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# Identification of Amino-Acids Residues for Key Role in Dextransucrase Activity of *Leuconostoc mesenteroides* B-742CB

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**Abstract** Dextransucrase (DSRB742) from *Leuconostoc mesenteroides* NRRL B-742CB is a glucosyltransferase that catalyzes the synthesis of dextran using sucrose, or the synthesis of oligosaccharides when acceptor molecules, like maltose, are present. The DSRB742 gene (*dsrB742*) was cloned and the properties were characterized. In order to identify critical amino acid residues, the DSRB742 amino acid sequence was aligned with glucosyltransferase sequences, and three amino acid residues reported as sucrose binding amino acids in *Streptococcus* glucosyltransferases were selected for site-directed mutagenesis experiments. Asp-533, Asp-536, and His-643 were independently replaced with Ala or Asn. D533A and D536A dextransucrases showed reduced dextran synthesis activities, 2.3% and 40.8% of DSRB742 dextransucrase, respectively, and D533N, D536N, H643A, and H643N dextransucrases showed complete suppression of dextran synthesis activities altogether. Additionally, D536N dextransucrase showed complete suppression of oligosaccharide synthesis activities. However, modifications at Asp-533 or at His-643 retained acceptor reaction activities in the range of 8.4% to 21.3% of DSRB742 acceptor reaction activity. Thus at least two carboxyl groups of Asp-533 and Asp-536, and His-643 as a proton donor, are essential for the catalysis process.

**Key words:** Dextransucrase, catalytic site, *Leuconostoc mesenteroides*, site-directed mutagenesis, acceptor reaction

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Dextrans make up a class of polysaccharides that are D-glucans of various structures with contiguous α-1→6 glycosidic linkages in the main chains and α-1→2, α-1→3, or α-1→4 branch glycosidic linkages, depending on the specificity of the particular dextransucrase [27]. The enzymes responsible for the synthesis of these dextrans from sucrose are known as dextransucrases, glucansucrases, and glucosyltransferases. The enzymes are elaborated by various bacterial species from two genera, *Leuconostoc* and *Streptococcus*. Dextransucrases are of industrial interest as they are used to synthesize dextran and oligosaccharides that function as prebiotics to encourage growth of beneficial bacteria in food or skincare products [5, 14, 31]. The enzymatic mechanism for synthesizing dextran has been extensively studied by Robyt and co-workers [27, 28]. Using a pulse of $^{14}$C-sucrose and a chase with non-labeled sucrose, Robyt *et al.* [28] found that *Leuconostoc mesenteroides* B-512F dextransucrase added D-glucopyranosyl units from sucrose to the reducing-end of a growing dextran chain. A covalent glucopyranosyl-enzyme intermediate and a covalent dextranyl-enzyme intermediate are formed during synthesis and the glucopyranosyl group is added to the reducing-end of the dextranyl group. A two-site insertion mechanism was proposed for the elongation of the dextran chain [27, 28]. Parnaik *et al.* [25] isolated a covalent glucosyl-enzyme intermediate for *S. sanguis* GTF-S dextransucrase, and Mooser and Iwaoka [21] also isolated a glucosyl-enzyme intermediate for *S. sorbrinus* GTF-S dextransucrase.

Although there are several GTFs, no three-dimensional structures are available, therefore only limited information concerning structure/function relationships is known. Mooser *et al.* [20, 21] demonstrated that an Asp-residue in GTFI and GTFS from *S. sobrinus* is involved in the formation of a glucosyl-enzyme intermediate. Kato *et al.* [4, 32] showed that homologous Asp-451 in GTFB from *S. mutans* is essential for catalytic activity. MacGregor *et al.* [13] postulated that this residue was equivalent to the Asp-residue important for catalysis in enzymes of the α-amylase superfamily. Monchois *et al.* [17] reported that mutations at Asp-511 and Asp-551 of *L. mesenteroides* B-512F dextransucrase completely suppressed both dextran and oligosaccharide synthesis activities. Moreover, chemical modifications of dextransucrase from *L. mesenteroides* NRRL B-512F with various agents have also shown that histidine residues are important for enzyme activity [1]. Homologous histidine is thought to stabilize the glucosyl-enzyme complex in the α-amylase or cyclodextrin glucanotransferase families [13].

*L. mesenteroides* B-742 produces two kinds of dextransucrases when grown on sucrose. One enzyme synthesizes dextran containing α-1→4 branch linkages and the other synthesizes dextran with a very high degree (~50%) of single α-1→3 branched glucose residues [27]. Kim *et al.* [10, 24] cloned and sequenced two dextransucrase genes from B-742CB (*dsrB742*; GenBank Accession No. AF294469 and *dsrB*). 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 [19]. A 70% similarity with *L. mesenteroides* B-512FMCM dextransucrase [30] and a 45-56% similarity with *Streptococcal* glucansucrases (GTFs) [18, 33, 35] were also observed. To further investigate the catalysis mechanism of GTF, it is necessary to identify other amino-acid residues that may be strongly involved in enzyme activity. With this in mind, substitution mutants of Asp-533, Asp-536, and His-643 of DSRB742 have been constructed and the potential amino acid residues of *L. mesenteroides* B-742CB dextransucrase (DSRB742) potentially involved in dextran synthesis and acceptor reaction are reported. The three target amino acids were chosen based upon the proposed mechanism of catalysis of dextransucrase [28], and upon sequence comparisons between GTF and cyclodextrin glucosyltransferases or α-amylases [13]. They were also reported to be involved in sucrose binding of *Streptococcus* glucosyltransferase [21].

*E. coli* DH5α and *E. coli* BL21(DE3)pLysS were used as plasmid hosts and for site-directed mutagenesis. Cultures were grown in Luria-Bertani (LB) medium containing ampicillin (50 μg/ml) and agar (1.5%) as required. The recombinant *E. coli* DH5α (KACC 95014 containing *dsrB742*; Korean Agricultural Culture Collection, Suwon, Korea) was maintained in LB agar medium with ampicillin (50 μg/ml) and 2% glucose or sucrose (w/v) [17]. Plasmid pDSRB742 contains the intact *dsrB742* gene under the control of its own promoter. Restriction enzymes were purchased from Takara (Shiga, Japan), Promega (U.S.A.), or Poscochem (Pohang, Korea). T4 DNA ligase, Klenow fragment, calf intestinal alkaline phosphate, and Ex *Taq* polymerase were purchased from Takara (Shiga, Japan). A site-directed mutagenesis kit, QuickChange™, was purchased from Stratagene (U.S.A.). Thin-layer chromatography glass plates were purchased from Whatman Int. Ltd. (U.K.). Routine DNA manipulation, including plasmid purification and *E. coli* transformation, was performed as described by Maniatis *et al.* [15]. Plasmid pDSRB742 was used as the template for mutagenesis. The mutagenesis was performed using a set of mutagenic oligonucleotides (For D533A; 5' GATGGGATACGTGTCACTGCTGTCGATAATGTT 3', for D533N; 5' GATGGGATACGTGTCAATGCTGTCGATA-ATGTT 3', for D536A; 5' CGTGTCGATGCTGTCGCTA-ATGTTGACGCTGAT 3', for D536N; 5' CGTGTCGAT-GCTGTCAATAATGTTGACGCTGAT 3', for H643A; 5' TACAGCTTTGTTCGCGCAGCCGATAGTGAAGTA 3', for H643N; 5' TACAGCTTTGTT CGCGCAAACGATA-GTGAAGTA 3'-Underlined nucleotides are codons for mutated amino acid) with a Quick-change site-directed mutagenesis kit (Stratagene, U.S.A.). After transformation into *E. coli* BL21(DE3)pLysS with mutated plasmid DNA from polymerase chain reactions, recombinant colonies were selected on LB medium plates containing 5% sucrose and 50 μg/ml ampicillin in difference of phenotype. The nucleotides of the selected recombinants were sequenced to confirm the mutations. Then the 300 bp *Acc*I-*Nsi*I fragment containing the mutated positions (533 and 536) or the 230 bp *Nsi*I-*Nhe*I fragments containing the change of His-643 to Asn or Ala on *dsrB742* were subcloned into either *Acc*I-*Nsi*I treated or *Nsi*I-*Nhe*I treated intact pDSRB742. The DNA sequencing was performed using a standard sequencing strategy with an ABI Prism™ BigDye™ Terminator Cycle Sequencing Kit for ABI377 PRISM in a GeneAmp 9600 thermal cycler (Perkin Elmer) and ABI377 PRISM (Applied Biosystems).

Dextransucrase proteins were expressed in *E. coli* BL21(DE3)pLysS cells which had been freshly transformed with the plasmids containing the *dsrB742* gene or mutated *dsrB742* genes. Overnight cultures of bacteria were inoculated in fresh LB broth supplemented with ampicillin (50 μg/ml), and grown with shaking at 28°C until the absorbance at 600 nm reached 0.4-0.5. The cells were incubated with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 28°C, and then the cells were kept overnight at 4°C. The cells were harvested and washed with 20 mM sodium acetate buffer (pH 5.2) containing 1 mM $CaCl_2$. The cell pellets were resuspended in the same buffer and disrupted by sonication in an ice bath. The

lysate supernatant was used to determine dextransucrase activity. Kim and Robyt [6] have reported the adsorption of dextransucrase on Sephadex beads (Pharmacia, Sweden). For more efficient, easy, and rapid purification of mutant glucansucrases, the method was modified slightly. One-fourth volume of 50% (v/v) slurry of Sephadex G-200 beads was added to the cell-free extract, and mixed gently for 4 h at 4°C. The beads were then loaded into a column, and washed five times with an equal volume of 20 mM Na-acetate buffer (pH 5.2). Finally, the adsorbed enzymes were eluted by applying an equal volume of 5% dextran T-40. The active fractions were collected and incubated with immobilized dextranase to remove the dextran T-40 for 3–4 h at room temperature. The dextranase-treated fraction was then dialyzed and concentrated to 10-fold using a Centricon (30k cut-off, Amicon, U.S.A.) and used as the purified enzyme preparation. Activity was assayed by measuring fructose release in the presence of 100 mM sucrose using the TLC analysis method [with the Public Domain NIH Image Program developed at the U.S National Institute of Health; 10, 12, 22, 30]. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol fructose per min at 28°C. Acceptor reactions were carried out at 28°C in 20 mM Na-acetate buffer (pH 5.2) with 100 mM sucrose and maltose [2, 7–9].

Based upon the proposed mechanism of catalysis of dextransucrase [28], and upon sequence comparisons between GTF and cyclodextrin glucosyltransferases or α-amylases [13], three targets for site-directed mutagenesis were chosen: Asp-533, Asp-536, and His-643 (Fig. 1). In

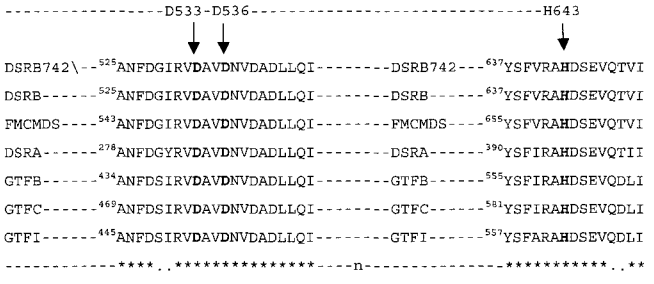


**Fig. 1.** Comparisons of amino acid sequences in the regions chosen for site-directed mutagenesis in *dsrB742*.
The amino acid sequences were deduced from glucansucrase genes of *L. mesenteroides* B-742CB, *dsrB742* [10]; *L. mesenteroides* NRRL B-1299, *dsrB* [16]; *L. mesenteroides* B-512FMCM, *fmcmds* [30]; *L. mesenteroides* B-1299, *dsrA* [19]; *S. mutans* GS5, *gtfB* [33], *gtfC* [35]; and *S. downei* Mfe28, *gtfI* [18].

all GTFs and dextransucrases of known sequence, these amino acid residues are always conserved. In contrast to other conserved amino acid residues, these were also chosen because previous studies showed that they might be involved in enzyme activity [1, 13, 17, 20, 23]. To determine whether amino acids Asp-533, Asp-536, and His-643 were essential for catalytic activities, both Asp and His residues were changed independently to Asn or Ala residues, allowing removal of the carboxyl group of the amino acid residue or conformational changes with a smaller size amino acid. His-643 was changed to Asn in order to retain the positive charge but not mimic the hydrogen binding of His or to Ala in order to remove the positive charge. SDS-PAGE analysis revealed similar

**Table 1.** The relative activity and acceptor reaction efficiency of each dextransucrase prepared from each clone.

| Enzyme sources[a] | Enzyme reaction | Sucrose consumed [%][b] | Acceptor products produced [%][c] | Relative dextransucrase activity[d] | Acceptor reaction efficiency[e] |
|---|---|---|---|---|---|
| DSRB742 | S[f] | 61 | 3.5 | 100 | 100 |
| (No mutation) | A[g] | 100 | 28.6 | 164 | |
| D533A | S | 1.4 | N | 2.3 | 21.3 |
| | A | 8.9 | 6.1 | 14.6 | |
| D533N | S | 0 | N | 0 | 8.4 |
| | A | 3.8 | 2.4 | 6.2 | |
| D536A | S | 24.9 | N | 40.8 | 7.7 |
| | A | 27.3 | 2.2 | 44.8 | |
| D536N | S | 0 | N | 0 | 0 |
| | A | 0 | 0 | 0 | |
| H643A | S | 0 | N | 0 | 17.1 |
| | A | 10.2 | 4.9 | 16.7 | |
| H643N | S | 0 | N | 0 | 19.9 |
| | A | 12.5 | 5.7 | 20.5 | |

[a]All proteins were bound onto Sephadex G-200.
[b]Relative amount of sucrose consumed when all sucrose was consumed in DSRB742 dextransucrase acceptor reaction digest.
[c]Acceptor reaction products formed; % of total carbohydrate in reaction digest.
[d]Relative amount of fructose released; 100% is the amount of fructose released by DSRB742 dextransucrase digest with sucrose only.
[e]The relative amount of oligosaccharides in digest; 100% is the amount of oligosaccharides produced by DSR742CB dextransucrase.
[f]"S" means dextransucrase reaction digest with sucrose only.
[g]"A" means dextransucrase acceptor reaction with sucrose and maltose; N: No reaction performed.

levels of expression for the electrophoretic forms of dextransucrase in wild-type and mutants (0.66–0.68 mg/ml). In order to detect activity, gels were incubated in 50 g/l sucrose. The replacements of Asp-533 and Asp-536 to Ala reduced, but not completely eliminated, the synthesis of dextran (Table 1). However, individual replacement of Asp-533, Asp-536, and His-643 with Asn, and His-643 with Ala resulted in a protein retaining no dextran synthesis activities. Asp-536 of DSRB742 is homologous to the Asp residues identified by Mooser *et al.* [20] as part of the sucrose binding sites of *S. sobrinus* GTF, and identified by Monchois *et al.* [17] to Asp-551 of *L. mesenteroides* B-512F DSRS, shown by site-directed mutagenesis to be essential. The fact that the change of the carboxyl group to an amide group is sufficient to completely inhibit the enzyme activity strongly suggests that Asp-533 and Asp-536 are also part of a sucrose binding site in DSRB742. However, Asp-536 is more essential to maintain DSRB742 activity, as a mutation of Asp-533 to Asn or to Ala allows significant residual activity to be maintained for dextran production to be detectable. Also in this region, Glu-484 in DSRB742, always conserved in GTF or dextransucrase sequences [18, 30, 33, 35], may be another candidate for carrying a carboxyl group important for maintaining enzyme activity. As the level of expression of DSRB742 and mutant genes is similar (data not shown), this indicated that strong reduction in activity was not due to a problem of expression but really due to mutations. Fu and Robyt [1] showed that two imidazolium groups of histidine were essential for dextran synthesis by chemical modification of the enzyme with diethyl pyrocarbonate and photo-oxidation with Rose Bengal dye. They postulated that two imidazolium groups donate their hydrogen ions to the leaving fructose units and the resulting imidazole group in a second step becomes reprotonated by abstracting a proton from the 6-hydroxyl group of the glucosyl-enzyme intermediate, thereby facilitating the nucleophilic attack and the formation of the α-1→6 linkage. Thus, the imidazole group also becomes reprotonated for the next reaction with sucrose. His-643 is also essential for enzyme activity. His-643 is homologous to conserved histidine in other enzymes acting on carbohydrates like glucosyltransferases, cyclodextrin glucosyltransferases, and α-amylases [13], where the histidine residue seems to be involved in the catalysis process and acts as a proton donor. A similar role may be attributable to His-643 in DSRB742.

To check whether the mutations altered transferase activity as well as polymer synthesis, oligosaccharide synthesis assays were carried out in the presence of sucrose and maltose. In addition to catalyzing the synthesis of dextran from sucrose, dextransucrase also catalyzes the transfer of glucose from sucrose to other carbohydrates that are present or are added to the reaction mixture. The added carbohydrates are called 'acceptors' and the reactions are called 'acceptor reactions' [11, 29]. When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor-products is usually produced. In the presence of sucrose, the introduction into the reaction medium of molecules like maltose, isomaltose, and *O*-α-methylglucoside shifted the pathway of glucan synthesis towards the production of oligosaccharides. The addition of maltose in the reaction digest activates the reaction and accelerates the reaction velocity. It may be explained by a change in the limiting step occurring during the acceptor reaction [26]. In the presence of maltose, the formation of the glucosyl-enzyme complex then becomes the limiting step instead of the polymer transfer in the case of the glucan synthesis reaction. All mutants, except D536N, showed acceptor reaction with different efficiencies (Table 1). No oligosaccharides were observed with D536N mutant. All mutants showed similar or increased acceptor reaction efficiencies compared to dextran synthesis activities, except D536A: it gave significantly lower acceptor reaction efficiency compared to dextran synthesis activity (7.7% acceptor reaction compared to 44.8% dextran synthesis activity of DSRB742). Other mutants showed oligosaccharides synthesis activities ranging from 8.4% to 21.3% of DSRB742. Both D533N and H643 mutants showed oligosaccharide synthesis activities even though there was no dextran synthesis activity. All glucansucrases were bound onto Sephadex G-200. Thus, mutations of DSRB742 and resulting mutants did not alter the glucan-binding capacities of the enzymes, indicating that mutations of amino acid residues located in the catalytic domain of DSRB742 did not remove the glucan-binding capacities of the enzyme, thus showing that the mutations had no effect on the overall conformation of the DSRB742. According to proposed mechanisms explaining dextran synthesis [1] and oligosaccharide synthesis [34], a mutation at Asp-533 or Asp-536 should completely suppress dextran synthesis activity but it should still be possible to measure the production of oligosaccharides. The mutation of Asp-533 showed the synthesis of oligosaccharides, but not by D536N. Thus, it is possible that, unlike the DSRS of *L. mesenteroides* B-512F, DSRB742 has a separate maltose-binding site from the sucrose-binding site.

In this report, carboxyl groups and imidazole groups important for enzyme activity have been localized in the DSRB742 sequence. However, the exact identification of the roles of these residues in the catalytic process is not possible. Other conserved amino acid residues will also contribute to sucrose binding and conversion but they can not be identified by simple sequence alignments. Recently, an *E. coli* transformant (pDSRB742CK) has been obtained from the DSRB742 clone using ultrasoft X-rays for the expression of a dextransucrase [3]. 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 & 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. Based on these mutagenesis results, 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 the dextransucrase gene for catalytic and glucan binding site and the determination of a crystal structure are in progress.

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