JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Levan Fructotransferase from *Arthrobacter oxydans* J17-21 Catalyzes the Formation of the Di-D-fructose Dianhydride IV from Levan

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A new levan fructotransferase (LFTase) isolated from *Arthrobacter oxydans* J17-21 was characterized for the production of difructose dianhydride IV (DFA IV). LFTase was purified to apparent homogeneity by Q-Sepharose ion exchange chromatography, Mono-Q HR 5/5 column chromatography, and gel permeation chromatography. The enzyme had an apparent molecular mass of 54000 Da. The optimum pH for the enzyme-catalyzed reaction was pH 6.5, and the optimum temperature was observed at 45 °C. The LFTase was activated by the presence of $CaCl_2$ and EDTA-2Na but inhibited strongly by $MnCl_2$ and $CuSO_4$ at 1 mM and completely by $FeSO_4$ and Ag_2SO_4 at 1 mM. A bacterial levan from *Zymomonas mobilis* was incubated with an LFTase; final conversion yield from the levan to DFA IV was 35%. Neither inulin, levanbiose, sucrose, dextran, nor starch was hydrolyzed by LFTase. DFA IV was very stable at acidic pH and high temperature, thus indicating that DFA IV may be suitable for the food industry and related areas.

KEYWORDS: ***Arthrobacter oxydans*; DFA IV; levan fructotransferase; levan-hydrolyzing enzyme**

INTRODUCTION

Fructan, one of the most highly distributed biopolymers in nature, is a homopolysaccharide composed of D-fructofuranosyl residues joined by β-(2,6) and β-(2,1) linkages. Two types of fructan, distinguished by the type of linkage present, are inulin and levan (*1*, *2*). Several endoinulinases (EC 3.2.1.7) and closely related enzymes were reported to produce fructooligosaccharides, cyclo inulo-oligosaccharides, and di-D-fructofuranose dianhydrides (DFAs), which are cyclic forms of difructose (*1*, *2*). Two types of DFAs can be formed when inulin is used as the substrate for enzyme reactions: DFA I, α-D-fructofuranose-β-D-fructofuranose 2′,1;2,1′-dianhydride, and DFA III, α-D-fructofuranose-β-D-fructofuranose 2′,1;2,3′-dianhydride (*1*, *3*). In the case of the levan-degrading enzyme, levan is degraded to D-fructose, levanbiose, sucrose, levan oligomers, or low molecular weight levan by the hydrolytic activity of levanase, levansucrase, or levan fructotransferase (LFTase) from some plants and microorganisms (*1*, *3*). The latter enzyme, LFTase, hydrolyzes levan to DFA IV (α-D-fructofuranose-β-D-fructofuranose 2′,6:6,2′ dianhydride). DFA IV has half the sweetness of sucrose and is not hydrolyzed by invertase and inulinase; it is therefore well suited for use in low-calorie foods (**Figure 1**). Due to its structural characteristics, DFA IV can accommodate various materials including calcium and iron to form chelated complexes (*3*, *4*). These complexes increase mineral absorption in the small intestine of animals and stimulate the growth of lactic acid-producing bacteria in the large intestine of rats (*3*, *4*). Therefore, production of DFA IV using LFTase has attracted increasing interest from the food and pharmaceutical industries.

The chemical procedure for the production of DFA IV includes the heating of sucrose at high temperature (>100 °C). After the reaction mixture has cooled to room temperature, DFA IV can be recovered. However, this procedure is inefficient due to the formation of several isomers of DFAs (*5*, *6*). Therefore, an LFTase with hydrolyzing and cycling activities would be appropriate for efficient production of DFA IV.

In the present work, we report the isolation and production of an LFTase from soil bacteria. The physicochemical properties of the enzyme were characterized, and DFA IV was produced by treating a levan solution with LFTase alone. The stability of

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10.1021/jf026207o CCC: $25.00
Published on Web 03/26/2003

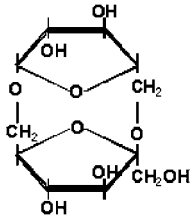

Figure 1. Chemical structure of DFA IV.

the resulting product, DFA IV, was also compared with that of levan at high temperature and acidic pH condition.

MATERIALS AND METHODS

Materials. Bacterial levan (MW 6×10^6 Da) from *Zymomonas mobilis* was prepared in a laboratory scale as described previously (*7*), and obtained from RealBioTech Co. Ltd. (Daejon, Korea). Chemicals for electrophoresis were purchased from Bio-Rad, Sigma, or Takara. All other chemicals were of analytical reagent grade unless otherwise stated.

Screening and Identification of Microorganisms Producing LFTase. Bacterial strains isolated from Korean soil were tested for levan-hydrolyzing activity on an agar plate (medium A) containing 1% (w/v) levan, 0.3% (w/v) yeast extract, 0.3% (w/v) $NaCO_3$, 0.05% (w/v) $MgSO_4$, 0.02% $MnCl_2$, 0.1% K_2HPO_4, and 2% agar in dH_2O. The strains positive for levan-hydrolyzing activity were screened for measuring the DFA IV-forming activity by HPLC analysis, as described previously (*7*). One of the LFTase-producing strains capable of producing LFTase in a stable manner was selected and used for this study. Physiological and biochemical characteristics of the isolate were tested by following the *Bergey's Manual of Systematic Bacteriology* (Vol. II), as described previously (*8*). All cultivations were done at 30 °C. Cellular fatty acid composition was determined by using the method described previously (*8*).

Purification of LFTase. *Arthrobacter oxydans* J17-21 was grown in medium A. Two hundred milliliters of culture was transferred to 20 5 L flasks containing 1 L of liquid medium and cultured at 30 °C for 40 h, with agitation at 100 rpm. Cells were removed from the culture by centrifugation (4000 rpm) at 4 °C for 30 min, and the supernatant was used for fractionation of extracellular LFTase by 80% (w/v) $(NH_4)_2SO_4$ at 4 °C overnight. The precipitate was collected by centrifugation, resuspended in 20 mM sodium phosphate buffer (pH 6.5) (buffer A), dialyzed against buffer A, and then thoroughly centrifuged. The supernatant obtained was applied to a Q-Sepharose FF anion exchange column (2.5×500 mm, Pharmacia, Piscataway, NJ) previously equilibrated with buffer A. The column was washed thoroughly with buffer A, and LFTase bound to the column was eluted by a linear gradient (0−0.5 M) of NaCl in buffer A at the flow rate of 0.45 mL/min. The eluted enzyme fractions (6.5 mL) were concentrated by Centriplus (Amicon, Beverly, MA) and dialyzed thoroughly against buffer A. The dialyzed solution was then loaded on a Mono-Q HR 5/5 column (Pharmacia) previously equilibrated with buffer A, and the column was washed thoroughly with buffer A. The adsorbed enzyme was eluted using a linear gradient (0−0.5 M) of NaCl in buffer A at a flow rate of 0.5 mL/min. The active fractions for LFTase were collected and concentrated. After dialysis against buffer A, the enzyme solution was further purified by FPLC chromatograpy using a pre-equilibrated Superose 12 column with buffer A containing 0.1 M NaCl. Elution was performed at a flow rate of 0.3 mL/min, and the active fractions for LFTase were pooled and concentrated. After dialysis against buffer A, the enzyme solution was used as a purified LFTase. For molecular weight estimation of the purified enzyme, either sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (*9*) or gel permeation chromatography using Superose 12 column was performed, and the gel was visualized by staining with Coomassie brilliant blue. Buffer A containing 0.1 M NaCl was used as an elution buffer at flow rate of 0.2 mL/min. The void volume of the column was measured with blue dextran.

Assays of LFTase Activity. LFTase activity was assayed at 45 °C in 50 mM sodium phosphate buffer (pH 6.5). The amount of reducing sugar compounds released from levan was determined according to the Nelson−Somogyi method (*10*). One unit of LFTase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose per minute (*8*). Assay of LFTase activity by measurement of the reducing sugar production was based on the levan hydrolysis reaction; thus, DFA IV, oligosaccharides, and fructose also contributed to the enzyme activity. Quantitative analysis of DFA IV was done by HPLC (Beckman, Fullerton, CA) as follows. HPLC was performed under the following conditions: column, Shodex Ionpack KS-802 (Showa Denko Co., Tokyo, Japan); detector, RI detector (Beckman Coulter, Fullerton, CA); solvent system, distilled water, 100%; flow rate, 0.4 mL/min; room temperature. Protein concentration of enzymes was determined using the method described by Bradford in which a Bio-Rad protein assay kit was used (*11*).

Amino Acid N-Terminal Sequencing of the Purified Enzyme. To determine the N-terminal amino acid sequence, the protein bands on the SDS-PAGE gel were electrophoretically transferred to an Immobilon-PVDF membrane (Millipore, Schwalbach, Germany). The blotted PVDF membrane was stained with Coomassie brilliant blue, and the selected band was subjected to sequencing. The protein blotted onto the membrane was used for N-terminal amino acid sequence analysis using Procise 491 automatic sequencer (Applied Biosystems, Foster City, CA).

Purification and Characterization of the Reaction Product. The crude enzyme prepared by $(NH_4)_2SO_4$ precipitation was used for the production of DFA IV from levan. After enzyme reaction, 3 volumes of ethanol was added to the reaction mixture, and the mixture was centrifuged at 5000 rpm. The supernatant was collected and concentrated with a rotary evaporator (45 °C). To the concentrated solution was inoculated bakery yeast, which was cultured to remove sugars, and finally cells were removed by centrifugation. To crystallize the purified product, 3 volumes of ethanol was added to the solution and stored for 2 days at 4 °C. White crystals formed were collected, air-dried, and used for the characterization, as described previously (*8*). A ^{13}C nuclear magnetic resonance (NMR) spectrum was run at 400 MHz with a Bruker Avance 400 superconducting FT NMR spectrometer (Rheinstetten, Germany). A sample of DFA IV was dissolved in D_2O solution.

Analysis of Thermal Properties and Acid Stability. The thermal properties of the purified DFA IV and levan were observed using a differential scanning calorimeter (DSC, Shimadzu DSC-50, Kyoto, Japan) and a thermogravimetric analyzer (TGA, Shimazu TGA-50). On a weighed platinum pan, 10 mg each of purified DFA IV and levan was placed, covered, and reweighed. These samples were heated from 25 to 220 °C (700 °C for TGA) at a 10 °C/min increment in a nitrogen atmosphere. For the digestion by artificial gastric juice, 0.4 mL of a 5% (w/v) aqueous solution of levan or DFA IV was incubated with 0.2 mL of artificial gastric juice. The artificial gastric juice consisted of 2 g of NaCl and 7 mL of concentrated HCl (35%, v/v) in 1 L of solution. Hydrolysis reactions were terminated at various time intervals (0, 1, 2, 4, 6, and 16 h) by additions of 50 mM NaOH solution.

RESULTS AND DISCUSSION

Isolation and Identification of LFTase-Producing Microorganisms. Levan-degrading microorganisms from soil samples were collected from the solid media, and the products were analyzed by acid hydrolysis and HPLC analysis. Each single colony was subjected to screening after incubation in screening medium broth for 3 days, and the formation of DFA IV from levan was determined by HPLC analysis. About 350 isolates showed levan-hydrolyzing activity onto agar plates containing levan. On the basis of the retention times of the products, eight colonies were isolated as DFA IV producers. Strain J17-21 produced a relatively high level of DFA IV and showed the LFTase enzyme activity in a stable manner. As a consequence, this strain was chosen for further experiments. LFTase produced was an extracellular enzyme as found from other LFTase-producing bacteria (*12*−*14*).

Table 1. Cellular Fatty Acid Composition of *A. oxydans* J17-21

fatty acid	fatty acid composition (%, w/w)
13-methyltetradecanoic acid	14
12-methyltetradecanoic acid	51
14-methylpentadecanoic acid	8
hexadecanoic acid	4
14-methylhexadecanoic acid	6

Table 2. Summary of Purification Steps of LFTase from *A. oxydans* J17-21

step	total protein (mg)	activity (units)	specific activity (units/mg)	yield (%)	purification factor
culture supernatant	480.0	7400	15	100.0	1.0
$(NH_4)_2SO_4$ precipitation	153.8	3855	25	52.6	1.7
Q-Sepharose	4.3	2776	646	37.5	43.1
Mono-Q	0.21	246	1158	3.3	77.2
gel permeation	0.13	179	1432	2.0	95.5

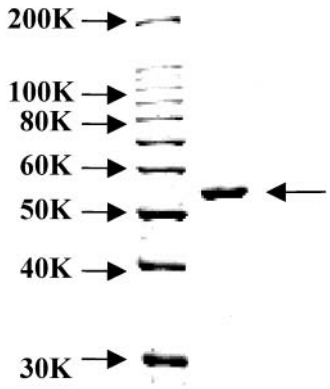

Figure 2. Determination of molecular weight of LFTase purified from *A. oxydans* J17-21 by SDS-PAGE: (lane 1) 10 kDa protein ladder (Gibco BRL); (lane 2) purified LFTase from *A. oxydans* J17-21.

Strain J17-21 represented a morphology as pleomorphic forms; most cells were rod-shaped in young cultures. As growth increased, however, the rod-shaped cells became shorter and were eventually replaced by oval-shaped cells. The strain exhibited the major characters of *Arthrobacter* sp. with Gram-positive and nonmotility behaviors. This strain appeared to be positive for oxygen requirement, catalase production, urease, and acetoin production and a negative for methyl red test, oxidase test, H_2S production, nitrate reduction, citrate utilization, and indole production. The predominating cellular fatty acids were 12-methyltetradecanoic acid (15:0 anteiso) and 13-methyltetradecanoic acid (15:0 iso) (**Table 1**). The cellular fatty acid components of strain J17-21 showed 77.3% similarity with *A. oxydans*. Consequently, the bacterium strain was designated *A. oxydans* J17-21.

Purification of LFTase from *A. oxydans* J17-21. The results of the purification of LFTase are summarized in **Table 2**. LFTase was purified 95.5-fold. We could obtain 0.13 mg (570 μL) of the purified LFTase enzyme with a specific activity of 1432 units/mg of protein from 480 mg of protein, which were obtained from 20 L culture broths. As shown in **Figure 2**, the purified LFTase gave a single band on SDS-PAGE with the molecular weight of ~54000 Da.

Properties of the Purified LFTase. The optimum reaction pH for LFTase was 6.5 in a sodium phosphate buffer (**Figure 3A**). Enzyme stability was tested by incubation at 4 °C for 22 h in buffers of various pH values, and it was stable over a wide range (pH 5.0−11.0). The optimum temperature for enzyme activity was at 45 °C (**Figure 3B**). To examine the temperature stability, the enzyme reaction mixture was incubated at various temperatures for 2 h in the same buffer without levan, and then the remaining activity was assayed at 45 °C for 30 min. The enzyme showed maximum stability at temperatures below 40 °C, at which ~87% of its original activity could be retained. To determine the hydrolysis pattern of the enzyme, monosaccharides (glucose, galactose, and fructose), disaccharides (sucrose, lactose, cellobiose, and levanbiose), oligosaccharides (raffinose, kestose, nystose, and fructosyl-nystose), or polysaccharides (levan, inulin, dextran, and starch) were reacted with the purified LFTase for 12 h at 37 °C, and subsequently, the products were analyzed by HPLC. Among the carbohydrates, LFTase was active only toward levan and did not release any detectable reducing sugar compounds from other substrates tested, indicating that the purified LFTase was a levan-degrading enzyme.

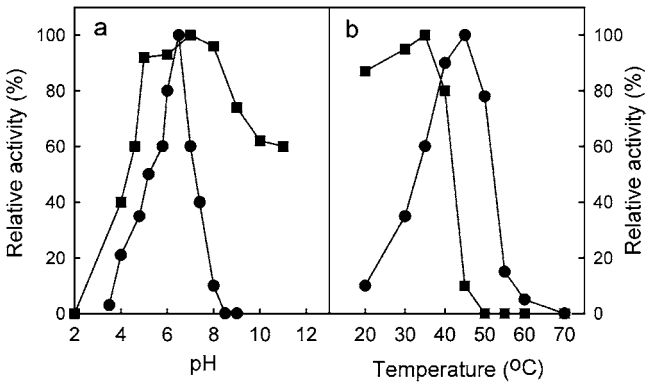

Figure 3. Characteristics of LFTase: (a) activity (●) and stability (■) of LFTase at various pH values; (b) activity (●) and stability (■) of LFTase at various temperatures.

Table 3. Effect of Metal Ions and Chemicals on the Relative Activity of LFTase from *A. oxydans* J17-21

metal ion/ chemical (1 mM)	relative activity[a] (%)	metal ion/ chemical (1 mM)	relative activity[a] (%)
none	100.0	$FeSO_4$	1.0
$MnCl_2$	56.1	$CuSO_4$	63.9
$MgCl_2$	90.8	Ag_2SO_4	0.0
$NaCl_2$	90.0	$HgSO_4$	15.8
$CaCl_2$	136.4	EDTA-2Na	127.7
$CoCl_2$	83.7	SDS	90.1
$NiCl_2$	71.0	urea	83.4
$ZnCl_2$	98.0		

[a] The relative activity of an enzyme compared with the activity without the presence of metal or chemicals.

Effects of Metal Ions and Chemicals on LFTase Activity. The effects of different metal ions on LFTase activity were examined under the standard assay conditions using 1 mM metal ions. As shown in **Table 3**, complete inhibition occurred in the presence of $FeSO_4$, $HgSO_4$, and Ag_2SO_4, and strong inhibition was seen in $MnCl_2$, $CuSO_4$, and $NiCl_2$. These results are also in good agreement with the fact that Ca^{2+} is required for the enhancement of the LFTase activity of *A. ureafaciens* K2032 (*8*), but different from LFTase activity of *Microbacterium* sp. AL-210 enhanced by 10 mM of $MgCl_2$ (*13*). Enzyme activity was not inhibited by the presence of a 1 mM concentration of the metal-chelating agent EDTA.

NH_2-Terminal Amino Acid Sequencing of LFTase. The N-terminal 20 amino acid residues of the purified LFTase were

Table 4. H_2N-Terminal Amino Acid Sequence Alignment of LFTase

Source	H_2N-Terminal sequence
A.oxydance J17-21	A Q-G-S Q-X-A-V-Y X-M T P-P-S-G-W-L-X
A.ureafaciens K2032	S-A P-G-S L-X-A V-Y H-M T P-X-L-X-D-P-G
A.nicotinovorans GS-9	H-A Q-A-S L-R-A I-Y H-M T P-P-S-G-W-L-C
Microbacterium sp. AL-210	A-A S-G-S L-R-A V-Y H-M T P-P-S-G-W-L-C

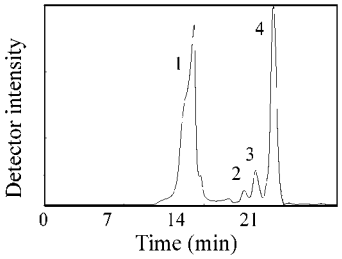

Figure 4. Conversion efficiency from levan to DFA IV using levan and an LFTase. A 5% levan solution was incubated with 10 units of enzyme at 45 °C. Peaks: 1, limited levan; 2 and 3, oligosaccharides; 4, DFA IV.

determined as follows: AQGSQXAVYXMTPPSGWLXD. As shown in **Table 4**, the N-terminal amino acid sequence of the LFTase from *A. oxydans* J17-21 represented high similarity with those of both *A. nicotinovorans* GS-9 and *Microbacterium* sp. AL-210; the seven conserved amino acid residues were found together with that of *A. nicotinovorans* GS-9, *A. ureafaciens* K2032, and *Microbacterium* sp. AL-210 (*8*, *12*−*14*). The first residue of the LFTase from *A. oxydans* J17-21 was, however, alanine, which was different from the others which had serine or histidine as the starting amino acid, except *Microbacterium* sp. AL-210.

Mode of Levan-Degrading Reaction Catalyzed by LFTase. To gain further information on the enzymatic products formed from levan by LFTase, enzyme reaction was performed in the mixture containing 5% (w/v) levan in 50 mM sodium phosphate buffer (pH 6.5) at 45 °C. After 48 h of the reaction, enzymatic reaction reached the steady state. Final products contained DFA IV (35%) and limited levan (55%) as the main products (**Figure 4**). Oligosaccharides and fructose were also found as the minor products. The conversion yield (35%) of *Z. mobilis* levan to DFA IV with LFTase from *A. oxydans* J17-21 was lower than that of *Serratia levanicum* levan by LFTase isolated from *A. nicotinovorans* GS-9 (73%) (*13*). It has been suggested that DFA IV production by LFTase is terminated when the enzyme meets the β-2,1 branch point in levan (*3*). On the basis of this hypothesis, they suggested that low-branched levan is the most suitable substrate for DFA IV production (*3*). Therefore, the difference in the conversion yields between the present study and other studies might be explained by the different source of levans used; the degrees of branching of *Z. mobilis* levan and *S. levanicum* levan are 12 and 6%, respectively. Further works in the production of linear levan and its application for the production of DFA IV are warranted.

DFA IV was isolated from the reaction mixture and analyzed by HPLC, NMR, and DSC. Hydrolysis of the purified product under the conditions of 0.1 N HCl and 100 °C generated fructose only in HPLC analysis. The product, DFA IV, showed a melting point at 180.38 °C (**Figure 5**). ^{13}C NMR analysis showed six main resonances at 103.5, 81.2, 77.1, 72.1, 60.7, and 59.6 ppm. The same result was observed with DFA IV, which was reported by Saito and Tomita (*3*). The above results clearly suggest that the major reaction product formed from levan by enzymatic reaction was a DFA IV.

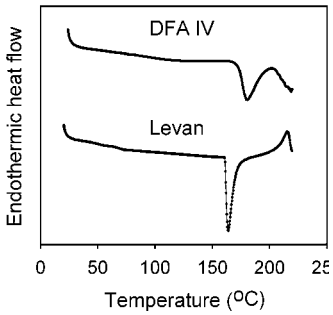

Figure 5. DSC endothermic curve of the purified DFA IV and levan.

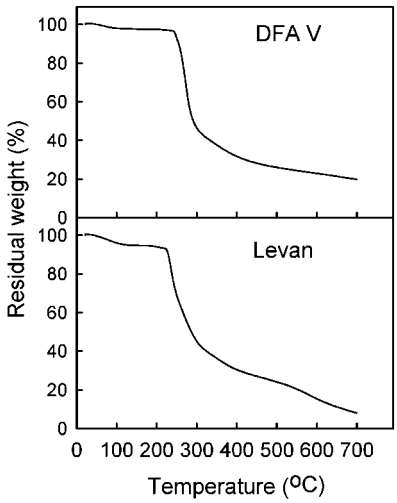

Figure 6. TGA curves of DFA IV and levan.

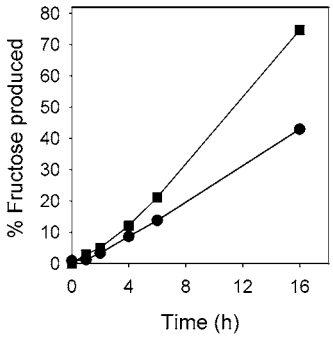

Figure 7. Hydrolysis of DFA IV (■) and levan (●) by acid treatment. Five percent solutions were incubated with acid at 37 °C for up to 16 h, and then the proportions of fructose were determined using HPLC.

Analysis of Thermal Properties and Acid Tolerance of DFA IV. In **Figure 6**, the TGA curves show that the weights of DFA IV and levan change according to temperature in a single step. A 2−5% weight loss between 60 and 100 °C occurred, suggesting that the occurrence of evaporation or boiling of water accompanied the temperature change. It was determined that the dissociation of DFA IV occurred between 290 and 330 °C due to the weight loss of 50−60%. The effects of acid treatment on levan and DFA IV were evaluated. When levan and DFA IV solutions were incubated with artificial gastric juice (0.25% as the final concentration, pH 1.4), the hydrolysis reaction progressed in a linear fashion; amounts of fructose released from DFA IV were 1.3% (in 1 h), 3.4% (in 2 h), 8.7% (in 4 h), 13.8% (in 6 h), and 43.0% (in 16 h), respectively (**Figure 7**). DFA IV was more stable in acid hydrolysis and

gradually reduced during the incubation. The formation rates of fructose generated from levan and DFA IV were 4.42%/h in levan and 2.60%/h in DFA IV. At pH 5.0, no or little fructose was released from DFA IV in extended incubation for 30 days at 4 °C. These results indicated that the structure of DFA IV is very stable in acidic conditions and, thereby, it has favorable properties for use as a food material.

In conclusion, an LFTase was characterized with respect to enzymatic properties and the production of DFA IV. LFTase showed very limited substrate specificity and had relatively stable enzymatic properties. Results from heat and acid stability experiments on DFA IV provide a strong indication of its potential use as a food material.

ACKNOWLEDGMENT

We gratefully acknowledge RealBioTech Co. Ltd, for generously providing DFA IV and levan samples.

LITERATURE CITED

(1) Franck, A.; Leenheer, L. De. In *Biopolymers. Vol. 6, Inulin*; De Baets, S., Vandamme, E. J., Steinbuchel, A., Eds.; Wiley-VCH Verlag: Weinheim, Germany, 2002; pp 439−480.

(2) Rhee, S. K.; Song, K. B.; Kim, C. H.; Park, B. S.; Jang, E. K.; Jang, K. H. In *Biopolymers. Vol. 5, Levan*; De Baets, S., Vandamme, E. J., Steinbuchel, A., Eds.; Wiley-VCH Verlag: Weinheim, Germany, 2002; pp 351−377.

(3) Saito, K.; Tomita, F. Difructose anhydrides: their mass-production and physiological functions. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1321−1327.

(4) Saito, K.; Hira, T.; Suzuki, T.; Hara, H.; Yokota, A.; Tomita, F. Effects of DFA IV in rats: calcium absorption and metabolism of DFA IV by intestinal microorganisms. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 655−661.

(5) García, F. J. M.; Gadelle, A.; Defaye, J. Difructose dianhydrides from sucrose and fructooligosaccharides and their use as building blocks for the preparation of amphiphiles, liquid crystals, and polymers. *Carbohydr. Res.* **1994**, *265*, 249−269.

(6) Christian, T. J.; Field. R. J.; Parker, B. A.; Manley-Harris, M. Kinetics of formation of di-D-fructose dianhydrides during thermal treatment of inulin. *J. Agric. Food. Chem.* **2000**, *48*, 1823−1837.

(7) Jang, K. H.; Song, K. B.; Kim, C. H.; Chung, B. H.; Kang, S. A.; Chun, U. H.; Choue, R. W.; Rhee, S. K. Comparison of characteristics of levan produced by different preparations of levansucrase from *Zymomonas mobilis*. *Biotechnol. Lett.* **2001**, *23*, 339−344.

(8) Song, K. B.; Bae, K. S.; Lee, Y. B.; Lee, K. Y.; Rhee, S. K. Characteristics of levan fructotransferase from *Arthrobacter ureafaciens* K2032 and difructose anhydride IV formation from levan. *Enzyme Microb. Technol.* **2000**, *27*, 212−218.

(9) Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(10) Somogyi, M. Notes on sugar determination. *J. Biol. Chem.* **1952**, *195*, 19−23.

(11) Bradford, M. M. A rapid sensitive method for quantification of microgram quantities of protein utilizing the principle of protein of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(12) Kuniji, T. Production of a non-reducing fructotrisaccharide from levan in the culture of *Arthrobacter ureafaciens*. *Agric. Biol. Chem*. **1989**, *53*, 2275−2276.

(13) Saito, K.; Yokoda, A.; Tomita, F. Molecular cloning of levan fructotransferase from *Arthrobacter nicotinovorans* GS-9 and its expression in *Escherichia coli. Biosci., Biotechnol., Biochem.* **1997**, *61*, 2076−2079.

(14) Cha, J.; Park, N. H.; Yang, S. J.; Lee, T. H. Molecular and enzymatic characterization of a levan fructotransferase from *Microbacterium* sp. AL-210. *J. Biotechnol*. **2001**, *91*, 49−61.

Received for review December 9, 2002. Revised manuscript received February 11, 2003. Accepted February 11, 2003. This work has been supported by a grant (M10015000009-02B210000813) from the Ministry of Science and Technology, Republic of Korea. We deeply appreciate this financial assistance.

JF026207O