

Water-soluble and water-insoluble glucans produced by *Escherichia coli* recombinant dextranases from *Leuconostoc mesenteroides* NRRL B-512F

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Abstract

Two dextranase genes, *dsrS* and *dsrT5*, from *Leuconostoc mesenteroides* NRRL B-512F were expressed in *Escherichia coli*, and recombinant *dsrT5* dextranase was shown to produce a water-insoluble glucan. In contrast, native dextran from *L. mesenteroides* B-512F is water-soluble. The water-insoluble glucan was shown by ¹³C NMR and glycosyl-linkage composition analysis to contain about 50% 6-linked Glcp and 40% 3-linked Glcp. The 'primitive' B-512F strain is suggested to have produced water-insoluble glucan containing 3-linked Glcp. The glucans produced by dextranases expressed in *E. coli* contained 4-linked Glcp, as shown by glycosyl-linkage composition analysis. The amount of 4-linked Glcp was increased when the truncated, water-insoluble, glucan-producing dextranase, which does not have C-terminal repeating units, was added to the water-soluble, glucan-producing dextranase. Trace amounts of 4-linked Glcp were also detected in the dextran obtained from the B-512F culture supernatant, in dextran produced by dextranase purified from the B-512F strain culture supernatant, and in clinical dextran. The results of glycosyl-linkage composition analysis suggest that dextranases produce 4-linked Glcp as well as 6- and 3-linked Glcp. © 2001 Published by Elsevier Science Ltd.

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1. Introduction

Dextran is a glucan that is mainly composed of (1 → 6)-linked α-D-Glcp and is synthesized from sucrose by various strains of *Leuconostoc mesenteroides* and by *Streptococcus* species. Three genes encoding dextran-

sucrases (EC 2.4.1.5), *dsrS* from *L. mesenteroides* NRRL B-512F, and *dsrA* and *dsrB* from strain NRRL B-1299, have been cloned.^{1–4} The genes *dsrS* and *dsrB* encode dextranases that produce (1 → 6)-linked α-D-Glcp^{2,4} and *dsrA* encodes a dextranase that produces (1 → 6)- and (1 → 3)-linked α-D-Glcp.³ Some *L. mesenteroides* strains such as B-1299 produce dextran with (1 → 2)-linked α-D-Glcp, and strains B-742 and B-1254 pro-

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duce dextran with (1→4)-linked α -D-Glcp in addition to (1→6) and (1→3)-linked α -D-Glcp.^{5–7} However, the enzymes that catalyze the formation of the (1→2) or (1→4)-linked α -D-Glcp in dextran have not been identified. Dextran from *L. mesenteroides* B-512F contains 95% (1→6)-linked α -D-Glcp and 5% (1→3)-linked α -D-Glcp. Only one kind of dextranase has been found in the B-512F strain.⁸ The characteristics of the enzyme expressed by the *dsrS* gene (DSRS) were reported to be similar to the characteristics of the extracellular dextranase produced by the B-512F strain.² We have isolated a gene, *dsrT*, encoding a dextranase-like protein from the B-512F strain; the identity of the nucleotide sequences of the open reading frames of *dsrT* and *dsrS* was about 50%.⁹ The gene *dsrT* encodes a truncated dextranase of deduced molecular weight 112,000; the truncation results from the deletion of five nucleotides in the open reading frame. It is

speculated that the *dsrT* gene had two CAGAT repeats next to each other at the putative deletion point, and that during DNA replication, homologous recombination occurred between the tandem CAGAT sequence. The *dsrT* gene product does not synthesize dextran, but the insertion of the five nucleotides CAGAT in the *dsrT* gene (*dsrT5*) resulted in an enzyme (DSRT5) that is 52,000 daltons larger than the putative protein product of *dsrT*, and that has dextranase activity.⁹

We have analyzed the structure of glucan produced by DSRT5 dextranase and compared it to that of the native dextran produced by *L. mesenteroides* B-512F.

2. Results and discussion

Analysis of glucans by ^{13}C NMR.—The ^{13}C NMR spectra of the water-soluble fraction of DSRS glucan (S-DSRS glucan) and the water-insoluble fraction of DSRT5 glucan (I-DSRT5 glucan), were measured. Clinical dextran was used as a control. The glucans were dissolved in 0.5 M NaOH. All samples were reduced with NaBH_4 to convert the reducing terminals into D-glucitol residues to prevent the base-catalyzed degradation of the reducing terminals.¹⁰ The assignments in the ^{13}C NMR spectra of linear (1→6)- α -D-glucan, (1→3)- α -D-glucan, and other linkages of *L. mesenteroides* dextran T40 and *Streptococcus sobrinus* glucans were based on those made by Shimamura.¹⁰ Based on these assignments, the signals in the ^{13}C NMR spectra of clinical dextran, S-DSRS glucan, and I-DSRT5 glucan were assigned. As shown in Fig. 1, the chemical shifts of S-DSRS glucan and clinical dextran were similar to each other. No signal was observed for 3-Glcp in clinical dextran or S-DSRS glucan, while signals were observed for 3-Glcp in I-DSRT5 glucan. Monchois et al. reported that DSRS produced dextran mainly composed of (1→6)-linked α -D-Glcp, as shown by ^{13}C NMR analysis.² Our ^{13}C NMR spectrum of S-DSRS glucan agreed with their data. The structure of DSRT5 glucan is assumed to be different from that of native B-512F dextran. About 90% of DSRT5

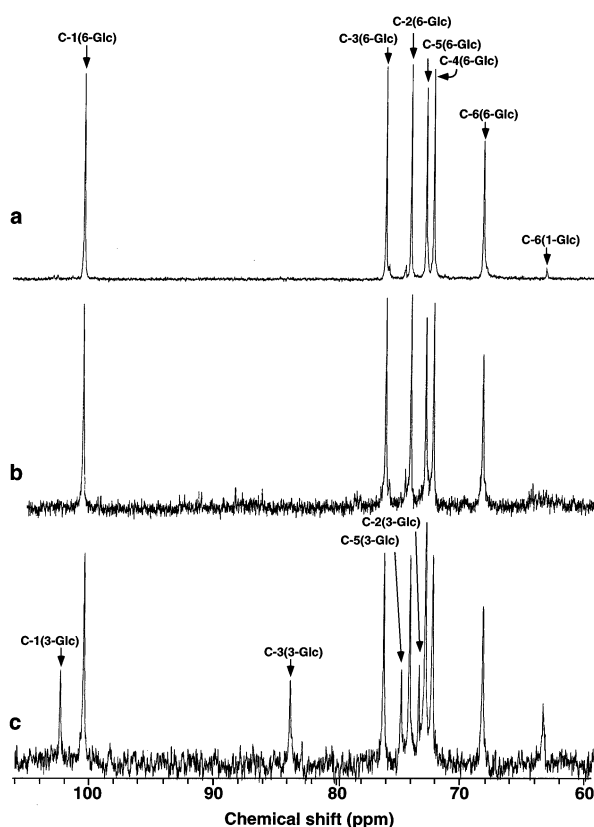


Fig. 1. ^{13}C NMR spectra of glucans. S-DSRS glucan and I-DSRT5 glucan were prepared, reduced and lyophilized. The ^{13}C NMR spectra of glucan samples and clinical dextran were measured as described in Section 3. a, clinical dextran; b, S-DSRS glucan; c, I-DSRT5 glucan.

Table 1
Mole percentages of linkages in glucans

Glucan	Fraction (%)	T-Glcp	3-Glcp	4-Glcp	6-Glcp	3,6-Glcp	4,6-Glcp
Clinical dextran	soluble	11	1	1	80	7	—
Native B-512F dextran	soluble	9	—	1	81	8	1
B-512F DS dextran	soluble	10	—	—	77	12	1
S-DSRS glucan	soluble (90)	12	1	13	64	9	1
I-DSRS glucan	insoluble (10)	9	3	14	66	7	1
S-DSRS+DSRT glucan	soluble (90)	11	3	18	55	11	2
I-DSRS+DSRT glucan	insoluble (10)	12	9	14	57	7	1
S-DSRS+DSRT5 glucan	soluble (10)	10	3	21	60	5	1
I-DSRS+DSRT5 glucan	insoluble (90)	5	32	1	54	7	1
S-DSRT5 glucan	soluble (10)	8	19	15	41	15	2
I-DSRT5 glucan	insoluble (90)	4	43	—	47	5	1

—, trace amounts. DS; dextransucrase.

glucan was water-insoluble because of the high concentration of 3-linked Glcp. The difference between the structures of DSRS glucan and native B-512F dextran could not be determined based on the ^{13}C NMR spectra.

Glycosyl-linkage composition analysis of the glucans.—Glycosyl-linkage composition analysis was performed on the glucans produced by the DSRS, DSRT5, DSRS + DSRT5, or DSRS + DSRT proteins (Table 1). Clinical dextran, native B-512F dextran, and dextran produced by purified B-512F dextransucrase (B-512F DS dextran) were used as controls. The ratio of 6-linked Glcp to 3,6-linked Glcp in clinical dextran, native B-512F dextran, and B-512F DS dextran was about 9:1, which was close to the value expected for the reported structure of native B-512F dextran.⁸ In all three authentic dextrans, trace amounts of 3-, 4-, and 4,6-linked Glcp were also observed.

Since DSRS is thought to be the sole dextransucrase in the B-512F strain,² DSRS glucan was expected to be structurally similar to the three authentic dextrans. However, glycosyl-linkage composition analysis showed that the proportion of 6-Glcp in S- and I-DSRS glucans was about 10% less than that in B-512F dextran, while 4-linked Glcp, which were detected in only trace amounts in B-512F dextran, accounted for 15% of the bonds in DSRS glucans. When DSRT, the truncated dextransucrase, was added to DSRS, the proportion of 6-linked Glcp was reduced. DSRT lacks the C-terminal repeating units completely and has weak sucrose-cleaving activity

but does not synthesize dextran.⁹ Thus, although DSRT does not have transferase activity, it may interact with the intact DSRS enzyme and affect the transfer reaction of DSRS.

I-DSRT5 glucan contains 50% 6-linked Glcp and 40% 3-linked Glcp. Glycosyl-linkage analysis indicated that DSRT5 glucan is different from native B-512F dextran. When glucan was produced by the DSRS and DSRT5 mixture, the amounts of 6-linked Glcp and 3-linked Glcp were intermediate between those of DSRS glucan and DSRT5 glucan, but DSRS + DSRT5 glucan was mostly water insoluble. 4-Linked Glcp was also observed in DSRT5 and DSRS + DSRT5 glucans, but most of the 4-linked Glcp were in the soluble fraction. Both S-DSRT5 and S-DSRS + DSRT5 glucans contain fewer linear 3-linked Glcp than I-glucans. We found a dextransucrase-like gene, *dsrT*, in the *L. mesenteroides* B-512F strain, and assumed that the strain originally had two dextransucrases, DSRS and DSRT5.⁹ The structure of the 'primitive' B-512F dextran should be different from that of the current B-512F dextran. If DSRT5 were active in the B-512F strain, the dextran produced should include (1 → 6) and (1 → 3)- α -D-glucan.

Some dextrans from the genus *Leuconostoc* have 2- or 4-linked Glcp. It has been unclear why 2- or 4-linked Glcp are contained in some *Leuconostoc* dextrans. The glucans produced by the dextransucrases expressed in *E. coli*, DSRS and DSRT5, contained 4-linked Glcp.

Trace amounts of 4-linked Glcp were also found in the authentic clinical dextran, native B-512F dextran, and B-512F DS dextran. Purified B-512F dextranase was a homogeneous enzyme protein with molecular mass of 170 kDa, but dextran produced by the B-512F dextranase contained 4-linked Glcp. Our findings suggest that *Leuconostoc* dextranase can produce at least 4-linked Glcp besides 6- and 3-linked Glcp.

Digestion of glucans by various glucanases.—The extent of digestion of glucans by endodextranase was examined. All glucans were fragmented to various extents by endodextranase, indicating that they contain (1 → 6)-linked α -D-Glcp (Fig. 2). B-512F dextran was digested 80% as much as clinical dextran. S-DSRS glucan was digested 30%, while I-DSRS glucan was digested only 10% as much as clinical dextran, although DSRS glucans contained about 65% 6-linked Glcp. The 35% non-6-linked Glcp residues may give DSRS glucans resistance to enzymic digestion. I-DSRT5 glucan contained less 6-linked Glcp than S-DSRS glucan (Table 1), but was digested by endodextranase to almost the same extent as S-DSRS glucan (Fig. 2). The amounts of T-Glcp, 3,6-Glcp, and 4-Glcp in I-DSRT5 were less than those in S-DSRT5, S-DSRS, and I-DSRS glucans (Table 1). S-DSRT5 glucan contained about 40% 6-linked Glcp (Table 1), but was not effectively digested by endodextranase (Fig. 2). The proportions of T-Glcp and 3,6-Glcp in S-DSRT5

glucan were higher than those in I-DSRT5 glucan, and the highest proportion of 4-linked Glcp was found in S-DSRT5 glucan. Thus, I-DSRT5 glucan seemed to be less branched and less resistant to enzymatic digestion than S-DSRT5 glucan.

The glucans were also digested by glucodextranase, Novozym 234 (an α -(1 → 3)-glucanase) and alpha amylase. Glucodextranase did not effectively digest S-DSRS, I-DSRS, S-DSRT5, or I-DSRT5 glucans (Fig. 2). Novozym 234 digested I-DSRT5 glucan efficiently endodextranase but did not digest other glucans. Alpha amylase did not digest any glucans. Gel-filtration of alpha amylase-treated S-DSRS and native B-512F dextran on Sepharose 6B and TSK gel G3000 SWXL revealed that their molecular weights were not changed (both were < 4,000,000). Enzymatic digestion did not show the existence of 4-linked Glcp in the glucans. S-DSRS, I-DSRS, and S-DSRT5 were not digested by alpha amylase, even they contained about 15% 4-linked Glcp. The structure of these glucans is resistant to alpha amylase digestion, as found for endodextranase digestion.

Different characteristics of glucans were also demonstrated by the reaction with concanavalin A. S-DSRS, I-DSRS, and S-DSRT5 glucans formed precipitates with concanavalin A, whereas clinical dextran, native B-512F dextran, and I-DSRT5 glucan did not. Endodextranase-resistant glucans precipitated well with concanavalin A.

We reported that *dssS* mRNA was expressed in the B-512F strain when the strain was cultured with sucrose, and that its level was five times higher than that of *dssT* mRNA, as shown by Northern blotting.⁹ DSRS is likely to exist in the B-512F strain. However, glucans produced by recombinant DSRS had different characteristics from native B-512F dextran, as shown by glycosyl-linkage analysis and enzyme digestion patterns. The molecular mass of DSRS deduced from the nucleotide sequence was almost the same as that of native B-512F dextranase, but that of DSRS determined by SDS-PAGE was 30 kDa larger than that of native B-512F dextranase.⁹ It is unclear if the tertiary structures of native DSRS existing

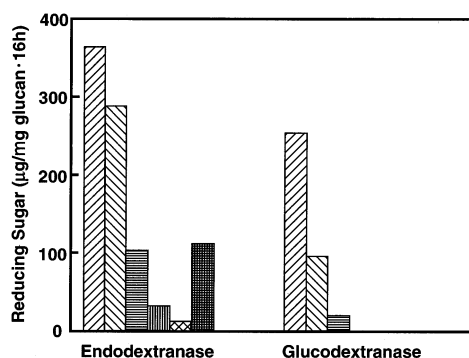


Fig. 2. Digestion of the glucans by dextranases. Clinical dextran and DSRS or DSRT5 glucans (200 μ g each) were digested with endodextranase and glucodextranase. The reducing sugar produced was determined colorimetrically as described in Section 3. ▨, clinical dextran; ▩, B-512F dextran; ■, S-DSRS glucan; □, I-DSRS glucan; ▤, S-DSRT5 glucan; ■, I-DSRT5 glucan.

in the B-512F strain and of recombinant DSRS expressed in *E. coli* are the same. The ratio of the linkages in glucans may be changed by differences of the structures of dextransucrases.

L. mesenteroides B-512F strain probably had at least two different types of dextransucrases, DSRS, which produces water-soluble glucan, and DSRT5, which produces water-insoluble glucan. Holt and Côté found that many polymer-producing *Leuconostoc* strains, including B-512F, produce more than one kind of dextransucrase, as shown by analysis of randomly amplified polymorphic DNA.¹¹ Both glucosyltransferases that synthesize water-soluble glucan and those that synthesize water-insoluble glucan are required for maximal plaque formation of oral streptococci.¹² Glucans produced by oral streptococci adhere to smooth tooth surfaces and facilitate aggregation of bacteria. The dextransucrases of *L. mesenteroides* may also have evolved in order to make various kinds of dextrans, or this organism may have discarded certain kinds of enzymes in order to produce glucans suitable for bacterial colonization in the natural environment.

3. Experimental

Enzyme assay and protein concentration.—The dextransucrase activity was assayed colorimetrically by the Nelson–Somogyi procedure¹³ by measuring the release of reducing sugar from sucrose with glucose as a standard as described previously.¹⁴ One unit of dextransucrase releases 1 μ mole of reducing sugar from sucrose per min. The protein concentration was measured by using the BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

Expression of gene product DSRS, DSRT, and DSRT5 proteins in E. coli BL21(DE3).—The plasmids pDSRT, pDSRT5, and pDSRS were constructed as described before.⁹ pET23d as a control, and the plasmids carrying *dsrS*, *dsrT*, and *dsrT5*, named pDSRS, pDSRT, pDSRT5, respectively, were used to transform *E. coli* BL21(DE3) cells. Fresh single colonies grown on Luria–Bertani plates containing 200

μ g/mL ampicillin were inoculated in 3 mL of Luria–Bertani broth containing 200 μ g/mL ampicillin and cultured at 37 °C overnight with shaking. These cultures were inoculated in 150 mL of the same broth at a ratio of 1:60 and the broth was incubated for 2 h at 37 °C. Then 0.5 mM isopropyl β -D-1-thio-galactopyranoside was added and the cells were cultivated for 8 h at 30 °C. The cells were then pelleted by centrifugation at 1,500g for 10 min at 4 °C, suspended in 30 mL of 20 mM NaOAc (pH 5.2) containing 30% glycerol and centrifuged again at 6,000g for 10 min at 4 °C. Cells were resuspended in 16 mL of 20 mM NaOAc (pH 5.2) containing 30% glycerol and lysed by sonication. The supernatant was then obtained by centrifugation at 10,000g for 10 min, dialyzed against 20 mM NaOAc (pH 5.2) and used as the enzyme solution.

Preparation of glucans.—DSRS glucan and DSRT5 glucan were produced by incubation of the dialyzed DSRS or DSRT5 protein (0.01 to 0.02 unit/mL) in 100 mL of 10% sucrose in 20 mM NaOAc (pH 5.2) at 30 °C for 8 h. DSRS + DSRT5 glucan was produced by using equal dextransucrase units of both DSRS and DSRT5 proteins in the incubation. DSRS + DSRT glucan was produced by using equal masses of both DSRS and DSRT proteins in the incubation. B-512F DS dextran was prepared by using the purified dextransucrase (0.01 to 0.02 unit/mL) prepared as described before.¹⁵ After the incubation, an equal volume of EtOH was added to the solution with stirring, and then the precipitate was collected by centrifugation. The precipitate was suspended in water at a concentration of 1% (wet weight/volume). Precipitation with 50% EtOH was repeated 3 times. The resulting precipitate was dissolved in 1 M NaOH, and then neutralized with HCl. The glucan solution was dialyzed against water. The dialyzate was centrifuged at 10,000g for 10 min and the supernatant and precipitate were separated and lyophilized. The supernatant fraction was designated ‘S-glucan’ and the precipitate was designated ‘I-glucan’. Native B-512F dextran was prepared from the culture supernatant as described by Jeanes.¹⁶

¹³C NMR analysis of glucan.—The reducing glucosyl residues of lyophilized glucans were reduced to their corresponding alditols by

treatment with 4% NaBH₄ in 0.5 M NaOH. ¹³C NMR analysis was done as described by Shimamura.¹⁰ Reduced glucans (25 mg) were dissolved in 0.5 mL of 0.5 M NaOH containing 10% D₂O and 3 mg of sodium-4,4-dimethyl-4-silapentane-1-sulfonate (DSS), ultrasonicated for 15 min and transferred into 5-mm sample tubes (Shigemi BS001). ¹³C NMR spectra were recorded at 100.6 MHz with 23 744 scans for clinical dextran, 40 000 scans for S-DSRS glucan, and 41 280 scans for I-DSRT5 glucan, using a Varian Unity-plus 400 NMR spectrometer with a Varian 400 AutoSw PFG probe (4NUC/40-182 MHz), with complete proton decoupling, at 35 °C, with rotation at 20 rps.

Glycosyl-linkage composition analysis.—Lyophilized glucans (< 1 mg) were permethylated as described by York et al.¹⁷ To measure the relative amounts of per-*O*-acetylated alditols, the reaction products were dissolved in acetone and analyzed by gas chromatography (Shimadzu GC-14A with C-R6A Chromatopac) with an SP-2330 (0.25 mm × 30 m) column (Supelco). Identification of each per-*O*-acetylated alditol was performed by gas chromatography–mass spectrometry (Hewlett Packard HP6890 Series GC System with Benchtop Quadrupole Mass Spectrometer JEOL Automass System II) with an SP-2330 (0.25 mm × 30 m) column (Supelco).

Digestion of glucans by various glucanases.—Glucans (200 µg each in a 400-µL reaction mixture) were incubated at 30 °C for 16 h with endodextranase (Worthington Biochemical Co.) (2 units) in 50 mM Sodium phosphate (pH 6.0), glucodextranase (Wako Chemical Co.) (2 units) in 50 mM Sodium phosphate (pH 6.0), Novozym 234 (Novo Nordisk) (0.07 mg) in NaOAc (pH 5.5), or with alpha amylase from porcine pancreas (Sigma) (2 units) in 50 mM Sodium phosphate (pH 6.9). One unit of dextranase releases 1 µmol of isomaltose per min. One unit of alpha amylase releases 1 mg of maltose per 3 min. The reaction was terminated by adding 150 µL of 0.5 M NaOH. The amount of reducing sugar produced was measured colorimetrically by the Nelson–Somogyi procedure¹³ using glucose as a standard.

Molecular weight distribution.—Gel-filtration column chromatography of glucans was performed using a Sepharose 6B column (2 × 86 cm) which was eluted with water, and total sugar was measured as described before.¹⁸ HPLC gel filtration was performed with a Tsk gel G3000 SWXL (7.8 × 300 mm) column (Tosoh) eluted with 20 mM Sodium phosphate (pH 7.6)–0.2M NaCl–0.02% NaN₃ and monitored with an RI (RI830) detector as described before.¹⁸

Precipitation test with concanavalin A.—Concanavalin A from jack bean was purchased from Honen Co. Glucan (0.2 to 1 mg/mL) was tested for the formation of a precipitate with 2 mg/mL concanavalin A in 50 mM NaOAc (pH 5.2) or in 50 mM Sodium phosphate (pH 7.0) at 25 °C for 10 min, and the increase of turbidity was measured at 420 nm as described before.¹⁹ The blank value was the sum of the absorbances of the glucan solution and concanavalin A solution. Water-insoluble glucans were first dissolved in 0.1 M NaOH and then neutralized with HCl before concanavalin A treatment.

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