Evolution of a dextransucrase gene for constitutive and hyper-production and for synthesis of new structure dextran

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ABSTRACT

After irradiation of a cloned dextransucrase gene (*dsrB742*) with ultrasoft X-ray, an *E. coli* transformant (pDSRB742CK) was first developed for the expression of an extracellular dextransucrase, having increased activity and the synthesis of a highly branched dextran. Seven nucleotides of the parent gene (*dsrB742*) were changed in the nucleotide sequences of *dsrB742ck*. Among them, four nucleotides were changed at the ORF of *dsrB742*, resulting in a 30 amino acids deletion in the N-terminal of DSRB742 dextransucrase. The activity of DSRB742CK dextransucrase in culture supernatant was approximately 2.6 times higher (0.035 IU/ml) than that of the DSRB742 clone. The pDSRB742CK clone produced DSRB742CK dextransucrase when grown both on a sucrose medium (inducibly) and on a glucose medium (constitutively). The DSRB742 clone did not produce dextran constitutively on a glucose medium. DSRB742CK dextran had 15.6% branching and 2.7-times higher resistance to dextranase hydrolysis compared to DSRB742 dextran. ^{13}C-NMR showed that DSRB742CK dextran contained α-(1→3) branch linkages that were not present in DSRB742 dextran.

INTRODUCTION

Dextransucrases [EC 2.4.1.5] are extracellular enzymes that synthesize dextrans from sucrose. 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 medium. From *L. mesenteroides* B-742, Kim and Robyt isolated a constitutive dextransucrase mutant, *L. mesenteroides* B-742CB [1]. Kim *et al.* [2] cloned and sequenced a dextransucrase gene from *L. mesenteroides* B-742CB (*dsrB*742). The nucleotide sequence of *dsrB*742 shows one open reading frame

(ORF) composed of 4,524 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, a 70% similarity with *L. mesenteroides* B-512FMCM dextransucrase. The expressed and purified enzyme (DSRB742) from the clone showed similar enzymatic properties, but with the inability to form a significant amount of α-(1→3) branch linkages compared to that of B-742CB dextransucrase. 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 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.

MATERIALS AND METHODS

Ultransoft X-ray irradiation onto *pdsrB742*. To develop novel mutants, *pdsrB742* was irradiated with ultrasoft X-ray. Ultrasoft X-ray was obtained from the LIGA (Lithography, Galvanoformung-electroplating, Abformung-replication) beamline in the Pohang Accelerator Laboratory (Pohang, Korea). An aluminum target was placed inside the exposure chamber and the ultrasoft X-ray was generated by irradiating this target with a white beam. In this experiment, the Al K ray was corresponded to energy of 1.487 KeV. After irradiation, the *dsrB742* in each well was transformed to *E. coli* strain DH5 and transformants were plated onto LB medium supplemented with ampicillin (50μg/ml) and 2% sucrose (w/v).

Immuno-detection of dextran prepared by clones. To detect dextransucrase activity, the SDS-PAGE of native protein was conducted and the proteins were transferred to PVDF membranes. Membranes were then incubated at 28℃ for overnight with 200 mM sucrose dissolved in 20 mM sodium acetate buffer (pH 5.2) and Western blots were performed [3]. 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.

Analysis of branching dextran. Dextran was prepared with the reaction of dextransucrase (0.2 U/ml) with 100 mM sucrose (pH 5.5). The reaction was allowed to proceed at 28℃

until the sucrose had been completely consumed. Dextran was prepared from the reaction digests by ethanol precipitation. Dextranase (*Penicillium* dextranase; 1.0 unit; Sigma Co., U.S.A) was added to the carbohydrate solution and allowed to react at 37℃ for 3 hrs. 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℃, 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).

NMR analysis of dextran. The linkage composition of the dextran synthesized by DSRB742CK was analyzed by using ^{13}C-NMR spectrometry. 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 [4]

RESULTS AND DISCUSSIONS

Selection of a mutated dextransucrase gene. 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. pDSRB742CK showed 2.6-times higher dextransucrase activity per mg protein, compared to that of the parent clone, pDSRB742.

The nucleotide sequence of *dsrB742* shows one ORF composed of 4,524 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* showed seven DNA base differences comparing to that of *dsrB742*; three nucleotide deletions and one nucleotide substitution. 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. 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. 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.

Immunological detection of dextran. The dextran synthesizing activity of DSRB742CK dextransucrase was 2.6 times higher than that of DSRB742 dextransucrase (Fig. 1) based on Western blot analysis using anti-dextran antibody. The slight increase in protein production in culture supernatant of pDSRB742CK (1.05 times) compared to that of pDSRB742 clone and the activity increase of 2.6-times suggests that the 30 amino acids in the N-terminal region significantly affects the specific activity of the dextransucrase.

Composition of dextranase hydrolyzates of dextran. The dextran prepared from DSRB742CK was hydrolyzed with *Penicillium* dextranase and the hydrolyzate composition was compared with that of DSRB742 dextran. 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.

Structural analysis of glucan using ^{13}C-NMR. The branch linkage was analyzed by using ^{13}C-NMR. No signal was observed for C3-OS in DSRB742 dextran, while C3-OS signals for α-(1→3) linkages were observed in DSRB742CK dextran. 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. As of yet, there is no detailed molecular explanation and information for the synthesis of branch linkages by different dextransucrases. Studies on DSRB742CK the structure of crystalline enzyme could give a more complete picture of branch formation and their regioselectivity.

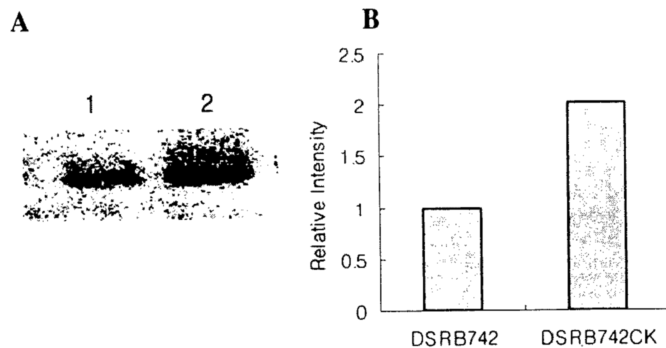

Fig. 1. Detection of dextran production by Western blot analysis.
Detection of dextran synthesized by proteins from culture supernatants of pDSRB742 (lane 1 in 2A and DSRB742 in 2B) and pDSRB742CK (lane 2 in 2A) and DSRB742CK in 2B).

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