Glucan Binding Regions of Dextranucrase from *Leuconostoc mesenteroides* NRRL B-512F

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We isolated glucan-binding peptides of a dextranucrase from *Leuconostoc mesenteroides* B-512F. The dextranucrase was bound to DEAE-Sephadex A-50, Sephadex G-100, and mutan from Streptococcus mutans. Mild trypsin digestion dissociated the enzyme and glucan binding. In the presence of ammonium sulfate, several peptides were bound to glucan after trypsin digestion. Four main mutan-binding peptides were obtained by this method, and those amino acid sequences were analyzed. One of them was identical with the dextran-binding peptide that contains lysine, which was previously isolated by differential chemical modification with o-phthalaldehyde. We also found mutan-binding peptides in sucrose- and dextran-binding regions and a lysine-rich region. Also, there was a peptide similar in sequence to glucan-binding A-repeat of streptococcal glucosyltransferases.

**Key words:** dextranucrase; dextran; α-glucan; glucan-binding; *Leuconostoc mesenteroides*

Dextranucrase (EC 2.4.1.5) is produced by several strains of *Leuconostoc*. It has a strong affinity to its product dextran and makes an aggregated form. Some of the *Streptococcus* strains produce glucosyltransferases (GTFs). Glucan which was produced by GTFs has been considered to contribute colonization on teeth surfaces. Glucan-binding domain was found in the C-terminal domain of streptococcal GTFs. This domain contains a series of direct repeating units and its function was suggested to be glucan-binding. Recently, *L. mesenteroides* NRRL B-1299 dextranucrase gene (*dxaA*) was isolated and sequenced. The deduced amino acid sequence showed a series of repeats (A to D) in the C-terminal domain. We have reported that the structures of *Leuconostoc mesenteroides* NRRL B-512F dextranucrase and streptococcal GTFs had high similarity with each other. We isolated an A-repeat similar peptide, and also suggested different glucan-binding domains in B-512F dextranucrase other than C-terminal repeats by a differential chemical modification by o-phthalaldehyde with and without dextran.

In this report, glucan-binding regions of B-512F dextranucrase were isolated by a complex formation with glucans and subsequent tryptic digestion.

**Materials and Methods**

**Materials.** DEAE-Sephadex A-50, Sephadex G-100, and Sepharose 6B were purchased from Pharmacia Fine Chemicals; sequence grade trypsin (cat. no. 1047841) was from Boehringer Manheim Biochemica.

**Preparation of Dextranucrase from B-512F.** *L. mesenteroides* NRRL B-512F was cultured with 2% sucrose at 30°C overnight by the method described. Cells were discarded by centrifugation at 8,000 x g for 20 min at 4°C. Dextranucrase was purified by a slightly modified method of that previously described.

The culture supernatant was fractionated with 30% polyethylene glycol 6000 (PEG 6000) as described previously and the precipitations were dissolved in 20 mM acetate buffer (pH 5.2) containing 30% glycerol (buffer A). The dissolved PEG fraction was put on a DEAE-Sephadex A-50 column (3.5 x 21 cm) previously equilibrated with buffer A. The column was washed with 500 ml of buffer A. The enzyme bound to the column was then eluted with the same buffer containing a 0–4 M guanidine-HCl linear gradient. Small samples of each fraction were dialyzed against buffer A, and enzyme activities were measured. Active fractions were pooled and volume was reduced by freeze-drying. Guanidine was removed by Sepharose CL-6B (3.0 x 68.0 cm) gel filtration with buffer A as described previously. Active fractions were collected and freeze-dried, and used as a purified dextranucrase.

**Analytical methods.** Dextranucrase activity was measured by the release of reducing sugar from sucrose as in the method of Kobayashi and Matsuda.

Protein was measured by a fluorescence method, or by the absorbance at 280 nm. The absorbance coefficient of dextranucrase was 4.52% cm⁻¹. Sugars and polysaccharides were measured by the phenol-sulfuric acid method.

**Preparation of mutan.** Mutan was prepared from *Streptococcus mutans* culture supernatant as described by Imai et al.

**Isolation of glucan-binding peptides from dextranucrase.** 1) Isolation of dextran-binding peptides, using DEAE-Sephadex A-50 or Sephadex G-100 as immobilized dextran gels was done as the modified method of Kobayashi et al. Purified dextranucrase (2.5 mg) was put on Sephadex G-100 or DEAE-Sephadex A-50 column (12 x 40 mm) previously equilibrated by 20 mM
acetate buffer (pH 5.2) and kept at 4°C overnight. Then, 1.3 ml of 100 mM Tris-HCl (pH 8.5) was added to the gel. Subsequently, 1 μg of trypsin in 0.5 ml of 100 mM Tris-HCl (pH 8.5) was added to the column and kept at 37°C for 3 h. DEAE-Sephadex A-50 was washed with 5 ml of 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.0) containing 0.3 mM NaCl. Sephadex G-100 was washed with 5 ml of 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.0). Then, DEAE-Sephadex A-50 was washed with 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.0) containing 0.3 mM NaCl and 5 mM guanidine-HCl, and Sephadex G-100 was washed with 6 mM guanidine-HCl. Sephadex-binding peptides solution was collected. 2) Isolation of mutan-binding peptides was done as follows. In a 1.5-ml microtube, 2.5 mg of dextranucrase and 2.5 mg of mutan were suspended with 250 μl of 20 mM of acetate buffer (pH 5.2) and kept at 4°C for overnight. Then, 167 μl of 500 mM Tris-HCl (pH 8.5) and 1 μg of trypsin were added to the suspension, and incubated at 37°C for 3 h. After that, the pH was adjusted at 5.2 by HCl and 200 μl of 200 mM acetate-buffer (pH 5.2) was added. The supernatant was removed by centrifugation and precipitates were washed by 1 ml of water twice. To precipitates, 100 μl of 6 M guanidine-HCl was added, mixed, and kept at 4°C overnight. The supernatant was collected by centrifugation and used as a mutan-binding peptide solution. To increase the affinity of dextranucrase for mutan, 25 mg of additional mutan and 2 mM (NH₄)₂SO₄ were optionally added to the mutan-dextranucrase suspension and kept at 4°C overnight after the pH adjustment at 5.2 following the trypsin treatment.¹⁹

Amino acid sequences of peptides from dextranucrase. Peptides were separated by HPLC (Jasco PU-980) with a C₁₈ reversed-phase column (TSK gel ODS-120T) by 0.8 ml/min of 0 to 80% acetonitrile containing a 0.05% trifluoroacetic acid (TFA) linear gradient for 60 min. Absorbance at 220 nm was monitored by UV spectrometer (Jasco UV-970). Peaks were collected and rechromatographed by HPLC with the same column by 0.6 ml/min of 0 to 60% acetonitrile containing a 0.05% TFA linear gradient for 120 min and collected the peptides again. Isolated peptides were sequenced by a protein sequencer (ABI 477A).

Results

Isolation of glucan-binding peptides from dextranucrase

Dextranucrase has affinities to Sephadex G-100, DEAE-Sephadex A-50, or mutan. Purified dextranucrase (2.5 mg) was put on Sephadex G-100, DEAE Sephadex A-50 (12 × 40 mm), or 2.5 mg of mutan and kept at 4°C. When the Sephadexes or mutan were washed with buffer A, no dextranucrase activity was detected in the washings. All dextranucrase bound to these glucans in this condition. However, when dextranucrase was cleaved with trypsin in the presence of Sephadex G-100, DEAE-Sephadex A-50, or mutan, the enzyme was digested to small peptides. No trypsin-resistant fragment was detected by SDS-PAGE or by HPLC with a C₁₈ reversed-phase column. To increase the affinity of peptides to α-glucan, 2 mM of (NH₄)₂SO₄ was added to the reaction mixtures after trypsin digestion.

In a trypsin-treated dextranucrase-mutan mixture, at least three peptides were decreased in the supernatant after (NH₄)₂SO₄ treatment (Fig. 1b) compared with Fig. 1a. Those peptides were designated peptides 1, 2, and 3, respectively. Mutan was washed three times and no peptide peak was observed in the supernatant (Fig. 1c). A few peaks were eluted from mutan by 6 M of guanidine-HCl. Peptides 1, 2, and 3, which decreased in the supernatant after (NH₄)₂SO₄ treatment were eluted by guanidine (Fig. 1d). Those peptides were thought to bind to mutan in the presence of (NH₄)₂SO₄ and dissociated from glucan by guanidine-HCl. Another big peak, peptide 4, was observed after guanidine treatment. The amount of this peptide increased in the supernatant after an addition of (NH₄)₂SO₄. This indicates that (NH₄)₂SO₄ did not increase the affinity of peptide 4 to mutan. In this point, peptide 4 and other mutan-binding peptides 1, 2, and 3 were different.

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Fig. 1. HPLC Mapping of the Trypsin Digests of the Mutan-binding Dextranucrase.

Purified dextranucrase (2.5 mg) and mutan (2.5 mg) were incubated and treated with trypsin. Ten % of supernatant was put on TSK gel ODS-120T and peptides were separated by 0.8 ml/min of 0 to 80% acetonitrile containing 0.05% trifluoroacetic acid (TFA) linear gradient for 60 min (a). Dextranucrase was incubated with mutan and treated with trypsin in the same condition as (a). Then, 25 mg of mutan and 2 mM (NH₄)₂SO₄ were added to the mixture. Ten % of supernatant was taken and peptides were analyzed by HPLC as described above (b). The mutan precipitated at the treatment (b) was washed with water and peptides in washings were analyzed as described above (c). Guanidine-HCl (6 M) was added to the mutan precipitated at the treatment (c) and released peptides in the supernatant were analyzed as described above (d). Experimental details were described in Materials and Methods.
Effects of \((\text{NH}_4)_2\text{SO}_4\) on mutan-binding in dextranucrase. As shown in Fig. 2b, glucan was not added to the enzyme before trypsin digestion. After trypsin treatment, mutan was added, then mutan-binding peptide peaks of 1, 3, and 4 were eluted by guanidine, but peptide 2 had disappeared. The affinity of peptide 2 to mutan should be weaker than that of peptides 1, 3, and 4. When dextranucrase was treated by trypsin without the protection of mutan, the secondary or tertiary structure of peptide 2 region might be changed and mutan-binding activity of peptide 2 be decreased. When 2 M \((\text{NH}_4)_2\text{SO}_4\) and mutan were added before trypsin digestion, mutan-binding peptides were eluted in broad peaks and shifted to more hydrophobic regions. The larger size of peptide fragments seemed to have increased and the number of peptides also seemed to have increased. The affinity of dextranucrase to mutan increased by the additional \((\text{NH}_4)_2\text{SO}_4\) in early stages, and lower affinity regions also bound strongly to mutan and became resistant to trypsin.

We isolated peptides 1, 2, 3, and 4 and analyzed their amino acid sequences.

Amino acid sequences of mutan-binding peptides from \(B-512\) dextranucrase

Peptides 1, 2, 3, and 4 were re-chromatographed as described in materials and methods. Their sequences are shown in Table I. Peptide 3 were separated into two peptides, peptide 3-1 and peptide 3-2. Peptide 4 did not show any amino acid peaks in the Edman degradation cycle, that indicated peptide 4 was not a peptide peak.

Comparison of mutan-binding peptides and known sequences of dextranucrases and glucosyltransferases

As shown in Table II, peptide 3-2 was 56% similar to a peptide from \(B-512\) dextranucrase we previously isolated and identified as the A-repeat sequence.\(^1\) Peptide 2 was almost identical with one of the dextran-binding lysine containing regions, peptide C,\(^12\) but the fifth amino acid of peptide C was lysine while that of peptide 2 was serine. Deduced amino acid sequences of streptococcal GTFs and \(L.\) mesenteroides B-1299 dextranucrase in databases GenBank and EMBL were searched, and only one peptide-2 similar sequence was found in every enzyme. Peptide 2 and Peptide C were likely to be the same peptide rather than two almost identical sequences in the different positions of dextranucrase. Either the fifth amino acid of peptide 2 or peptide C could have been misread. Peptide 1 was similar to the region of 14 to 43 amino acids toward the N-terminal from one of the sucrose-and-dextran-binding lysine containing regions. Peptide 3-1 was located between the active-site aspartate\(^20\) and dextran-binding lysine containing regions.\(^12\) Previously, no substrate-binding lysine was isolated in this region, but the location of peptide 3-1-homologous region in streptococcal GTFs was the most lysine-rich one.\(^12\)

Discussion

Dextranucrases are known to make a highly aggregated form with dextran.\(^21\) It has a strong affinity for dextran, and we have reported the enzyme purification procedure using DEAE-Sephadex A-50 as an affinity ligand.\(^1\) However, when trypsin was added to dextran-glucan (DEAE-Sephadex A-50, Sephadex G-100, or mutan) complex, no trypsin-resistant peptide was observed. In \(Streptococcus\) \(sobrinus\), trypsin-resistant fragments were reported in GTF-I\(^22\) and GTF-5.\(^2\) Wong et al.\(^22\) digested GTF-I with trypsin under the conditions of trypsin: GTF-I = 1:500 (wt/wt) for 3 h at 37°C. Kobayashi et al.\(^23\) digested GTF-I and GTF-S at trypsin:GTF = 1:100 to 10000 for 20 h at 37°C.\(^3\) Wong et al.\(^3\) isolated a 42-kDa glucan-binding fragment, and Kobayashi et al.\(^3\) isolated 55-kDa glucan-binding fragments from GTF-I and GTF-Sd and smaller peptides from GTF-Si. Our conditions of trypsin digestion were:trypsin:dextranucrase = 1:2500 for 3 h at 37°C, which was milder than the conditions in GTFs. We did not detect even any small pieces of glucan-binding peptide fragment. We
Table II. Comparison of Mutan-binding Peptides, Previously Reported Peptides of B-512F Dextranucrase,1,2) Deduced Amino Acid Sequences of Streptococcal GTFs,7)8) and L. mesenteroides B-1299 Dextranucrase.13)

<table>
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<tr>
<th>Enzyme</th>
<th>Origin</th>
<th>Amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>DS</td>
<td>L.m.B-512F Peptide 1 (mutan-bind)</td>
<td>IFLTTQNTDYSNHNA</td>
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<tr>
<td>DS</td>
<td>L.m.B-129914)</td>
<td>(48) QYVSELFISIGNKHNAV</td>
</tr>
<tr>
<td>GTF-S</td>
<td>S.m.gffD</td>
<td>(210) DIVTTRSNLKKYBQV</td>
</tr>
<tr>
<td>GTF-I</td>
<td>S.m.gffB</td>
<td>(222) QOLKEKNNDYTPHNIQ</td>
</tr>
<tr>
<td>DS</td>
<td>Peptide 2 (mutan-bind) L.m.B-512F</td>
<td>ALHASGIQAI</td>
</tr>
<tr>
<td>DS</td>
<td>Peptide C (OPA dex-bind) L.m.B-512F12)</td>
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<tr>
<td>DS</td>
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<td>(734) ALHASGIQAIINDWPDQIYMN</td>
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<tr>
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<td>(949) ALHAKGIQVADWPDQIYMN</td>
</tr>
<tr>
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<td>S.m.gffB</td>
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</tr>
<tr>
<td>DS</td>
<td>Peptide 3-1 (mutan-bind) L.m.B-512F</td>
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<tr>
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<td>DS</td>
<td>Peptide 5 (A-repeat) L.m.B-512F17)</td>
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<tr>
<td>GTF-I</td>
<td>S.m.gffB (A-repeat 1st)</td>
<td>(1095) WYFEDNNGYM</td>
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</table>

previously reported that B-512F dextranucrase forms a looser aggregation than streptococcal GTFs.1) An affinity to glucan of B-512F dextranucrase should be weaker than that of streptococcal GTFs.

Addition of (NH₄)₂SO₄ to the reaction mixture increased its affinity of dextranucrase to glucan and we got four main mutan-bound peptide fragments (Fig. 1). One of them, peptide 3-2, was identified as a part of the A-repeat (Table II). Glucan-binding domains exist in the C-terminals of streptococcal GTFs. Those domains have a series of repeated sequences that have been classified into different classes, A, B, C, and D7,23-29 and they are known as the “YG” repeat29) which has been thought to have a function of glucan-binding. Usually, known GTFs have three to seven repeating units in their C-terminals. The combinations of A to D repeats are different from each other, but every GTF of which encoded DNA sequence was known to have an A-repeat. “YG” repeats are also observed in C-terminals of other carbohydrate-binding proteins, i.e., Clostridium difficile toxins,20) a Streptococcus mutans glucan-binding protein,21) Streptococcus pneumoniae lysins,22) and a Streptococcus sobrinus dextranucrase inhibitor.23) A dextranucrase gene (dsr A) from Leuconostoc mesenteroides B-1299 also showed five A-repeats and three C-repeats in its C-terminal.29)

Peptide 3-2, a mutan-binding peptide which we obtained from B-512F dextranucrase was 33 to 44% similar to the first (the most N-terminal) A-repeats of B-1299 dextranucrase, S. mutans GTF-S and GTF-I. Especially, the eighth glycine, which is conserved in every A-repeat, and a highly conserved third tyrosine were in peptide 3-2. One of the tryptic peptide fragments we previously isolated from B-512F dextranucrase was 56% homologous to peptide 3-2, but not exactly the same sequences each other. That indicates that B-512F dextranucrase also has at least two A-repeat sequences and suggested the existence of direct repeating units which have a role of dextran-binding in B-512F dextranucrase.

In many other reports mentioned about glucan-binding in streptococcal GTFs, only C-terminal repeating units have been studied. We have suggested two different dextran-binding regions other than C-terminal repeats in B-512F dextranucrase by chemical modification with o-phthalaldehyde.12) One was assumed to be about 100 to 200 amino acids toward the N-terminal regions and the other was about 400 to 500 amino acids toward the C-terminal regions from active site carboxyl amino acids.20,25) Those positions of glucan-binding ly- sine regions were deduced from the similarities between B-512F dextranucrase and streptococcal GTFs. N-termi- nal one was also suggested to bind to not only dextran but also sucrose.12) Both dextran-binding lysine regions were not located in the C-terminal direct repeat regions. One of them was about 800 to 900 amino acids and the other was about 100 to 200 amino acids N-terminal from glucan-binding repeating units.12) Comparing those pep- tides with the mutan-binding peptides isolated from dextranucrase, peptide 1 was suggested to be in a sucrose and dextran-binding lysine-containing N-terminal region and peptide 2 was in a dextran-binding lysine-con- taining region near the C-terminal repeats (Table 2). Peptide 3-1, which was isolated as a mutan-binding peptide was not detected by o-phthalaldehyde modification,12) but it was assumed to be located in the most lysine-rich region in the enzyme deduced from the similarities with GTFs. Lysine residues should be important for glucan-binding in peptide 1, peptide 2, and peptide 3-1.

Lis et al. suggested the toward N-terminal sequences from glucan-binding domain are important for glucan- binding.29) They constructed a deletion derivative of S. mutans GTF-S lacking those sequences and observed the derivative showed weak binding of glucan, but did
not detect any glucan-binding activity in these sequences by themselves.

We detected glucan-binding regions other than glucan-binding repeating units by stimulating glucan-binding activity with \((\mathrm{NH}_4)_2\mathrm{SO}_4\). \textit{L. mesenteroides} dextran-sucrase was weaker by the attack of trypsin in the presence of glucan than streptococcal GTFs, but there was no difference expected in the structure of glucan-binding domains between them. Glucan-binding sites in non-direct repeated regions may decide the strength of bindings on dextran-sucrases or GTFs to glucans.

References