Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 26 (2003) 167–176
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Directed evolution of a dextransucrase for increased constitutive activity and the synthesis of a highly branched dextran

Hee-Kyoung Kang [a], Eun-Seong Seo [b], John F. Robyt [c], Doman Kim [a,b,*]

[a] *Engineering Research Institute, Chonnam National University, Gwang-Ju 500-757, South Korea*
[b] *Faculty of Applied Chemical Engineering and Institute for Catalysis, Chonnam National University, Gwang-Ju 500-757, South Korea*
[c] *Laboratory of Carbohydrate Chemistry and Enzymology, Iowa State University, Ames, IA 50011, USA*

Received 18 March 2003; received in revised form 16 May 2003; accepted 29 May 2003

Abstract

An *Escherichia coli* transformant (pDSRB742CK) was obtained from the DSRB742 clone by using ultrasoft X-rays for the expression of a dextransucrase. The enzyme differed in several aspects from DSRB742 dextransucrase: it (1) was constitutive; (2) was extracellular; (3) had 2.6 times greater activity (0.035 IU/ml and 0.23 IU/mg); and (4) synthesized a highly (15.6%) α-(1 → 3) branched dextran. Seven nucleotides of the parent gene (*dsrB742*) were changed in the nucleotide sequence; four nucleotides were changed in the open reading frame (ORF) that resulted in a 30 amino acid deletion in the N-terminus.

Keywords: Dextransucrase; Directed bacterial evolution; Constitutivity; *Leuconostoc mesenteroides* mutants; Dextran; *E. coli* dextransucrase transformants

1. Introduction

Dextransucrases (glucansucrases or glucosyltransferases) (EC 2.4.1.5) are extracellular enzymes that synthesize dextrans from sucrose. Different kinds of glucansucrases are produced by different strains of *Leuconostoc* or *Streptococcus*. Dextrans are mainly composed of α-(1 → 6) linked chains of D-glucose units with varying amounts, types, and arrangements of branch linkages, such as α-(1 → 2), α-(1 → 3), and α-(1 → 4) to the main chain, depending on the particular kind of dextransucrase [1,2].

The *Leuconostoc* species require sucrose in the culture medium as an inducer for the elaboration of glucansucrases, whereas the *Streptococcus* species glucansucrases are produced constitutively by growing on a glucose or fructose medium [3]. One exception is the constitutive mutants developed by Kim and Robyt from *L. mesenteroides* NRRL B-512FM [3–6], B-742 [7], B-1355 [3], and B-1299 [8]. These mutants produce glucansucrases in a glucose or fructose medium as a carbon source instead of sucrose.

It has been previously reported that *L. mesenteroides* B-742 produces two kinds of dextransucrases when grown on sucrose. One enzyme synthesizes a dextran containing α-(1 → 4) branch linkages and the other synthesizes a dextran with very high degree (∼50%) of single α-(1 → 3) branched glucose residues [1,6,7,9,10]. From *L. mesenteroides* B-742 (B-742), Kim and Robyt isolated a constitutive dextransucrase mutant, *L. mesenteroides* B-742C. After further mutation of B-742C, two different constitutive mutants, *L. mesenteroides* B-742CA and *L. mesenteroides* B-742CB (B-742CB), were isolated [7].

* Corresponding author. Tel.: +82-62-530-1844; fax: +82-62-530-1849.
E-mail address: dmkim@chonnam.ac.kr (D. Kim).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2003.05.006

Table 1
The dextransucrase genes isolated from *L. mesenteroides*

Strain	Gene	Protein	Dextran[a]	Size (aa)	M_w[b] (10^3)	Reference
L. mesenteroides NRRL B-512F	*dsrS*	DSRS	5% α-(1 → 3), 95% α-(1 → 6)	1527	170	[43]
L. mesenteroides B-512FMCM	*fmcmds*	FMCMDS	5% α-(1 → 3), 95% α-(1 → 6)	1527	170	[14]
L. mesenteroides NRRL B-1299	*dsrA*	DSRA	15% α-(1 → 3), 85% α-(1 → 6)	1290	146	[12]
	dsrB	DSRB	5% α-(1 → 3), 95% α-(1 → 6)	1508	167	[13]
	dsrE	DSRE	ND	2835	313	[44]
L. mesenteroides B-742CB	*dsrB742*	DSRB742	5% α-(1 → 3), 95% α-(1 → 6)	1508	168	[11]
L. mesenteroides NRRL B-1355	*dsrC*	DSRC	ND	1477	165	[45]

Characterizations of dextran were achieved by using dextransucrase expressed in *E. coli*.
[a] Structure of the produced dextran. ND, not determined. Percentages are of total linkages in glucan structure.
[b] Molecular weight deduced from protein sequences.

B-742CB produces extracellular dextransucrases on 2% glucose with higher activity than that produced by B-742 on 2% sucrose medium. Seven genes coding for dextransucrase from *L. mesenteroides* has been cloned (Table 1).

Kim et al. [11] cloned and sequenced a dextransucrase gene from B-742CB (*dsrB742*; GenBank accession no. AF294469). Unlike other genes, the *dsrB742* gene encodes an extracellular dextransucrase in *Escherichia coli*. However, the dextransucrase primarily synthesizes an α-(1 → 6) linked dextran with 3–5% α-(1 → 3) branch linkages [12]. The nucleotide sequence of *dsrB742* shows one open reading frame (ORF) composed of 4524 bp encoding the dextransucrase. The amino acid sequence of B-742CB dextransucrase (DSRB742) shows a 50% similarity with DSRA from *L. mesenteroides* B-1299, which synthesizes a dextran containing 87% α-(1 → 6) linkages and 13% α-(1 → 3) branch linkages [12,13], a 70% similarity with *L. mesenteroides* B-512FMCM dextransucrase from [14] and a 45–56% similarity with *Streptococcal* glucansucrases (GTFs) [15–18]. The expressed and purified enzyme (DSRB742) from the clone showed similar enzymatic properties, such as: acceptor reactions, molecular size, optimum pH and temperature to that of B-742CB dextransucrase, but with the inability to form a significant amount of α-(1 → 3) branch linkages compared to that of *L. mesenteroides* B-742CB dextransucrase [19]. Until now, there has not been a report of the isolation or development of a mutant or clone that elaborates a constitutive, extracellular dextransucrase, capable of synthesizing a highly branched dextran.

In a previous study [4], we developed a novel mutation method for the hyper-production of dextransucrase by using vacuum ultraviolet radiation or ultrasoft X-rays. The electromagnetic radiation that lies between the UV and X-ray is called ultrasoft X-ray and its energy ranges from 10 eV to 2 keV [20]. In this paper, we report the directed evolution of a B-742CB dextransucrase gene (*dsrB742*) that elaborates a novel extracellular dextransucrase gene (*dsrB742ck*) after ultrasoft X-ray irradiation, producing a dextransucrase of increased activity and synthesis of a highly branched dextran.

2. Experimental

2.1. Bacterial strains, vector, and culture condition

The recombinant *E. coli* DH5α (KACC 95014 containing *dsrB742* or KACC 95013 containing *dsrB742ck*; Korean Agricultural Culture Collection, Suwon, Korea) was maintained in a Luria–Bertani (LB) agar medium with ampicillin (50 μg/ml) and 2% glucose or sucrose (w/v) [11,19]. *E. coli* DH5α and a pGEM-3Zf(−) (Promega, USA) vector were used for all the cloning steps [19]. The nucleotide sequence of the *dsrB742ck* has been deposited in the GenBank database under accession no. AY280636.

2.2. Ultrasoft X-ray irradiation onto pdsrB742

To develop novel mutants, *pdsrB742* was irradiated with ultrasoft X-ray. Ultrasoft X-ray was obtained

from the lithography, galvanoformung-electroplating, abformung-replication (LIGA) beamline in the Pohang Accelerator Laboratory (Pohang, Korea) using a specially constructed chamber. An aluminum target was placed inside the exposure chamber and the ultrasoft X-ray was generated by irradiating this target with a white beam. The chamber was filled with helium (He) gas which is effectively transparent to the ultrasoft X-rays. The binding energies of Al orbital K(1), L(1), L(2), and L(3) were 1559.6, 117.8, 73.1, and 72.7 eV, respectively. If an electron of the K-shell is taken out by the incident photon, an electron from a neighboring orbital moves to that empty orbital. The difference in the orbital energies is emitted as a photon of the energy range of the ultrasoft X-ray. In this experiment, the Al K ray was corresponded to energy of 1.487 keV. The DNA (20 μl of a 50 μg/ml concentration) was allocated in the well of a 96-well micro plate (Nalge NUNC International, USA), and ultrasoft X-ray was evenly exposed. In this irradiation, 6.4×10^6 photons/s were irradiated to each sample in a well. After irradiation, the *dsrB742* in each well was transformed to *E. coli* strain DH5α and transformants were plated onto LB medium supplemented with ampicillin (50 μg/ml) and 2% sucrose (w/v).

2.3. DNA manipulation

Routine DNA manipulation, including plasmid purification and *E. coli* transformation, was performed as described by Maniatis et al. [21]. The plasmid DNA was isolated from an overnight culture of *E. coli* using the alkaline lysis method.

2.4. Purification of dextransucrase

The recombinant *E. coli* DH5α was cultivated in LB medium containing 2% glucose or sucrose and 50 μg/ml ampicillin for 27 h at 28 °C. The culture supernatant was collected by centrifugation (11,000 × *g*, 15 min) and it was concentrated with polyethylene glycol (PEG, $M_w = 1500$, 50% (w/v) in H_2O) to a final concentration of 20%. The extracellular activity was recovered in the dextran-rich phase [22]. To remove the dextran bound to enzyme, *Penicillium* dextranase (1 U/ml, Sigma, St. Louis, MO, USA) was added to enzyme solution and stirred for 1 h at 24 °C. The dextranase-treated enzyme was then dialyzed against 20 mM sodium acetate buffer (pH 5.4) that contains 1 mM $CaCl_2$. The dialyzate was loaded onto a DEAE-sepharose column (2.5 cm × 10 cm) equilibrated with the same buffer. The column was washed with 250 ml buffer, and the adsorbed protein was eluted with a linear gradient of NaCl (0–0.5 M) in the buffer. The fractions exhibiting dextransucrase activity were pooled and dialyzed against a 20 mM sodium acetate buffer (pH 5.4). The resulting dialyzate was applied to phenyl-sepharose column (2 cm × 13 cm) equilibrated with the same buffer. The dextransucrase fraction was eluted with a linear gradient of decreasing ionic strength of sodium acetate buffer (starting from 20 to 0 mM). The amount of protein was determined by Bradford method [23] using bovine serum albumin as a standard. The dextransucrase activity was determined by analyzing the rate of fructose released from sucrose (200 mM in 20 mM Na-acetate buffer; pH 5.2; 24 °C) [24]. The quantity of fructose on the TLC plates was analyzed with a densitometer as described previously [3,7,25].

2.5. Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli [26]. The protein was stained with Coomassie Brilliant Blue R-250. The dextransucrase activities were detected by incubating the gels in 200 mM sucrose for overnight and followed by staining using a periodic acid-Schiff (PAS) procedure [27].

2.6. Immuno-detection of dextrans prepared by clones

To detect dextransucrase activity, the SDS-PAGE of native protein (without heating) was conducted and the proteins were transferred to PVDF membranes. Membranes were then incubated at 28 °C for overnight with 200 mM sucrose dissolved in 20 mM sodium acetate buffer (pH 5.2). Dextran synthesized was detected using a rabbit polyclonal antibody of dextran as detailed below. Protein samples from pDSRB742 and pDSRB742CK clones prepared on sucrose medium were diluted in equal amounts (0.635 μg/lane), and Western blots were performed [28]. Dextrans were detected by using a rabbit polyclonal antibody

prepared with dextran which is specific for α-(1 → 6) linked dextran composing the majority of *L. mesenteroides* 512F. Serum containing anti-dextran antibodies was used at 1:300 dilutions. Peroxidase-conjugated anti-rabbit-IgG (conjugate of goat–rabbit-IgG with (γ)-horseradish, Amersham Pharmacia Biotech, USA) was used at a dilution of 1:7500 (1.3 μl/10 ml). Following incubation with the antibodies, membranes were washed five times with Tris-buffered saline solution (TBS; 20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween 20). The antigen–antibody complex was detected with secondary antibody and an ECL Western blotting detection system (Amersham Pharmacia Biotech).

2.7. Analysis of branching of dextran

Dextran was prepared with the reaction of an equal volume of dextransucrase (0.2 U/ml) with 100 mM sucrose (pH 5.5). The reaction was allowed to proceed at 28 °C until the sucrose had been completely consumed. Dextran was prepared from the reaction digests by ethanol precipitation (up to 67% of the original volume, v/v). The precipitates were dried and dissolved in a 20 mM sodium acetate buffer (pH 5.5). Dextranase (*Penicillium* dextranase; 1.0 U) was added to the carbohydrate solution and allowed to react at 37 °C for 3 h. Dextranase hydrolyzes dextran to give D-glucose, isomaltose, isomaltotriose, and branched oligosaccharides ranging from a tetrasaccharide (3^3-α-D-glucopyranosyl isomaltotriose) to hexasaccharides (3^3-isomaltosyl isomaltotetraose and 3^3-D-glucopyranosyl isomaltopentaose). It previously has been shown by methylation analysis and ^{13}C NMR that dextran synthesized by *L. mesenteroides* B-512F dextransucrase, reaction with sucrose at pH 5.5 and 28 °C, gave a dextran with 5% α-(1 → 3) branch linkages [29,30]. The degree of branching was estimated by obtaining the ratio of branched products [branched oligosaccharides + unhydrolyzed dextran] to unbranched products [D-glucose + isomaltooligosaccharides] times the factor, 20.24, obtained by postulating 5% branching for a dextran synthesized by dextransucrase from 0.1 M sucrose at pH 5.5 and 28 °C, for example, [5% branching/ratio 0.247 = 20.24]. The carbohydrate components (D-glucose, isomaltose, branched oligosaccharides, and unhydrolyzed dextran at the origin) were separated and quantitatively determined by thin-layer chromatography, using an imaging densitometer (BioRad, model GS 710) with glucose standards (50–2000 ng) [31]. Aliquots (1–5 μl) were placed onto 20 cm × 20 cm Whatman K5F TLC plates; the TLC plate was irrigated at 22 °C, using two ascents (18 cm path length each) of 20:50:15 volume proportions of nitromethane:1-propanol:water. The carbohydrates were visualized on the plate by rapidly dipping the plate into a solution containing 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine and 5% (v/v) H_2SO_4 in methanol, dried, and heated at 120 °C for 10 min [25].

2.8. NMR analysis of dextran

The linkage composition of the dextran synthesized by DSRB742CK was analyzed by using ^{13}C NMR spectrometry. The dextran was dissolved in DMSO (10 mg/ml) at 20 °C. The ^{13}C NMR spectra were obtained at 500 MHz and at 125 MHz on an AMX-500 (Bruker, Germany). Chemical shifts were measured (and reported in parts per million) downfield from TMS, the external standard. The linkage assignments in the ^{13}C NMR spectra of linear α-(1 → 6)-D-glucan and α-(1 → 3)-D-glucan were made based on report by Shimamura [32].

3. Results and discussion

3.1. Selection of a mutated dextransucrase gene

Among the glucansucrases, *L. mesenteroides* NRRL B-742 produces two extracellular dextrans from sucrose, namely L-dextran, which is precipitated at an ethanol concentration of 39%, and S-dextran, which is precipitated at an ethanol concentration of 45% [6]. Fraction S-dextran contained 50% α-(1 → 3) branches and fraction L-dextran contained 14% α-(1 → 4) branch linkages and about 1% α-(1 → 3) branch linkages [9,10]. One dextransucrase gene from *L. mesenteroides* NRRL B-742CB has already been cloned [11]. Unlike other genes, the *dsrB742* gene encodes an extracellular dextransucrase in *E. coli*. However, it synthesizes a dextran with a relatively low degree of branching containing 95–97% of α-(1 → 6) linkages and 3–5% α-(1 → 3) branch linkages [12].

Table 2
Comparison of dextransucrase activities of pDSRB742 and pDSRB742CK clones

Dextransucrase	Specific activity (U/mg)[a]	Relative amount of protein (%)[b]
DSRB742	12.6	100
DSRB742CK	29.2	107

[a] Specific activity is shown as units per milligram of protein purified from the sucrose culture supernatant from each clone.

[b] Relative amount of protein was calculated compared with the amount of protein in pDSRB742 clone culture supernatant as a control.

Table 3
Comparison of dextransucrase activities produced by pDSRB742-CK in different expression conditions

Expression conditions[a]	Enzyme activity (U/ml)	Relative amount of protein produced (%)[b]
Sucrose	0.035	100
Glucose	0.019	54
IPTG induction	0.004	12

[a] Activity was determined from culture supernatant of *E. coli* DH5α harboring pDSRB742CK cultivated in LB medium containing 2% sucrose without IPTG induction or 2% glucose without IPTG induction or from cell lysate prepared from LB medium with IPTG induction.

[b] Relative amount of protein was calculated compare with the amount of protein in pDSRB742CK clone sucrose culture supernatant as a control.

The ultrasoft X-ray irradiation affected the viability of dextransucrase gene (*dsrB742*); the longer the exposure to irradiation, the higher was the percent of conversion of supercoiled DNA to relaxed DNA. Ninety percent conversion was detected after 25 min exposure with 1.487 keV energy with an aluminum target. The exposed DNA was transformed and about 500 colonies were found, forming mucous dextran on an agar plate containing sucrose. These were selected and analyzed for the production of dextransucrase on a sucrose or glucose medium. A constitutive and hyper-producing dextransucrase *E. coli* transformant (pDSRB742CK) was obtained. The purified DSRB742CK dextransucrase showed 2.3 times higher activity per milligram of protein, compared to that of the parent clone, DSRB742 dextransucrase (Table 2).

The nucleotide sequence of *dsrB742* shows one ORF composed of 4524 bp encoding dextransucrase (GenBank database under accession no. AF294469). The deduced amino acid sequence gave a calculated molecular mass of 168.4 kDa. The nucleotides sequence of the *dsrB742ck* dextransucrase gene showed seven DNA base differences comparing to that of *dsrB742* dextransucrase gene (Fig. 1); three nucleotide deletions (at nucleotides 705, 706, and 713) and one nucleotide substitution (at nucleotide 4406; from G to T). Therefore, the start codon appears at 30 amino acids downstream of DSRB742 dextransucrase. There are two nucleotide sequence differences at the promoter region (at nucleotides 602 and 613) and one nucleotide sequence difference at the RBS site at nucleotide 685.

DSRB742 dextransucrase is produced without isopropyl-β-D-galactose (IPTG) induction on sucrose medium, but not on glucose medium (Table 3). pDSRB742CK did produce dextransucrase on glucose medium (0.019 U/ml) as well as on a sucrose medium (0.035 U/ml). These values are higher than that of IPTG-induced cell extract (0.004 U/ml), yet the approximate specific activity of each preparation was same. Two general mechanisms are suggested for the introduction of protein into the liquid medium of *E. coli* [33]. One is a two-stage translocation using active transporters in the cytoplasmic membrane followed by passive transport through the outer membrane. Passive transport is achieved through either external or internal destabilization of the *E. coli* structural components [34,35]. The other is transplantation (type I secretion/ABC transport, fusion protein/β-auto transport, specific signal peptide and over expression) of proteins capable of active transport over one or both of the membranes. This involves the transplantation of secretion mechanisms into the *E. coli* cell from pathogenic *E. coli* as well as from other species [33,36,37]. A detailed study of the potential secretion mechanism and involving component of DSRB742 and DSRB742CK dextransucrase is in progress.

It has been reported that a constitutive dextransucrase mutant, *L. mesenteroides* B-512FMCM, produced 13 times higher activity than that of the parent strain, *L. mesenteroides* B-512FM. Furthermore, 1000 times higher amount of dextransucrase was produced than that of the industrial-dextran producing strain, *L. mesenteroides* B-512F [4]. *L. mesenteroides* B-512FMCM is a completely constitutive mutant for

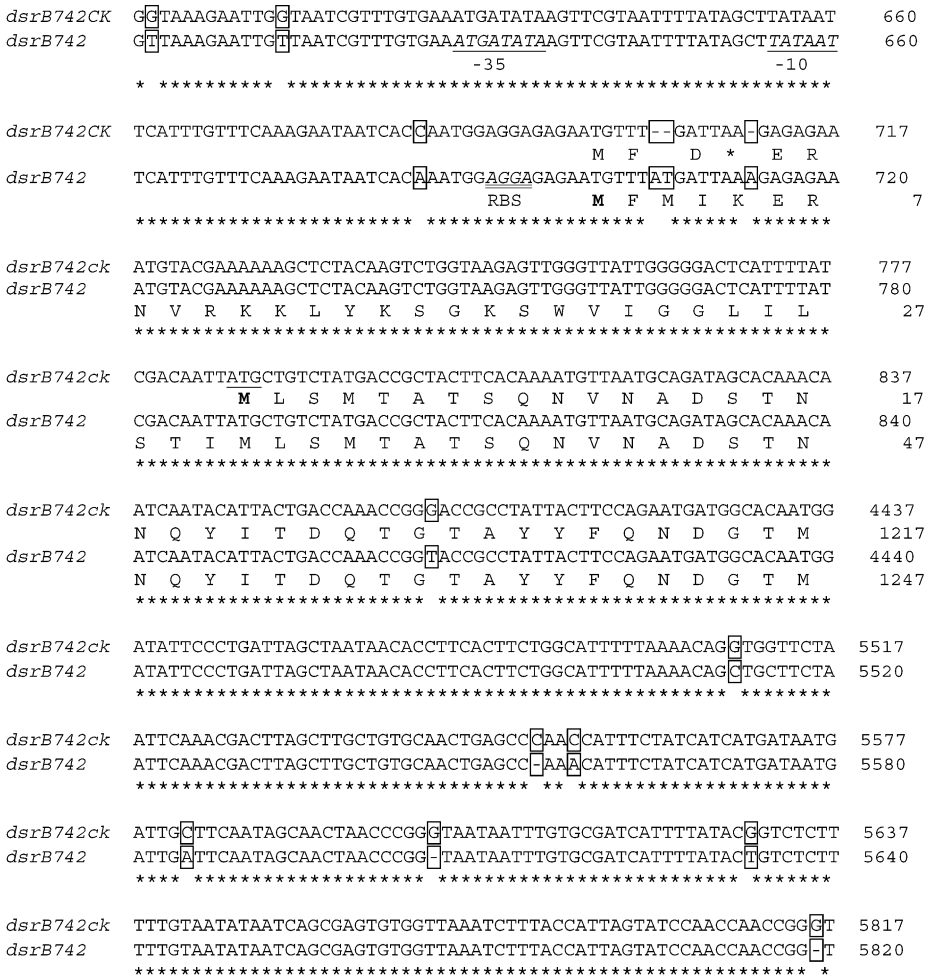

Fig. 1. Base substitutions of the mutant (pDSRB742CK) developed using the irradiated DNA. Boxed letters indicate changed nucleotides. Underlined nucleotides represent the −35 and −10 regions for presumptive promoter. Double underlined nucleotides correspond to the putative ribosome-binding sites. The nucleotide sequence of the *dsrB742ck* dextransucrase gene has been deposited in the GenBank database under accession no. AY280636.

the production of dextransucrase. There is a single nucleotide change in the promoter region and two amino acid changes in the structural gene from that of the parent *L. mesenteroides* NRRL B-512F [14]. Whether the single substitution in the promoter affects the high production of dextransucrase in *L. mesenteroides* B-512FMC remains to be determined. The change in the nucleotides in the promoter region in *dsrB742ck* could affect the pattern of expression and the amount of protein produced. A detailed study of the role of *dsrB742ck* promoter is in progress.

3.2. Immunological detection of dextran

The dextran synthesizing activity of DSRB742CK dextransucrase was 2.3 times higher than that of DSRB742 dextransucrase (Fig. 2A and B; Table 2) based on Western blot analysis using anti-dextran antibody. The slight increase in protein production in culture supernatant of pDSRB742CK (1.07 times, Table 2) compared to that of pDSRB742 clone and the activity increase of 2.3 times suggests that the 30 amino acids in the N-terminal re-

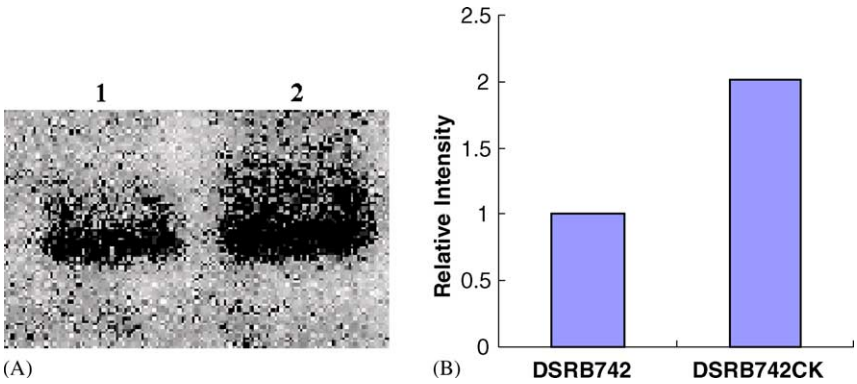

Fig. 2. Detection of dextran production by Western blot analysis. Detection of dextran synthesized by proteins from culture supernatants of pDSRB742 (lane 1 in (A) and DSRB742 in (B)) and pDSRB742CK (lane 2 in (A) and DSRB742CK in (B)). Proteins were separated in an 8% polyacrylamide gel and subsequently transferred to PVDF membranes. The filters were incubated overnight at 28 °C in 200 mM sucrose. The membrane was briefly rinsed in TBS-T and incubated with anti-dextran-antibody as described in Section 2.

gion significantly affects the specific activity of the dextransucrase.

3.3. Active dextransucrase staining

Electrophoresis analyses and in situ activity assays were performed with sucrose culture supernatants. The molecular weights of the active bands of DSRB742CK and DSRB742 dextransucrases were 164.1 and 168.6 kDa, respectively (Fig. 3). Even when we applied the same amount of each protein, the intensity of each active band was significantly different. For DSRB742CK, there were higher molecular weight activity bands. These higher forms are likely the aggregated forms of a dextransucrase, as previously reported from *L. mesenteroides* B-512F, B-1299, B-1355 and B-742 [3,5,7,38]. The DSRB742CK dextransucrase, prepared using glucose, gave a single activity band of 164.1 kDa (data not shown). Thus, dextransucrase, prepared on sucrose culture had bound dextran that could play a role in the formation of multiple aggregated dextransucrase forms [5].

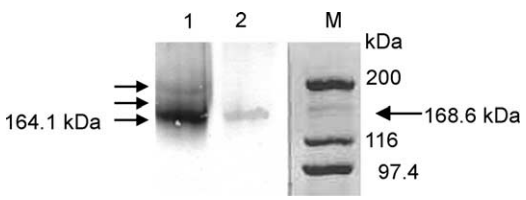

Fig. 3. Activity staining of dextransucrases from pDSRB742 and pDSRB742CK clones grown on 2% sucrose. Each band was stained by periodic acid-Schiff staining procedure after sucrose reaction. The arrow indicated the activity bands of DSRB742 and DSRB742CK dextransucrases. Lane 1, PAS staining of DSRB742CK dextransucrase after reaction with sucrose; lane 2, PAS staining of DSRB742 dextransucrase after reaction with sucrose; M, molecular mass marker (kDa).

3.4. Composition of dextranase hydrolyzates of dextran

The structure, size, and the position and degree of branch linkages are dependent on the specific

Table 4
Dextranase/TLC determination of the degree of branching of dextrans synthesized by DSRB742 and DSRB742CK dextransucrases[a]

	DSRB742 dextran	DSRB742CK dextran
Mono-saccharide (M, %)	35.4	29.5
Isomaltodextrins (IMD, %)	42.2	27.1
Branched isomaltodextrins (BIM, %)	19.1	23.8
Unhydrolyzed dextran (UHD, %)	3.3	19.6
[BIM + UHD] ratio	0.29	0.77
[M + IMD] percent branching[b]	5.9	15.6

[a] Hydrolysis reactions were conducted at 37 °C for 3 h.
[b] Branching for dextran synthesized from 0.1 M sucrose was assumed to be 5% (from [23,24]). A branching conversion factor was obtained by dividing 5% by the ratio, 0.247, giving 20.24. The percent branching for DSRB742 and DSRB742CK dextrans was obtained by multiplying their ratio by the conversion factor.

glucansucrases that is elaborated by a specific gene. The genome also determines whether the organism requires an inducible medium with sucrose as the carbon source to elaborate the enzyme or elaborates the enzyme in a constitutive medium that can use glucose or fructose as the carbon source. The structure, size, and the position and degree of branching in the glucans are dependent on the specific glucansucrases that is elaborated by a specific gene. Glucansucrase encoding genes from *L. mesenteroides* code for enzymes that have an average molecular mass of 170 kDa. Key elements in the structure of the enzyme that determine the structure of the glucans have not been determined and remain unclear. The cloning and development of new and unique glucansucrases genes and the determination of the structures of the glucans products as well as the structures of the acceptor products and the catalytic parameters of the enzymes are necessary in improving and understanding the reaction mechanisms of the *Leuconostoc* and *Streptococcus* glucansucrases.

The dextran prepared from DSRB742CK was hydrolyzed with *Penicillium* dextranase and the hydrolyzate composition was compared with that of DSRB742 dextran (Table 4). Both hydrolyzates were primarily composed of glucose, isomaltose, and branched isomaltodextrins. DSRB742CK dextran contained 15.6% branching and showed 2.7 times higher resistance to *Penicillium* endodextranase hydrolysis compared to that of DSRB742 dextran. Thus, DSRB742CK dextran contains much higher degrees of branching than previously reported before for dextrans synthesized by dextransucrase-producing clones [13,39].

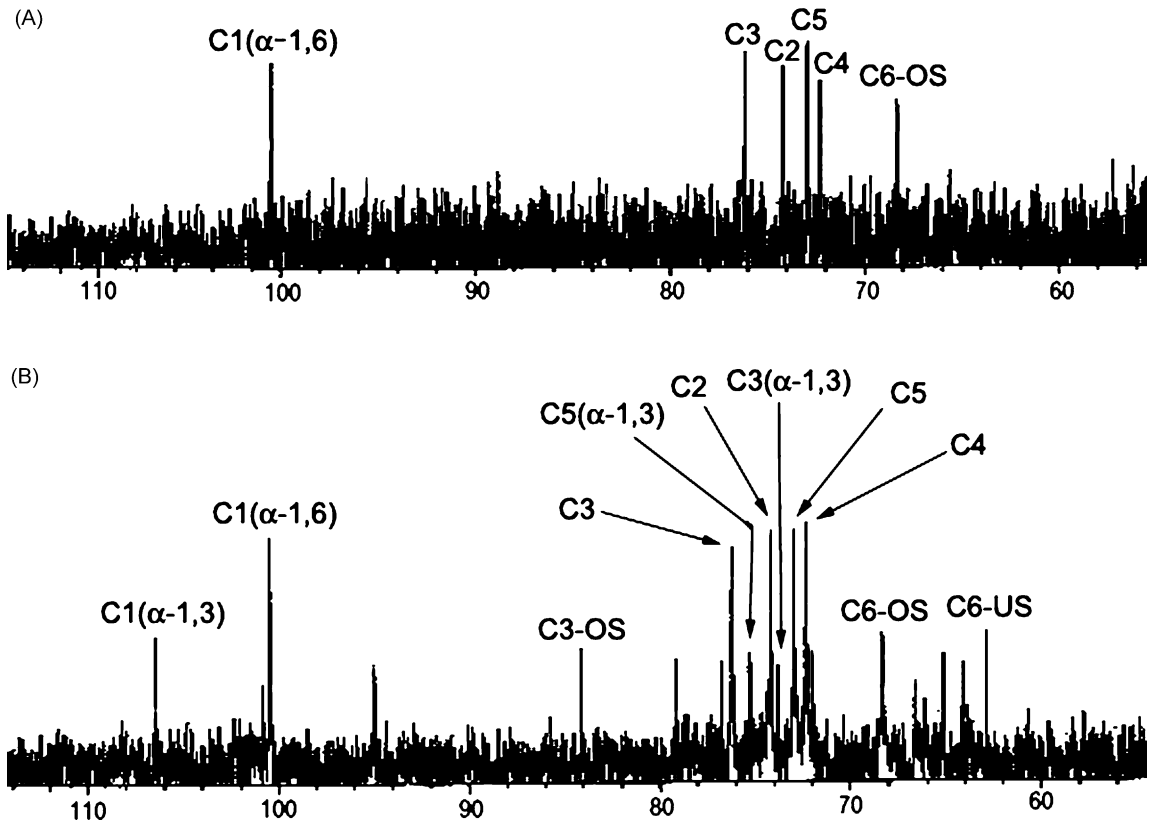

Fig. 4. ^{13}C NMR analysis of the dextrans synthesized by DSRB742 and DSRB742CK dextransucrases produced on sucrose media. PPM is relative to tetramethylsilane. US, unsubstituted carbons; OS, *O*-unsubstituted carbons. (A) DSRB742 dextran synthesized by dextransucrase produced on sucrose medium; (B) DSRB742CK dextran synthesized by dextransucrase produced on sucrose medium.

When various carbohydrates, in addition to sucrose, are present in glucansucrase reaction digests, some of the glucosyl groups are transferred to the carbohydrates to make branched oligosaccharides and are diverted from the formation of glucan. Branched oligosaccharides resist hydrolysis by digestive enzymes in animals and humans because of the configuration of their glycosidic bonds. They selectively stimulate intestinal microflora such as *Bifidobacterium* sp., *Lactobacillus* sp., or *Bacteroides* sp. as prebiotic agents [40,41] and can have uses in dermocosmetic applications [42].

3.5. Structural analysis of glucans using ^{13}C NMR

The branch linkage was analyzed by using ^{13}C NMR. The ^{13}C NMR spectrum signals of DSRB742 and DSRB742CK dextrans were assigned as shown in Fig. 4. No signal was observed for C3-OS in DSRB742 dextran, while C3-OS signals for α-(1 → 3) linkages were observed in DSRB742CK dextran. Until now different clones, such as DSRB742 [11], DSRS (from *L. mesenteroides* NRRL B-512F dextransucrase) [19], FMCMDS (from *L. mesenteroides* B-512 FMCM) [14] and DSRB (from *L. mesenteroides* B-1299) [13], produced primarily linear dextrans that were composed of α-(1 → 6) linkages when grown in a sucrose containing media. A clone, DSRA (from *L. mesenteroides* B-1299), synthesizes a dextran containing 13% α-(1 → 3) linkages [12] using cell extract and sucrose reaction after IPTG induction. pDSRB742CK is the first clone that makes highly branched dextran in sucrose culture supernatant without IPTG induction or in glucose medium and is evolved from a gene producing a dextransucrase of linear dextran. The DSRB742 dextransucrase amino acid sequence is very different from those of DSRS, FMCMDS, DSRA and DSRB dextransucrases: the amino acid sequence similarities of DSRB742 dextransucrase with DSRS, FMCMDS, DSRA and DSRB dextransucrases ranged from 50 to 70%. As of yet, there is no detailed molecular explanation and information for the synthesis of branch linkages by different dextransucrases. Studies on DSRB742CK dextransucrase and the structure of the crystalline enzyme could give a more complete picture of branch linkage formation and their regioselectivity.

4. Conclusions

In this paper, we report the first direct evolution of a dextransucrase gene that encodes for a new dextransucrase (DSRB742CK) that is elaborated constitutively with high activity per milliliter of culture supernatant and catalyzes the synthesis of highly α-(1 → 3) branched dextran. The nucleotide changes in the promoter region contributed a 7% increase of protein expression. The higher activity (2.3 times) and the higher degree of branch formation (2.7 times) were closely related by the nucleotide changes in ORF. This gene was expressed in the culture supernatant constitutively growing on glucose as the carbon source. To determine which specific nucleotide(s) of the promoter region is related to the constitutivity of dextransucrase and which amino acids are involved in branch formation, molecular evolution and/or site-directed mutagenesis of dextransucrase gene and the determination of a crystal structure must be performed.

Acknowledgements

This work was supported by 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Science and Technology (Grant MG02-0301-004-1-0-0) and Genofocus Co. Ltd., Republic of Korea.

References

[1] J.F. Robyt, Adv. Carbohydr. Chem. Biochem. 51 (1995) 133.
[2] A.W. Jeanes, A.W. Haynes, C.A. Williams, J.C. Rankin, E.H. Melvin, M.J. Austin, J.E. Cluskey, B.E. Fisher, H.M. Tsuchiya, C.E. Rist, J. Am. Chem. Soc. 76 (1954) 5041.
[3] D. Kim, J.F. Robyt, Enzyme Microb. Technol. 17 (1995) 689.
[4] D. Kim, D.W. Kim, J.H. Lee, K.H. Park, L.M. Day, D.F. Day, Biotechnol. Technol. 11 (1997) 319–321.
[5] D. Kim, J.F. Robyt, Enzyme Microb. Technol. 16 (1995) 1010.
[6] J.F. Robyt, in: Encyclopedia of Polymer Science and Engineering, fourth ed., Wiley, New York, 1986, p. 752.
[7] D. Kim, J.F. Robyt, Enzyme Microb. Technol. 16 (1994) 659.
[8] D. Kim, D.W. Kim, J. Microbiol. Biotechnol. 9 (1999) 219.
[9] F.R. Seymour, R.D. Knapp, S.H. Bishop, A. Jeanes, Carbohydr. Res. 68 (1979) 123.
[10] F.R. Seymour, E.C.M. Chen, S.H. Bishop, Carbohydr. Res. 68 (1979) 113.
[11] H.S. Kim, D. Kim, H.J. Ryu, J.F. Robyt, J. Microbiol. Biotechnol. 10 (2000) 559.

[12] V. Monchois, R.M. Willemot, M. Remaud-Simeon, C. Croux, P. Monsan, Gene 182 (1996) 23.
[13] V. Monchois, M.R. Simeon, P. Monsan, R.M. Willemot, FEMS Microbiol. Lett. 159 (1998) 307.
[14] H.J. Ryu, D. Kim, D.W. Kim, Y.Y. Moon, J.F. Robyt, Biotechnol. Lett. 22 (2000) 421.
[15] J.A. Banas, R.R.B. Russell, J.J. Ferretti, Infect. Immun. 58 (1990) 667.
[16] J. Ferretti, M. Gilpin, R. Russell, J. Bacteriol. 169 (1987) 4271.
[17] T. Fujiwara, Y. Terao, T. Hoshino, S. Kawabata, T. Ooshima, S. Sobue, S. Kimura, S. Hamada, FEMS Microbiol. Lett. 161 (1998) 331.
[18] G. Mooser, A. Hefta, R. Paxton, J. Shively, T. Lee, J. Biol Chem. 266 (1991) 8916.
[19] M.R. Park, H.J. Ryu, D. Kim, J.Y. Choe, J.F. Robyt, J. Microbiol. Biotechnol. 11 (2001) 628.
[20] E.E. Koch, Handbook on synchrotron radiation, third ed., North-Holland, New York, 1983, p. 103.
[21] E.F. Maniatis, E.F. Fritsch, J. Sambrook, Molecular cloning, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989, p. 1.25.
[22] J. Sun, S. Wanda, R. Curtiss, J. Bacteriol. 177 (1995) 1703.
[23] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[24] A.W. Miller, S.H. Eklund, J.F. Robyt, Carbohydr. Res. 147 (1986) 119.
[25] R. Mukerjea, D. Kim, J.F. Robyt, Carbohydr. Res. 292 (1996) 11.
[26] U.K. Laemmli, Nature 227 (1997) 680.
[27] A.W. Miller, J.F. Robyt, Anal. Biochem. 156 (1986) 357.
[28] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. 76 (1979) 4350.
[29] J.W. Van Cleve, W.C. Schaefer, C.E. Rist, J. Am. Chem. Soc. 78 (1956) 4435.
[30] A. Jeanes, F.R. Seymour, Carbohydr. Res. 74 (1979) 31.
[31] J.F. Robyt, R. Mukerjea, Carbohydr. Res. 251 (1994) 187.
[32] A. Shimamura, Carbohydr. Res. 185 (1989) 239.
[33] A. Shokri, A.M. Sanden, G. Larsson, Appl. Microbiol. Biotechnol. 60 (2003) 654.
[34] P. Cornelis, Curr. Opin. Biotechnol. 11 (2000) 450.
[35] N. Yokota, T. Kuroda, S. Matsuyama, H. Tokuda, J. Biol. Chem. 274 (1999) 30955.
[36] M.A. Blight, C. Chervaux, I.B. Holland, Curr. Opin. Biotechnol. 5 (1994) 468.
[37] I.R. Hederson, F. Navarro-Garcia, J.P. Nataro, Trends Microbiol. 6 (1998) 370.
[38] D. Kim, J.F. Robyt, Enzyme Microb. Technol. 17 (1995) 1050.
[39] S.W. Botchway, D.L. Stevens, M.A. Hill, T.J. Jenner, P. O'neill, Radiat. Res. 148 (1997) 317.
[40] Z. Djouzy, C. Andrieux, V. Pelenc, S. Somarriba, F. Popot, F. Paul, P. Monsan, O. Szylit, J. Appl. Bacteriol. 79 (1995) 117.
[41] P. Valette, V. Pelenc, Z. Djouzy, C. Andrieux, F. Paul, P. Monsan, O. Szylit, J. Sci. Food Agric. 62 (1993) 121.
[42] P. Monsan, F. Paul, FEMS Microbiol. Rev. 16 (1995) 187.
[43] V. Monchois, M. Remaud-Simeon, R.R.B. Russel, P. Monsan, R.M. Willemot, Appl. Microbiol. Biotechnol. 48 (1997) 465.
[44] S. Bozonnet, M. Dols-Laffargue, E. Fabre, S. Pizzut, M. Remaud-Simeon, P. Monsan, R.M. Willemot, J. Bacteriol. 184 (2002) 5753.
[45] M. Arguello-Morales, M. Remaud, S. Pizzut, P. Sarcabal, R. Willemot, P. Monsan, FEMS Microbiol. Lett. 182 (2000) 81.