Role of the Two Catalytic Domains of DSR-E Dextranucrase and Their Involvement in the Formation of Highly α -1,2 Branched Dextran

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The *dsrE* gene from *Leuconostoc mesenteroides* NRRL B-1299 was shown to encode a very large protein with two potentially active catalytic domains (CD1 and CD2) separated by a glucan binding domain (GBD). From sequence analysis, DSR-E was classified in glucoside hydrolase family 70, where it is the only enzyme to have two catalytic domains. The recombinant protein DSR-E synthesizes both α -1,6 and α -1,2 glucosidic linkages in transglucosylation reactions using sucrose as the donor and maltose as the acceptor. To investigate the specific roles of CD1 and CD2 in the catalytic mechanism, truncated forms of *dsrE* were cloned and expressed in *Escherichia coli*. Gene products were then small-scale purified to isolate the various corresponding enzymes. Dextran and oligosaccharide syntheses were performed. Structural characterization by ¹³C nuclear magnetic resonance and/or high-performance liquid chromatography showed that enzymes devoid of CD2 synthesized products containing only α -1,6 linkages. On the other hand, enzymes devoid of CD1 modified α -1,6 linear oligosaccharides and dextran acceptors through the formation of α -1,2 linkages. Therefore, each domain is highly regiospecific, CD1 being specific for the synthesis of α -1,6 glucosidic bonds and CD2 only catalyzing the formation of α -1,2 linkages. This finding permitted us to elucidate the mechanism of α -1,2 branching formation and to engineer a novel transglucosidase specific for the formation of α -1,2 linkages. This enzyme will be very useful to control the rate of α -1,2 linkage synthesis in dextran or oligosaccharide production.

From sucrose, *Leuconostoc mesenteroides* NRRL B-1299 produces a very uncommon dextran containing 61% α -1,6 glucosidic linkages in the linear chain and 28% α -1,2 linkages at branching points (11). The dextranucrase catalyzing the formation of this unusual dextran also produces glucooligosaccharides (GOS) by acceptor reaction. In the presence of a maltose acceptor, the glucosyl moiety of sucrose is transferred to maltose at the cost of polymer synthesis. This results in the formation of two different GOS families: the α -1,6 GOS series and the α -1,2 GOS series (5). The α -1,6 GOS series includes linear GOS possessing only α -1,6 linkages and a maltose residue at the reducing end. These oligosaccharides can also be produced by *L. mesenteroides* NRRL B-512F dextranucrase (12). The α -1,2 GOS series consists of α -1,6 GOS bearing an additional glucosyl residue linked by an α -1,2 linkage at the nonreducing end of the molecule or at the branching points. GOS containing α -1,2 linkages are resistant to enzymatic digestion in animals and humans and have been shown to possess prebiotic properties (3, 20, 22, 25). They are commercialized for application in animal and human nutrition, as well as in dermocosmetics.

To investigate the mechanism of highly α -1,2 branched dex-

tran and GOS formation, three genes from *L. mesenteroides* NRRL B-1299 were cloned and expressed in *Escherichia coli*, all encoding glucansucrases. They were named *dsrA* (16), *dsrB* (17), and *dsrE* (2). Of the three gene products obtained, only DSR-E was found to catalyze the synthesis of both α -1,6 and α -1,2 glucosidic linkages (2). Sequence comparison revealed that DSR-E belongs to glycoside hydrolase family 70 (8), like all other glucansucrases from lactic acid bacteria. However, the primary structure of this unusual enzyme shows striking features (2). First, the protein molecular weight is about twice the average molecular weight of the other glucansucrases from family 70. In addition, DSR-E possesses two catalytic domains (CD1 and CD2) instead of the one usually found in this family. These domains are separated by a long domain, homologous to the C-terminal domain of dextranucrases, often called the glucan binding domain (GBD). CD1 extends from amino acids 248 to 1141 and CD2 extends from amino acids 1980 to 2835. They show 45% identity and 65% similarity. Both catalytic domains contain the highly conserved amino acids (7, 8) proposed to be involved in the glucosyl enzyme intermediate formed from sucrose cleavage via the same α -retaining mechanism employed by the enzymes from families 13 and 70 (2, 9). Asp 527 in CD1 and Asp 2210 in CD2 were proposed to play the role of the nucleophile in each independent domain; Glu 565 in CD1 and Glu 2248 in CD2 were also assigned the role of general acid-base catalyst. From this analysis, it appeared that both domains were probably fully active. In addition, in the vicinity of the catalytic residues, CD2 contains stretches of sequence that diverge from the consensus sequence usually found in all glucansucrases that mainly catalyze the formation of α -1,6 linkages. For this reason, specificity for the synthesis of

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TABLE 1. Oligonucleotides used to amplify different truncated forms of *dsrE* (accession no. AJ430204)

Name	Location	Sequence ^a
pBAD-SP/VZ-dir	677–706	5'-GCCATGGCAAATACGATTGCAGTTGACACG-3'
pBAD-VZ/CD1-dir	1304–1334	5'-GCCATGGACGGTAAAACCTATTTCTTGACG-3'
pBAD-CD1/GBD-dir	3986–4012	5'-CAGTCCATGGGTGAAAAACAAGCAC-3'
pBAD-GBD/CD2-dir	6500–6522	5'-ACCATGGATATGTCTACTAATGC-3'
pBAD-CD1/GBD-rev	3971–3991	5'-TAACTGTTTAGGCAAGAATCC-3'
pBAD-GBD/CD2-rev	6479–6505	5'-TAATGTATTAGTGAATAAGTATTCACC-3'
pBAD-ent-rev	9047–9070	5'-AATTTGAGGTAATGTTGATTATC-3'

^a NeoI sites are underlined. Boldface letters show punctual mutations added in comparison with the native sequence.

the α -1,2 linkage was suggested to be related to the presence of CD2. Otherwise, the particularly long GBD of DSR-E (839 amino acids) is composed of 41 consecutive repeats, homologous to the cell wall binding units (2, 21). From these observations, it has been suggested that the structure of the GBD resembles that of the C-terminal choline binding domain of autolysin LytA from *Streptococcus pneumoniae* (6), showing a solenoid fold adapted to interaction with a glycan chain. The DSR-E variable zone (VZ) also shows peculiarities. Several studies demonstrated that the deletion of this nonconserved region does not affect enzyme activity (1, 19), but the VZ of DSR-E contains a 14-amino-acid unit called S, repeated 11 times, that has never been identified before and that is possibly involved in the catalytic mechanism (2).

As a consequence, compared to all the other glucansucrases from family 70, DSR-E shows many distinctive features that raise numerous questions, including the following. What is the involvement of the second catalytic domain of DSR-E in the catalytic mechanism? Can these catalytic domains be fully active independently? What are the functions of the long GBD and of the variable zone?

In this report, to address these questions, DSR-E and five truncated variants of DSR-E have been characterized. The functions of the different domains of the protein are discussed with respect to the structures of the products they form from sucrose.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain TOP10 was used as the host for TOPO cloning of PCR products in pBAD/TOPO Thiofusion (Invitrogen) and for the overproduction of complete (2) and truncated DSR-E enzymes.

Luria-Bertani broth was used as the culture medium, and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. Ampicillin was used at a concentration of 100 μ g ml⁻¹. *L. mesenteroides* NRRL strain B-1299 was cultured in 40 g of sucrose liter⁻¹, 20 g of yeast extract liter⁻¹, 20 g of K₂HPO₄ (pH 6.9) liter⁻¹ (adjusted with orthophosphoric acid), and 0.01 g of MnSO₄(H₂O) liter⁻¹ at 30°C.

Molecular techniques. *E. coli* plasmid isolation and *L. mesenteroides* NRRL B-1299 genomic DNA purification were carried out using the QiaPrep Spin Plasmid kit and the Blood and Cell Culture DNA Maxi kit (QIAGEN), respectively. General procedures for DNA manipulation were performed by standard methods (14). Restriction and modification enzymes were purchased from New England Biolabs or Gibco BRL and used according to the recommendations of the manufacturers.

Construction of expression plasmids bearing the truncated forms of *dsrE*. Cloning of the complete *dsrE* gene into the pBAD/TOPO Thiofusion vector in fusion with sequences encoding the thioredoxin tag at the 5' end and the histidine tag at the 3'-terminal end and under the control of the pARA promoter has been described previously (2).

(i) **Amplification of truncated forms of *dsrE*.** The primers presented in Table 1 were designed from sequence analysis of *dsrE* and the pBAD/TOPO Thiofusion vector and enabled the amplification of the following sequences from *L. mesenteroides* NRRL B-1299 genomic DNA (Table 2): *dsrE*- Δ (VZ), encoding the protein Δ (VZ), which corresponds to DSR-E with the VZ deleted; *dsrE*- Δ (CD2), encoding the protein Δ (CD2) (DSR-E devoid of the second catalytic domain); *dsrE*-CD1, encoding only the first catalytic core; *dsrE*-GBD-CD2, encoding the two C-terminal domains; and *dsrE*-CD2, encoding only the second catalytic core. In all the constructs, a NeoI restriction site was introduced in the forward primers to eliminate, if necessary, the thioredoxin tag at the 5' end.

Reactions were carried out in a final volume of 50 μ l containing genomic DNA (100 ng), primers (300 nM each), deoxynucleoside triphosphates (200 μ M each), Expand High Fidelity buffer (1 $\times$) containing MgCl₂ (1.5 mM), and 2.6 U of Expand High Fidelity polymerase (Roche Boehringer Mannheim). A Perkin-Elmer thermal cycler, model 2400, was used following the cycling parameters recommended by the enzyme manufacturer.

(ii) **Cloning of truncated *dsrE* fragments.** The resulting PCR products were purified (QIAquick gel extraction kit; QIAGEN) and ligated into the pBAD/TOPO Thiofusion vector. The presence of a thioredoxin tag located at the

TABLE 2. Schematic primary/structure of DSR-E and truncated forms constructed (accession no. CAD22883)

Protein name	Encoded protein	Theoretical mass (kDa) (without thioredoxin)	Primers used
DSR-E		312	pBAD-SP/VZ-dir pBAD-ent-rev
Δ (VZ)		291	pBAD-VZ/CD1-dir pBAD-ent-rev
Δ (CD2)		217	pBAD-SP/VZ-dir pBAD-GBD/CD2-rev
CD1		103	pBAD-VZ/CD1-dir pBAD-CD1/GBD-rev
GBD-CD2		192	pBAD-CD1/GBD-dir pBAD-ent-rev
CD2		98	pBAD-GBD/CD2-dir pBAD-ent-rev

N-terminal ends of the recombinant proteins was expected to enhance translation and improve solubilization. In the particular case of the plasmid harboring the *dsrE*-GBD-CD2 insert, higher levels of expression were reached without the thioredoxin tag, thanks to *Nco*I restriction and self-ligation. The results reported concerning this variant were obtained from the plasmid from which the thioredoxin-encoding fragment had been deleted.

Several clones were selected after transformation of chemically competent *E. coli* TOP 10 cells (Invitrogen) and checked by restriction digestion to select one plasmid harboring the coding sequence in the proper orientation. The plasmid selected was then verified by DNA sequencing.

(iii) DNA sequencing. All the inserts were subsequently sequenced (Genome Express, Grenoble, France) to determine whether mutations had been introduced by PCR. After comparison of the recombinant sequences with the genomic sequences, no mutations were found in four of them, *dsrE*- Δ (VZ), *dsrE*-CD1, *dsrE*-GBD-CD2, and *dsrE*-CD2. A single mutation leading to the F1411L protein variant occurred in *dsrE*- Δ (CD2). The mutation occurred in a junction sequence between two repeated motifs of the GBD and was thus ignored.

Preparation of crude cell extracts. (i) Recombinant enzymes. *E. coli* cells were grown at 30°C in Luria broth-ampicillin medium to an absorbance at 600 nm of 0.5. Arabinose was added to a final concentration of 0.002% (wt/vol), and the cells were allowed to grow for 6 additional hours. Then, the cells were harvested by centrifugation. The cell pellets were resuspended to a final A_{600} of 80 in 20 mM sodium acetate buffer, pH 5.4, supplemented with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication, and whole-cell extract was used for enzyme characterization.

(ii) Native enzymes. In order to produce native *L. mesenteroides* NRRL B-1299 dextranases, a 50-ml culture of the strain was performed at 30°C for 6 h (the end of the exponential growth phase). The cell-bound enzymes were harvested by centrifugation and resuspended in 5 ml of 20 mM sodium acetate buffer, pH 5.4. As described previously (2), this preparation contains four enzymes with molecular masses ranging from 195 to 283 kDa, all exhibiting the ability to form α -1,2 linkages.

Enzyme assay. The glucanase activity of the cellular extract was determined at 30°C by monitoring the release of fructose from sucrose using the dinitrosalicylic acid method (24). One unit is defined as the amount of enzyme that catalyzes the formation of 1 μ mol of fructose min^{-1} at 30°C in 20 mM sodium acetate buffer (pH 5.4)–0.34 mM CaCl_2 with 100 g of sucrose liter^{-1} .

SDS-PAGE analysis and zymograms. Tenfold-diluted crude cell extracts with activities ranging from 0 to 40 mU, depending on the construction considered, were loaded on 3 to 8% NuPAGE Tris-acetate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen). After migration, the gel was either submitted to colloidal blue staining for total protein detection or transferred to 20 mM sodium acetate buffer for glucanase renaturation. Incubation of the gel in 100 g of sucrose solution liter^{-1} in 20 mM sodium acetate buffer (pH 5.4)–0.34 mM CaCl_2 –0.1% Triton X-100 allowed polymer synthesis *in situ*. Dextran was then detected by Schiff staining (15).

Dextran synthesis or modification with crude cell extract and purification. For polymer synthesis, enzymatic reactions were performed for 24 h in the presence of 100 g of sucrose liter^{-1} , 0.5 U of recombinant enzyme ml^{-1} , 20 mM sodium acetate buffer, and 0.34 mM CaCl_2 . α -1,6 dextran (70,000 kDa; Sigma) was modified by the variant GBD-CD2 over 12 h in the presence of 100 g of sucrose liter^{-1} , 1 U of crude enzyme ml^{-1} , 20 mM sodium acetate buffer (pH 5.4), 0.34 mM CaCl_2 , and 50 g of dextran liter^{-1} . Dextrans were precipitated with ethanol (50% [vol/vol]), and recovered in water before the next precipitation in ethanol. The procedure was repeated four times before the dextrans were freeze-dried.

^{13}C nuclear magnetic resonance (NMR) analysis of polymer. Quantitative ^{13}C spectra were recorded using the INVGATE Bruker sequence, with 90° orientation pulse length (6.5 μ s), 15,000-Hz spectral width, 16,000 data points, 0.54 s acquisition time, and a relaxation delay of 2.5 s: 60,000 scans were accumulated. The amounts of the different glucosidic linkages were determined by measuring the relative intensities of the corresponding anomeric carbon signals by both integration of peak areas and excision of the chart paper and weighing.

Enzyme small-scale purification. For each *E. coli* transformant expressing a *dsrE* variant, optimized concentrations of crude cell extract were loaded on SDS-PAGE gels (3 to 8% NuPAGE Tris-Acetate; Invitrogen) (crude cell extracts were diluted 10- or 20-fold to allow the best separation). The first track of the gel was dedicated to the molecular weight standard (Broad-range prestained precision protein standard; Bio-Rad). After migration, the gel was cut into two parts. The first part, corresponding to the track of the standard and one track of sample, was stained by colloidal blue. The other part of the gel, corresponding only to tracks containing sample, was submitted to protein renaturation in 20

FIG. 1. SDS-PAGE profiles of recombinant DSR-E and truncated forms produced by *E. coli* TOP10. Lanes: M, broad-range prestained precision protein standard (Bio-Rad); 1, DSR-E, 325 kDa; 2, Δ (VZ), 304 kDa; 3, Δ (CD2), 230 kDa; 4, CD1, 116 kDa; 5, GBD-CD2, 192 kDa; 6, CD2, 111 kDa.

mM sodium acetate buffer. Then, the gel was reconstituted. The part stained with colloidal blue allowed us to determine the position of the entire recombinant protein of the expected size. Then, the other part was blind cut at the corresponding site. This cut band was used for acceptor reaction catalysis under the conditions described below.

Acceptor reactions with crude cell extracts or micropurified enzymes. Acceptor reactions were performed with crude cell extracts containing 0.5 U of recombinant enzyme or the micropurified protein band extracted from SDS-PAGE/ml (in a 1-ml total volume).

(i) Maltose acceptor. Maltose acceptor reactions were carried out at 30°C in 20 mM sodium acetate buffer (pH 5.4)–0.34 mM CaCl_2 , with 200 g of sucrose liter^{-1} and 100 g of maltose liter^{-1} (sucrose/acceptor [S/A] ratio = 2).

(ii) α -1,6 GOS acceptor. The mixtures of α -1,6 GOS, with degrees of polymerization ranging from 3 to 6 (DP3 to -6), used as acceptors were previously synthesized by maltose acceptor reactions catalyzed by *L. mesenteroides* NRRL B-512F dextranase (12). The reactions were performed at 30°C in 20 mM sodium acetate buffer, pH 5.4, and 0.34 mM CaCl_2 . Two different S/A ratios (wt/wt) of 4 and 0.615 were tested. At an S/A ratio of 4, the final reaction mixture had the following composition: 200 g of sucrose liter^{-1} , 15.2 g of panose (α -D-glucopyranosyl-1,6-D-maltose, or α -1,6 GOS DP3) liter^{-1} , 21.6 g of α -1,6 GOS DP4 (α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6- α -D-maltose) liter^{-1} , 12.0 g of α -1,6 GOS DP5 (α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6-D-maltose) liter^{-1} , and traces of DPs of ≥ 6 . At an S/A ratio of 0.615, the sucrose concentration was decreased to 75 g liter^{-1} , and α -1,6 GOS was used at the following concentrations: 15.8 g of DP3 liter^{-1} , 43.7 g of DP4 liter^{-1} , 41.2 g of DP5 liter^{-1} , and 21.3 g of DP6 liter^{-1} .

After 20 h (the theoretical reaction time necessary for total sucrose depletion), the reaction was stopped by heating the mixture at 95°C for 10 min.

Oligosaccharide analysis. Acceptor reaction products were analyzed by either reverse-phase high-performance liquid chromatography (HPLC) or high-performance anion-exchange chromatography (HPAEC). Reverse-phase HPLC was carried out on a Hewlett-Packard system equipped with a C_{18} column (Ultrasep 100; 6 μ m; 5 by 300 mm; Bischoff Chromatography), using ultrapure water as the eluant at 0.5 ml min^{-1} . Oligosaccharides were detected by refractometry. HPAEC was carried out using a Dionex Carbo-Pack PA10 column, a mobile phase (150 mM NaOH) at 1 ml min^{-1} flow rate with a sodium acetate gradient (6 to 180 mM in 30 min), and a Dionex ED40 module with a gold working electrode and an Ag-AgCl pH reference to perform the detection. In addition to sucrose, fructose, glucose, maltose, leucrose (α -D-glucosyl-1,5-D-fructose), α -1,6 GOS ranging from DP3 to DP6, and α -1,2 GOS were used as standards. α -1,2 GOS DP4 (α -D-glucopyranosyl-1,2- α -D-glucopyranosyl-1,6-D-maltose) and DP5 (α -D-glucopyranosyl-1,2- α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6-D-maltose) were synthesized and isolated as previously described (5) and also used as standards. Oligosaccharides were identified from their retention times and quantified from their response factors.

Percentage of glucosyl units transferred from sucrose to water, fructose, α -1,6,

TABLE 3. Glucosidic linkages of dextrans synthesized by different variants of DSR-E, determined by quantitative ^{13}C NMR analysis

Protein	Proportion of glucosidic linkages (%)			
	α -1,6	α -1,2	α -1,3	α -1,4
<i>L. mesenteroides</i> NRRL B-1299	61	28	12	0
DSR-E	81	5	10	3
Δ (VZ)	82	10	6	2
Δ (CD2)	86	0	11	3

or dextran; α -1,2 GOS composition of the final reaction medium; and oligosaccharide yields (Y_{GOS}) were determined from the following equations: percentage of glucosyl units transferred to water = $100 \times \text{glucose (millimolar)}/\text{sucrose consumed (millimolar)}$; percentage of glucosyl units transferred to fructose = $100 \times \text{leucrose (millimolar)}/\text{sucrose consumed (millimolar)}$; percentage of glucosyl units transferred to α -1,6 GOS in the final reaction medium = $100 \times \text{total } \alpha$ -1,2 GOS (millimolar)/sucrose consumed (millimolar); percentage of glucosyl units transferred to dextran = $100 - \text{percentage of glucosyl units transferred to water; } \alpha$ -1,2 GOS composition = $100 \times \text{mass of } \alpha$ -1,2 GOS/mass of total GOS; Y_{GOS} = mass of final GOS/($0.474 \times \text{mass of sucrose consumed} + \text{mass of acceptor consumed}$).

RESULTS

Construction of truncated forms of *dsrE* gene. Plasmids for high-level overexpression of truncated *dsrE* genes were first constructed, checked by DNA sequencing, and expressed in *E. coli*.

Truncated DSR-E variants (Table 2) were successfully overproduced, as shown on the SDS-3 to 8% PAGE gel (Fig. 1). The protein sizes were in agreement with the expected sizes. Enzyme assays of crude cell extracts using dinitrosalicylic acid confirmed that, except for CD2 alone, all the variants possessed a glucosyltransferase activity in the presence of sucrose as a unique substrate. The levels of activity obtained for each crude cell extract were 580 U/liter of culture for the entire DSR-E, 635 U/liter for Δ (VZ), 169 U/liter for Δ (CD2), 38 U/liter for GBD-CD2, and 2 and 0 U/liter for CD1 and CD2 alone, respectively.

Zymograms detecting polymer synthesis activity were analyzed in parallel. They revealed that CD2, with or without GBD, did not produce any polymer. The other forms, except for CD1, were active. The zymograms also revealed the presence of several active bands of lower molecular weight than the expected one. This may be due to proteolytic degradation, mRNA instability, secondary translation sites, or premature

release of ribosomes. Western blot experiments using anti-thioredoxin or anti-His antibodies indicated that all degraded forms harbored only one of the two tags, showing that degradation proceeds from both extremities and thus prevents the isolation of the complete forms on the Ni column (data not shown).

However, SDS-PAGE gels (Fig. 1) showed that nondegraded recombinant proteins are preponderant in each crude enzyme extract. In addition, no glucansucrases are naturally produced by *E. coli*. This allowed these extracts to be used for polymer synthesis.

Actions of DSR-E and its truncated forms on sucrose: polymer synthesis. Polymer synthesis was performed in the presence of 100 g of sucrose liter $^{-1}$ and the various crude enzyme extracts except for CD1 and CD2 alone, which were not sufficiently active. The glucans obtained were analyzed by ^{13}C NMR spectroscopy. The various signals were assigned as described by Seymour and Knapp (23). The relative contents of the different glucosidic linkages of the polymer were then calculated and compared to the data obtained for the glucan synthesized by the native enzymes (Table 3).

All the glucans produced are dextrans, since they possess >50% α -1,6 linkages, ranging from 81% for the complete form to 86% for the Δ (CD2) form.

In addition to α -1,6 glucosidic bonds, α -1,2 linkages were also observed in dextran synthesized by the complete form of DSR-E. Surprisingly, the percentage of α -1,2 linkages was low (5%). Characteristic α -1,3 and α -1,4 ^{13}C anomeric signals were also detected. They represented 10 and 3%, respectively, of the overall glucosidic bonds. The variant Δ (VZ) synthesized α -1,2 bonds more efficiently than the complete enzyme: indeed, the polymer produced was composed of 10% α -1,2 glucosidic bonds versus 5% for the complete DSR-E. Moreover, no α -1,2 signal was detected on spectra corresponding to enzymatic forms devoid of the second catalytic domain. Finally, GBD-CD2 was incapable of producing any polymer. Seventy-eight and 16% of the glucosyl residues coming from the sucrose were transferred to water (sucrose hydrolysis) or to previously released fructose to form leucrose (α -1,5 glucosylfructose), respectively (Table 4). Then, acceptor reactions were performed to further characterize the capacity of each variant to transfer glucose units on various acceptors and to determine yields and the type of linkage synthesized.

Actions of DSR-E and its truncated forms in the presence of maltose acceptor. First, GOS synthesis was performed with

TABLE 4. Products formed with or without acceptors by GBD-CD2^a

Initial concn (g · liters $^{-1}$)			% of glucosyl units transferred from glucose to:			α -1,2 GOS composition (%) of final reaction medium (wt)	Synthesis yield of α -1,2 GOS (Y_{α -1,2 GOS) (%)
Sucrose	Maltose	α -1,6 GOS	α -1,6 dextran (70,000 mw)	Water (hydrolysis)	Fructose (leucrose synthesis)	α -1,6 GOS or dextran through α -1,2 linkage formation	
100				78	16		
200	100			65	25		
200		50		32	28	40	31
75		122		19	23	58	72
100			50	30		70	

^a Reactions were carried-out using 0.5 U of crude cell extract ml $^{-1}$, except for the dextran acceptor-reaction (1 U · ml $^{-1}$), at 30°C in 20 mM sodium acetate buffer, pH 5.4. When the total of the percentages of glucosyl units transferred differed from 100, small quantities of unidentified sucrose isomers and/or maltooligosaccharides were encountered but were not quantified.

FIG. 2. Reverse-phase HPLC chromatograms of the oligosaccharides synthesized by the different crude truncated forms of DSR-E in the presence of sucrose and maltose. The enzymes used were (a) native *L. mesenteroides* NRRL B-1299 dextranucrase, (b) complete DSR-E, (c) DSR-E with the variable zone deleted [Δ (VZ)], (d) DSR-E with the second catalytic domain deleted [Δ (CD2)], (e) the first catalytic domain alone (CD1), (f) CD2 linked with GBD (GBD-CD2), and (g) the second catalytic domain alone (CD2). F, fructose; M, maltose; S, sucrose; P, panose, or α -D-glucopyranosyl-1,6- α -D-maltose; α -1,6 GOS DP4, α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6- α -D-maltose; α -1,6 GOS DP5, α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6- α -D-glucopyranosyl-1,6- α -D-maltose; α -1,2 GOS DP4, α -D-glucopyranosyl-1,2- α -D-glucopyranosyl-1,6- α -D-maltose; α -1,2 GOS DP5, α -D-glucopyranosyl-1,2- α -D-glucopyranosyl-1,6- α -D-maltose. The profiles obtained with crude or micropurified enzyme are similar (not shown).

TABLE 5. Synthesis yields of α -1,6 GOS, α -1,2 GOS, and total GOS in the presence of 200 g of sucrose liter⁻¹ and 100 g of maltose liter^{-1a}

Protein	Oligosaccharide yield (%)		Total oligosaccharide yield (%)	Sucrose consumption (%)
	$Y_{\alpha-1,6 \text{ GOS}}$	$Y_{\alpha-1,2 \text{ GOS}}$		
<i>L. mesenteroides</i> NRRL B-1299	32.6	26.4	59	100
DSR-E	68.2	11.7	79.9	100
Δ (VZ)	59.2	21.4	80.6	100
Δ (CD2)	84.2	0.0	84.2	100
CD1	57.3	0.0	57.3	20
GBD-CD2	0.0	0.0	0.0	73
CD2	0.0	0.0	0.0	7

^a Reactions were carried out using the different truncated forms of DSR-E, with 0.5 U of crude cell extract ml⁻¹, at 30°C in 20 mM sodium acetate buffer, pH 5.4.

each variant using 200 g of sucrose liter⁻¹ and 100 g of maltose liter⁻¹. Fig. 2 shows the different HPLC profiles from which the oligosaccharide synthesis yields were calculated (Table 5). CD1 and CD2 show very low sucrose consumption rates: only 20 and 7%, respectively, were consumed after 20 h of reaction. GOS with α -1,2 linkages are synthesized only by the variants possessing both catalytic domains. Another observation concerns the variant devoid of the variable zone, for which a twofold-increased α -1,2 GOS yield was observed compared to DSR-E. Surprisingly, variants containing only the second catalytic domain did not produce any GOS from sucrose and maltose; most of the glucosyl moieties were transferred to water (65%) or fructose (25%) (Table 4). To understand the absence of GOS synthesis, α -1,6 GOS mixtures were tested as acceptors for GBD-CD2.

Action of GBD-CD2 in the presence of α -1,6 GOS and dextran acceptors. (i) **α -1,6 GOS acceptor.** The HPAEC chromatograms presented in Fig. 3 show that in the presence of 200 g of sucrose liter⁻¹ and 50 g of α -1,6 GOS acceptors liter⁻¹, the variant GBD-CD2 used almost all the α -1,6 GOS as an acceptor to form products whose retention times are similar to those of α -1,2 GOS. This shows that GBD-CD2 transfers one glucosyl residue to the nonreducing end of each α -1,6 GOS through the formation of an α -1,2 linkage. Moreover, no α -1,6 GOS was produced, as indicated by the calculation of the α -1,2 GOS yield, which reached 31%. This low result can be explained by the high level of glucosyl transfer to water (32%) or fructose (28%) (Table 4). However, under these conditions, α -1,2 GOS represented 89% of the total final GOS mixture. Using a lower sucrose/acceptor ratio (75 g of sucrose liter⁻¹ and 122 g of α -1,6 GOS liter⁻¹) reduced hydrolysis and leucrose synthesis. Consequently, an α -1,2 GOS yield of 72% was reached and α -1,2 GOS represented 60% of the total GOS mixture.

In parallel to the acceptor reactions carried out with the crude extracts, reactions were performed with the constructs purified from SDS gels and thus devoid of any active degraded proteins. The profiles of the synthesized products were similar to those obtained with the crude extracts, confirming that the activity of the degraded forms can be ignored (data not shown).

(ii) **Linear dextran acceptor.** α -1,6 dextran with an average molecular weight of 70,000 was used as an acceptor. NMR

FIG. 3. HPAEC chromatograms of the oligosaccharides synthesized by the truncated form GBD-CD2 of DSR-E in the presence of sucrose and α -1,6 GOS.

spectra of the dextran, before and after the acceptor reaction (shown in Fig. 4A and B, respectively), revealed that GBD-CD2 significantly modified the acceptor. Indeed, spectrum B displayed all the chemical shifts characteristic of the α -1,2 branched dextran. At 97.17 ppm, the anomeric carbon that participates in the α -1,2 linkage appears. At 98.62 and 96.34 ppm, the anomeric carbons corresponding to glucosyl units involved in α -1,6 linkages, carbon 2 free or linked to another glucose unit, respectively, are encountered. The presence of the α -1,2 linkage is further confirmed by the signal at 76.61 ppm, which corresponds to carbon 2 involved in α -1,2 linkages. From the integration of the different anomeric signals, the α -1,2 content was estimated to be 32%.

DISCUSSION

The structural organization of DSR-E with two potentially active catalytic domains, CD1 and CD2, is highly unusual in glycoside-hydrolase family 70 enzymes. In addition, the enzyme was shown to catalyze the formation of both α -1,6 and α -1,2 glucosidic linkages (2). This prompted us to investigate the role of each domain with regard to its specificity in order to elucidate the mode of highly α -1,2 branched dextran forma-

tion. For this purpose, truncated forms of DSR-E were constructed and their activities were characterized.

Specificity of each catalytic domain. The data presented here demonstrate that each catalytic domain exhibits a specific function. Indeed, all the truncated enzymes devoid of CD2 essentially synthesized α -1,6 linkages (and small amounts of α -1,3 and α -1,4 linkages in dextran polymer). Interestingly, the GBD-CD2 variant catalyzes glucosyl transfers from sucrose to dextran (or the α -1,6-GOS acceptor) exclusively through α -1,2 linkage synthesis. The α -1,2 linkage is a very rare glucosidic linkage in nature. For this reason, α -1,2-GOS are highly resistant to digestive enzyme attack. CD2 is the only catalytic site described up to now that is able to synthesize this type of linkage from sucrose.

Functions of the glucan binding domain and the variable zone. Analysis of maltose acceptor reaction products formed by the entire recombinant DSR-E revealed that this construct synthesizes mainly α -1,6 GOS and very few α -1,2 GOS. This indicates that CD1 possesses a higher catalytic efficiency than CD2 in the complete construct. Also, both catalytic cores (CD1 and CD2) can be independently active. However, alone, both of them showed very low activity. In addition, they were very unstable, as shown by the very low sucrose consumption observed in all of the assays. Remarkably, the truncated forms with GBD either at the C-terminal or N-terminal end are much more active, suggesting that this domain is important for maintaining catalytic core efficiency and stability. This is consistent with similar results obtained for other family 70 glucansucrases (10, 13, 18). The C-terminal domains of all these enzymes usually contain repeats, showing homology with the cell wall binding repeat motif of DSR-E. In some cases, deletion of these repeats was shown to affect glucansucrase stability or catalytic efficiency. This was particularly marked for dextran-sucrase from *L. mesenteroides* NRRL B-512F (18). Interestingly, the N-terminal variable zone of DSR-E also modulates enzyme activity. When this region was deleted, DSR-E catalyzed the formation of α -1,2 GOS more efficiently, suggesting that the relative activity of CD1 versus that of CD2 was lower. The variable zone thus contributes to enhancing CD1 catalytic efficiency. It can be suggested that the repeated S motifs may be involved in this function.

Roles of the two domains in the synthesis of *L. mesenteroides* NRRL B-1299 α -1,2 branched dextran. To synthesize products harboring both α -1,6 and α -1,2 linkages, the DSR-E catalytic

FIG. 4. ^{13}C NMR analysis of the dextran synthesized by dextran-sucrases from *L. mesenteroides* NRRL B-512F before (A) and after (B) modification by the variant GBD-CD2 of DSR-E.

domains clearly must work together. From the specificity displayed by each domain, CD1 is proposed to be involved in the formation of the α -1,6 linear dextran, which will be used as an acceptor by CD2 acting as an α -1,2 branching enzyme. However, dextran produced by the recombinant DSR-E is less branched than dextran synthesized by native *L. mesenteroides* NRRL B-1299 dextranases. It contains only 5% α -1,2 linkages versus ~30% usually found in the native polymer (4). Consistent with this observation is the low α -1,2 GOS yield obtained with recombinant DSR-E by the maltose acceptor reaction. With a value of 11%, it is half the α -1,2 GOS yield reached with the native enzymes. SDS-PAGE analysis of the extracellular enzymes produced by *L. mesenteroides* NRRL B-1299 revealed that this strain produces several forms synthesizing α -1,2 linkages (2). The various active forms identified were attributed to active degradation products of DSR-E. We have seen that recombinant truncated forms of DSR-E can be more efficient than the complete enzyme in catalyzing α -1,2 linkage synthesis. This is the case for Δ (VZ) and GBD-CD2. We can thus assume that the degradation occurring during glucanase production by the DSR-E parental strain indeed generates enzymatic forms that are more efficient than whole DSR-E for catalyzing α -1,2 linkage synthesis.

Characteristics of the α -1,2 branching enzyme: GBD-CD2. Although GBD-CD2 possesses a catalytic domain showing ~44% identity with the catalytic domains of other family 70 glucanases, this enzyme possesses functional properties that distinguish it from family 70 enzymes. First, GBD-CD2 must not be named glucanase. It is in fact incapable of synthesizing a glucan polymer from sucrose alone, and under these conditions it catalyzes only sucrose hydrolysis and leucrose formation. The presence of leucrose indicates that fructose can be used as an acceptor of glucosyl moieties. More striking is the fact that glucose is not recognized as an acceptor: no glucodisaccharide or higher glucooligosaccharides were detected in the medium. In addition, maltose, which is known as one of the best acceptors for glucanases, is not glucosylated by GBD-CD2. This suggests that the acceptor binding site of GBD-CD2 is significantly different from the acceptor sites of all other glucanases. GBD-CD2 acts as an efficient transglucosidase only in the presence of α -1,6 GOS or dextran acceptors. All the α -1,6 GOS tested as acceptors were modified by the addition of an α -1,2 linkage at their nonreducing ends, and no branched product was detectable after enzyme action. However, modification of the dextran acceptor clearly showed that the enzyme is really efficient in transforming the linear dextran into an α -1,2 branched product. The dextran modified by GBD-CD2 showed an α -1,6 linear chain in which, statistically, one of two glucose units is α -1,2 glucosylated. In the presence of these acceptors, hydrolysis and leucrose formation is highly reduced, showing that α -1,2 branching synthesis is the natural function of CD2. The absence of α -1,2 branching points in modified α -1,6 GOS can be attributed to the small size of the acceptors, and it would be very informative to define the minimal size required to observe α -1,2 branching formation. From these results, it can be concluded that the GBD-CD2 active site really is designed to accommodate long dextran. On this basis, the enzyme can be qualified as an α -1,2 branching enzyme. Finally, alteration of the reaction conditions, in particular the sucrose/acceptor ratio, enabled the rate

of transformation of α -1,6 GOS to be modulated, with α -1,2 GOS representing 90% of the total GOS content at an S/A ratio of 4 versus 60% at an S/A ratio of 0.625. With the native enzyme, the proportion of α -1,2 GOS in the total GOS mixture never reached values higher than 45% under the best conditions (data not shown). Moreover, this suggests that dextran modification can also be controlled by the ratio of sucrose donor to dextran acceptor. Finally, the question that remains is what structural features are responsible for the rather unusual specificity of GBD-CD2. It is obviously difficult to answer this question in the absence of a three-dimensional structure. However, in sequence comparisons with family 70 glucanases, it has been noted that CD2 shows stretches of sequence differing from the consensus sequence in the vicinity of the amino acids involved in glucosyl enzyme formation (2). These fragments are strongly suspected to be implicated in enzyme specificity (2) and are obviously good candidates for site-directed mutagenesis aimed at changing enzyme specificity.

Dextranase DSR-E shows a structure that is unique in glucoside hydrolase family 70, essentially due to the presence of two catalytic domains, CD1 and CD2. This unique structure makes it a key enzyme for the formation of the highly α -1,2 branched dextran produced by the native *L. mesenteroides* NRRL B-1299 strain. Indeed, for the first time, the α -1,2 branching mechanism was demonstrated to be an acceptor reaction, catalyzed by a highly specialized domain, CD2, of DSR-E. Indeed, DSR-E possesses both a polymer elongation site (CD1) and a second catalytic site specific for branching formation (CD2). Moreover, CD2, associated with GBD, is fully active independently and presents very promising properties for the modification of linear dextran or high-yield production of prebiotic α -1,2 GOS. The structure-function relationship of this catalyst must now be further investigated to deepen our understanding of the structural elements controlling glucanase specificity. Its ability to use other acceptors will also be examined to develop new compounds of pharmaceutical and/or nutritional interest.

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