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Enzymatic Characterization of a Recombinant Levansucrase from *Rahnella aquatilis* ATCC 15552

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Abstract A 1.25 kb DNA fragment including the *lscR* gene, which encodes a levansucrase of *Rahnella aquatilis* ATCC 15552, was subcloned into a high-expression vector, pET-29b, and the recombinant enzyme was overexpressed in *Escherichia coli*. Most of the levansucrase activity was detected in the cytoplasmic fraction after induction with isopropyl β-D-thiogalactoside. The recombinant enzyme with a tag of six histidine residues at the C-terminus was purified 146-fold by affinity and gel-filtration chromatographies. The molecular mass of the purified LscR was approx. 49 kDa as determined by SDS-PAGE. The optimum pH and temperature of this enzyme for levan formation was pH 6.0 and 30°C, respectively. The optimum substrate concentration for levan formation was 300 mM sucrose. Levan formation was increased by the increase of the enzyme concentrations. Maxium yield of levan formation at optimum substrate concentration, pH, and temperature after 24 h of reaction was approximately 80%.

Key words: Fructooligosaccharide, levansucrase, levan, *Rahnella aquatilis*

Levansucrases are a family of bacterial enzymes with β-fructofranosyl fructotransferase activity (EC 2.4.1.10) [6]. They can produce different types of D-fructose poly-(levan) and oligomers (1-kestose and nystose) from sucrose. Levan is a β-2,6-linked fructose homopolymer with some β-2,1-linked branching points. Levan produced in some plants is known to serve as an osmoprotectant, and recently, the transgenic tobacco plant containing a levansucrase gene has been demonstrated to exhibit an increased tolerance to drought stress [21]. Commercial interest in the production of levan has been emphasized in recent years. The high solubility and the low viscosity of an aqueous levan solution would make it a suitable substitute for arabic gum [10]. Levan has also been shown to have certain biological functions, such as the promotion of necrosis and tumor inhibition [17]. Application of levan as an emulsifier, formulation aid, stabilizing thickener, surface-finishing agent, and carrier for color or flavor in the fields of food, cosmetics, and pharmaceuticals, has been suggested [4]. Therefore, the efficient production of levan by bacterial levansucrase has a great potential for its industrial application. Belghith *et al.* [3] reported the optimal conditions for levan production by using *Zymomonas mobilis* levansucrase. Previously, the levansucrase gene (*lscR*) from *Rahnella aquatilis* ATCC 15552 was cloned and the structural feature of the enzyme was examined by comparison with other levansucrases [13].

In the present work, the high-level expression of the recombinant *R. aquatilis* levansucrase in *E. coli* was achieved and the optimum conditions for levan formation and substrate specificity were investigated by using the purified recombinant enzyme.

Materials and Methods

Materials

Sucrose, glucose, and fructose were obtained from Sigma (St. Louis, MO, U.S.A.). Levan was prepared in a laboratory scale as previously described [8]. *E. coli* BL21(DE3) and pET-29b (Novagen) were used as the host and the vector for protein overexpression, respectively. *Taq* DNA polymerase and dNTPs for PCR amplification were purchased from Takara (Shiga, Japan) and Kieselgel 60 F_{254} TLC plates from Merck (Darmstadt, Germany). Other chemicals, including isopropyl β-D-thiogalactoside (IPTG) and kanamycin, were purchased from Sigma. A Hi-Trap

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chelating affinity column and Sephacryl S-100 media were purchased from Amersham Bioscience. Oligonucleotide primers for PCR were synthesized by Bioneer (Cheongwon, Korea). All buffers were prepared in Millipore Milli-Q water.

Construction of the Expression Vector pETLSU

LscR was cloned and sequenced previously [13]. A plasmid, pTRLSU, carrying a 1.25 kb fragment of the *lscR* gene was constructed [13]. Based on the *lscR* sequence, two oligonucleotide primers, LsuNdeI-F (5'-AGGACC-CATATGACAAATTTAA-3') and LsuXhoI-R (5'-TCTC-CGCTCGAGATTTAAAATAA-3'), were designed to carry the *Nde*I and *Xho*I recognition sites, respectively (underlined). These oligomers and pTRLSU were used as the template to amplify the 1.25 kb *Nde*I-*Xho*I fragment, which carries the *lscR* gene, using PCR under standard conditions as follows: one cycle of denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and extra extension at 72°C for 7 min. The PCR-amplified DNA fragment was subcloned into the expression vector, pET-26b, to yield pET-LSU. The LscR protein expressed from the expression vector pET-LSU was a fusion protein, including six histidine residues tagged on to the C-terminus for rapid and convenient purification. The recombinant plasmid containing *lscR* was transformed into *E. coli* BL21(DE3) cells.

Expression and Purification of Recombinant LscR

E. coli BL21(DE3) cells harboring the pET-LSU were grown in 500 ml of LB broth supplemented with 30 μg/ml of kanamycin at 37°C to an OD_{600} of 0.8–1.0 before induction by 1 mM IPTG. To maximize the production of LscR, incubation was further continued for 3–4 h after the induction. The induced cells were harvested and the supernatant was saved at 4°C for the analysis of the media fraction. In order to isolate levansucrase secreted into the periplasmic space, the washed cell pellet was resuspended in 30 mM Tris-HCl, containing 20% (w/v) sucrose, pH 8.0. EDTA was added to a final concentration of 1 mM and the suspension was incubated on ice for 10 min with gentle agitation. The cell pellet collected by centrifugation was thoroughly resuspended in the same volume of an ice-cold 5 mM $MgSO_4$. The resuspended cells were incubated for 10 min on an ice bath with gentle agitation and then centrifuged for 20 min at 4°C. The supernatant, which is the osmotic shock fluid containing periplasmic proteins, was recovered and dialyzed extensively against 20 mM phosphate buffer (pH 7.0) before analysis of the periplasmic fraction. Osmotic shocked cells, that appear round instead of rod-shaped under a light microscope, were used to isolate cytoplasmic levansucrase present as the soluble or insoluble form. The cells were resuspended in an ice-cold 20 mM phosphate buffer (pH 7.0), at 5 ml per gram wet weight, and sonicated four times (15 pulses each). The supernatant after centrifugation (8,000 ×g, 10 min) was collected and saved for the analysis of soluble, cytoplasmic protein fraction, and purified using a 1 ml Hi-Trap chelating column. The cell debris were resuspended in an ice-cold 20 mM phosphate buffer (pH 7.0) and saved for the analysis of insoluble cytoplasmic fraction (inclusion bodies). Hi-Trap chelating column was charged with 100 mM $NiSO_4$ and equilibrated with 20 mM sodium phosphate and 0.5 M NaCl (pH 7.4). After unbound proteins had been washed out, the protein was eluted with the same buffer containing 30 mM imidazole solution. The fractions showing high activity were collected, pooled, dialyzed against 100 mM sodium phosphate buffer (pH 7.0), and then concentrated by ultrafiltration. These fractions were applied to a Sephacryl S-100 gel filtration column (1.5×75 cm) equilibrated with the same buffer. The fractions showing enzyme activity were pooled and concentrated. The purity of active LscR was examined by SDS-PAGE.

Enzyme Assays

Sucrose hydrolysis activity was determined at 37°C by measuring absorbance at 575 nm according to the DNS method [19]. Reaction mixtures (1 ml) contained 0.5 ml diluted enzyme solution and 0.5 ml of 1 M sucrose in 100 mM sodium phosphate buffer (pH 7.0). Samples were withdrawn after 2 h, and 3 ml of DNS reagent was added to stop the reactions. Glucose and fructose formed were quantified by monitoring the development of color by heating at 100°C for 5 min. One unit of sucrose-hydrolyzing activity was defined as the amount of enzyme required for the release of 1 mg of reducing sugar equivalent to glucose per hour under the assay conditions. Levan-forming activity was determined at 37°C by measuring the change in turbidity at absorbance 540 nm [2, 13], and the amount of levan formed was calculated from an established standard curve of levan concentration versus absorbance. Reaction mixtures (1 ml) contained 0.5 ml diluted enzyme solution and 0.5 ml of 1 M sucrose in 100 mM sodium phosphate buffer (pH 7.0). One unit of levan-forming activity was defined as the amount of enzyme required for the formation of 1 mg of levan per hour under the assay conditions.

Substrate Specificity of Levansucrase

To determine the substrate specificity of the purified enzyme, 10% sucrose was replaced with the same amount of various kinds of sugars. The substrate and the enzyme (1 unit) in 100 mM sodium phosphate buffer (pH 6.0) were incubated at 37°C for 6 h. The reaction products were analyzed by the TLC method as previously described [24].

Analytical Methods

The protein was determined by the method of Bradford using a protein quantification kit (BioRad, Richmond, CA,

U.S.A.), with bovine serum albumin as the standard protein [5]. Denatured proteins were separated in 12% SDS polyacrylamide gels [16] and stained with Coomassie Blue R-250. The molecular mass of levansucrase was estimated by comparison with standard protein markers (Elpis-Biotech, Korea). TLC analysis of sugars was done as previously described [24]. An aliquot of the reaction mixture was spotted onto silica gel 60 F_{254} plates 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 were quantitatively determined on the TLC plate by densitometry [22] and confirmed by HPLC as well. For HPLC, the reaction mixtures were filtered through a membrane filter (0.2 μm pore size, Gelman Sciences) and analyzed with a Sugar-Pac column (65×300 mm, Waters, U.S.A.). The elution was performed at a flow rate of 0.5 ml/min. The detection of the products was monitored with a 410 differential refractometer (Waters) at 85°C. Fructose, glucose, sucrose, 1-kestose, nystose, and levan were used as standards.

RESULTS AND DISCUSSION

Overexpression and Cellular Localization of LscR in *E. coli*

pTRLSU, a plasmid carrying a 1.25 kb PCR fragment of the *lscR* gene, was constructed and the enzyme activity was detected in *E. coli* cells containing pTRLSU [13]. Since the expression level of the recombinant enzyme was insignificant, the expression plasmid pET-LSU (7.72 kb), in which the *lscR* gene with a tag of six histidine residues at the C-terminus was expressed under the control of the T7*lac* promoter on pET-29b, was constructed. This construct was confirmed by sequencing and then *E. coli* BL21(DE3) cells harboring pET-LSU were examined for enzyme expression. After induction by IPTG, the expression of the enzyme reached its maximum level within 4 h, whereas *E. coli* cells carrying pET-29b without the *lscR* gene did not show any enzyme activity, thus indicating that the levansucrase activity of the recombinant *E. coli* cells originated from *R. aquatilis* ATCC 15552. The enzyme activity (2.86 units/ml) of the recombinant *E. coli*/pET-LSU was found to be increased 7.6-fold, as compared with that of *E. coli*/pTRLSU (0.38 units/ml). Most of the enzyme activity (>94%) was found in the cytoplasmic fraction, indicating that the expressed LscR was localized in an intracellular region (Table 1). No activity was found in the culture supernatant.

Table 1. The cellular localization of LscR expressed in *E. coli* BL21(DE3) cells carrying pET-LSU.

Fraction	Activity (units/ml)	Relative activity (%)
Culture supernatant	0	0
Periplasmic	0.18	6.2
Cytoplasmic	2.68	93.5
Insoluble	0.01	0.3

Relative activity shows the activity of each fraction as a percentage of the total activity.

Purification of LscR

LscR tagged with an extension of six histidine residues at the C-terminus was purified by Hi-Trap chelating affinity chromatography. The recombinant LscR was tightly bound to the column and eluted at high imidazole concentration. Minor contaminants of other *E. coli* proteins were removed by Sephacryl S-100 gel filtration chromatography. The increase in specific activity of the LscR indicated an 146-fold purification of the enzyme (Table 2). The enzyme after gel filtration appears to be homogeneous, as shown by SDS-PAGE (Fig. 1, lane 3). Approximately 1.6 mg of protein was obtained with a recovery yield of 61%. The specific activity of the purified enzyme increased to 142.1 units/mg. The molecular mass of the recombinant LscR was estimated to be approximately 49 kDa by SDS-PAGE. The molecular mass of the purified LscR with six histidines at the C-terminus was very close to the calculated molecular mass of the deduced amino acid sequence of LscR. The molecular mass of LscR was similar to the levansucrases from *Rahnella aquatilis* ATCC33071 (46 kDa) [23], *Zymomonas mobilis* ZM1 (46 kDa) [25], *Erwinia herbiocola* (46 kDa) [7], *Pseudomonas syringae* (45 kDa) [11], and *Pseudomonas aurantiaca* S-4380 (47 kDa) [12].

Enzymatic Characteristics of LscR for Levan Formation

The reaction products from the enzyme reaction were examined and analyzed by TLC and HPLC (Fig. 2). Levan, glucose, fructose, and 1-kestose increased gradually as the incubation time was prolonged. Although the yield

Table 2. Purification of the recombinant LscR from *E. coli* BL21(DE3) cells carrying pET-LSU.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free extract	386	374.4	0.97	100	1
Hi-Trap chelation	4.9	281.2	57.4	75.1	59.1
Sephacryl S-100	1.62	230	142.1	61.4	146.4

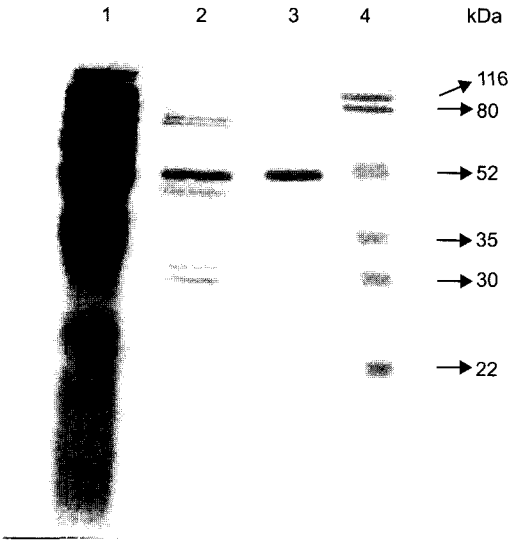

Fig. 1. SDS-PAGE analysis of the purified LscR.
The protein samples are analyzed by SDS-PAGE on 12% polyacrylamide gel. Lane 1, total cell extract of induced BL21(DE3) cells; lane 2, eluate from Hi-Trap chelating column; lane 3, eluate from Sephacryl S-100 gel-filtration column; lane 4, molecular mass standards.

was lower than levan, the production of 1-kestose and nystose, which are one and two fructosyl residues bound at the β-2,1 position of sucrose, respectively, indicated that the enzyme mainly catalyzed the fructosyl transfer at the β-2,6 position, but also transferred the fructosyl residue at the β-2,1 position. The pH dependence of LscR for levan formation was investigated at various pHs, and the optimum pH was found at around 6.0 for both levan formation and sucrose hydrolysis, similar to other levansucrases (Fig. 3A). There was no difference in the pH profile between the sucrose hydrolysis activity and levan-forming activity. Although the levansucrase of *R. aquatilis* JCM-1683 was reported to be stable at pH 9.0 [20], LscR was stable from pH 5.0 to 6.0, but was inactivated at above pH 8.0. The effect of temperature on both levan formation (transfructosylation) and sucrose hydrolysis was examined, and the results are shown in Fig. 3B. The optimum temperature for levan formation and sucrose hydrolysis was 30 and 45°C, respectively. Levan formation was more favorable at between 25 and 40°C. Similar variation of optimum temperature for both activities was reported for levansucrases of *Bacillus subtilis* [9], *R. aquatilis* JCM-1683 [20], and *Zymomonas mobilis* [18]. With respect to the optimum temperature for the transfructosylation activity of the enzyme, the levansucrases were very different. The optimum temperatures for levan formation were 0°C (*Z. mobilis*) [3], 18°C (*P. syringae*) [11], 30°C (*R. aquatilis* ATCC 33071) [15, 26], and 40°C (*Bacillus natto*) [1].

The effect of substrate concentrations on levan formation was also investigated. As shown in Fig. 4, the optimum substrate concentration for levan formation was 300 mM,

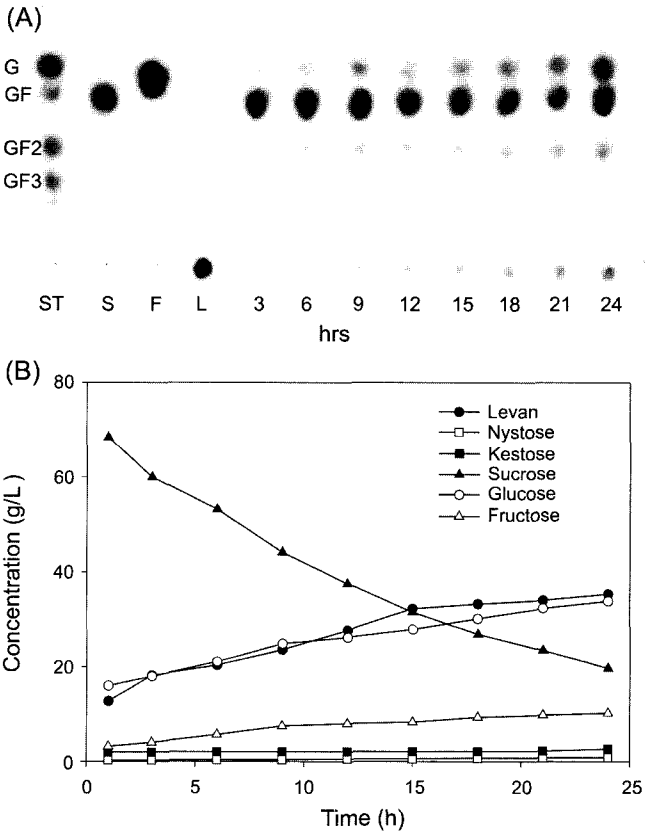

Fig. 2. Time courses for the production of the reaction products from sucrose by LscR.
The enzyme (200 μg/ml) was incubated with 300 mM sucrose in 100 mM phosphate buffer (pH 6.0) at 30°C for indicated time intervals. TLC and HPLC analyses were performed as described in Materials and Methods. (A) TLC analysis. ST, standard sugars (G, glucose; GF, sucrose; GF_2, 1-kestose; GF_3, nystose); S, sucrose; F, fructose; L, levan. (B) HPLC analysis of the reaction products shown in (A).

and this concentration was quite different from that of levansucrase from *R. aquatilis* ATCC 33071 (555 mM) [15]. In the present study, the amounts of levan formed were drastically decreased at above 500 mM, however, the sucrose hydrolysis activity was continuously increased in proportion to the increase of the substrate concentration. The decrease of the levan formation at high sucrose concentration has been suggested to be due to relative increase of fructooligosaccharides (FOS) instead of levan [27]. The effect of enzyme concentration on levan formation was also examined. Various amounts of the enzyme (20–200 μg/ml) were added to the reaction mixtures containing 300 mM sucrose in 100 mM phosphate buffer (pH 6.0), and the mixture was incubated at 30°C. Levan formation was enhanced by the increase of the enzyme concentrations (data not shown). Levan formation was fast at the initial stage, but the rate was gradually decreased as the reaction proceeded. Total amounts of levan formation were not significantly increased after 24 h of incubation. Maxium

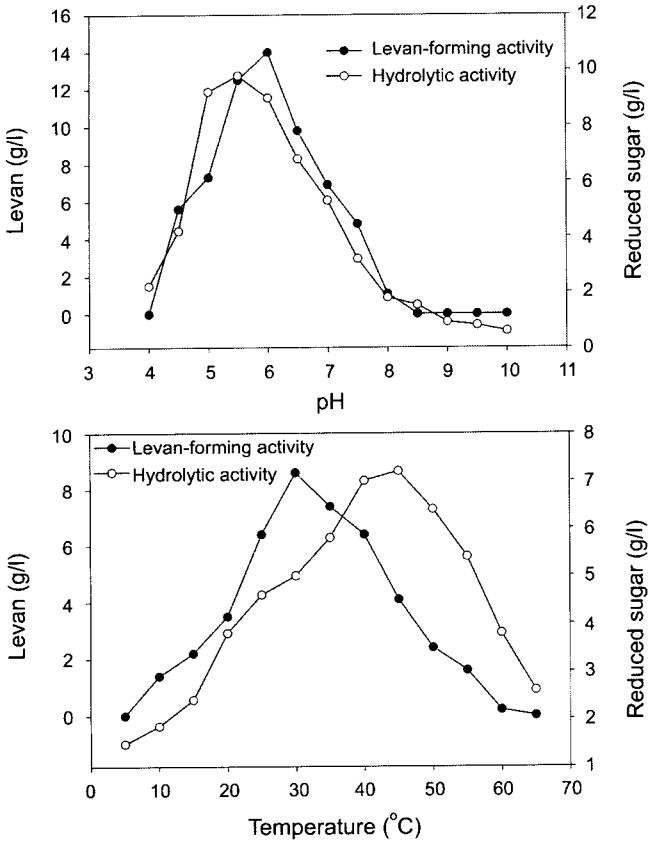

Fig. 3. pH and thermal properties of the purified LscR.
For determination of optimal pH (A), the standard assay was employed in the following buffers: 0.1 M sodium acetate buffer (pH 3–5.5), phosphate buffer (pH 6–7.5), Tris-HCl (pH 8–9), and boric acid-NaOH buffer (pH 9.5–10). The reaction mixture containing 0.5 ml of the enzyme (20 μg/ml) and 0.5 ml of 1 M sucrose in 100 mM buffer solution was incubated at 37°C. For determination of the optimal reaction temperature (B), the reaction mixtures containing 0.5 ml of the enzyme (20 μg/ml) and 0.5 ml of 1 M sucrose in 100 mM phosphate buffer (pH 6.0) were incubated at various temperatures. Sucrase activity was assayed after 30 min and levan-forming activity was measured after 6 h of incubation.

yield of levan formation at optimum substrate concentration, pH, and temperature after 24 h of reaction was approx. 80%. The yield obtained here was higher than that of any other reported levansucrases.

Substrate Specificity of LscR

The purified LscR showed very limited substrate specificity. When reacted with various kinds of sugars other than sucrose, the enzyme was active only towards raffinose (Fig. 5). An interesting feature of LscR is the fact that it can also synthesize FOS, although the amounts of these products are lower than that of levan. A minor product synthesized by the enzyme with sucrose was 1-kestose (GF_2) (Fig. 5, lane 1c). Other unidentified FOS, with sizes similar to nystose (GF_3) and 1-fructosyl nystose (GF_4), were observed, when raffinose was used as a substrate (Fig. 5, lane 6f and 6g). The production of the unidentified

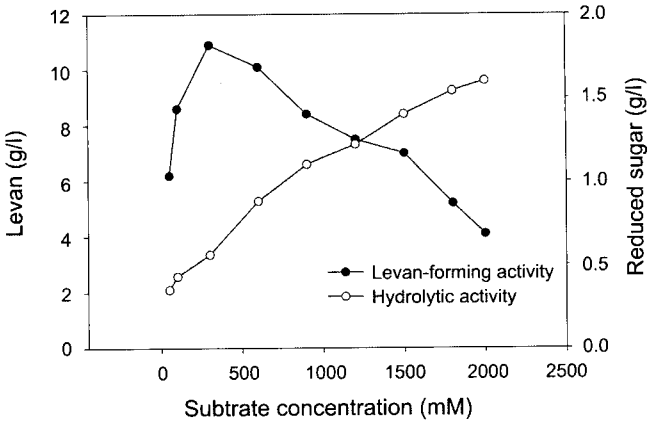

Fig. 4. Effect of substrate concentration on the activity of LscR.
The enzyme (20 μg/ml) was incubated at 37°C in 100 mM phosphate buffer (pH 6.0) with various substrate (50 mM to 2 M) concentrations. Samples were taken after 6 h and the activity was measured as described in Materials and Methods.

FOS was most likely due to the transfer of the fructosyl residue of raffinose to another raffinose by cleaving the α-(2→1) linkage between glucose and fructose. The identification of these unidentified reaction products should be carried out to confirm the above possibility. Previously, Kim *et al.* [14] reported that methyl fructoside was formed by the transfructosylation reaction of levansucrase with sucrose and methanol. The site of methylation was suggested to be the second carbon atom on fructose, based on the analysis of NMR spectra. Therefore, we assume that the unidentified reaction products are formed by the linkage of C2-OH of the fructose residue cleaved from sucrose or

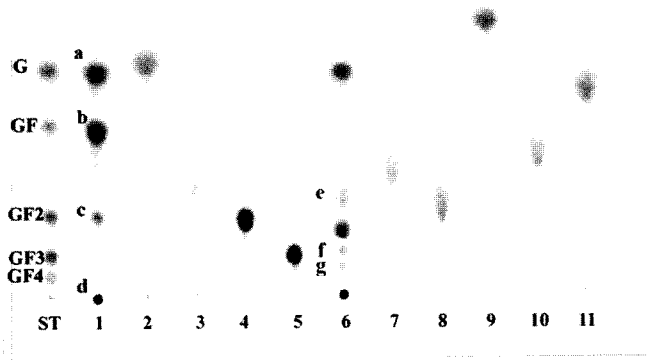

Fig. 5. TLC analysis of the reaction products from various substrates with purified LscR.
The enzyme reaction was conducted with 10% substrate under standard assay conditions. Lanes 1, sucrose; 2, arabinose; 3, trehalose; 4, 1-kestose; 5, nystose; 6, raffinose; 7, cellobiose; 8, melibiose; 9, xylose; 10, maltose; 11, galactose. a, glucose/fructose; b, sucrose; c, 1-kestose; d, levan; e, f, and g, unidentified FOS; ST, standard sugars (G, glucose; GF, sucrose; GF_2, 1-kestose; GF_3, nystose; GF_4, 1-fructosyl nystose).

raffinose to the C1-OH of the fructofranosyl residue of raffinose. Clarification of the chemical structures of the unidentified reaction products is necessary for the production of novel FOS as well as elucidation of the transfructosylation mechanism of the enzyme. The chemical structures of the unidentified FOS are currently under investigation.

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