Aggregated Form of Dextranases from *Leuconostoc mesenteroides* NRRL B-512F and Its Constitutive Mutant

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Purified dextranases [EC 2.4.1.5], DSW-D and DSW-G, from *Leuconostoc mesenteroides* B-512F were obtained from affinity chromatography with DEAE-Sephadex A-50 by elution with clinical dextran and guanidine-HCl, respectively. DSM-G was purified from the B-512F mutant strain SH 3002, which produces dextranase constitutively. Although the sugar contents of the purified enzymes were different, their molecular masses by SDS-PAGE were all 170 kDa. DSW-D and DSW-G were highly aggregated and all the activities were eluted at the void volume (Vv) on Sepharose 6B, while the DSM-G was eluted at 1.2 × Vv volume. On rechromatography, DSM-G was separated into three peaks corresponding to the aggregated form, monomeric form, and partially digested form, respectively. The aggregation of *Leuconostoc* dextranase was lower than that of streptococcal glucosyltransferases, but the structures of these enzymes had high homology with each other.

Several strains of *Leuconostoc* and *Streptococcus* produce dextranase (EC 2.4.1.5), which catalyzes the transfer of 2→6-glucosyl groups from sucrose to form dextran. Glucosyltransferases (GTFs) from *Streptococcus* species are strongly aggregated with dextran and enhance bacterial colonization of smooth enamel surfaces of teeth. Another role of the dextran bond to the enzyme was suggested to be as a stabilizer of the enzyme molecule. Streptococcal GTFs are constitutively expressed. However, the *Leuconostoc* dextranase is induced by sucrose during cultivation, and dextran is produced together with the enzyme. Therefore, carbohydrate-free dextranase has not been purified in high yield before. The B-512F dextranase was purified by using affinity for the Sephacryl G-100 gel, but the yield was only 1.6%. Miller et al. purified low carbohydrate content (1–100 μg/mg protein) dextranase in 30–50% yield by using additional dextranase treatment, but dextran was not completely removed. We have purified a sugar-free dextranase for the first time using the *L. mesenteroides* B-512F mutant strain SH 3002, which produces dextranase constitutively. In this report, we describe the differences of the aggregated form of the *Leuconostoc* dextranases in the presence and absence of dextran.

Materials and Methods

**Materials.** DEAE-Sephadex and Sepharose 6B were purchased from Pharmacia Fine Chemicals; clinical dextran was provided by Meito Sangyo Co. Sequencing grade trypsin (cat. no. 1047841) and lysyl endopeptidase were from Boehringer Mannheim Biochemica and Wako Pure Chemical Industries Ltd., respectively. Pyroglycumen amineopeptidase (cat. No. 7321) and a protein N-terminal deblocking kit (cat. No. 7315) were purchased from Takara.

**Enzyme assay.** Dextranase activity was measured by the release of reducing sugar from sucrose as described previously.

**Protein.** Protein was measured by a fluorescence method, or by the absorbance at 280 nm. The absorbance coefficient of dextranase was 4.52% cm⁻¹ (10⁻¹).

**Sugar.** Sugars and polysaccharides were measured by the phenol–sulfuric acid method.

**Purification of Dextranase from L. mesenteroides 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 × g for 20 min at 4°C. The following purification steps were done at 4°C.

**Step 1.** The culture supernatant was fractionated on polyethylene glycol 6000 (PEG 6000), and the precipitate at the concentration of 30 g of PEG 6000/L was collected by centrifugation at 8,000 × g for 20 min. The precipitates were redissolved in 20 mM acetate buffer (pH 5.2) containing 30% glycerol (buffer A).

**Step 2.** The dissolved PEG fraction was put directly on a DEAE-Sephadex A-50 column (3.5 × 21 cm) previously equilibrated with buffer A. The column was washed with 500 ml of the buffer and proteins were eluted with the same buffer containing a 0–2 M NaCl linear gradient and the effluent was discarded. At this stage, more than 96% of the enzyme was still absorbed in the gel. The enzyme bound to the column was then eluted by circulation with 300 ml of 0.5% clinical dextran, 2% Tween in buffer A for one day. The effluent was concentrated about 10-fold by ultrafiltration using a UK-10 membrane (Advantec Toyo).

**Step 3.** The enzyme was put on a Sepharose CL-6B (3.0 × 68.0 cm) column previously washed with 100 ml of buffer A, and active fractions were pooled and freeze-dried (DSW-D).

**Step 4.** After 0.5% clinical dextran–2% Tween elution (step 2), the enzyme that remained on the DEAE-Sephadex A-50 column was eluted with guanidine–HCl in buffer A at concentrations of 0 to 4 M in a linear gradient. Small samples of each fraction were dialyzed against buffer A, and enzyme activities were measured.

**Step 5.** Active fractions were pooled and the volume was reduced by freeze-drying. Then the enzyme was put on a Sepharose 6B column as described in step 3, and active fractions were collected and freeze-dried (DSW-G).
It was best to complete the purification steps within two weeks to avoid proteolysis. Degraded small proteins gradually increased and the yield of enzyme activity was decreased with time.

Purifications of dextranases from mutant SH 3002. The L. mesenteroides constitutive mutant SH 3002 was cultured with 2% fructose at 30°C overnight by the method described. The following purification steps were done at 4°C.

Step 1. The culture supernatant was put on a DEAE-Sephadex A-50 column. After elution with a 0-2 M NaCl linear gradient, the enzyme retained in the column was eluted with 0.4 M guanidine-HCl. The enzyme activities were measured as described above, and active fractions were collected.

Step 2. The volume of active fractions were reduced by freeze-drying and put on a Sepharose CL-6B column as described above (DSM-G). It was also best to complete the purification steps within two weeks to avoid proteolysis as for the enzyme from wild type.

Gel electrophoresis. Polyacrylamide gel electrophoresis (Native-PAGE) was done as described by Maurer with 7.5% gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli with 7.5% gels. A molecular weight (MW) marker kit was purchased from Pharmacia (code No. 17-0615-01). Protein was stained with Coomassie brilliant blue or with a silver staining kit (Wako Pure Chemical Industries Ltd.).

Activity stain. Native PAGE was done as above. The gel was washed twice with 20 mM acetate buffer (pH 5.2) for 3 min each time, then incubated with 12.5% sucrose in the same buffer at 30°C for 1 h. After incubation, the gel was washed with distilled water and fixed with 10% acetic acid–40% methanol solution. The formation of dextran was detected by PAS staining.

N-Terminal deblocking of dextranase. The N-terminal pyroglutamyl group was deblocked with pyroglutamyl amidepeptidase by the method of Podell and Abrahm. The N-terminal acylamino group was deblocked with an N-terminal deblocking kit by the method of Tsunawata et al.

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<th>Table I. Purification of Dextranase from Leuconostoc mesenteroides NRRL B-512F</th>
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<tr>
<td>1. Culture sup.*</td>
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<td>2. 30%PEG</td>
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<td>3. DEAE-Sephadex A-50 (eluted by dextran)</td>
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<td>4. Sepharose 6B (DSW-D)</td>
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<td>5. DEAE-Sephadex A-50 (eluted by guanidine)</td>
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<td>6. Sepharose 6B (DSW-G)</td>
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* The volume of culture supernatant was 1 liter.

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<th>Table II. Purification of Dextranase from Constitutive Mutant SH3002 from Leuconostoc mesenteroides NRRL B-512F</th>
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* The volume of culture supernatant was 1 liter.

* N.D.; Not detected.

Fig. 1. SDS–PAGE of the Purified B-512F Dextranase.

DSW-D, DSW-G, and DSM-G were purified as described in Materials and Methods. DSW-D (0.1 µg), DSW-G (0.05 µg), and DSM-G (0.05 µg) were denatured in 1% SDS and 0.5% mercaptoethanol by 10-min boiling and put on the gel. SDS-PAGE was done for 60 min with 20 mA, and protein was stained with Coomassie brilliant blue. (a) DSW-D, (b) DSW-G, (c) DSM-G.
Activity staining of DSW-G and DSM-G gave only one active band (Fig. 2). Although DSW-G had 100 μg sugar/mg of protein, and DSM-G had no dextran, electrophoretic mobilities of DSW-G and DSM-G were the same (Fig. 2). Dissociated B-512F-dextranase was not obtained by gel chromatography, but dextranase seemed to tend to dissociate in an electric field. No significant differences were observed between DSW-D, DSW-G, and DSM-G, judged by the mobilities on SDS– and native-PAGE.

We tried to sequence the N-terminal amino acids of wild-type and mutant dextranase but could not, suggesting the possibility of their being N-terminally blocked proteins. However, the N-terminal amino acids of DSW-D and DSW-G could still not be detected after deblocking treatment using pyroglutamate aminopeptidase and acylamino acid-releasing enzyme. Nucleotides of the GTF genes of several Streptococcus strains were sequenced, but their N-terminal amino acids have not been assigned from the enzyme proteins.

**Aggregated forms of DSW-D, DSW-G, and DSM-G**

Elution patterns of dextranases from a Sepharose 6B column are shown in Fig. 3. Both DSW-D and DSW-G containing certain amounts of dextran were eluted at $V_0$ volume. However, in the case of DSM-G, which contained no sugar, dextranase activity was eluted at 1.2 × $V_0$ volume (molecular mass of 170 kDa) (Fig. 3b), corresponding to the monomer form. The peak of active fractions indicating a bar in Fig. 3b were collected and freeze-dried, and the enzyme was again put on the Sepharose 6B column. The active fractions were separated into three fractions, that is, $V_0$, 1.2 × $V_0$, and 1.5 × $V_0$. Those elution volumes had molecular masses of higher than 1000, 170, and 66 kDa, respectively (Fig. 3). The specific activities of each form were 11, 5.4, and 5.1 units/mg protein, respectively. During the freeze-drying treatment after guanidine was removed by gel filtration, the monomers of dextranase were assumed to be aggregated again and eluted at the $V_0$ volume (Fig. 3c). When dextranase from SH 3002 was concentrated by 0 to 30% ammonium sulfate fractionation, all of the activity was eluted at the $V_0$ volume on Sepharose 6B column chromatography (data not shown). Therefore the dextranase seemed to be aggregated even in the absence of dextran, and the monomer form would reaggregate spontaneously during the storage.

We could not obtain the active monomeric form of wild-type dextranases by gel chromatography. Dextran could not be eliminated from the B-512F enzyme preparation completely even by guanidine treatment. Reaggregation would be accelerated by dextran, and all the enzyme must have rapidly aggregated again during gel filtration when guanidine was removed. Only when DSW-G was treated with 0.1% NaOH, the 170-kDa protein peak was detected by gel filtration (data not shown). Rapid aggregation was also observed by gel chromatography of DSM-G mixed with clinical dextran (Fig. 3b). All the

![Fig. 2. Activity Staining of B-512F and Mutant SH 3002 Dextranases. Native PAGE was done as described in Materials and Methods. Proteins were stained with a silver staining kit. The enzyme dextran-forming activity was detected by incubation with sucrose and subsequent PAS staining as described in Materials and Methods. DSW-G (0.1 μg) and DSM-G (0.05 μg) were put on the gel. Protein staining of the culture supernatant of DSW-G (a), DSW-G (b), PAS staining of the dextran synthesized by the culture supernatant of DSW-G (c), DSM-G (d).](image)

![Fig. 3. Elution Pattern of B-512F and Mutant SH 3002 Dextranases from a Sepharose 6B Column. Activity was measured for the reducing sugar as described in Materials and Methods. (a) B-512F enzyme □, DSW-D: ○, DSW-G, (b) SH3002 enzyme ▲, DSM-G: ●. 0.5% clinical dextran was added to the enzyme after DEAE-Sephadex fractionation. (c) Rechromatography of DSM-G. The volume of each fraction was 5 ml. The protein concentrations (μg/ml) of the peak fractions were (a) 8.2 (□, $V_0$) and 14.4 (○, $V_0$), (b) 9.3 (▲, 1.2 × $V_0$) and 3.6 (●, $V_0$), (c) 1.6 ($V_0$), 2.9 ($V_0$), 1.2 × $V_0$, and 2.4 ($V_0$, 1.5 × $V_0$), respectively.](image)
activities were eluted at the V₀ volume and there was no distinct enzyme peak observed at 1.2 • V₀ volume (Fig. 3b )

Comparison of internal amino acid sequences of DSW-G and streptococcal GTFs

As the strength of aggregated forms of Leuconostoc dextranase and streptococcal glucosyltransferases (GTFs) were different, we thought that there must be some differences between their structures. Both of them were high molecular mass (150 to 170 kDa) monomeric enzymes, but no direct comparison of the structures have been made. Therefore, Leuconostoc dextranase was digested with proteases and internal sequences were analyzed. DSW-G was used for the protein analysis. The sequences of 1 to 7 peptides were analyzed (Table III). When the sequences were compared with the deduced amino acid sequences of GTFs from Streptococcus mutans, 21, 22 5 of 7 peptide fragments were about 50 to 70% homologous. However, the remaining two peptides showed no significant homology to S. mutans GTFs (Table III).

Discussion

Dextranases from B-512F and its constitutive mutant SH 3002 were purified almost to homogeneity as shown in SDS-PAGE (Fig. 1). PEG 6000 treatment and affinity chromatography worked well for purification. Miller et al. 4) purified B-512F dextranase and Mayer 23) purified streptococcal GTF-S and GTF-I, but found it difficult to avoid proteolysis during purification. Mayer added SDS in all purification steps to dissociate the enzyme and inhibit the proteolytic action, but proteolysis occurred during purification. 23) We obtained only one active form by this procedure and purified enzymes were stable at -80°C.

Dextranases are known to make a highly aggregated form with dextran. 23) Dextran seemed to stimulate dextranase to make aggregated forms rapidly and tightly. On the contrary, streptococcal GTFs are known to be highly aggregated even in glucan-free form. 23, 24) On gel electrophoresis, GTF-I from Streptococcus sanguis remained immobile at the top of the gel. 23) When the enzyme was put on a Sepharose 6B column, only 3% of the enzyme activity was eluted at the V₀ volume and the remaining activity was detected in the top 1 cm of the column. 24) In this case, SDS was required to dissociate the aggregated forms. 23, 24) Compared with the streptococcal enzymes, Leuconostoc dextranase should form a looser aggregation than streptococcal GTFs.

The 66-kDa active form was obtained from DSM-G (Fig. 3c) and we thought that it was a proteolyzed enzyme. Kobayashi and Matsuda reported that the monomeric form of B-512F dextranase was 65 kDa. 3, 6) The molecular mass of the protein we obtained was almost the same size with their 'putative monomeric form.' There is still some possibility that it was the real monomeric form of the enzyme. The purified dextranase we obtained was easily digested during storage at 4°C. Miller et al. reported that dextranase from L. mesenteroides B-512F was a 177-kDa monomeric protein. 4) They also observed that a smaller form of 158 kDa was increased and 177-kDa form was decreased during storage. The known GTFs were approximately 147 kDa (GTF-S from S. downei 18) to 173 kDa (GTF-I from S. sobrinus 13) and those GTFs were proved to be monomeric form from genetic information. 19, 19) Mayer obtained a fully active proteolyzed GTs from S. sanguis of 158 kDa, which was about 18 kDa smaller than the native form. 24) Abo et al. showed that GTF-I that lost all the C-terminal glucan-binding region still had sucrose activity. 25) The exact region that requires for expressing enzyme activity in N-terminal is still unclear. From their information about the sizes of enzymes, we thought the 66-kDa peak of purified DSM-G was likely to be a partially deleted dextranase that still had activity, but more information on amino acid sequences of 170- and 66-kDa enzymes is required.

The molecular masses of GTFs were similar to that of B-512F dextranase, 170 kDa, and both streptococcal GTFs and Leuconostoc dextranases were monomers. Five internal amino acid sequences of B-512F dextranase and Peptide 8, which we previously isolated as the second essential carboxyl acid-containing peptide, 25) were 50 to 70% homologous to GTFs of S. mutans, and all of the peptides except Peptide 2 were hydrophilic (Table III). Streptococcal GTFs show about 50% overall identity, 19, 20) and have been reported to be highly hydrophilic. 19, 20) Both S. mutans GTF-S and GTF-I regions homologous to Peptide 5 were identified as A-repeats of C-terminal glucan-binding domains, 50-22) which has been found in all GTFs sequenced to date. 20) Peptide 5 of DSW-G must be a part of the A-repeat. There are two types of aggregated forms of Peptide 1 and 2 were located by endopeptidase digestion. Peptides 3-5 were obtained by trypsin digestion.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>M. mesenteroides</th>
<th>Streptococcus mutans</th>
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<tbody>
<tr>
<td>Peptide 1</td>
<td>DQVPRVYYGDLQYDGSYMA</td>
<td>(648) DSITRLYYGDMSYDDGQYMA</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>ALHAGKQIAIDPS</td>
<td>(949) ALHSDKQIOVIADW</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>(917) ALHSKGIKVMDADW</td>
<td>AFGTRTKNDKADN</td>
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<tr>
<td>Peptide 4</td>
<td>(484) AHYGVSDKSNKAI</td>
<td>(470) AAKGIIKNDKAAN</td>
</tr>
<tr>
<td>Peptide 5</td>
<td>(334) ISAQSTSWL</td>
<td>(334) ISOQTOQTQW</td>
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<td>Peptide 6</td>
<td>(334) ISAQKQTQSA</td>
<td>LMYDFNQLQ</td>
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<td>Peptide 7</td>
<td>(1127) WYYFNDDNGHM</td>
<td>(1096) WYYFDNREGYM</td>
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<td>Peptide 8</td>
<td>(424) NDDNSNPPVQA</td>
<td>LQEDNSNVVVEA</td>
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<tr>
<td>Peptide 9</td>
<td>(424) NDVDNSPPVQA</td>
<td>NDVDNSNPVQA</td>
</tr>
</tbody>
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Peptides 1-5 were obtained by trypsin digestion. Peptides 6-8 were obtained by endopeptidase digestion. * From S. mutans gtfD gene. 21 * From S. mutans gtfB gene. 22 * The peptide containing second essential carboxylic acids. 83
dextranuescrases. One is the simple protein complex, and the other is the dextran mediated protein complex. The glucan-binding site may be important for making the aggregated form in the presence of dextran, and the structure of glucan-binding domains must be similar in *Leuconostoc* and streptococcal enzymes.

From these observations, we assumed that the protein structures of B-512F dextranucrese and streptococcal GTFs had similarities. However, the strength of aggregated form of B-512F enzyme was weaker than those of the GTFs.

References