPLC, and the result revealed that the polymer consisted solely of fructose. These results demonstrated that the fructan produced by *P. aurantiaca* S-4380 was the levan-type fructan.

Methylation Analysis

Methylation analysis showed that the major methylated product of levan produced by *P. aurantiaca* S-4380 was 1,3,4-tri-*O*-methyl-D-fructose with minor amounts of 3,4-di-*O*-methyl-D-fructose and 1,3,4,6-tetra-*O*-methyl-D-fructose in the molar ratio of 89.3:4.8:5.9, respectively (Fig. 3). 1,3,4,6-Tetra-*O*-methyl-D-fructose indicates the reducing end of branched chains, and 3,4-di-*O*-methyl-D-fructose occurs from β-2,1-branched fructose residues. The main linkage was C-2,6, and branching occurs at C-1. The degree of branching was 6%, and average length of the repeating unit was 19. Lanes 1 and 3 in Fig. 3 show the methylation analysis of *P. aurantiaca* S-4380 and *S. levanicum*. In both levans, the proportions of 3,4-di-*O*-methyl-D-fructose were low, indicating that the degree of branching of the two levans was lower than that of *Z. mobilis* levan.

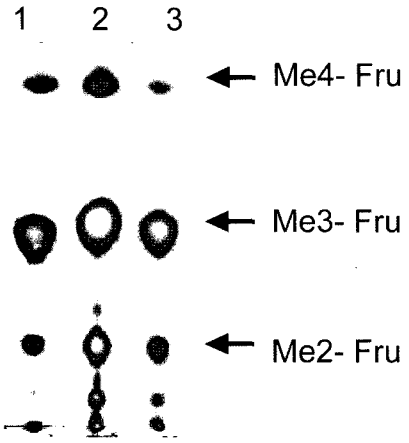

Fig. 3. Thin-layer chromatography of methylated products.
Lane 1, levan from *P. aurantiaca* S-4380; lane 2, levan from *Z. mobilis*; lane 3, levan from *S. levanicum*. Me4-fru, 1,3,4,6-tetra-*O*-methyl-D-fructose; Me3-Fru, 1,3,4-tri-*O*-methyl-D-fructose; Me2-Fru, 3,4-di-*O*-methyl-D-fructose.

Expression of *lscA* in *E. coli*

To develop an efficient enzymatic process for levan production, the levansucrase overexpression in *E. coli* and a purification procedure were developed. The modified levansucrase carrying the His-affinity tag in its C-terminal was overexpressed up to 40% of the total cell protein in *E. coli*, and the enzyme was purified by a single step using metal affinity chromatography with more than 95% homogeneity (Fig. 4), resulting in 62% of the theoretical yield (Fig. 5). The purified levansucrase showed optimal levan formation activity at 0°C and pH 5.6.

Formation of DFA IV from Levan

Levans obtained from *P. aurantica* S-4380 levan and the recombinant *E. coli* strain were used as substrates for the

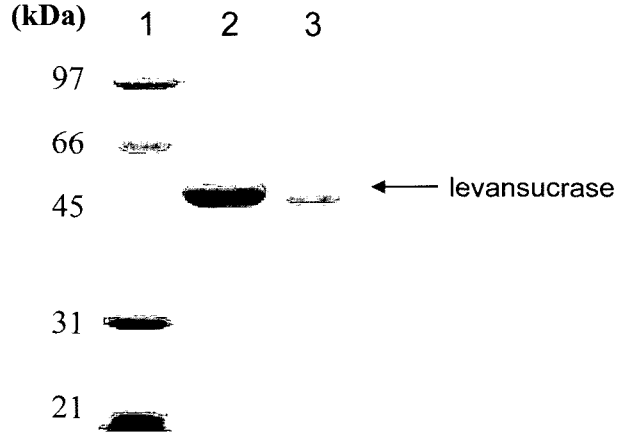

Fig. 4. SDS-PAGE analysis of levansucrase produced in *E. coli* BL21(DE3) containing pELK1. Protein standards are indicated on the left.
Lane 1, molecular weight marker; lane 2, before purification; lane 3, purified levansucrase.

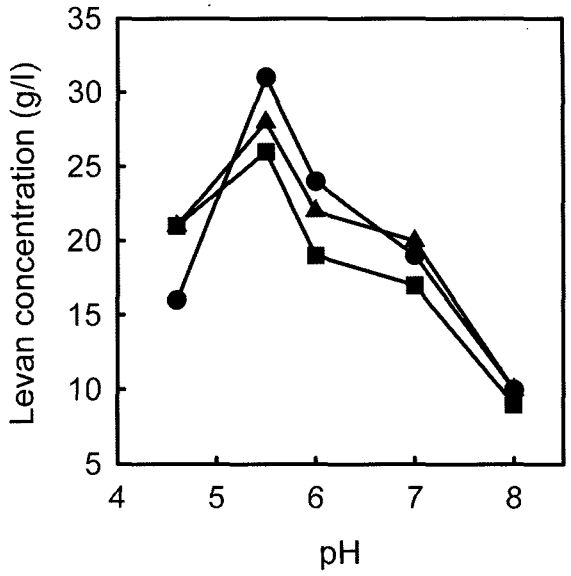

Fig. 5. Effect of temperature and pH on levan formation.
The assays were carried out under standard conditions for 140 h at either 0°C, 4°C, or 10°C, and the pH was varied from 4.6 to 8.0. ●: 0°C; ▲: 4°C; ■: 10°C.

LFTase reaction [10]. In parallel, some commercially available bacterial levans were also analyzed. Since LFTase is not commercially available, the *A. ureafaciens* K2032 LFTase was prepared from our laboratory stocks. The levan content in the reaction mixture was decreased, and the DFA IV and oligosaccharide content were increased as the reaction time progressed. Final products contained DFA IV as the main product together with small amounts of levan, oligosaccharides, and fructose (Fig. 6). After 60 h of reaction, the enzymatic reaction reached the steady state. Figure 7 shows that the final conversion yields from levan to DFA IV were 38.9% in *Z. mobilis*, 53.0% in *S. levanicum*, and 59.0% in *P. aurantiaca* S-4380 levan. Among various

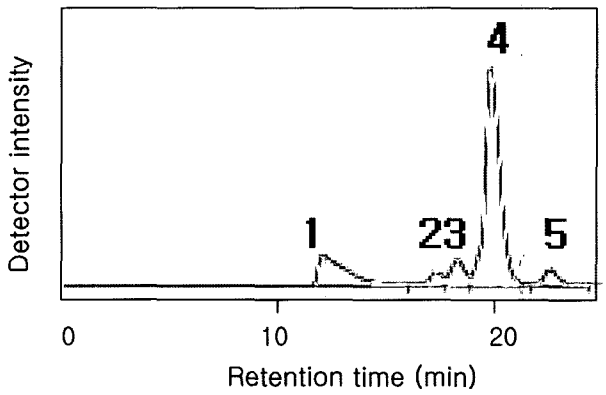

Fig. 6. Typical HPLC chromatogram of enzymatic products using LFTase and *P. aurantiaca* S-4380 levan.
Five % levan solution was incubated with 10 U of enzyme at 37°C for 60 h. 1, Limited-levan; 2 and 3, oligosaccharides; 4, DFA IV; 5, fructose.

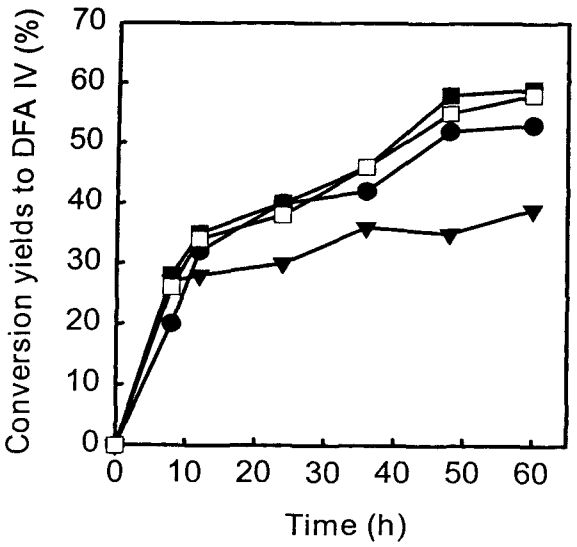

Fig. 7. Conversion yields from levan to DFA IV using four bacterial levans and LFTase.
Five % levan solution was incubated with 10 U of enzyme at 37°C for up to 60 h. ■, *P. aurantiaca* S-4380 levan; □, *E. coli* BL21(DE3) containing pELK1 levan; ●, *S. levanicum* levan; ▼, *Z. mobilis* levan.

carbohydrates (levan, inulin, xylan, starch, sucrose, fructose, cellulose, raffinose, cellobiose, lactose, and maltose), levan gave about 20-folds higher conversion efficiency than the others [21]. It has been suggested that the DFA IV production by LFTase is stopped when the enzyme meets the β-2,1 branch point in levan [19]. Therefore, the proportion of undigested levan indicates the ratio of β-2,1/β-2,6 in the levan [19, 22]. Based on this hypothesis, the above authors suggested that linear levan or low-branched levan is suitable for DFA IV production by LFTase [19, 21, 22].

Taken together, the results of methylation analysis of levan and the LFTase hydrolysis implied that the strain *P. aurantiaca* S-4380 used in this study is one of the low-branched levan-producing bacteria reported, and thus might be used for the DFA IV production. It is quite possible that the recombinant levan obtained in this work has a potency equivalent to the potency of levan produced by *P. aurantiaca* S-4380, since both levans gave similar efficiency to convert to DFA IV (Fig. 7).

In conclusion, we described herein the production of large quantities of recombinant levan by an *E. coli* strain and efficient purification of this levan. The high-level production of low-branched levan may be useful for efficient production of DFA IV. Currently, studies on the purification and crystallization process for the production of DFA IV are in progress.

REFERENCES

1. Baik, B. H., Y. W. Lee, and Y. B. Lee. 1997. Antianemic agent containing iron and difructose. US patent 5,700,832.
2. Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Defaye, J., A. Gadelle, and C. Pedersen. 1985. The behaviour of D-fructose and inulin towards anhydrous hydrogen fluoride. *Carbohydr. Res.* **136:** 53–65.
4. Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle, U.S.A.
5. Han, Y. W. and M. A. Clarke. 1990. Production and characterization of microbial levan. *J. Agric. Food Chem.* **38:** 393–396.
6. Jang, E. K., K. H. Jang, I. Koh, I. W. Kim, S. H. Kim, S. A. Kang, C. H. Kim, S. D. Ha, and S. K. Rhee. 2002. Molecular characterization of the levansucrase gene from *Pseudomonas aurantiaca* S-4380 and its expression in *Escherichia coli*. *J. Microbiol. Biotechnol.* **12:** 603–609.
7. Jang, K. H., K. B. Song, C. H. Kim, B. H. Chung, S. A. Kang, U. H. Chun, R. W. Choue, and S. K. Rhee. 2001. Comparison of characteristics of levan produced by different preparations of levansucrase from *Zymomonas mobilis*. *Biotechnol. Lett.* **23:** 339–344.
8. Kang, S. A., K. Hong, K. H. Jang, S. Kim, K. H. Lee, B. I. Chang, C. H. Kim, and R. Choue. 2004. Anti-obesity and hypolipidemic effects of dietary levan in high fat diet-induced obese rats. *J. Microbiol. Biotechnol.* **14:** 796–804.
9. Kang, S. A., J. C. Lee, Y. M. Park, C. Lee, S. H. Kim, B. I. Chang, C. H. Kim, J. W. Seo, S. K. Rhee, S. J. Jung, S. M. Kim, and K. H. Jang. 2004. Secretory production of *Rahnella aquatilis* ATCC 33071 levansucrase expressed in *Escherichia coli*. *J. Microbiol. Biotechnol.* **14:** 1232–1238.
10. Kim, C. H., E. K. Jang, S. H. Kim, K. H. Jang, S. A. Kang, K. B. Song, O. S. Kwon, and S. K. Rhee. 2005. Molecular cloning of levan fructotransferase gene from *Arthrobacter ureafaciens* K2032 and its expression in *Escherichia coli* for the production of difructose dianhydride IV. *Lett. Appl. Microbiol.* **40:** 228–234.
11. Kim, D. M. and D. W. Kim. 1999. Facile purification and characterization of dextransucrase from *Leuconostoc mesenteroides* B-512FMCM. *J. Microbiol. Biotechnol.* **9:** 219–222.
12. Kim, H. J., H. E. Park, M. J. Kim, H. G. Lee, J. Y. Yang, and J. H. Cha. 2003. Enzymatic characterization of a recombinant levansucrase from *Rahnella aquatilis* ATCC 15552. *J. Microbiol. Biotechnol.* **13:** 230–235.
13. Kim, M. G., C. H. Kim, J. S. Lee, K. B. Song, and S. K. Rhee. 2000. Synthesis of methyl β-D-fructoside catalyzed by levansucrase from *Rahnella aquatilis*. *Enzyme Microbiol. Technol.* **27:** 646–651.
14. Kim, M. J., H. E. Park, H. K. Sung, T. H. Park, and J. Cha. 2005. Action mechanism of transfructosylation catalyzed by *Microbacterium laevaniformans* levansucrase. *J. Microbiol. Biotechnol.* **15:** 99–104.
15. Kojima, I., T. Saito, M. Iizuka, N. Minamiura, and A. Ono. 1993. Characterization of levan produced by *Serratia* sp. *J. Ferm. Bioeng.* **75:** 9–12.

16. Lindberg, B., J. Lönngren, and J. L. Thompson. 1973. Methylation studies on levans. *Acta Chem. Scand.* **5:** 1819–1821.
17. Oh, I. K., S. H. Yoo, I. Y. Bae, J. Cha, and H. G. Lee. 2004. Effects of *Microbacterium laevaniformans* levans molecular weight on cytotoxicity. *J. Microbiol. Biotechnol.* **14:** 985–990.
18. Rochelle, P. A., J. C. Fry, R. J. Parkes, and A. Weightman. 1992. DNA extraction for 16S rRNA gene analysis to determine genetic diversity in deep sediment communities. *FEMS Microbiol. Lett.* **100:** 59–66.
19. Saito, K., H. Goto, and A. Yokota. 1997. Purification of levan fructotransferase gene from *Arthrobacter nicotinovorans* GS-9 and production of DFA IV from levan by the enzyme. *Biosci. Biotech. Biochem.* **61:** 1705–1709.
20. Saito, K., T. Hira, T. Suzuki, H. Hara, A. Yokota, and F. Tomita. 1999. Effects of DFA IV in rats: Calcium absorption and metabolism of DFA IV by intestinal microorganisms. *Biosci. Biotech. Biochem.* **63:** 655–661.
21. Saito, K. and F. Tomita. 2000. Difructose anhydrides: Their mass-production and physiological functions. *Biosci. Biotechnol. Biochem.* **64:** 1321–1327.
22. Saito, K., A. Yokoda, and F. Tomita. 1997. Molecular cloning of levan fructotransferase from *Arthrobacter nicotinovorans* GS-9 and its expression in *Escherichia coli*. *Biosci. Biotech. Biochem.* **61:** 2076–2079.
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning. A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, New York, U.S.A.
24. Sanger, F., S. Nichlen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.
25. Song, K. B., K. S. Bae, Y. B. Lee, K. Y. Lee, and S. K. Rhee. 2000. Characteristics of levan fructotransferase from *Arthrobacter ureafaciens* K2032 and difructose anhydride IV formation from levan. *Enzyme Microbiol. Technol.* **27:** 212–218.
26. Song, K. B., H. Belghith, and S. K. Rhee. 1996. Production of levan, a fructose polymer, using an overexpressed recombinant levansucrase. *Ann. N. Y. Acad. Sci.* **799:** 601–607.
27. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned gene. *Meth. Enzymol.* **185:** 60–89.
28. Tanaka, K., F. Yamaguchi, and S. Kusui. 1989. Action of levan fructotransferase of *Arthrobacter ureafaciens* on levan and phlein. *Agric. Biol. Chem.* **53:** 1203–1211.