J. Microbiol. Biotechnol. (2005), **15**(1), 99–104

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Action Mechanism of Transfructosylation Catalyzed by *Microbacterium laevaniformans* Levansucrase

KIM, MIN-JEONG, HAE-EUN PARK, HEE-KYUNG SUNG, TACK HYUN PARK, AND JAEHO CHA*

Department of Microbiology, Pusan National University, Busan 609-735, Korea

Received: March 2, 2004
Accepted: June 14, 2004

Abstract *Microbacterium laevaniformans* levansucrase synthesized various hetero-oligosaccharides by transferring fructosyl residue from sucrose to various saccharides as acceptors. The acceptor specificity test showed that reducing saccharides were more favorable acceptors than nonreducing saccharides. The transfructosylated product, fructosyl galactose, was produced in the presence of D-galactose as an acceptor. The chemical structure of the resulting fructosyl galactose was analyzed by yeast invertase and NMR, and identified as *O*-α-D-galactosyl-(1→2)-β-D-fructofuranoside. These results indicate that the main transfructosylation activity of the enzyme is to make nonreducing transferred products via a transfer of fructosyl residue to acceptor molecules having reducing group. When nonreducing sugars, such as methyl α-D-glucoside and methyl α-D-galactoside, were used as an acceptor, the transfer product was also formed in spite of the reducing group blocked with methyl group. The fact that no transfer product was formed with sugar alcohols as acceptors was suggested to be due to marked conformational difference of acceptors.

Key words: Levansucrase, transfructosylation, hetero-oligosaccharide

Fructans are produced by microorganisms and some higher plants and composed of fructose polymers attached to sucrose [7, 14]. They are implicated in drought and frost tolerance in plants [12, 17], however, little is known of their function in bacteria. Generally, fructans are divided into levan (β-2,6) and inulin (β-2,1), depending on fructose linkage. In plants, biosynthesis of fructan is carried out by a series of fructosyltransferases which have different specificities [2], however, levan synthesis is catalyzed by a single enzyme. Levansucrase belongs to glycoside hydrolase (GH) family 68 and synthesizes levan from sucrose by the reaction: sucrose+acceptor→glucose+fructosyl-acceptor. At low sucrose concentrations, it functions as a hydrolase with water as acceptor, whereas it adds fructosyl units to a growing levan chain at higher substrate concentrations [3]. The reaction has been known to occur via a Ping-Pong mechanism, involving the formation of a transient fructosyl-enzyme intermediate [4]. Tertiary structure of *Bacillus subtilis* levansucrase at a resolution of 1.5 Å has recently been elucidated and strongly supports a fructosyl-enzyme intermediate [10].

In addition to the levan formation, the enzyme also catalyzes fructosyl transfer from sucrose to numerous acceptors other than levan, including mono- and oligosaccharides and short-chain acylalcohols [9, 11, 13, 18]. It is important to elucidate the action mechanism of transfructosylation catalyzed by levansucrase to define the accurate chemical structures of transferred fructosyl derivatives. Although there have been considerable attempts to produce fructosyl derivatives, the transfructosylation mechanism of this enzyme is still poorly understood.

In the present work, the acceptor specificity of levansucrase from *Microbacterium laevaniformans* was investigated using various acceptors, and an essential structure of acceptor product catalyzed by the enzyme was evaluated.

Materials and Methods

Materials

Sugars and other chemicals, including methyl α-D-glucose, methyl α-D-galactose, xylitol, lactitol, and mannitol, were from Sigma (St. Loius, MO, U.S.A.). Invertase (Grade V, from *Saccharomyces cerevisiae*), β-galactosidase (from *Escherichia coli*), and α-glucosidase (type I, from *Saccharomyces cerevisiae*) were purchased from Sigma. Levan was prepared by laboratory-scale production using levansucrase from *Microbacterium laevaniformans* [13].

*Corresponding author
Phone: 82-51-510-2196; Fax: 82-51-514-1778;
E-mail: jhcha@pusan.ac.kr

Kieselgel 60 F_{254} TLC plates were purchased from Merck (Darmstadt, Gemrmany).

Microbial Strain and Culture Condition

Microbacterium laevaniformans ATCC 15953 was grown aerobically at 30°C in medium containing 3% sucrose, 1% bactopeptone, 0.3% yeast extract, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. When appropriate, medium was solidified with 20 g/l of agar. All media were autoclaved for 15 min at 121°C. Sugars were autoclaved separately.

Preparation of Levansucrase

Levansucrase was prepared as previously described [13]. The microorganism was grown for 24 h at 30°C, and the culture was then diluted two volumes with 20 mM sodium phosphate buffer (pH 6.0). The cells were harvested, resuspended in 20 mM sodium phosphate buffer (pH 6.0), and disrupted by ultrasonication at 4°C. The cell debris was removed by centrifugation, and levansucrase was precipitated out with 60% ammonium sulfate. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.5), dialyzed against the same buffer, and loaded onto a DEAE-Sepharose Fast Flow column (2.5×10 cm, Pharmacia, Uppsala, Sweden). The eluted enzyme fraction by 1 M NaCl gradient was concentrated by ultrafiltration (10 kDa MWCO, Amicon) and further purified by a Sephacryl S-100 column (1.5×75 cm, Pharmacia, Uppsala, Sweden). The active fractions were pooled, concentrated, and used as the enzyme solution for further analysis.

Enzyme Assays

One ml reaction mixture containing 5% sucrose in 20 mM sodium phosphate buffer (pH 6.0) and the enzyme solution was incubated at 30°C. Levan-forming activity was determined by measuring the change in turbidity at 540 nm [1]. One unit (U) of enzyme activity was defined as the amount of enzyme needed for the formation of 1 mg levan per h. Activity of an invertase was measured at 55°C for 12 h. The reaction was carried out using various acceptor reaction mixtures and 5 U enzyme solution in 20 mM sodium phosphate buffer (pH 6.0). Activities of β-galactosidase and α-glucosidase were assayed at 37°C for 12 h. The reaction was carried out using lactose or maltose acceptor reaction mixture and 5 U enzyme solution, respectively.

Substrate and Acceptor Specificity of Levansucrase

To determine the substrate specificity of the enzyme, sucrose was replaced with various kinds of sugars. The reaction mixture containing 10% substrate and 1 U enzyme solution in 20 mM sodium phosphate buffer (pH 6.0) was incubated at 30°C for 24 h, and the reaction products were analyzed by TLC [15]. For the acceptor specificity of the enzyme, various acceptors were added to the enzyme reaction. The transfer products, produced by incubating 5 U levansucrase and 5% sucrose in 20 mM sodium phosphate buffer (pH 6.0) with 5% acceptors at 30°C for 6 h, were analyzed by TLC.

Isolation of the Transfer Product

The reaction products were concentrated by membrane filter (Biomax-30K, Millipore), freeze-dried, and then concentrated. The concentrate was loaded onto a recycling preparative LC column (JAI Co., Japan), equilibrated with distilled water, and the column was eluted at a flow rate of 3.0 ml/min, which is equal to a column pressure of 18 kgf/cm^2. The fraction having the reaction product was isolated and applied to NMR.

Analytical Methods

Denatured proteins were separated in 12% SDS polyacrylamide gels and stained with Coomassie Blue R-250. Sugars produced by the enzyme reaction were analyzed by TLC [8]: An aliquot of the reaction mixture was spotted onto a silica gel 60 F_{254} plate with a capillary tube, and the chromatogram was then developed with a solvent system of butanol/acetic acid/water (5:4:1, v/v/v). After irrigating twice, the TLC plate was dried and visualized by dipping it into 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine, 5% (v/v) sulfuric acid in methanol, followed by heating at 120°C for 10 min. The sugars on the TLC plate were quantitated by densitometry [15]. The ^1H-^1H COSY nuclear magnetic resonance spectrum was recorded at 500 MHz with a Unity Inova-500 NMR system (Varian, U.S.A.). The probe temperature was kept at 20°C, and chemical shifts were

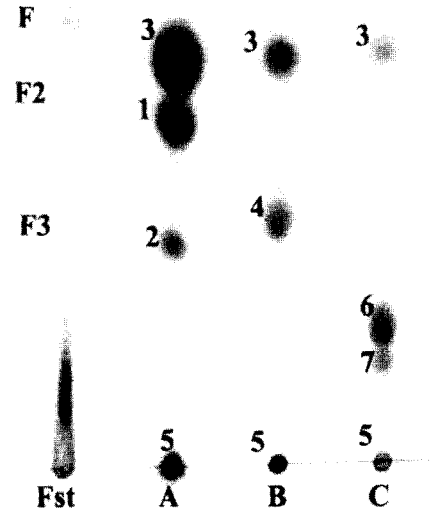

Fig. 1. TLC analysis of the reaction products produced by *M. laevaniformans* levansucrase with sucrose (A), raffinose (B), and stachyose (C) as substrates.

F_{ST}, partial hydrolysate of standard levan (f, fructose; f2, levanbiose; f3, levantriose); 1, sucrose; 2, 1-kestose; 3, glucose/fructose; 4, raffinose; 5, levan; 6, stachyose; 7, fructosyl stachyose.

measured by reference to the chemical shift in D_2O without internal standard.

RESULTS AND DISCUSSION

Substrate Specificity of *M. laevaniformans* Levansucrase

Levansucrase produces levan when sucrose is used as a substrate. In order to examine the catalytic mechanism of levansucrase in detail, the enzyme was reacted with mono-, di-, tri-, and tetrasaccharides which have diverse glycosidic linkages. TLC analysis showed that levansucrase produced levan and other fructo-oligosaccharides when sucrose, raffinose, and stachyose were used (Fig. 1). The rest of the sugars used for substrates did not give rise to any reaction products (Table 1), implying that the enzyme cleaves the α-glucose-(1→2)-β-fructose linkage. However, the enzyme did not cleave 1-kestose or nystose which has the same α-glucose-(1→2)-β-fructose linkage. These results indicate that the enzyme specifically hydrolyzes saccharides with terminal sucrose residues to synthesize levan.

Acceptor Specificity of *M. laevaniformans* Levansucrase

The transfructosylation activity of levansucrase has an interesting feature in terms of production of hetero-oligosaccharides with a fructose residue. Although the yield was relatively lower than levan (β-2,6 linked fructose polymer), the production of 1-kestose and nystose, in which one and two fructosyl units are bound at the β-2,1 position of sucrose, respectively, indicates that the enzyme catalyzes fructosyl transfer at the β-2,1 position as well as β-2,6 position. When various compounds were added as acceptors, the enzyme transferred the fructosyl moiety of sucrose to other fructose acceptor compounds. Table 2 shows the acceptor specificity of the enzyme. The enzyme showed broad acceptor specificity: Reducing saccharides were more favorable acceptors than nonreducing saccharides, hydrogenated saccharides, and alcohols. In general, the good acceptor substrates were saccharides having a pyranose ring in their chemical structure, as we reported previously [13]. When saccharides having terminal fructose unit were used as acceptors, lactulose, levanbiose, and palatinose were better acceptors than 1-kestose and nystose. This result suggests that saccharides having a reactive hemiacetal hydroxyl group are preferred acceptors. Similar mechanism was also observed in *Arthrobacter* sp. β-fructofuranosidase [5]: The main transfer products to reducing saccharides by the action of transglycosylation of *Arthrobacter* sp. β-fructofuranosidase were nonreducing oligosaccharides with a fructosyl residue linked to a hemiacetal hydroxyl groups. When a nonreducing sugar such as methyl α-glucoside and methyl α-galactoside were used as acceptors, the transfer product was also formed, even though the reducing group is blocked with methyl group. This result was also consistent with the results observed in *Arthrobacter* sp. β-fructofuranosidase [5].

Interestingly, mannose which has a pyranose ring was not used as an acceptor, unlike other monosaccharides such as glucose and galactose. This result was not in agreement with the result of another group, in which mannosucrose was synthesized by *Bacillus subtilis* levansucrase [16]. It has been known that the transfer specificities of fructosyltransferase family enzymes are dependent on the structure of an acceptor. The study of the acceptor specificity of *Arthrobacter*

Table 1. Substrate specificity of *M. laevaniformans* levansucrase.

Substrate	Structure	Activity
D-Arabinose		-
D-Xylose		-
D-Glucose		-
D-Fructose		-
D-Galactose		-
Sucrose	**α-Glc *p*-(1→2)-β-Fru *f***	+++
Melibiose	α-Gal *p*-(1→6)-Glc *p*	-
Cellobiose	β-Glc *p*-(1→4)-Glc *p*	-
Maltose	α-Glc *p*-(1→4)-Glc *p*	-
Trehalose	α-Glc *p*-(1→1)-α-Glc *p*	-
Lactulose	β-Gal *p*-(1→4)-Fru *f*	-
Levanbiose	β-Fru *f*-(2→6)-Fru *f*	-
Palatinose	α-Glc *p*-(1→6)-Fru *f*	-
1-Kestose	α-Glc *p*-(1→2)-β-Fru *f*-(1→2)-β-Fru *f*	-
Raffinose	α-Gal *p*-(1→6)-**α-Glc *p*-(1→2)-β-Fru *f***	+++
Nystose	α-Glc *p*-(1→2)-β-Fru *f*-(1→2)-β-Fru *f*-(1→2)-β-Fru *f*	-
Stachyose	α-Gal *p*-(1→6)-α-Gal *p*-(1→6)-**α-Glc *p*-(1→2)-β-Fru *f***	+

Abbreviations: Glc*p*, glucopyranoside; Fru*f*, fructofuranoside; Gal*p*, galactopyranoside.
Activity is expressed as: more than 50%, +++; less than 30%, +; not detected, -.

Table 2. Acceptor specificity of *M. laevaniformans* levansucrase.

Acceptor	Transfer product	Predicted structure of transfer product
D-Arabinose	(+)	α-Ara *p*-(1→2)-β-Fru *f*
D-Xylose	(+)	α-Xyl *p*-(1→2)-β-Fru *f*
D-Galactose	GaF (++)	α-Gal *p*-(1→2)-β-Fru *f*[a]
D-Mannose	ND	
Melibiose	MeF (+++)	α-Gal *p*-(1→6)-α-Glc *p*-(1→2)-β-Fru *f*[b]
Cellobiose	CF (+++)	β-Glc *p*-(1→4)-α-Glc *p*-(1→2)-β-Fru *f*
Maltose	MF (+++)	α-Glc *p*-(1→4)-α-Glc *p*-(1→2)-β-Fru *f*[c]
Trehalose	ND	
Lactose	LF (+++)	β-Gal *p*-(1→4)-α-Glc *p*-(1→2)-β-Fru *f*[d]
Lactulose	(+)	β-Gal *p*-(1→4)-Fru *f*-(1→2)-β-Fru *f*
Levanbiose	(+)	β-Fru *f*-(2→6)-Fru *f*-(1→2)-β-Fru *f*
Sophorose	(+)	β-Glc *p*-(1→2)-α-Glc *p*-(1→2)-β-Fru *f*
Palatinose	(+)	α-Glc *p*-(1→6)-Fru *f*-(1→2)-β-Fru *f*
1-Kestose	ND	
Nystose	ND	
Methyl glucoside	(+)	β-Fru *f*-(2→6)-MGlc *p*
Methyl galactoside	(+)	β-Fru *f*-(2→6)-MGal *p*
Xylitol	(+/-)	
Lactitol	(+/-)	
Mannitol	ND	

Abbreviations: Glc*p*, glucopyranoside; Fru*f*, fructofuranoside; Gal*p*, galactopyranoside; Ara*p*, arabinopyranoside; Xyl*p*, xylopyranoside; MGlc*p*, methyl glucopyranoside; MGal*p*, methyl galactopyranoside. Transfer efficiency is expressed as a fructosyl product area/sucrose area by TLC densitometry: more than 50%, +++; 30 to 50%, ++; less than 30%, +; below 10%, +/-; not detected, ND.

[a]Confirmed by invertase and NMR.

[b]Confirmed by NMR (Ref. 13).

[c]Confirmed by invertase and α-glucosidase.

[d]Confirmed by invertase and β-galactosidase.

sp. β-fructofuranosidase revealed that an equatorial hydroxyl groups at C2 and C3 on chair conformation are necessary for an acceptor [6]. In the case of mannose, having the axial hydroxyl group at C2, the steric hindrance around the C2 with fructose residue coming from donor molecule is expected. The observation that there was no transfer product with sugar alcohols has been suggested to be due to a marked conformational difference of acceptors in the aqueous solution.

Purification and Characterization of Fructosyl Oligosaccharides

The transfructosylated products produced with various acceptors were isolated and purified by recycling HPLC.

Table 3. Enzymatic hydrolysis products of transfructosylated products synthesized by *M. laevaniformans* levansucrase.

Transfer product	Invertase	β-Galactosidase	α-Glucosidase
GaF	Gal, Fru		
MeF	Mel, Fru		
CF	Cel, Fru		
LF	Lac, Fru	Gal, Suc	
MF	Mal, Fru		2Glc, Fru

Abbreviations: Gal, galactose; Mel, melibiose; Cel, cellobiose; Lac, lactose; Mal, maltose; Fru, fructose; Suc, sucrose; Glc, glucose.

The chemical structure of each purified fructosyl oligosaccharide was then examined by yeast invertase, *E. coli* β-galactosidase, and yeast α-glucosidase (Table 3). The galactose transfer product (GaF) and melibiose transfer product (MeF) were incubated with invertase, and the reaction products were analyzed by TLC (Fig. 2). GaF was hydrolyzed by yeast invertase to give rise to galactose and fructose in the molar ratio of 1:1. Also, MeF was hydrolyzed to melibiose and fructose in the molar ratio of 1:1. All other reaction products were also hydrolyzed by invertase into acceptor and fructose molecules. These results indicate that C2-OH of the fructose residue from sucrose is linked to the acceptors, because an invertase specifically catalyzes the hydrolysis of the terminal nonreducing β-fructofuranoside residues in β-fructofuranosides.

The structure of GaF was confirmed by NMR spectroscopy. ^1H-^1H COSY spectra of GaF in D_2O at 20°C revealed that C2-OH of fructose linked to the C1-OH of galactose (Fig. 3). The lactose transfer product (LF) and the maltose transfer product (MF) were incubated with β-galactosidase and α-glucosidase, respectively. As shown in Table 3 and Fig. 2, LF was hydrolyzed to galactose and sucrose in the molar ratio of 1:1, and MF was hydrolyzed to glucose and fructose in the molar ratio of 2:1. These results indicate

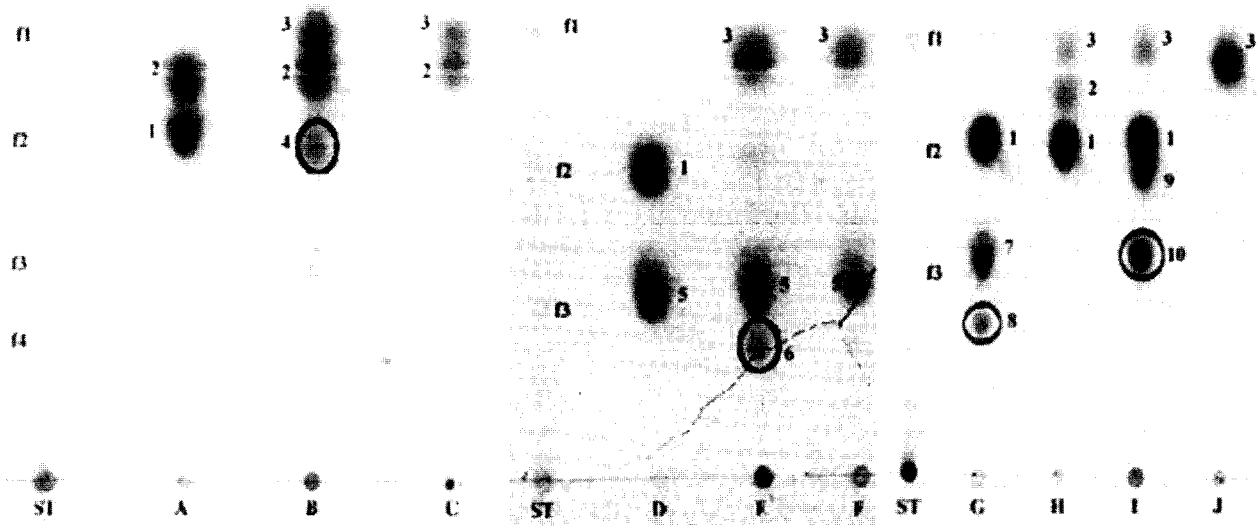

Fig. 2. Hydrolysis of the transfructosylated products of *M. laevaniformans* levansucrase by invertase, β-galactosidase, and α-glucosidase.

Numbered circles indicate the transfructosylated products. ST, partial hydrolysate of standard levan (f, fructose; f2, levanbiose; f3, levantriose; f4, levantetraose); lane A, before the acceptor reaction with galactose; lane B, after the acceptor reaction with galactose; lane C, after invertase treatment; lane D, before the acceptor reaction with melibiose; lane E, after the acceptor reaction with melibiose; lane F, after invertase treatment; lane G, after the acceptor reaction with lactose; lane H, after β-galactosidase treatment; lane I, after the acceptor reaction with maltose; lane J, after α-glucosidase treatment. 1, sucrose; 2, galactose; 3, glucose/fructose; 4, galactose acceptor product; 5, melibiose; 6, melibiose acceptor product; 7, lactose; 8, lactose acceptor product; 9, maltose; 10, maltose acceptor product.

the presence of a β-galactosidic linkage in LF and two α-glucosidic linkages in MF. Furthermore, this result confirms a β-2,1 glycosidic linkage between acceptor and fructose residue by the enzyme reaction. These results, therefore, led us to conclude that the transfructosylation activity of *Microbacterium levaniformans* levansucrase produces nonreducing transferred products via transfer of fructosyl residue from sucrose to acceptor molecules which have a reducing group.

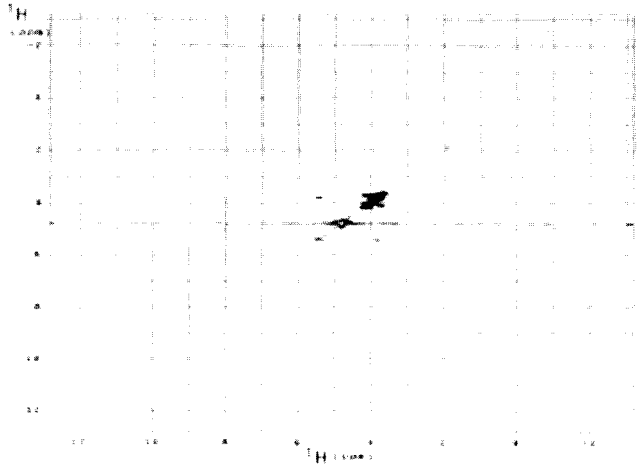

Fig. 3. ^{1}H-^{1}H COSY spectrum of fructosyl galactose synthesized by *M. laevaniformans* levansucrase in D_2O at 20°C.

Acknowledgments

This work was supported by the Korea Research Foundation Grant (KRF-2001-041-G00068). The authors wish to thank Kwang Jae Shon at the Pusan National University Labcenter for the measurement of NMR spectrum. T. H. Park is the recipient of graduate student fellowships supported by the Brain Busan 21 Project in 2003.

References

1. Ananthalakshmy, V. K and P. Gunasekaran. 1999. Overproduction of levan in *Zymomonas mobilis* by using cloned *sacB* gene. *Enzyme Microb. Technol.* **25:** 109-115.
2. Cairns, A. J. 1993. Evidence for the *de novo* synthesis of fructan by enzymes from higher plants; a reappraisal of the SST/FFT model. *New Phytologist* **123:** 15-24.
3. Chambert, R., G. Treboul, and R. Dedonder. 1974. Kinetic studies of levansucrase of *Bacillus subtilis*. *Eur. J. Biochem.* **41:** 285-300.
4. Chambert, R. and G. Gonzyl-Treboul. 1976. Levansucrase of *Bacillus subtilis*: Kinetic and thermodynamic aspects of transfructosylation processes. *Eur. J. Biochem.* **62:** 55-64.
5. Fujita, K., N. Kuwahara, T. Tanimoto, K. Koizumi, M. Iizuka, N. Munamiura, K. Furuichi, and S. Kitahata. 1994. Chemical structures of hetero-oligosaccharides produced by *Arthrobacter* sp. K-1 β-fructofuranosidase. *Biosci. Biotech. Biochem.* **58:** 239-243.

6. Fujita, K., K. Hara, H. Hashimoto, and S. Kitahata. 1990. Purification and some properties of β-fructofuranosidase I from *Arthrobacter* sp. K-1. *Agric. Biol. Chem.* **54:** 913-919.
7. Hendry, G. A. F. and R. K. Wallace. 1993. The origin, distribution and evolutionary significance of fructans, pp. 119-139. *In*: Suzuki M, Chatterton N. J. (eds.). *Science and Technology of Fructans*. Boca Raton: CRC Press.
8. Kim, H., H.-E. Park, M.-J. Kim, H. G. Lee, J.-Y. Yang, and J. Cha. 2003. Enzymatic characterization of a recombinant levansucrase from *Rahnella aquatilis* ATCC 15552. *J. Microbiol. Biotechnol.* **13:** 230-235.
9. 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*. *Enz. Microb. Tech.* **27:** 646-651.
10. Meng, G. and K. Fütterer. 2003. Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. *Nat. Struct. Biol.* **10:** 935-941.
11. Ohtsuka, K., S. Hino, T. Fukushima, O. Ozawa, T. Kanematsu, and T. Uchida. 1992. Characterization of levansucrase from *Rahnella aquatilis* JCM-1683. *Biosci. Biotechnol. Biochem.* **56:** 1373-1377.
12. Park, J. M., S. Y. Kwon, K. B. Song, J. W. Kwak, S. B. Lee, Y. W. Nam, J. S. Shin, Y. I. Park, S. K. Rhee, and K. H. Paek. 1999. Transgenic tobacco plants expressing the bacterial levansucrase gene show enhanced tolerance to osmotic stress. *J. Microbiol. Biotechnol.* **9:** 213-218.
13. Park, H.-E., N. H. Park, M.-J. Kim, T. H. Lee, H. G. Lee, J.-Y. Yang, and J. Cha. 2003. Enzymatic synthesis of fructosyl oligosaccharides by levansucrase from *Microbacterium laevaniformans* ATCC 15953. *Enzyme Microb. Tech.* **32:** 820-827.
14. Pollock, C. J. and A. J. Cairns. 1991. Fructan metabolism in grasses and cereals. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42:** 77-101.
15. Song, E. K., H. Kim, H. K. Sung, and J. Cha. 2002. Cloning and characterization of a levanbiohydrolase from *Microbacterium laevaniformans* ATCC 15953. *Gene* **291:** 45-55.
16. Tanaka, T., S. Yamamoto, and T. Yamamoto. 1981. Structures of hetero-oligosaccharides synthesized by levansucrase. *J. Biochem.* **90:** 521-526.
17. Vijn, I. and S. Smeekens. 1999. Fructan: More than a reserve carbohydrate? *Plant Physiol.* **120:** 351-359.
18. Yanase, H., M. Iwata, R. Nakahigashi, K. Kita, and K. Tonomura. 1992. Purification, crystallization, and properties of the extracellular levansucrase from *Zymomonas mobilis*. *Biosci. Biotechnol. Biochem.* **56:** 1335-1337.