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Changes in linkage pattern of glucan products induced by substitution of Lys residues in the dextransucrase

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Abstract **Dextransucrase S (DSRS) is the only active glucansucrase that has been found in *Leuconostoc mesenteroides* NRRL B-512F strain. Native DSRS produces mainly 6-linked glucopyranosyl residue (Glc*p*), while *Escherichia coli* recombinant DSRS was observed to produce a glucan consisting of 70% 6-linked Glc*p* and 15% 3,6-Glc*p*. Lys residues were introduced at the N-terminal end of the core domain by site-directed mutagenesis. In glucans produced by the one-point mutants T350K and S455K, the amount of 6-linked Glc*p* was increased to about 85% of the total glucan produced, more similar in structure to native B-512F dextran. The double mutant T350K/S455K produced adhesive, water-insoluble glucan with 77% 6-linked Glc*p*, 8% 3,6-linked Glc*p* and 4% 2,6-linked Glc*p*. The T350K/S455K mutant exhibited a 10-fold increase in glucosyltransferase activity over those of the parental DSRS-His_6 and its T350K and S455K mutants. This is the first report demonstrating a change in the properties of a dextransucrase or a related glucosyltransferase through simple site-directed mutagenesis to create 2,6-linked Glc*p*.**

Keywords: Dextransucrase; Dextran; Glucansucrase; Glucan; Glucosyltransferase; Lysine residue

1. Introduction

Glucansucrases [EC 2.4.1.5] are produced by several kinds of *Leuconostoc*, *Streptococcus*, and *Lactobacillus* bacterial strains to catalyze the synthesis of α-D-glucan from sucrose. Glucansucrases that produce primarily α -1,6-D-glucan are called dextransucrases. *Leuconostoc mesenteroides* NRRL B-512F produces a high percentage (95%) of α-1,6-dextran. Frequently, different glucosidic linkages are found in glucan products; i.e., *Leuconostoc* strains produce glucans with α-1,6-linkages, frequently α-1,3-, and sometimes α-1,2- or α-1,4-linkages. [1]. *Streptococcus* strains produce glucan with α-1,6-linkages and/or α-1,3-linkages [2]. *Lactobacillus reuteri* 121 was reported to produce glucan with mainly α-1,4- and α-1,6-linkages [3]. The glucosidic linkage pattern of the glucan product differs depending on the kind of glucansucrase. However, the mechanisms as to what kinds and what proportions of the linkages are produced are unclear.

Glucansucrases are approximately 160 kDa in size and belong to the glycoside hydrolase family 70 (see http://afmb.cnrs-mrs.fr/CAZY/). A typical glucansucrase amino acid sequence includes a signal sequence, followed by a variable stretch of approximately 200 amino acids, conserved core region of about 900 amino acids (N-terminal catalytic core domain), and a series of direct repeating units of about 400 amino acids (C-terminal glucan-binding domain) [4]. In the highly-conserved core region, about 700 amino acids make up a predicted circularly permuted $(\beta/\alpha)_8$ barrel [5–7]. Catalytic Asp [7–9] and essential substrate-binding Gln [6] exist in this region. The N-terminal end of about 200 amino acids out of this $(\beta/\alpha)_8$ barrel are highly conserved among glucansucrases, but have not been found in other enzymes or proteins. The properties and functions of this region (glucansucrase specific-conserved region, GSC region) are unclear. In *Streptococcus downei* glucansucrase GTFI, Trp344 and His355, both of which are in the GSC region, were reported to be involved in the enzyme reaction [10]. It was also suggested that His355 plays a role in the binding to the subsite necessary for glucan and oligosaccharide elongation [10]. This region has been reported to contain an essential Asp511 residue at the border between the GSC region and the $(\beta/\alpha)_8$ barrel in the dextransucrase S (DSRS) of the *L. mesenteroides* NRRL B-512F strain, which could function to bind a sucrose molecule [11,12]. In one of our previous studies, a chemical modification of the Lys residues was performed on the native DSRS of the B-512F strain with *o*-phthalaldehyde in the presence and absence of sucrose, the sucrose analog sucrose monocaprate, and clinical dextran [13]. The results suggested the GSC region contained sucrose- and dextran-binding Lys-residues. Lys residues in the GSC region may have some role in substrate binding, but it is unclear whether or not some of the Lys residues contribute to the enzymatic reaction. The GSC regions of some glucansucrases from *Leuconostoc*, *Streptococcus*, and *Lactobacillus* are aligned, as shown in Fig. 1. Lys residues are indicated in white letters with a black background. Many of the Lys residues are not conserved in this region, but there are a few Lys residues conserved in most of the glucansucrases. In another one of our previous studies, DSRS was incubated with a glucan prepared from a *Streptococcus mutans* culture, and a glucan-binding peptide fragment was detected in the GSC region [14].

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Abbreviations: DSRS, dextransucrase S; *dsrS*, a gene encodes DSRS; GTF, glucosyltransferase; Glc*p*, glucopyranosyl residue; GSC region, glucansucrase specific-conserved region

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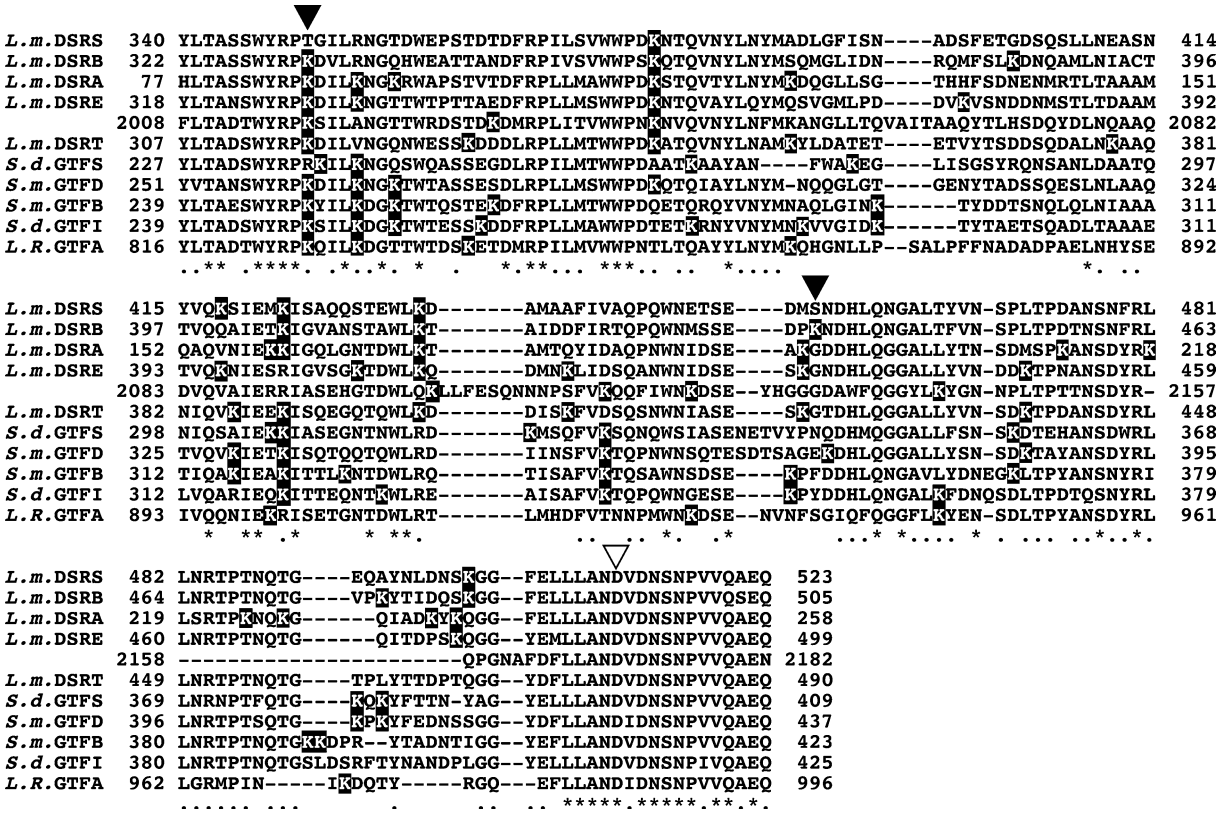

Fig. 1. Alignments of amino acid sequences and positions of Lys residues in the GSC region of various *Leuconostoc* dextransucrases and streptococcal glucosyltransferases. The deduced amino acid sequences of the GSC regions of *L. mesenteroides* dextransucrases are DSRS from the gene *dsrS* (I09598) [12,34], DSRB from *dsrB* (AF030129) [23], DSRA from *dsrA* (U38181) [35], DSRE from *dsrE* (AJ430204) [32] and DSRT from *dsrT* gene (AB020020) [18]. The streptococcal glucosyltransferase are GTFS from *gtfS* from *S. downei* (M30943) [36], GTFD from *S. mutans gtfD* (M29296) [37], GTFB from *S. mutans gtfB* (M17361) [38] and GTFI from *S. downei gtfI* (M17391) [39]. The *Lactobacillus* glucansucrase is GTFA from *L. reuteri gtfA* (AY697435) [3]. All were aligned by the GENETYX version 11.2 program (Software Development Co., Tokyo) as described in Section 2. DSRB produces α-1,6-glucan [23], DSRA produces α-1,6- and α-1,3-glucans [35] and DSRE produces α-1,2-branched linkage-containing α-1,6-glucan [32]. GTFS [36] and GTFD [37] produce water-soluble α-1,6-glucan. GTFI [39] and GTFB [38] produce water-insoluble α-1,3-glucan. GTFA [3] produce water-soluble α-1,4- and α-1,6-glucan. The positions of the Lys residues are indicated by white letters with a black background. Solid arrowheads are the positions of substituted Lys residues in the DSRS protein. The open arrowhead is an essential Asp residue [11,12].

Additionally, in our most recent study, chimeric enzymes constructed from two different recombinant dextransucrases that were exchanged their GSC regions each other produced glucans different from those of their parental enzymes [15]. It should be noted, however, that the positions of the glucan-binding Lys residues in the GSC region have not yet been identified, nor have the roles of the Lys residues in the GSC region been clarified. The positively charged amino acid residues of His, Arg, and Lys can potentially bind with sugars or other carbohydrates via hydrogen bonding. The Lys residue was reported to be one of the essential residues for sugar binding in a lectin from the freshwater Indian gastropod *Balamyia bengalensis* [16] and for carbohydrate binding in bovine β-lactoglobulin [17].

In this study, Lys residues were substituted in a recombinant C-terminal polyhistidine-tagged DSRS dextransucrase (DSRS-His_6) through site-directed mutagenesis. A possible role of the GSC region is speculated based on the changes in the linkage patterns of the glucan products of the DSRS-His_6 enzymes of the Lys-substituted mutant. The contributions of the Lys residues in the GSC region in terms of enzyme reactions and the determination of the kinds of glucosyl linkages made are discussed in this report.

2. Materials and methods

2.1. Construction of the DSRS-His_6 gene and the introduction of mutations

The plasmid pDSRS, carrying the complete *dsrS* gene in the form of pET23d, was constructed as described before [18]. To introduce the $(His)_6$-Tag at the C-terminal of the recombinant DSRS protein, an *Eco*RI/*Xho*I fragment of PCR-amplified DNA was used with pDSRS as a template with the primers 5′-GCCATGCTGTCACTGGTTTC-3′ and 5′-GCCTCGAGTGCTGACACAGC-3′, which were replaced in pDSRS to construct pDSRSH. Site-directed mutagenesis was carried out on pDSRSH with a Mutan-Super Express Km Kit (Takara).

2.2. Expression of pDSRSH and the purification of the gene product DSRS-His_6 and its mutants in E. coli *BL21(DE3)*

DSRS-His_6 and its mutant enzymes were produced as described before [18,19]. Cultured *E. coli* BL21(DE3) cells carrying pDSRSH or mutated plasmids were suspended in 20 mM Na-acetate (pH 5.2) containing 30% glycerol. The recombinant proteins were then extracted by sonication. The supernatant was obtained by centrifugation at $10\,000 \times g$ for 10 min, to which 6 M guanidine–HCl, 0.5 M NaCl, and 15 mM imidazole were added. The enzyme solution was put on a HiTrap™ 1 ml Chelating HP (Amercham Bioscience) column previously loaded with 0.1 M $NiSO_4$ and equilibrated by a 20 mM Na-acetate, 30% glycerol, 0.5 M NaCl, 6 M guanidine–HCl, 15 mM imidazole solution (pH 7.5). The column was washed with the same solution to

remove substances not tightly bound. The enzyme solution was then eluted with a linear gradient of the equilibrated solution and a 20 mM Na-acetate, 30% glycerol, 0.5 M NaCl, 6 M guanidine–HCl, 75 mM imidazole solution (pH 8.8). The protein solution was collected and then dialyzed against 20 mM Na-acetate (pH 5.2) and used as the enzyme solution.

2.3. Enzyme assay and protein concentration

The dextransucrase activity was represented in two different ways, as described before [20]. The enzyme solution (1–10 mU in 250 μl of reaction mixture) was incubated with 10% sucrose in 20 mM Na-acetate buffer (pH 5.2) at 30 °C. The sucrose cleaving activity was determined using the Nelson–Somogyi procedure by measuring the amount of reduced sugar released from sucrose with glucose as a standard, as described previously [20]. One unit of dextransucrase releases 1 μmol of reduced sugar from sucrose per minute. Dextran production was determined using the phenol–sulfuric acid method for the total sugar (glucan), which was precipitated with 67% ethanol followed by repeated precipitations to remove the low molecular weight sugars [20]. One unit of dextransucrase releases 1 μmol of glucose transferred from sucrose per minute. K_m values were determined using the Michaelis–Menten equation from the initial rate of the reaction determined at various sucrose concentrations from 10 to 100 mM, or at a fixed concentration of 200 mM sucrose with various clinical dextran (molecular weight 60 000) concentrations from 2 to 200 μM. The protein concentration was measured using a BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

2.4. Preparation of glucans

DSRS-His_6 and its mutants' glucans were produced by incubation of each purified enzyme (0.5–1 unit) in 50 ml of 10% sucrose in 20 mM Na-acetate (pH 5.2) at 30 °C for 8 h, as described previously [19]. The glucans produced were purified with a 50% ethanol precipitation, also described previously [19]. The resulting precipitate was dissolved in 1 M NaOH and then neutralized with HCl. This glucan solution was then dialyzed against water and lyophilized. A partially endodextranase digested glucan was prepared as follows: 100 mg of glucan was incubated with 3 units of endodextranase from *Penicillium* sp. (sigma) in 10 ml of 50 mM Na-phosphate (pH 6.0) solution at 37 °C for 2 h, followed by precipitation with 67% ethanol. The resulting precipitate was washed twice with 67% ethanol, dissolved in 1 M NaOH, neutralized with HCl, and then dialyzed against water and lyophilized.

2.5. ^{13}C NMR analysis of glucan

Lyophilized glucan (approximately 10 mg) was suspended in 0.5 ml of 0.5 M NaOD–D_2O in the presence of 0.05% sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. ^{13}C NMR spectra were recorded on a Bruker AVANCE 500 spectrometer equipped with a ^{13}C-{^1H} CryoProbe™ at 308 K. The assignments of the ^{13}C signals of the glucans were based on those made by Shimamura [21].

2.6. Glycosyl-linkage composition analysis

The lyophilized glucans (<1 mg) were permethylated and purified with a Sep-Pak C18 cartridge (Waters), as described by York et al. [22]. The purified permethylated glucans were then hydrolyzed, reduced, and acetylated [22]. The resulting partially *O*-acetylated and partially *O*-methylated alditols were analyzed by gas chromatography (Shimadzu GC-14A with C-R6A Chromatopac) and gas chromatography/mass spectrometry (Hewlett Packard HP6890 Series GC System with a Benchtop Quadrupole Mass Spectrometer JEOL Automass System II), both with an SP-2330 (0.25 mm × 30 m) column (Supelco) as described before [19].

3. Results and discussion

3.1. Introduction of Lys residues in the DSRS-His_6 enzyme

The 200 amino acid GSC region in the N-terminal catalytic domain is outside of the predicted circularly permuted $(\beta/\alpha)_8$ barrel and is not significant as an amino acid sequence in other enzymes and proteins, with the exception of glucansucrases (*Leuconostoc* dextransucrases, streptococcal glucosyltransferases, and *Lactobacillus* glucansucrase), in which its identity is conserved at about 50–70%. In our previous studies, the GSC region was identified as a sucrose and/or dextran-binding site [13,14] and therefore may affect the structure of glucan products [15]. The B-512F strains have two dextransucrase genes, *dsrS* and *dsrT*, although *dsrT* was reported to be inactive due to the deletion of the five nucleotide residues in its open reading frame, resulting in a truncated protein that is inactive [18]. *E. coli* recombinant DSRS-His_6 produced a glucan that, while primarily made up of 6-linked Glc*p*-like native DSRS, also contained 15% 3,6-linked Glc*p* (Table 1), which is more than that found in B-512F dextran. The migration of the SDS–PAGE of the recombinant DSRS in this study was different from that of the native DSRS, as observed before [18]. In addition, the recombinant DSRS had an increased affinity for DEAE Sephadex A-50 as compared with that of native DSRS. Native DSRS eluted in 1.5 M guanidine–HCl, but the recombinant DSRS needed 6 M guanidine–HCl for elution (data not shown). The reasons for these differences between the native and recombinant DSRS are still unclear. A recombinant dextransucrase of DSRB from the B-1299 strain was reported to produce only 6-linked Glc*p* [23]. There are five Lys residues at positions 378, 418, 423, 434, and 501 in DSRS and eight Lys residues at positions 332, 360, 385, 405, 416, 437, 476, and 483 in DSRB in their respective GSC regions (Fig. 1). Comparing the positions of the Lys residues in the GSC regions of DSRS and DSRB, there is no Lys residue in DSRB at the corresponding position of Lys418 in DSRS, and there are no Lys residues in DSRS at the corresponding positions of Lys332, Lys385, Lys437 or Lys476 in DSRB (Fig. 1). Lys332 and Lys437 in DSRB are conserved in other *Leuconostoc* dextransucrases (except DSRS) and largely conserved in streptococcal glucosyltransferases. The corresponding positions of these conserved Lys residues are Thr350 and Ser445 in DSRS.

The substitution of Lys for Thr350 and/or Ser445 in recombinant DSRS-His_6 was done by site-directed mutagenesis to make T350K, S455K and double mutant T355K/S455 K. The

Table 1
Glycosyl-linkage composition of glucans from recombinant DSRS-His_6 and mutant enzymes

Glucan	Glycosyl-linkage composition (mol%)							
	T-Glc*p*	2-Glc*p*	3-Glc*p*	4-Glc*p*	6-Glc*p*	2,6-Glc*p*	3,6-Glc*p*	4,6-Glc*p*
Wild type	10	0	3	2	70	0	15	0
T350K	9	0	0	2	84	0	5	0
S455K	7	0	1	0	86	0	6	0
T350K/S455K	10	0	0	1	77	4	8	0

The amounts of T-, 2-, 3-, 4-, 6-, 2,6-, 3,6-, 4,6-Glc*p* were calculated from the amounts of 2,3,4,6-tetra, 3,4,6-tri, 2,4,6-tri, 2,3,6-tri, 2,3,4-tri, 3,4-di, 2,4-di, and 2,3-di-*O*-methylated and partially *O*-acetylated glucitol, respectively.

specific activity of DSRS-His_6 was 0.038 U/mg of protein, while those of T350K, S455K and T350K/S455K were 0.044, 0.046 and 0.318 U/mg for sucrose cleaving activity, respectively. The specific activities for glucan formation in DSRS-His_6, T350K, S455K and T350K/S455K were 0.051, 0.034, 0.021 and 0.404 U/mg, respectively. While the specific activities of T350K and S455K mutants were almost the same that of the double mutant T350K/S455K was about 10 times higher than that of the wild-type enzyme. The K_m value for sucrose from the B-512F native dextransucrase was reported to be 46 mM at 30 °C [24]. That of DSRS-His_6 was 40.0 mM, that of T350K 55.6 mM, that of S455K 33.3 mM, and that of T350K/S455K 14.7 mM, all measured based on sucrose cleaving activity. The values determined based on glucan produced by DSRS-His_6, T350K, S455K, and T350K/S455K were 40.0, 29.0, 36.4, and 16.9 mM, respectively. When a *Leuconostoc* dextransucrase was incubated with various concentrations of dextrans at a fixed sucrose concentration, a downward biphasic plot was obtained, giving two K_m values from the two limbs of curves from Lineweaver–Burk plots [25]. With 200 mM sucrose, the K_m values for the clinical dextran of DSRS-His_6, T350K, S455K, and T350K/S455K were 3.5 and 36.4, 2.5 and 33.3, 5.7 and 28.6, and 2.3 and 40.0 μM, respectively, for sucrose cleaving activity. Similar K_m values for clinical dextran were observed in dextran formation activity (data not shown).

As shown in Fig. 1, there is a conserved WYRPK sequence in all glucansucrases in which the Lys is replaced by Thr at position 350 of DSRS. In addition, the Lys residue is replaced with Arg in *S. downei* GTFS (Accession No. M30973) and in *Streptococcus cricetus* GTFT (AB026123)[26], and with Ala in *Streptococcus sobrinus* GTFU (AB089438)[27] and in *Streptococcus salivarius* GTFJ (M64111)[28]. Position 455 in DSRS is less conserved, but is between the two conserved sequences SE and DHLQGG. There are 104 amino acids between positions 350 and 455 in DSRS, but the actual distance is unknown because the three-dimensional structures of glucansucrases are not available. Both of these positions should contribute to sucrose binding, and the substitution of Lys residues at positions 350 and 455 in DSRS should enhance the affinity to sucrose and the enzyme reactivity predicted from the K_m values of the mutants. A contribution of the Lys residues to glucan binding was, however, not observed at a significant level.

3.2. Characterization of glucans produced by Lys residue(s)-substituted DSRS-His_6 mutants

DSRS-His_6 and its mutants, T350K, S455K and T350K/S455K, were incubated with sucrose, and the subsequent glucan products were purified. The reaction products produced by the enzymes of DSRS-His_6, T350K and S455K made clear solutions, and the purified glucans were water-soluble. However, the products produced by T350K/S455K resulted in a very cloudy, and the purified glucan was water-insoluble. The 50% ethanol precipitate of this glucan exhibited an adhesive nature. This precipitate did not dissolve completely in <10 M NaOH, and remained self-adhesive. These characteristics were different from the other three glucans. Acceptor reactions were performed with 20% sucrose and 10% maltose in 20 mM Na-acetate (pH5.2). DSRS-His_6, T350K and S455K made water-soluble products, while only T350K/S455K made water-insoluble products whose precipitates also did not dissolve completely in alkali solution (data not shown).

To analyze the structure of the T350K/S455K glucan product, the glucan was suspended in 1 M NaOD, and its ^{13}C NMR spectrum was obtained (Fig. 2(b)) under high-resolution experimental conditions in which the suspended insoluble products were not observed due to the fast T2 relaxation resulting from the strong spin–spin interaction. As shown in Fig. 2(b), the soluble part of the T350K/S455K glucan contained a 6-linked Glc*p* substructure, while no signals consistent with other substructures were observed at a significant level. To obtain additional structural information from the ^{13}C NMR analysis, the T350K/S455K glucan was partially digested by endodextranase as described in Section 2. This results in a reduction in the molecular weight of the glucan, improving the solubility and decreasing the number of 6-linked Glc*p* bonds to emphasize the signals of other glycosyl linkages. As expected, the partially digested products increased their solubility in 0.5 M NaOD, although still made a cloudy solution. As shown in Fig. 2(c), small but clear signals were present at around δ104.5 and δ87, indicating the existence of C1 and C3 of 3-linked Glc*p* and/or 3,6-linked Glc*p*, respectively. The signal intensity of these linkages was about 3–4% of the 6-linked Glc*p* calculated from the spectra. The supernatant of the endodextranase-treated sample contained mainly 6-linked Glc*p*, but small signals at around δ105 and δ95, suggesting the existence of 3-linked Glc*p* and 2-linked Glc*p*, respectively, were also observed (data not shown). As there were limitations in characterizing the linkages of alkali-insoluble parts of the T350K/S455K glucan by high-resolution ^{13}C NMR, the glycosyl-linkage composition was analyzed.

The results of the glycosyl-linkage composition analysis performed on the glucans produced by the DSRS-His_6, T350K, S455K and T350K/S455K proteins are shown in Table 1. In both the T350K and S455K glucans, 3,6-linked Glc*p* was reduced to a structure similar to native B-512F dextran (95% 6-linked Glc*p* and 5% 3,6-linked Glc*p*). In T350K/S455K glucan, the level of 3,6-linked Glc*p* was 8%, and 2,6-linked Glc*p*, which was observed in neither DSRS-His_6 dextran nor native B-512F dextran at all, was present at 4%. The linkages in the insoluble parts, which could not be characterized by high-resolution ^{13}C NMR, were suggested to contain 3,6-linked Glc*p* and 2,6-linked Glc*p* by glycosyl-linkage composition analysis. α-D-Glucan becomes more water-soluble when the ratio of 6-linked Glc*p* is higher and becomes more water-insoluble when the ratio of the branch linkages of 3-linked Glc*p* and 3,6-linked Glc*p* is higher, which also causes an increase in adhesiveness [2]. In T350K/S455K glucan, 3,6-linked Glc*p* and 2,6-linked Glc*p* must make a more concrete structure that results in its insoluble and adhesive nature compared with native and other mutant glucans. It is interesting that Thr350 and Ser455 are far from each other in the amino acid sequence of DSRS, but the direction of the change in the enzymatic properties of both the T350K and S455K mutants is similar. The mutations in T350K and S455K may restore the *E. coli* recombinant dextransucrase structure to produce more α-1,6-and fewer α-1,3-branch linkages. Surprisingly, the substitution of the Lys residues at these positions simultaneously caused the T350K/S455K mutant to produce not only 3,6-linked Glc*p* but also 2,6-linked Glc*p*. Monchous et al. reported that H355V substitutions in *S. downei* glucansucrase GTFI increased the yield of glucan synthesis, and demonstrated that the oligosaccharide yield was reduced when maltotoriose or maltotetraose were used as acceptors. Based on this, they suggested that His355

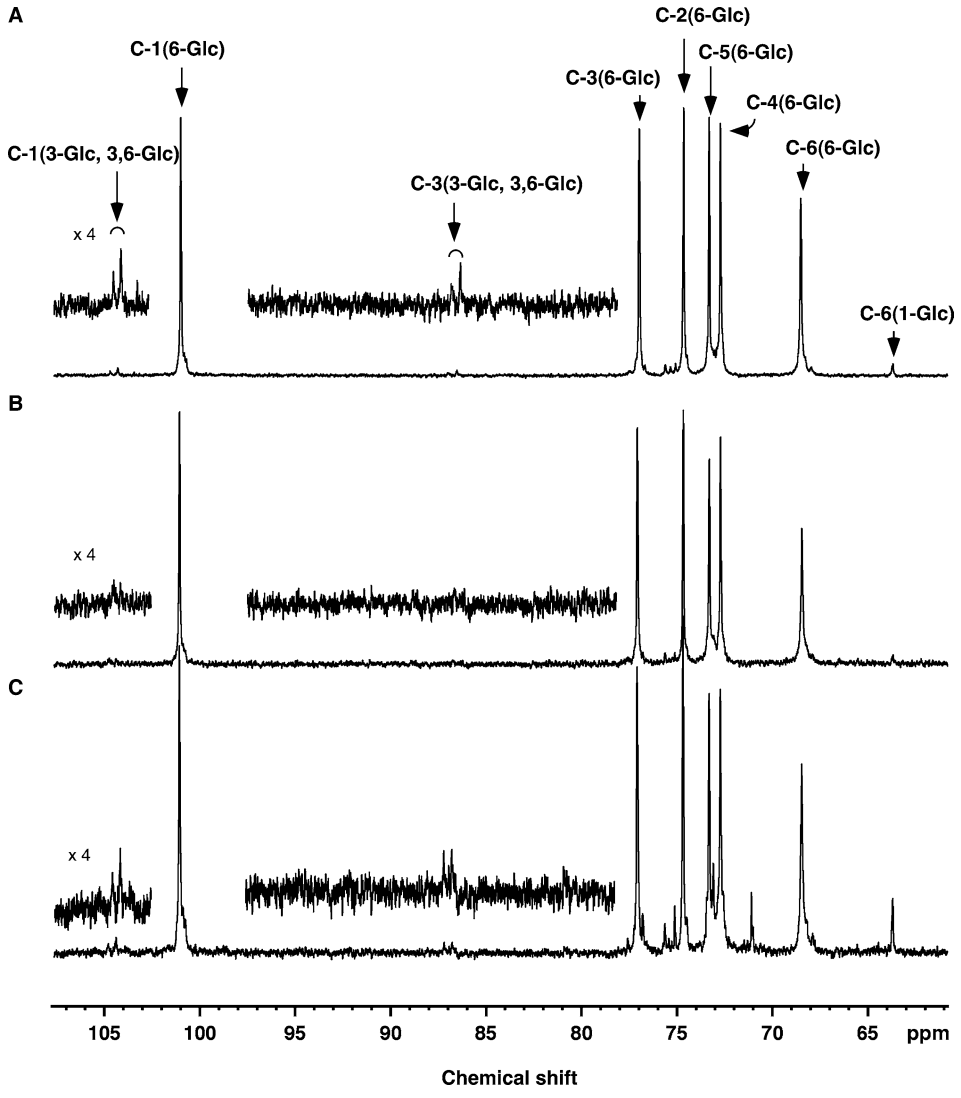

Fig. 2. ^{13}C NMR spectra of glucans. Glucans produced by recombinant wild-type dextransucrase (a), Glucans produced by recombinant T350K/S455K dextransucrase (b) Partially endodextranase digested glucans produced by recombinant T350K/S455K dextransucrase (c). The preparation of glucans and ^{13}C NMR^{13}C NMR spectra of glucan samples were measured as described in the text.

plays a role in the binding of longer acceptors [10]. The His355 position in GTFI is conserved in DSRS His458, which is close to Ser455. The structural changes around this position in the enzyme due to the S455K mutation were likely to have been enhanced by the simultaneous mutation in T355K, causing the T350K/S455K double mutant to produce a significantly different glucan from the original. These proportional changes in the amounts 6-linked Glc*p* and 3-linked Glc*p* in the glucan products have been observed by mutations in glucansucrases [29,30] and by C-terminal deletion [31]. The amounts 6-linked Glc*p*, 3-linked Glc*p*, and 4-linked Glc*p* in glucan products were also shown to change with the construction of chimeric enzymes [15]. However, changing a characteristic of one of these enzymes via mutation to enable it to synthesize any 2-linked Glc*p* is a first in terms of α-glucan synthesis.

3.3. Production of 2,6-linked Glcp by the T350K/455K DSRS-His$_6$ mutant

At the present time, dextransucrase DSRE produced from the *dsrE* gene of the B-1299 strain is the only known recombinant enzyme that produces 2-linked Glc*p* [32]. DSRE possesses the unique structure of two catalytic domains with a long glucan-binding domain between them. Bozonnet et al. showed the second catalytic domain in DSRE is unique: the highly conserved sequences NV and SEVQTVI found in common glucansucrases and in the first catalytic domain of DSRE are substituted with 2214FI and 2315KGVQEKV, respectively, in the second catalytic domain of DSRE [32]. They demonstrated the first catalytic domain of DSRE produced only 6-linked Glc*p*, while the second catalytic domain was responsible for forming 2-linked Glc*p* by making truncated mutant enzymes [33]. DSRS has only one catalytic domain, which has a primary structure commonly found in other glucansucrases, but yet only two amino acid substitutions enable the enzyme to synthesize 2,6-linked Glc*p* linkages like DSRE.

Table 1 suggests that the T350K/S455K DSRS mutant produced 2,6-linked Glc*p* and 3,6-linked Glc*p* in addition to 6-linked Glc*p* in small amounts (4% and 8%, respectively). The second catalytic domain of DSRE, which is responsible for forming 2-linked Glc*p*, has the longest sequence of

2128Y-2132G among *Leuconostoc* enzymes, suggesting this region is bulky and possibly flexible enough to accept the plane structure of 2-linked Glc*p*. The replacement of Ser445 in T350K-DSRS with Lys makes this region bulky, which allows T-350K-DSRS to produce 2-linked Glc*p* and additional 3,6-linked Glc*p*. The hydrogen-bonds formed by two Lys should also be important.

Remaud-Simeon et al. [29] reported that when the neutral amino acid Thr667 in DSRS was substituted for the positively charged amino acid Arg, α-1,3-linkages were reduced by 5% in the product. When the corresponding Thr residues in GTF-S were substituted with a carboxylic amino acid, the synthesis of α-1,3-linkages increased to 30% [30]. This position, which exists in the circularly permuted $(\beta/\alpha)_8$-barrel structure in the catalytic domain, has been suggested to be involved in glucan structure determination [29,30]. In this study, positions 350 and 455 in DSRS, which exist in the GSC region of the catalytic domain, were also observed to affect glucosyl linkage formation. Positively charged amino acids in these positions, mostly Lys residues in glucansucrases, seemed to contribute to sucrose binding and branch-linkage formation. To clarify the mechanism of the selectivity of glucosyl-linkage formation in glucansucrases, interactions between the GSC region, the predicted circularly permuted $(\beta/\alpha)_8$-barrel, and the glucan-binding repeating domain with donor and acceptor substrates must be studied.

References

[1] Robyt, J.F. (1995) Mechanisms in the glucansucrase synthesis of polysaccharides and oligosaccharides from sucrose. Adv. Carbohydr. Chem. Biochem. 51, 133–168.

[2] Hanada, N., Katayama, T., Kunimori, A., Yamashita, Y. and Takehara, T. (1993) Four different types of glucans synthesized by glucosyltransferases from *Streptococcus sobrinus*. Microbios 73, 23–35.

[3] Kralj, S., van Geel-Schutten, G.H., Rahaoui, H., Leer, R.J., Faber, E.J., van der Maarel, M.J.E.C. and Dijkhuizen, L. (2002) Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with α-(1→4) and α-(1→6) glucosidic bonds. Appl. Environ. Microbiol. 68, 4283–4291.

[4] Monchois, V., Lakey, J.H. and Russell, R.R.B. (1999) Secondary structure of *Streptococcus downei* GTF-I glucansucrase. FEMS Microbiol. Lett. 177, 243–248.

[5] MacGregor, E.A., Jesperson, H.M. and Svensson, B. (1996) A circularly permutated α-amylase-type α/β-barrel structure in glucan-synthesizing glucosyltransferases. FEBS Lett. 378, 263–266.

[6] Monchois, V., Vignon, M., Escalier, P.-C., Svensson, B. and Russell, R.R.B. (2000) Involvement of Gln937 of *Streptococcus downei* GTF-I glucansucrase in transition-state stabilization. Eur. J. Biochem. 267, 4127–4136.

[7] Tsumori, H., Minami, T. and Kuramitsu, H.K. (1997) Identification of essential amino acids in the *Streptococcus mutans* glucosyltransferases. J. Bacteriol. 179, 3391–3396.

[8] Devulapalle, K.S., Goodman, S.D., Gao, Q., Hemsley, A. and Mooser, G. (1997) Knowledge-based model of glucosyltransferase from the oral bacterial group of mutans streptococci. Protein Sci. 6, 2489–2493.

[9] Mooser, G., Hefta, S.A., Paxton, R.J., Shively, J.E. and Lee, T.D. (1991) Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two *Streptococcus sobrinus* α-glucosyltransferases. J. Biol. Chem. 266, 8916–8922.

[10] Monchois, V., Vignon, M. and Russell, R.R.B. (1999) Isolation of key amino acid residues at the N-terminal end of the core region *Streptococcus downei* glucansucrase, GTF-I. Appl. Microbiol. Biotechnol. 52, 660–665.

[11] Funane, K., Shiraiwa, M., Hashimoto, K., Ichishima, E. and Kobayashi, M. (1993) An active-site peptide containing the second essential carboxyl group of dextransucrase from *Leuconostoc mesenteroides* by chemical modification. Biochemistry 32, 13696–13702.

[12] Monchois, V., Remaud-Simeon, M., Russell, R.R.B., Monsan, P. and Willemot, R.-M. (1997) Characterization of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase (dextransucrase S) and identification of amino-acid residues playing a key role in enzyme activity. Appl. Microbiol. Biotechnol. 48, 465–472.

[13] Funane, K., Arai, T., Chiba, Y., Hashimoto, K., Ichishima, E. and Kobayashi, M. (1995) Sucrose- and dextran-binding sites of dextransucrase analyzed by chemical modification with *o*-phthalaldehyde. J. Appl. Glycosci. 42, 27–35.

[14] Funane, K., Ookura, T. and Kobayashi, M. (1998) Glucan binding domains of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Biosci. Biotechnol. Biochem. 62, 123–127.

[15] Funane, K., Ishii, T., Terasawa, K., Yamamoto, M. and Kobayashi, M. (2004) Construction of chimeric glucansucrases for analyzing substrate-binding regions that affect the structure of glucan products. Biosci. Biotechnol. Biochem. 68, 1912–1920.

[16] Banerjee, S., Chaki, S., Bhowal, J. and Chatterjee, B.P. (2004) Mucin binding miogenic lectin from freshwater Indian gastropod *Belamyia bengalensis*: purification and molecular characterization. Arch. Bioschem. Biophys. 421, 125–134.

[17] Hattori, M., Miyakawa, S., Ohama, Y., Kawamura, H., Yoshida, T., To-o, K., Kuriki, T. and Takahashi, K. (2004) Reduced immunogenicity of β-lactogloblin by conjugation with acidic oligosaccharides. J. Agric. Food Chem. 52, 4546–4553.

[18] Funane, K., Mizuno, K., Takahara, H. and Kobayashi, M. (2000) Gene encoding a dextransucrase-like protein in *Leuconostoc mesenteroides* NRRL B-512F. Biosci. Biotechnol. Biochem. 64, 29–38.

[19] Funane, K., Ishii, T., Matsushita, M., Hori, K., Mizuno, K., Takahara, H., Kitamurta, Y. and Kobayashi, M. (2001) Water-soluble and water-insoluble glucans produced by *Esherichia coli* recombinant dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F. Carbohydr. Res. 334, 19–25.

[20] Yokoyama, I., Kobayashi, M. and Matsuda, K. (1985) Purification of the dimeric form of dextransucrase from *Leuconostoc mesenteroides* strains NRRL B-1416 and B-1315. Agric. Biol. Chem. 19, 1385–1391.

[21] Shimamura, A. (1989) Use of ^{13}C-n.m.r. spectroscopy for the quantitative estimation of 3-*O*- and 3,6-di-*O*-substituted D-glucopyranosyl residues in α-D-glucans formed by the D-glucosyltransferases of *Streptococcus sobrinus*. Carbohydr. Res. 185, 239–248.

[22] York, W.S., Darvill, A.G., McNeil, M., Stevenson, T.T. and Albersheim, P. (1985) Isolation and characterization of plant cell walls and cell-wall components (Colowick, S.P. and Kaplan, N.O., Eds.), Methods Enzymol., vol. 118, pp. 3–40, Academic Press, New York.

[23] Monchois, V., Remaud-Simeon, M., Monsan, P. and Willemot, R.-M. (1998) Cloning and sequencing of a gene coding for an extracellular dextransucrase (DSRB) from *Leuconostoc mesenteroides* NRRL B-1299 synthesizing only a α(1,6) glucan. FEMS Microbiol. Lett. 159, 307–315.

[24] Kobayashi, M. and Matsuda, K. (1980) Characterization of the multiple form and main component of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Biochim. Biophys. Acta 614, 46–62.

[25] Kobayashi, M., Yokoyama, I. and Matsuda, K. (1984) Activation of dextransucrase from *Leuconostoc mesenteroides* by the substrate, dextran. Agric. Biol. Chem. 48, 221–223.

[26] Hanada, N., Isobe, Y., Aizawa, Y., Katayama, T., Sato, S. and Inoue, M. (1993) Nucleotide sequence analysis of the *gtfT* gene from *Streptococcus sobrinus* OMZ176. Infect. Immun. 61, 2096–2103.

[27] Hanada, N., Fukushima, K., Nomura, Y., Senpuku, H., Hayakawa, M., Mukasa, H., Shiroza, T. and Abiko, Y. (2002) Cloning and nucleotide sequence analysis of the *Streptococcus sobrinus gtfU* gene that produces a highly branched water-soluble glucan. Biochim. Biophys. Acta 15, 75–79.

[28] Giffard, P.M., Simpson, C.L., Milward, C.P. and Jacques, N.A. (1991) Molecular characterization of a cluster of at least two

glucosyltransferase genes in *Streptococcus salivarius* ATCC 25975. J. Gen. Microbiol. 137, 2577–2593.
[29] Remaud-Simeon, M., Willemot, R.-M., Sarcabal, P., Potocki de Montalk, G. and Monsan, P. (2000) Glucansucrase: molecular engineering and oligosaccharide synthesis. J. Mol. Catal. B10, 117–128.
[30] Shimamura, A., Nakano, Y.J., Mukasa, H. and Kuramitsu, H.K. (1994) Identification of amino acid residues in *Streptococcus mutans* glucosyltransferases influencing the structure of the glucan product. J. Bacteriol. 176, 4845–4850.
[31] Vickerman, M.M., Sulavik, M.C., Minick, P.E. and Clewell, D.B. (1996) Changes in the carboxyl-terminal repeat domain affect extracellular activity and glucan products of *Streptococcus gordonii*. Infect. Immun. 64, 5117–5128.
[32] Bozonnet, S., Dols-Laffargue, M., Fabre, E., Pizzut, S., Remaud-Simeon, M., Monsan, P. and Willemot, R.-M. (2002) Molecular characterization of DSR-E, an α-1,2 linkage-synthesizing dextransucrase with two catalytic domains. J. Bacteriol. 184, 5753–5761.
[33] Fabre, E., Bozonnet, S., Arcache, A., Willemot, R.-M., Vignon, M., Monsan, P. and Remaud-Simeom, M. (2005) Role of the two catalytic domains of DSR-E dextransucrase and their involvement in the formation of highly α-1,2 branched dextran. J. Bacteriol. 187, 296–303.
[34] Wilke-Douglas, M., Perchorowicz, J.T., Houck, C.M. and Thomas, B.R. (1989) Methods and compositions for altering physical characteristics of fruit products. *PCT Patent* WO 89/12386 (Dec. 28).
[35] Monchois, V., Willemot, R.-M., Remaud-Simeon, M., Croux, C. and Monsan, P. (1996) Cloning and sequencing of a gene coding for a novel dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 synthesizing only α(1,6) and α(1,3) linkages. Gene 182, 23–32.
[36] Gilmore, K.S., Russell, R.R.B. and Ferretti, J.J. (1990) Analysis of the *Streptococcus downei gtfS* gene, which specifies a glucosyltransferase that synthesizes soluble glucans. Infect. Immun. 58, 2452–2458.
[37] Honda, O., Kato, C. and Kuramitsu, H.K. (1990) Nucleotide sequence of the *Streptococcus mutans gtfD* gene encoding the glucosyltransferase-S enzyme. J. Gen. Microbiol. 136, 2099–2105.
[38] Shiroza, T., Ueda, S. and Kuramitsu, H.K. (1987) Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. J. Bacteriol. 169, 4263–4270.
[39] Ferretti, J.J., Gilpin, M.L. and Russell, R.R.B. (1987) Nucleotide sequence of a glucosyltransferase gene from *Streptococcus sobrinus* MFe28. J. Bacteriol. 169, 4271–4278.