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Functional, Genetic, and Bioinformatic Characterization of Dextransucrase (DSRBCB4) Gene in *Leuconostoc mesenteroides* B-1299CB4

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A gene encoding a dextransucrase (*dsrBCB4*) that synthesizes only α-1,6-linked dextran was cloned from *Leuconostoc mesenteroides* B-1299CB4. The coding region consisted of an open reading frame (ORF) of 4,395 bp that coded a 1,465-amino-acids protein with a molecular mass of 163,581 Da. The expressed recombinant DSRBCB4 (rDSRBCB4) synthesized oligosaccharides in the presence of maltose or isomaltose as an acceptor, plus the products included α-1,6-linked glucosyl residues in addition to the maltosyl or isomaltosyl residue. Alignments of the amino acid sequence of DSRBCB4 with glucansucrases from *Streptococcus* and *Leuconostoc* identified conserved amino acid residues in the catalytic core that are critical for enzyme activity. The mutants D530N, E568Q, and D641N displayed a 98- to 10,000-fold reduction of total enzyme activity.

Keywords: Dextransucrase, *Leuconostoc mesenteroides*, glucansucrase, oligosaccharides, dextran

Dextran is a polysaccharide consisting of glucose monomers linked mainly (95%) by α-1,6-linkages [4, 17], and has important medical applications in the production of fine chemicals, such as plasma substitutes and Sephadex, as well as being used by the food industry for texture improvement in milk drinks, yoghurt, and ice cream [26].

Dextransucrases (or glucansucrases) [E.C. 2.4.1.5] are produced by several strains of *Leuconostoc*, *Streptococcus*, and *Lactobacillus* to catalyze the synthesis of α-D-glucan from sucrose [2]. Glucansucrases that produce primarily α-1,6-D-glucan are called dextransucrases. For example, *L. mesenteroides* NRRL B-512F produces a high percentage (95%) of α-1,6-dextran. However, different glucosidic linkages are also frequently found in glucan products. *Leuconostoc* strains produce glucans with α-1,6-linkages, often α-1,3-linkages, and sometimes α-1,2- or α-1,4-linkages [17, 29], *Streptococcus* strains produce glucan with α-1,6-linkages and/or α-1,3-linkages [7], and *Lactobacillus reuteri* 121 has been reported to produce glucan with mainly α-1,4- and α-1,6-linkages [14]. As such, the glucosidic linkage pattern of the synthesized glucan differs according to the kind of glucansucrase.

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Glucansucrases catalyze two different reactions depending on the nature of the acceptor substrate: (i) hydrolysis, when water is used as the acceptor, and (ii) glucosyl transfer (transferase), which can be divided into (a) polymerization, when the growing glucan chain is used as the acceptor, and (b) oligosaccharide synthesis, when small oligosaccharides (*e.g.*, maltose, isomaltose) are used as the acceptor [12].

Virtually all glucansucrases possess a common pattern for their structural organization: their N-terminal end starts with (i) a signal peptide, followed by (ii) a highly variable stretch, (iii) a highly conserved catalytic or sucrose binding domain, and (iv) a C-terminal domain composed of a series of tandem repeats [23].

Amino acid sequence comparisons have also revealed that the catalytic core of glucansucrases has a $(\beta/\alpha)_8$ barrel structure similar to that found in members of the glycoside hydrolase (GH) 13 family (http://afmb.cnrs-mrs.fr/CAZY/). However, in glucansucrases, this $(\beta/\alpha)_8$ barrel structure is circularly permuted [6, 18], which is why glucansucrases are classified in the GH70 family (http://afmb.cnrs-mrs.fr/CAZY/).

Although the catalytic mechanism for the α-amylase GH13 family is already known [19, 36], the catalytic mechanism of glucansucrases remains to be explained [23]. The crucial amino acid residues for catalysis of the GH70 family have also been identified as D453 (putative catalytic nucleophile), E491 (putative acid/base catalyst), and D564 (putative transition-state stabilizer) in the glucansucrase (GTFI) from *S. downei* Mf28 [6, 18], where the equivalent invariable residues in the enzymes of the α-amylase GH13 family are D229, E257, and D328 (CGTase of *Bacillus circulans* 251) [37]. Among the enzymes in both families (GH13 and GH70), the first-mentioned Asp residue is involved in the formation of covalent glucosyl-enzyme complexes

[18, 25, 36]. The importance of this Asp residue has also been shown in other glucansucrases by site-directed mutagenesis experiments [6, 9, 22].

Kim and Robyt [11] developed a mutant constitutive for the dextransucrase from *L. mesenteroides* NRRL B-1299 using ethyl methane sulfonate. The mutant (*L. mesenteroides* B-1299CB4) produced both extracellular and cell-associated dextransucrases on glucose media with a higher activity (4.5 times) than that produced by the parental strain on sucrose. Based on endodextransucrase hydrolysis, the mutant B-1299CB4 dextransucrase produced a slightly different dextran when it was elaborated on a glucose medium and on a sucrose medium [11].

Accordingly, this mutation study identified the catalytic residues in DSRBCB4 based on alignments with glucansucrases from lactic acid bacteria [23]. A dextransucrase gene (*dsrBCB4*) was isolated from *L. mesenteroides* B-1299CB4, a constitutive mutant, and then sequenced and expressed in *E. coli*. A molecular and biochemical (analysis of main reactions catalyzed by rDSRBCB4) characterization of the α-1,6-only synthesizing dextransucrase is also presented.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Culture Media

Escherichia coli TOP10 (Invitrogen, U.S.A.) and a pGEM-T easy vector (Promega, U.S.A.) were used for the cloning, and the plasmid pRSETB (Invitrogen, U.S.A.) was used to express the *dsrBCB4* gene into *E. coli* BL21(DE3)pLysS (Invitrogen, U.S.A.). The *E. coli* was grown in an LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl], whereas LB_{AMP}, the LB medium containing ampicillin (100 μg/ml), was used with the pGEM-T easy vector and pRSETB vector to isolate and express the protein, respectively [32]. The *L. mesenteroides* B-1299CB4 was grown in an LW medium [0.5% (w/v) yeast extract, 0.5% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4{\cdot}7H_2O$, 0.001% (w/v) NaCl, 0.001% (w/v) $FeSO_4{\cdot}7H_2O$, 0.001% (w/v) $MnSO_4{\cdot}H_2O$, 0.013% (w/v) $CaCl_2{\cdot}2H_2O$] containing 2% sucrose at 28°C [11].

Cloning of *dsrBCB4* Gene from *Leuconostoc mesenteroides* B-1299CB4

The two primers, CB4-F (5'-ATAGGTAATGGTTTTTGAAGT-3') as the 5' forward primer and CB4-R (5'-CCATTAGCAGCCCTAAC-AGCG-3') as the 3' reverse primer, were designed based on the DNA sequence of the dextransucrase gene (*dsrB*) from *L. mesenteroides* B-1299 (Accession No. AF030129). The chromosomal DNA from *L. mesenteroides* B-1299CB4 was used as the template for the PCR. The PCR mixture was kept at 94°C for 5 min, subjected to 30 thermal cycles of 60 s at 94°C, 60 s at 52°C, and 4 min at 72°C, and then kept at 72°C for an additional 10 min. The desired products were purified using agarose gel electrophoresis and an AccPrep gel extraction kit. The PCR product was then cloned into a pGEM-T easy vector to construct the plasmid designated as DSRBCB4-pGEMT [1]. The sequencing of the insert in DSRBCB4-pGEMT was conducted by the Korea Basic Science Institute Gwang-Ju Branch. To avoid any PCR error, the product sequencing was performed three times.

Construction of Expression Vector for Recombinant DSRBCB4

The plasmid for rDSRBCB4 production in *E. coli* was constructed using a pRSETB vector. XhoI and NcoI sites were introduced to the 5' and 3' end of the *dsrBCb4* gene, respectively, by a PCR (100 μl) that was carried out using Ex *Taq* polymerase (TaKaRa, Japan), a primer set [CB4-XhoIF (5'-TCACAAACTCGAGATGCA-GATAGC-ACA-3') as the forward primer and CB4-NcoIR (5'-AAAA-AAGCCCATGGTTAATTGAGTAGG-3') as the reverse primer], and DSRBCB4-pGEMT as the template DNA. The PCR reaction mixture was kept at 94°C for 5 min, subjected to 30 thermal cycles of 60 s at 94°C, 60 s at 53°C, and 4 min at 72°C, and then kept at 72°C for an additional 10 min. Thereafter, the predicted product (4.4 kbp) was purified using agarose gel electrophoresis and an AccPrep gel extraction kit, hydrolyzed using XhoI (underlined at CB4-XhoIF) and NcoI (underlined at CB4-NcoIR), and ligated at the XhoI-NcoI site of pRSETB. Next, the *dsrBCB4* gene was constructed as a protein composed of A41 to A1505 without a signal peptide (M1 to N40). The insert in the constructed plasmid, designated as rDSRBCB4-pRSETB, was then sequenced. The nucleotide and deduced amino acid sequence of *dsrBCB4* have been submitted to the NCBI nucleotide sequence database under the accession number DQ497800.

Site-directed Mutagenesis of Putative Catalytic Residues of DSRBCB4

Based on the amino acid sequence alignments of DSRBCB4 with different glucansucrases, three (putative) catalytic residues were identified in rDSRBCB4 and the mutant proteins (D530N, E568Q, and D641N). The site-directed mutagenesis was performed using a Quik-Change kit (Stratagene, U.S.A.), and plasmid DSRBCB4-pRSETB used as the template DNA.The synthetic oligonucleotides are listed in Table 1. After completing the cyclic reaction, 10 units of a DpnI restriction enzyme were added directly to each amplification reaction. The mixture was then incubated at 37°C for 1 h to digest the parental (*i.e.*, nonmutated) supercoiled double-strand DNA, followed by transformation into Epicurian coli XL1-Blue and confirmation of the mutation by DNA sequencing.

Table 1. Oligonucleotides used for site-directed mutagenesis of *dsrBCB4*.

Mutation point	Sequence of oligonucleotide primer	Direction
D530N	5'-GGGATACGTGTCAATGCTGTCGATAATGTT-3'	Sense
	5'-AACATTATCGACAGCATTGACACGTATCCC-3'	Antisense
E568Q	5'-CTTTCAATTCTTCAAGATTGGAGCCATAACG-3'	Sense
	5'-CGTTATGGCTCCAATCTTGAAGAATTGAAAG-3'	Antisense
D641N	5'-CGCGCACACAATAGTGAAGTACAAACAGTC-3'	Sense
	5'-GACTGTTTGTACTTCACTATTGTGTGCGCG-3'	Antisense

Purification of rDSRBCB4

The *E. coli* BL21(DE3)pLysS harboring rDSRBCB4-pRSETB was grown by shaking in 1,000 ml of LB_{AMP} and 35 μg/ml chloramphenicol at 37°C until the A_{600} reached 0.5. The recombinant protein was induced by the addition of 1 mM IPTG, followed by incubation with vigorous shaking at 16°C for 18 h. The cells collected by centrifugation (8,000 ×*g* for 10 min, 4°C) were then resuspended in 50 ml of a 50 mM potassium phosphate buffer containing 150 mM NaCl (pH 7.2) and 5 mM β-mercaptoethanol, and broken by sonication (Ultrasonic Processor 250; Sonics and Materials, Inc., CT, U.S.A.; output 4, duty cycle 50% for 30-sec sonication, repeated 25 times on ice). The supernatant obtained by centrifugation (12,000 ×*g* for 15 min, 4°C) was used as a cell-free extract and the starting material for the purification. The purification was performed by column chromatography in a Ni-NTA Superflow column (Qiagen, Germany), in which the resin was prepared according to the supplier's manual and equilibrated with a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, 5 mM β-mercaptoethanol). After loading 50 ml of the cell-free extract and washing the column with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 5 mM β-mercaptoethanol), the non-absorbed fractions were eluted by a stepwise gradient using 20, 50, and 100 mM imidazole in the washing buffer. The 6× His-tagged protein was eluted using an elusion buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0, 1 mM β-mercaptoethanol), pooled, dialyzed against a 20 mM sodium acetate buffer (pH 5.2) containing 0.02% sodium azide, and concentrated using a Centricon Ultracel YM-100 (Millipore, Bedford, MA, U.S.A.). All the purification steps were carried out at 4°C.

The *E. coli* BL21(DE3)pLysS harboring D530N-, E568Q-, or D641N-pRSETB was grown in 1,000 ml of LB_{AMP}, and mutated protein induction was performed based on the addition of 1 mM IPTG at 16°C for 18 h, and the purification procedure conducted as mentioned above for rDSRBCB4.

Electrophoresis and Detection of Active Dextransucrase

The SDS-PAGE was performed as described by Laemmli [15]. The protein was stained with Coomassie Brilliant Blue R-250 and the dextransucrase activities were detected by incubating the gels in 200 mM sucrose overnight, followed by staining using a periodic acid-Schiff (PAS) procedure [20]. The protein concentration was determined using the method of Bradford [3] with crystalline bovine serum albumin (Sigma, U.S.A.) as the standard.

Enzyme Activity Assay

The rDSRBCB4 dextransucrase activity was assayed by measuring the fructose released in the presence of 100 mM sucrose at 28°C in a 20 mM sodium acetate buffer, pH 5.2, 1 mM $CaCl_2$. Samples of the reaction mixture were taken at predetermined time intervals and the enzyme reaction was stopped by adjusting the pH to 10.5 by the addition of 25 mM NaOH. The fructose concentration librated from sucrose was determined using the dinitrosalicylic acid method [34] or TLC analysis method with the Public Domain NIH Image Program (developed at the U.S. National Institutes of Health) [35]. One unit was defined as the amount of enzyme that catalyzed the formation of 1 μmol fructose per minute at 28°C.

Effects of pH, Temperature, and Metal Ions on rDSRBCB4

The effect of pH on the enzyme activity was determined by varying the pH between 3.0 and 8.0, where a sodium citrate buffer (20 mM) was used for pH 3.0 to 4.0, a sodium acetate buffer (20 mM) for pH 4.5 to 5.5, and a sodium phosphate buffer (20 mM) for pH 6.0 to 8.0. To determine the optimum pH, the enzyme (0.31 U/ml of reaction mixture) was incubated with 50 mM sucrose at various pHs at 30°C for 30 min. Thereafter, to determine the pH stability of the dextransucrase, the enzyme (0.41 U/ml of reaction mixture for pH treatment) was incubated at the indicated pH for 30 min at 4°C, and the residual activity (0.21 U/ml of reaction mixture) then determined under the standard reaction conditions at 28°C.

The effects of temperatures between 20°C and 70°C on the enzyme activity and stability were also studied. To determine the optimum temperature, the enzyme (0.31 U/ml of reaction mixture) was incubated with 50 mM sucrose at various temperatures at pH 5.2 in a 20 mM sodium acetate buffer for 30 min. The thermostability, expressed as the residual enzyme activity, was then determined by incubating the enzyme (0.46 U/ml of reaction mixture for thermal treatment) for 30 min at the designated temperature. After incubation, the residual enzyme activity (0.23 U/ml of reaction mixture) was assayed under the standard reaction conditions at 28°C.

The effects of various metal ions ($CaCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, NaCl, $MgCl_2$, $ZnCl_2$) and the chelating agent EDTA were determined at 30°C in a 20 mM sodium acetate buffer, pH 5.2, with 50 mM sucrose using 0.23 U/ml rDSRBCB4 with each chemical (1 mM in the final volume) [8]. After 30 min of incubation, the residual enzyme activity was assayed under the standard reaction conditions at 28°C.

Product Analysis Synthesized from Sucrose With or Without Acceptor

After the complete depletion of sucrose (100 mM, 18 h at 28°C and pH 5.2) by rDSRBCB4 (0.11 unit/ml) under the standard reaction conditions described above, the concentrations of fructose, glucose, and leucrose in the reaction mixture were determined using TLC [5, 16] and HPLC [24] analyses. rDSRBCB4 (0.11 unit/ml of reaction mixture) was incubated with 100 mM sucrose together with an acceptor (100 mM maltose or 100 mM isomaltose) for 18 h at 28°C and pH 5.2. The synthesized oligosaccharides were then analyzed using TLC and HPLC [5, 16, 24].

For glucan preparation, the purified rDSRBCB4 (0.11 unit/ml) was reacted with 100 mM sucrose in a 20 mM sodium acetate buffer (pH 5.2). The reaction was allowed to proceed at 28°C until the sucrose was completely consumed. Glucan was prepared from the reaction mixture by ethanol precipitation (up to 67% of original volume, v/v). The precipitates were dried and redissolved in a 20 mM sodium acetate buffer (pH 5.2).

NMR Analysis

The dried glucan was redissolved in dimethyl sulfoxide (DMSO) [0.4 ml, 99.8% ($^2H_6$)DMSO; Merck] at 20°C and the NMR spectra were obtained at 125 MHz for ^{13}C nuclei on an AMX-600 (Bruker, Karlsruhe, Germany). The chemical shifts were measured (reported in parts per million) downfield from tetramethylsilane, the external standard. The assignment of peaks was based on the report of Shimamura *et al.* [33].

Results and Discussion

Cloning and Sequencing of *dsrBCb4* from *L. mesenteroides* B-1299CB4

The dextransucrase gene from *L. mesenteroides* B-1299CB4 was cloned using sequence information for the corresponding

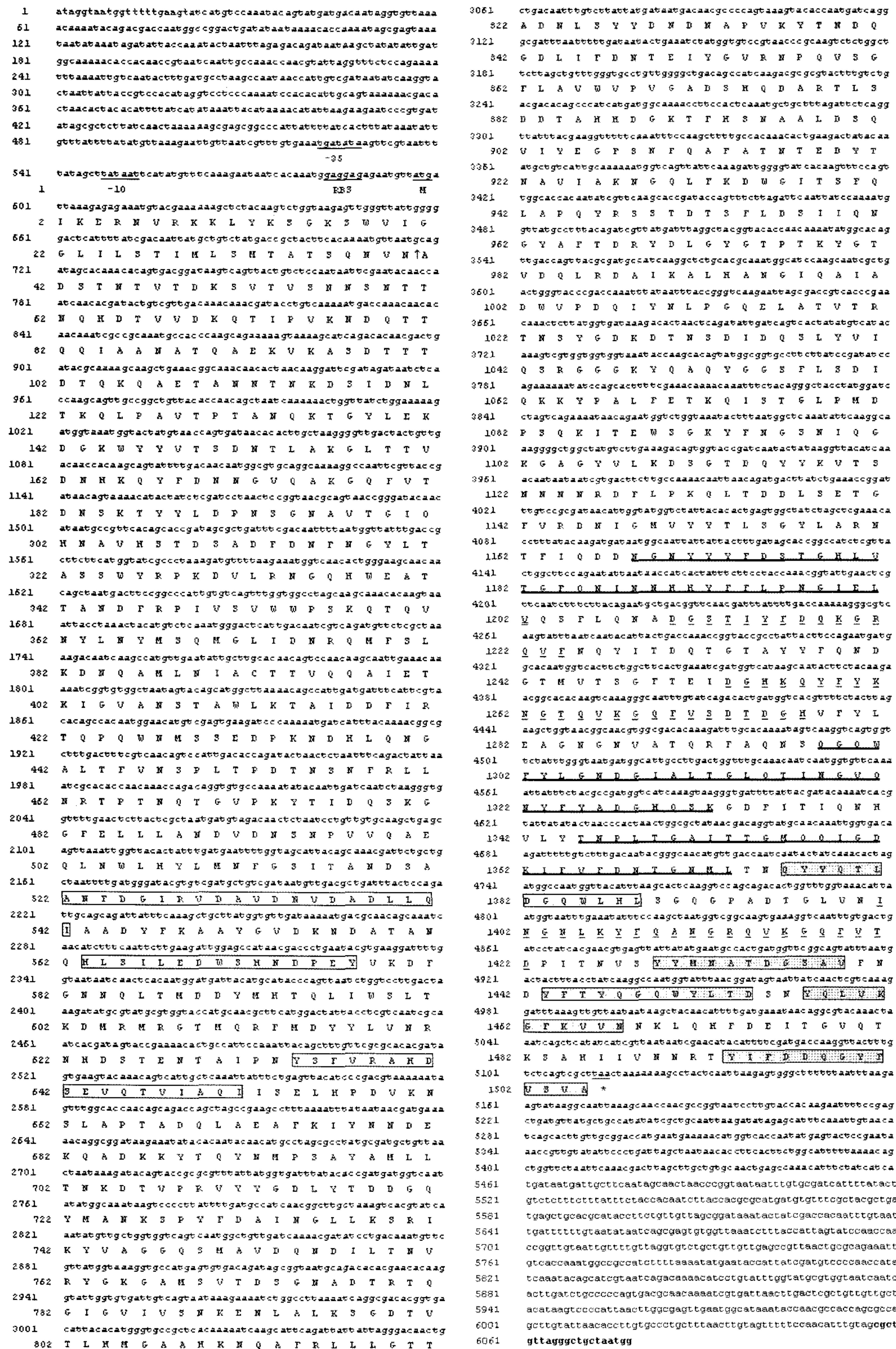

Fig. 1. Nucleotide sequence of *dsrBCB4* and deduced primary structure of dextransucrase from *L. mesenteroides* B-1299CB4.
The left-hand margin shows the nucleotide and amino acid numbers. The putative promoter sequence (−35 and −10; possible promoter region) and predicted ribosome-binding site (RBS) are underlined. The signal peptide-cleaving site predicted by Signal P [27] is indicated by a vertical arrow between N40 and A41. The boxes indicate conserved regions in the catalytic domain of dextransucrases from lactic acid bacteria. The shaded boxes indicate the YG repeats. The bold underlined amino acid residues represent the A repeats, whereas the discontinuous underlined amino acid residues represent the C repeats. The initiation and termination codons are underlined. The bold letters indicate the primer regions used for the *dsrBCB4* cloning. The nucleotide and deduced amino acid sequence of *dsrBCB4* have been submitted to the NCBI nucleotide sequence database under the accession number DQ497800.

Table 2. Summary of purification of rDSRBCB4 (1,000 ml culture)

Procedure	Protein[a] (mg)	Activity (U)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	141	327	2.32	1.00	100
Ni-NTA Superflow	16.5	153	9.29	4.00	46.9
Centricon Ultracel YM-100	10.6	147	13.9	5.98	45.1

[a]Measured by Bradford method [3].

gene (*dsrB*) from *L. mesenteroides* B-1299 [21]. The specific primers, CB4-F and CB4-R for the 5' and 3' end, respectively, were designed based on *dsrB*. A PCR product of 6,077 bp was obtained using the chromosomal DNA of *L. mesenteroides* B-1299CB4 as the template and sequenced (Fig. 1). When compared with the sequence of *dsrB,* the nucleotide sequence of *dsrBCB4* gene included three nucleotide deletions (at nucleotides between 941 and 942). Moreover, the Asp was deleted between K115 and D116, and the A500 was changed from Ser by one nucleotide substitution (at nucleotide 2,094; from T to G). When using the SignalP 3.0 (http://www.cbs.dtu.dk/servises/SignalP/) program [27] to recognize putative signal peptides, the cleavage site of a presumed signal peptide was identified between the N40 and A41 of DSRBCB4. The calculated molecular mass of the unmodified DSRBCB4 (1,505 amino acids) precursor was 168,060 Da, and the processing of the first 40 amino acids of DSRBCB4 during secretion resulted in the isolation of a 163,581 Da (1,465 amino acids) mature protein, with a theoretical pI of 4.90 (Fig. 1).

Expression of *dsrBCB4* Gene and Purification of rDSRBCB4

Active rDSRBCB4 was efficiently expressed using IPTG induction. A cell-free extract was prepared from 1,000 ml of the culture broth, where the dextransucrase activity and amount of protein were 327 U (100 mM sucrose as substrate) and 141 mg, respectively. The purified enzyme activity was 4-fold higher with a specific activity of 9.29 U/mg and yield of 46.9%. Meanwhile, after using a Centricon Ultracel YM-100, the enzyme activity was increased 5.98-fold with a specific activity of 13.9 U/mg of protein and yield of 45.1% yield. The purification procedures and results are summarized in Table 2.

Based on SDS-PAGE and a PAS staining analysis with rDSRBCB4, the molecular mass for the active bands of rDSRBCB4 was determined as 163 kDa (Fig. 2).

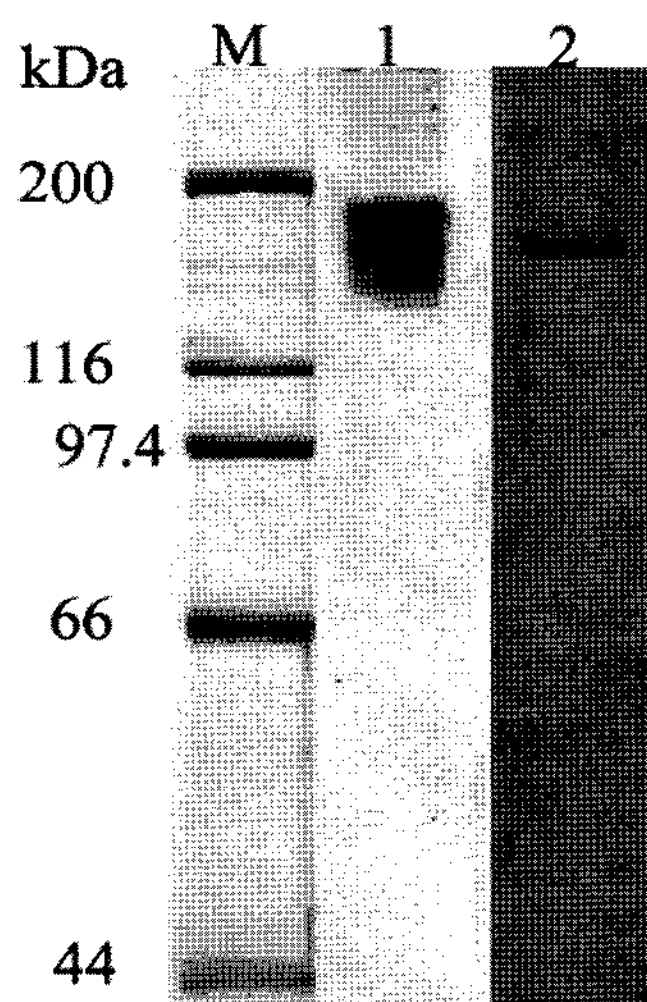

Fig. 2. SDS-PAGE and PAS-staining of purified rDSRBCB4.
The SDS-PAGE was performed using 7% gel. The purified protein (2 μg) was loaded onto the SDS-PAGE gel, electrophoresed, and visualized with PAS (lane 1) and CBB (lane 2) staining. Lane M, size marker.

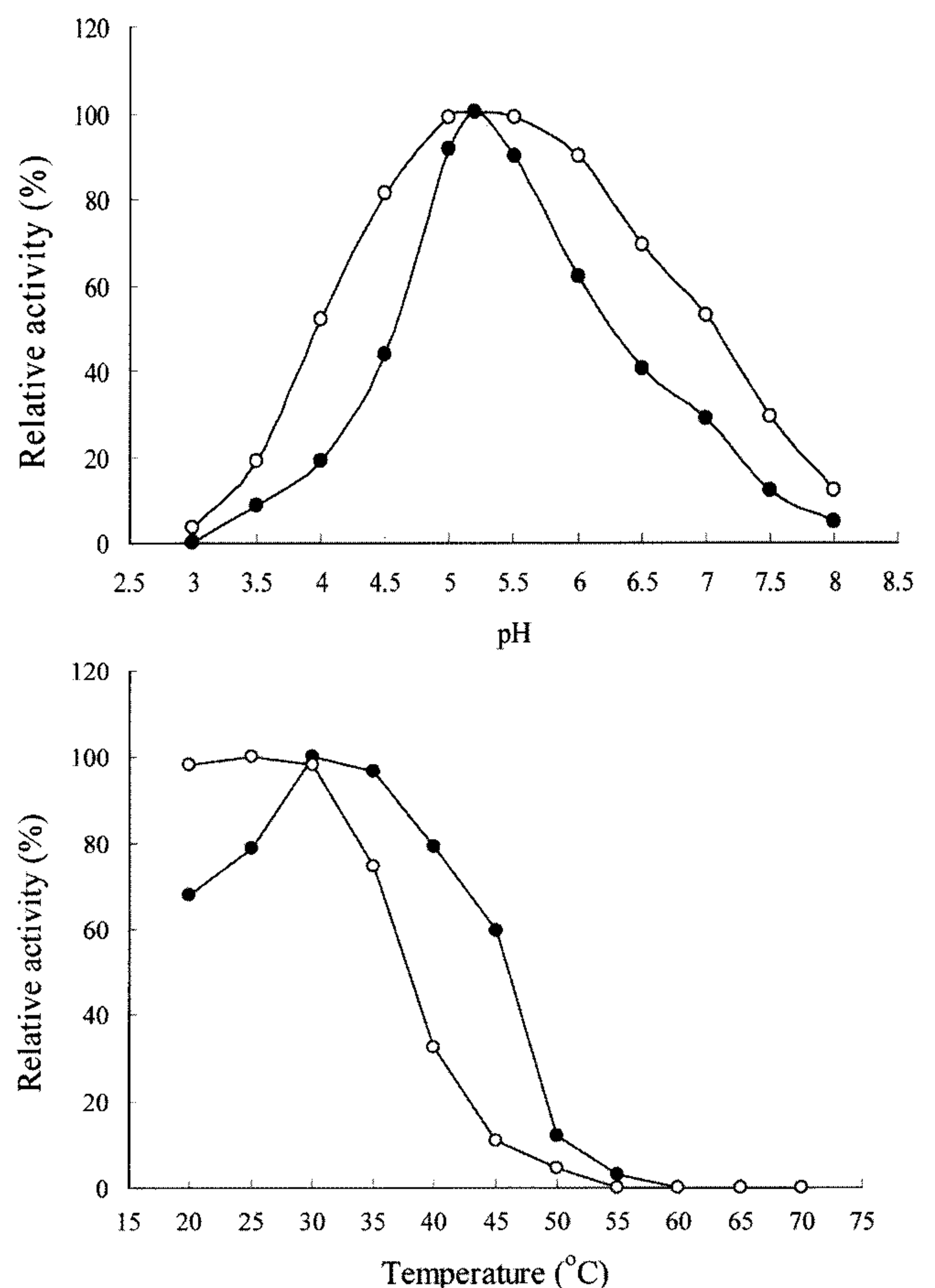

Fig. 3. Effects of pH (top) and temperature (bottom) on rDSRBCB4 activity.
The enzyme activity was determined at 28°C by measuring the amount of fructose released in 30 min from 50 mM sucrose by rDSRBCB4. ●, activity; ○, stability.

Table 3. Effect of metal ions and detergents (1 mM) on rDSRBCB4.

Compound	Relative activity (%)
Control (without any metal ion)	100±4
$CaCl_2$	189±17
$CuCl_2$	47±4
$FeCl_3$	39±6
$HgCl_2$	27±3
KCl	102±5
NaCl	102±2
$MgCl_2$	107±2
$ZnCl_2$	61±1
EDTA	83±5

Activity measurements done at 30°C in 20 mM sodium acetate buffer, pH 5.2, with 50 mM sucrose by 0.23 U/ml rDSRBCB4 after 30 min of incubation were taken as measures of enzymatic activity. Results are given as means±standard deviations (n=3).

Properties of rDSRBCB4

To define the experimental conditions for subsequent studies to examine the characteristics of the enzymes mutated in the putative catalytically important amino acid residues, the pH and temperature optima for rDSRBCB4 activity were examined. Although the optimal rDSRBCB4 activity was identified at pH 5.2 and 30°C (Fig. 3), the activity still remained relatively high (62% of original activity) at lower temperatures down to 15°C and relatively stable (over 80% of original activity) within a pH range of 5.0–6.0, but with a rapid decrease outside this range (Fig. 3).

The optimum pH and temperature for dextran formation by rDSRBCB4 was observed to be pH 5.2 and 30°C, respectively. A similar observation was also reported for the dextransucrase of *L. mesenteroides* B-512FM, at pH 5.2 and 28°C [10], although it differed from the optimum temperature reported for a recombinant dextransucrase from *L. mesenteroides* B-742CB, at 30°C [28].

Different metal ions had strongly varying effects on the activity of rDSRBCB4 (Table 3). Cu^{2+}, Fe^{3+}, Hg^{2+}, and Zn^{2+} ions and EDTA all significantly inhibited the enzyme activity. These ions were also previously found to inhibit the GTFB (glucansucrase for insoluble-glucan synthesis), GTFC (glucansucrase for insoluble/soluble glucan synthesis), and GTFD (glucansucrase for soluble-glucan synthesis) of *S. mutans* GS5 [38]. In contrast, Ca^{2+} and Mg^{2+} ions had a stimulating effect on the rDSRBCB4 enzyme activity, where Ca^{2+} had the highest stimulating effect with a 1.89-fold increase in activity.

Characteristics of Mutants in Putative Catalytic Amino Acid Residues

In vitro site-directed mutagenesis was applied to identify the essential amino acid residues providing the catalytic reaction. Based on amino acid sequence alignments of DSRBCB4 with different glucansucrases, residues D530, E568, and D641 were identified as a putative catalytic nucleophile, general acid/base catalyst, and transition state stabilizer, respectively (Fig. 1). In the *E. coli* host strain, the rDSRBCB4-mutated enzymes, D530N (38 mg/l), E568Q (44 mg/l), and D641N (28 mg/l), were expressed at levels similar to those in rDSRBCB4. Yet, an analysis of the purified proteins showed almost no activity, where D641N (1.8×10^{-4} U/mg) and E568Q (2.3×10^{-4} U/mg) showed about a 10,000-fold reduction of total enzyme activity, and the mutation of D530N resulted in an enzyme with a 98-fold reduction of total activity (0.16 U/mg) (Table 4), confirming that the essential catalytic residues of DSRBCB4 were D530, E568, and D641. D530 of DSRBCB4 was also found to be homologous to (1) the Asp residues identified by Mooser *et al.* [25] as part of the active site of GTFI and GTFS (glucansucrase from *S. downei* Mfe28), (2) D451 of GTFB from *S. mutans* GS5, (3) D453 of GTFI from *S. downei* Mfe28, and (4) D551 of DSRS, which has also been shown to be essential for activity [6, 9, 22]. E568 and D641 of DSRBCB4 were found to correspond to E491 and D564 in GTFI of *S. downei* Mfe28, respectively.

Table 4. Enzyme activities obtained from rDSRBCB4 and rDSRBCB4-mutated enzymes.

Enzyme	Specific activity (U/mg)[ab]	Relative activity (%)[c]
rDSRBCB4	13.9	100
D530N	0.16	1.15
E568Q	2.3×10^{-4}	1.7×10^{-3}
D641N	1.8×10^{-4}	1.3×10^{-3}

[a]The purified rDSRBCB4 and rDSRBCB4-mutated enzymes were quantified by the Bradford procedure using BSA as a standard [3].
[b]100 mM sucrose, pH 5.2, at 28°C.
[c]The specific activities were compared with that of the rDSRBCB4.

Products of rDSRBCB4 With Sucrose With or Without Acceptor

After the complete depletion of sucrose, rDSRBCB4 showed the following reaction-digest product distribution: dextran, 83.5%; leucrose [α-D-glucopyranosyl-1,5-β-D-fructofuranose], 10.4% (Table 5). In the presence of maltose, rDSRBCB4 formed panose [α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucopyranose] as the most abundant acceptor reaction product (from 100 mM sucrose and 100 mM maltose, approximately 33% panose was synthesized), indicating that an α-1,6-linkage was formed at the non-reducing end of maltose (Table 5). Moreover, rDSRBCB4 synthesized acceptor products, such as MP2 [α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucopyranose], MP3 [α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucopyranose], and MP4 [α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,6-α-D-glucopyranosyl-1,4-D-glucopyranose]. The linkage specificity of rDSRBCB4 was conserved in oligosaccharide synthesis,

Table 5. Products of rDSRBCB4 incubated with 100 mM sucrose, 100 mM sucrose/100 mM maltose, or 100 mM sucrose/100 mM isomaltose.

Substrate/acceptor	Products (%)				
100 mM sucrose[a]	Glucose		Leucrose		Glucan
	6.1±0.2		10.4±0.1		83.5±0.4
100 mM sucrose+ 100 mM maltose[b]	MP1	MP2	MP3	MP4	OS
	34.8±0.5	38.9±0.1	14.2±0.1	2.8±0.2	9.3±0.3
100 mM sucrose+ 100 mM isomaltose[c]	IM3	IM4	IM5	IM6	OS having high DP more 7
	17.8±0.2	25.4±0.3	18.3±0.1	10.4±0.6	28.1±0.3

[a]Percentages indicate the relative conversion of sucrose into glucan, leucrose, and glucose (hydrolysis).
[b,c]The total and individual oligosaccharide yields indicate the amount of [b]maltose (or [c]isomaltose) consumed as a percentages of the total amount of [b]maltose (or [c]isomaltose) initially presented reaction digest.
MP1, panose; MP2, 6^{II}-α-isomaltosyl maltose; MP3, 6^{II}-α-isomaltotriosyl maltose; MP4, 6^{II}-α-isomaltotetraosyl maltose; IM3, isomaltotriose; IM4, isomaltotetrose; IM5, isomaltopentaose; IM5, isomaltohexaose; OS, oligosaccharides. Results are given as means ± standard deviations (n=3).

and the oligosaccharides elongated at their nonreducing end [13]. When a D-glucose was transferred to the nonreducing end of a residue, the first product was also used as an acceptor to create the second reaction product, forming a homologous series. A panose or isopanose structure was also found when a D-glucose residue was transferred to a 6-OH group in a nonreducing or reducing residue of maltose, respectively [31].

When isomaltose was used as the acceptor, rDSRBCB4 synthesized isomaltotriose (17.8%), isomaltotetraose (25.4%), isomaltopentaose (18.1%), and isomaltohexaose (10.4%) (Table 5). This characteristic was similar to DSRS-synthesized isomaltotriose as the main product from an isomaltose acceptor reaction, followed by gradually lower amounts of isomaltooligosaccharides with an increased degree of polymerization (DP) of glucose [30].

Structure Analysis of Glucan Using NMR Analysis

rDSRBCB4 was incubated with sucrose, and the resulting glucans were purified. The reaction products produced by rDSRBCB4 made clear solutions, and the purified glucans were water-soluble. To analyze the glucan structure of rDSRBCB4, the glucan was dissolved in ($^2H_6$)DMSO at 70°C, and the ^{13}C-NMR spectrum obtained (Fig. 4). The

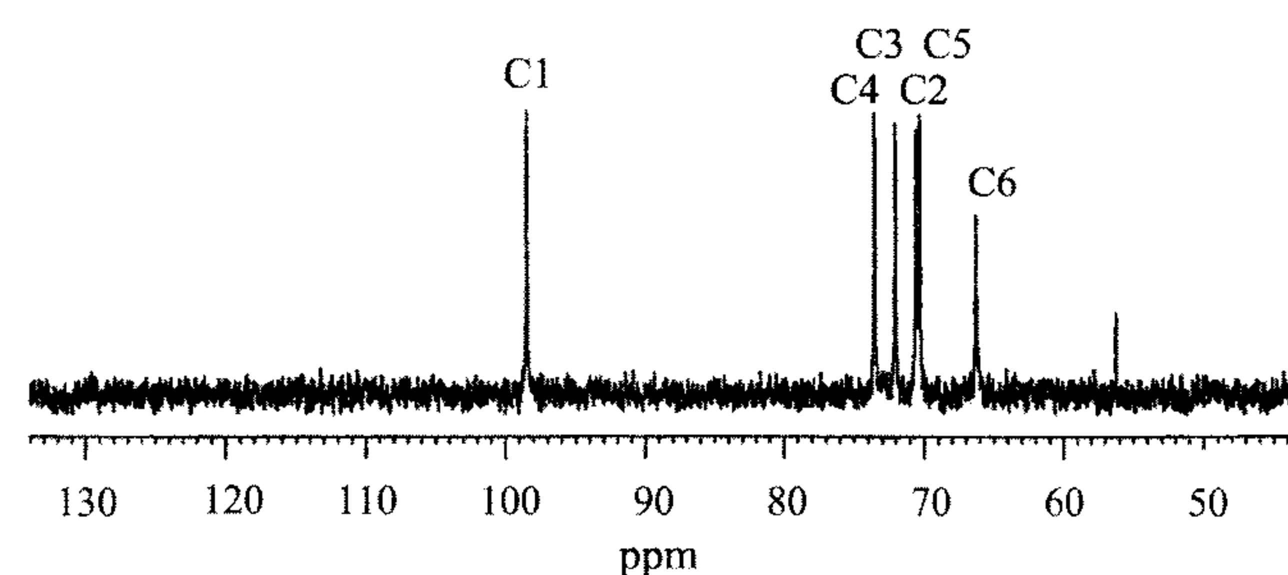

Fig. 4. ^{13}C-NMR spectrum of glucans synthesized by rDSRBCB4.

^{13}C-NMR spectrum of the polymer synthesized by rDSRBCB4 presented only signals of glucosyl residues linked through α-1,6-linkages (Fig. 4). In addition to signals corresponding to C-5, C-4, C-3, and C-2 in the 70.1 to 73.4 ppm area, signals arising at 98.2 and 66.1 ppm corresponded to C-1 involved in α-1,6-linkages and C-6 involved in α-1,6-linkages, respectively. Therefore, the polymer synthesized by rDSRBCB4 was a linear dextran.

The cloning approach used in the present study, based on the use of oligonucleotides, resulted in the isolation of *dsrBCB4*, similar to *dsrB*, which codes for an extracellular enzyme producing only α-1,6-linkages in *L. mesenteroides* NRRL B-1299. Nonetheless, although DSRB and DSRBCB4 exhibit similar characteristics (maltose accepter reaction, polymer structure by NMR analysis), their function in total dextran synthesis has yet to be determined. While different glucansucrases (GH70) have a highly similar catalytic core structure, they synthesize a diverse range of linear and branched α-glucans and oligosaccharides from sucrose, making it interesting to identify the amino acid residues that determine the linkage specificity in the poly- and oligosaccharides synthesized by DSRBCB4. Studies on the effect of amino acid substitutions on DSRBCB4 activity, and glucan and oligosaccharide synthesis, are currently in progress, and the results will hopefully facilitate the engineering of glucansucrases with a better control of glucan sizes, structures, and chemical properties.

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