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Some structural features of an insoluble α-D-glucan from a mutant strain of *Leuconostoc mesenteroides* NRRL B-1355

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Leuconostoc mesenteroides **strain NRRL B-1355 produces two soluble extracellular α-D-glucans from sucrose: alternan and dextran. An unusual mutant strain derived from NRRL B-1355 has recently been isolated which produces practically no soluble polysaccharide, but significant amounts of an insoluble D-glucan. Methylation analysis shows it contains linear (1→3) and (1→6) linkages as well as (1→2) and (1→3) branch linkages. The insoluble glucan was partially digestible by endodextranase, giving rise to a series of oligosaccharides, a high-molecular weight soluble fraction and an insoluble residue. Treatment of the soluble dextranase-limit fraction with an α(1→2) debranching enzyme led to further dextranase susceptibility. Methylation, FTIR and NMR analyses of the dextranase-treated fractions indicate a non-uniform structure with domains bearing similarities to *L. mesenteroides* strain NRRL B-1299 dextran and to insoluble streptococcal D-glucans.**

Keywords: glucosyltransferase; dextransucrase; alternansucrase; *Leuconostoc mesenteroides*; glucan; dextran; polysaccharide

Introduction

Many strains of *Leuconostoc mesenteroides* produce extracellular enzymes capable of producing dextrans and related d-glucans from sucrose. There is a great deal of strain variation in the structures of the dextrans [8]. Physical properties of the dextrans, particularly solubility and viscosity, are greatly influenced by structural features such as degree of branching, types of branch linkages and distribution of secondary linkages. The glucans are synthesized by enzymes known as glucansucrases, which have been isolated and studied for several strains of *L. mesenteroides*. In recent years, efforts have focused on obtaining mutants or producing clones which synthesize enhanced levels of certain enzymes [9–11,14,21]. One such enzyme with potential commercial applications is alternansucrase. This glucansucrase, produced by *L. mesenteroides* strain NRRL B-1355, synthesizes the polysaccharide alternan [2,4]. The same strain also produces dextransucrase, which synthesizes dextran from sucrose. There have been some successful efforts to produce mutants of this strain which produce only one of these two enzymes, for the purpose of synthesizing alternan or dextran more efficiently and to better understand the biosynthesis of these polysaccharides [14,20,21].

A mutant strain derived from *L. mesenteroides* NRRL B-1355 has recently been described which produces an insoluble glucan, little dextran and apparently no alternan [22]. Since the parent strain produces principally two soluble d-glucans, this was unexpected. Although few strains of *Leuconostoc* synthesize insoluble dextrans, the phenomenon is common among the closely related streptococci. It is generally believed that the insoluble glucans produced by streptococci involved in dental plaque play a role in adhesion of bacterial colonies to tooth surfaces. Compared to the wild-type strain, colonies of the mutant strain R1510 appear to be more adherent to surfaces such as glass. Some of the possible implications have been discussed in terms of the various glucansucrases produced by the mutants [22]. Since it is necessary to first understand the structure of the product before anything can be learned about its biosynthesis, we describe here some results of our structural studies on this unusual polysaccharide.

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Materials and methods

Leuconostoc mesenteroides strain R1510 has been described [22]. Cultures were grown at 28°C in a modified MRS medium (1.5 g polypeptone, 1.5 g beef extract, 1.5 g yeast extract, 20 g sucrose, 1 g Tween 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate heptahydrate, 0.05 g manganese sulfate monohydrate, 2 g dibasic potassium phosphate, 1 L water, pH 6.5) as previously described [22]. Cells and insoluble biomass were pelleted by centrifugation (approx. $3000 \times g$ for 10 min) and washed by resuspending them in 20 mM pH 5.4 sodium acetate buffer containing 0.01% sodium azide. After several washes, the insoluble biomass was suspended in a sodium acetate-buffered solution of sucrose (0.3 M) and allowed to incubate at 28°C for 24–48 h, whereupon a large increase in the amount of insoluble material was observed. The insoluble mass was collected as above, washed with distilled water several times, isolated by vacuum filtration on filter paper and dried *in vacuo* at 50°C. Microscopic exam-

ination and subsequent chemical analysis (phenol-sulfuric acid assay for total carbohydrate) indicated this insoluble material to be composed almost entirely of polysaccharide. This insoluble material (R1510-I) was subjected to structural analysis.

Dextran α(1–2) debranching enzyme was produced and isolated as described by Mitsuishi *et al* [15]. Endodextranase L was provided by Amano Corp (Troy, VA, USA). Endo-alternanase was purified as described by Biely *et al* [1]. Unless stated otherwise, these enzymes were used at pH 5.4 in 20 mM sodium acetate buffer containing 0.01% sodium azide. Digestions were achieved at room temperature (~25°C).

The phenol-sulfuric acid method was used for total carbohydrate analyses [5]. Reducing sugar values were obtained by an automated alkaline ferricyanide technique [7]. Methylation analyses were performed as previously described [19].

High-performance size-exclusion chromatography (HPSEC) was carried out using a Waters 625 LC system. Samples containing insoluble material were centrifuged to sediment the insoluble matter and aliquots of the supernatant fluid were filtered through 0.45-μm pore size syringe filters. The filtered sample was applied to either a Shodex KB-803 or KB-806M column, which was eluted at room temperature with water at a flow rate of 0.5 ml min^{-1}. All compounds were detected with a Waters 401 refractive index detector. Carbohydrates and other optically active compounds were selectively detected with a Shodex OR-1 optical rotation detector. Preparative gel-filtration chromatography was accomplished with a 2.5×75 cm column of Bio-Gel P-30 (BioRad Corp, Richmond, CA, USA), eluted with water by gravity flow at room temperature. Fractions of 5 ml each were collected and assayed for carbohydrate content.

Infrared spectra were obtained on samples in KBr pellets using an FTIR spectrometer (Model RFX-75, Analect, Irvine, CA, USA) equipped with a TGS detector. Absorbance data were acquired at 4 cm^{-1} resolution. Signals were averaged over 32 scans with no zero filling. Spectra are presented without baseline correction or other data manipulation. Nuclear-magnetic resonance (NMR) spectroscopy was performed in the ^{1}H-decoupled ^{13}C mode on solutions or suspensions in $^{2}H_2O$ as described previously [3].

Results

Figure 1 summarizes the enzymatic digestions and fractionations performed on the polysaccharides. Samples of the insoluble polysaccharide from *L. mesenteroides* R1510 (hereafter referred to as R1510-I) were suspended in sodium acetate buffer and treated with an excess of endodextranase. Material solubilized by dextranase was monitored by HPSEC until no further changes in the chromatographic elution profile were observed. The solubilized portion was separated by chromatography on Bio-Gel P-30 into two fractions: a fraction eluting in the void volume, representing a soluble polysaccharide portion (R1510-S) that was resistant to endodextranase, and a later-eluting portion, representing an oligosaccharide fraction (Fraction A). Since R1510-S elutes at the void volume of a P-30 column, it can be assumed to have a weight-average molecular weight greater than 30 000. Comparison of the reducing value of solutions of R1510-S with total carbohydrate content gave a number-average molecular weight of approximately 50 000. The endodextranase-solubilized polysaccharide fraction (R1510-S) was further treated with debranching enzyme (DBE) until no further release of glucose was observed. HPSEC analysis showed the presence of glucose and R1510-S′, a polysaccharide fraction of slightly lower molecular size than R1510-S (Figure 2). Upon treatment with an excess of endodextranase, R1510-S′ was completely converted to oligosaccharides (Fraction B). These enzymic digestions and the various fractions which result are summarized in Figure 1. Endo-alternanase, which is specific for $\alpha(1 \rightarrow 3),(1 \rightarrow 6)$ alternating sequences [1], had no detectable hydrolytic action on the insoluble R1510-1, insoluble R1510-R or soluble R1510-S.

Results of methylation analyses of each fraction appear in Table 1. The original insoluble dextran R1510-1 contains a relatively high 24% of non-reducing ends, due to branching through both $\alpha(1 \rightarrow 2)$ and $\alpha(1 \rightarrow 3)$ linkages. There is also a significant proportion (12%) of linear unbranched $\alpha(1 \rightarrow 3)$-linked residues. It is impossible to determine from methylation analysis alone whether these occur in sequences, as isolated linkages, or alternating with $\alpha(1 \rightarrow 6)$-linked residues. Absence of hydrolysis by endo-alternanase eliminates the latter possibility. Endodextranase hydrolyzed R1510-1 into three fractions, R1510-R, R-1510-S, and oligosaccharide Fraction A. This shows that many of the 46% of $\alpha(1 \rightarrow 6)$-linked residues occur in sequences long enough for the enzyme to bind and hydrolyze. Most of the $\alpha(1 \rightarrow 3)$-linked linear sequences remain in the insoluble fraction R1510-R, which contains 50% of such linkages. By contrast, R1510-S contains only 3% of $\alpha(1 \rightarrow 3)$-linked linear residues, but was enriched in $\alpha(1 \rightarrow 2)$ branch linkages compared to the starting material. Upon removal of these by debranching enzyme, R1510-S was converted to a polymer rich in linear sequences of $\alpha(1 \rightarrow 6)$ linkages (R1510-S′). Not surprisingly, R1510-S′ was completely hydrolyzed to oligosaccharide fraction B by endodextranase.

Satisfactory NMR spectra of the insoluble fractions could not be obtained. Fraction R1510-S, however, gave a clear spectrum (Figure 3). This spectrum is indistinguishable from that of *L. mesenteroides* NRRL B-1299 fraction S dextran, which contains a large proportion of single d-glucopyranosyl units linked $\alpha(1 \rightarrow 2)$ to the main $\alpha(1 \rightarrow 6)$-linked d-glucan chain [18].

Discussion

The insolubility of glucan R1510-I suggested the presence of contiguous $\alpha(1 \rightarrow 3)$ linked d-glucosyl units. This type of structure occurs in the streptococcal glucan known as mutan [6] and in *L. mesenteroides* NRRL B-523 dextran [8,17], both of which are insoluble in water. However, the FTIR spectrum of R1510-1 lacks an absorbance band at 820 cm^{-1}, which is characteristic of contiguous $\alpha(1 \rightarrow 3)$ linkages (Figure 4). On the other hand, methylation analysis indicates the presence of $\alpha(1 \rightarrow 3)$ linkages (Table 1). In addition, methylation analysis of R1510-1 indicates the

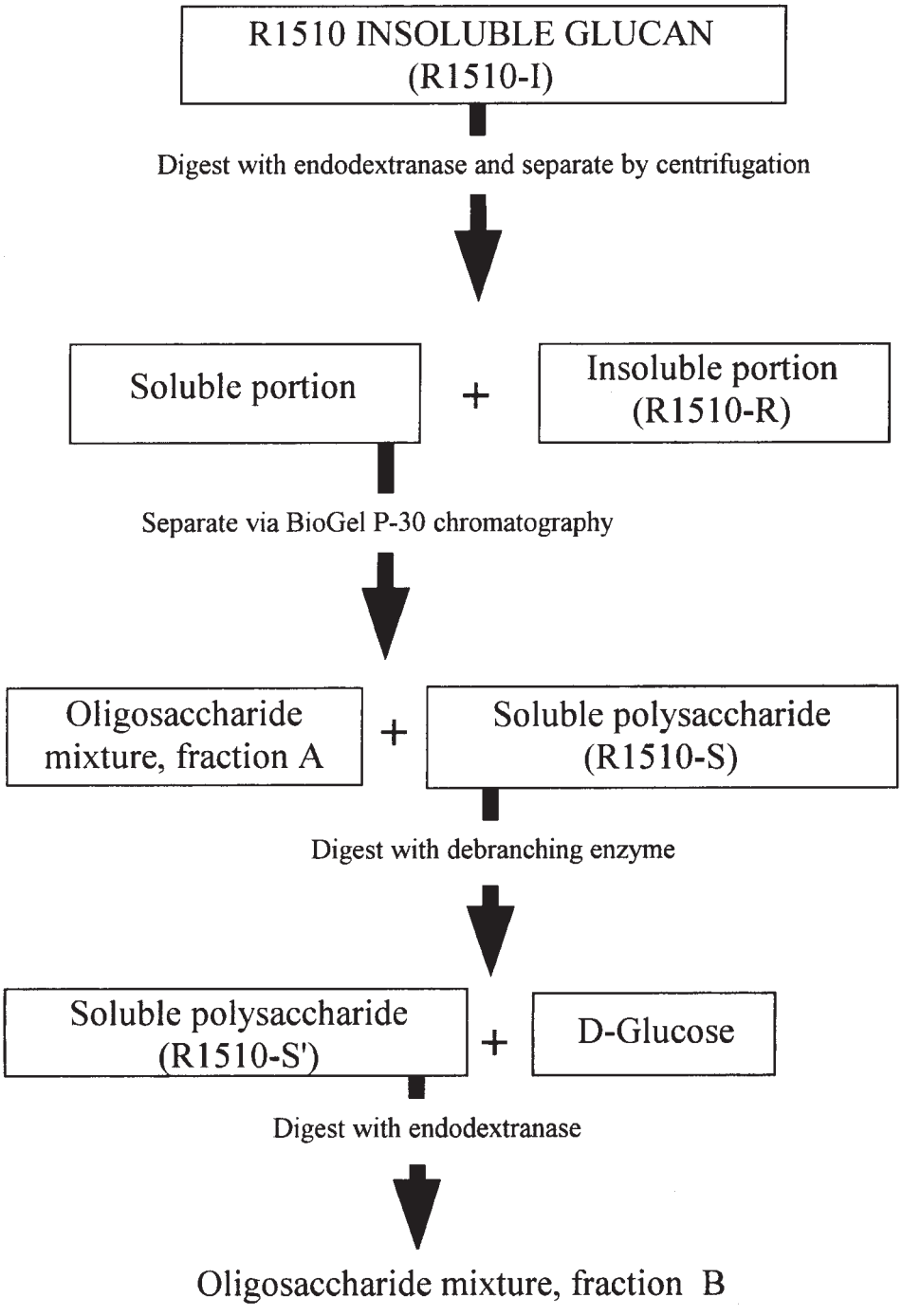

Figure 1 Scheme showing enzymatic digestions and fractionation of R1510 glucans.

presence of a relatively high proportion of branching through 2,6-disubstituted d-glucosyl units. This type of branching is characteristic of *L. mesenteroides* NRRL B-1299 dextrans [8,12,13], but has never been observed in strain B-1355, nor in any of its mutants.

The fact that R1510-1 is partially susceptible to hydrolysis by endodextranases indicates the presence of segments containing dextran-like sequences of $\alpha(1 \rightarrow 6)$ linked d-glucosyl units. Hydrolysis by endodextranase yields three fractions: a series of oligosaccharides similar to those obtained by dextranase hydrolysis of dextran (oligosaccharide fraction A), a soluble polysaccharide fraction (R1510-S) and a dextranase-resistant insoluble fraction (R1510-R). Fraction R1510-S is approximately 50 000 MW, with a degree of polymerization of approximately 300. Methylation analysis and ^{13}C-NMR spectroscopy of R1510-S show it to be very similar to *L. mesenteroides* NRRL B-1299 fraction S dextran [12], which contains single glucosyl unit branches linked $\alpha(1 \rightarrow 2)$ to the main $\alpha(1 \rightarrow 6)$-linked d-glucan chain. This is supported by degradation studies using $\alpha(1–2)$ debranching enzyme (DBE) [12,15]. Figure 2 shows that DBE hydrolyzed

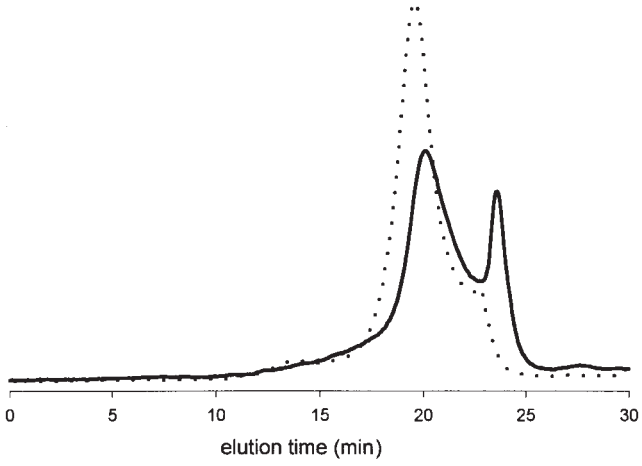

Figure 2 HPSEC profile of R1510-S (.....) and DBE-treated R1510-S (———). The column was Shodex KB-803. Detection was by optical rotation. The flow rate = 0.5 ml min^{-1}.

Table 1 Methylation analyses of various fractions from R1510

Sample	Mole percent of each major[a] peracetylated methylated monomer present				
	2,3,4,6-tetra-OMe	*2,4,6-tri-OMe*	*2,3,4-tri-OMe*	*2,4-di-OMe*	*3,4-di-OMe*
R1510-I	24	12	46	7	12
R1510-R	20	50	11	5	12
R1510-S	43	3	17	4	33
R1510-S′	9	2	79	5	4
Oligosaccharide fraction A	19	9	47	12	3

[a]Components ⩾ 2%.

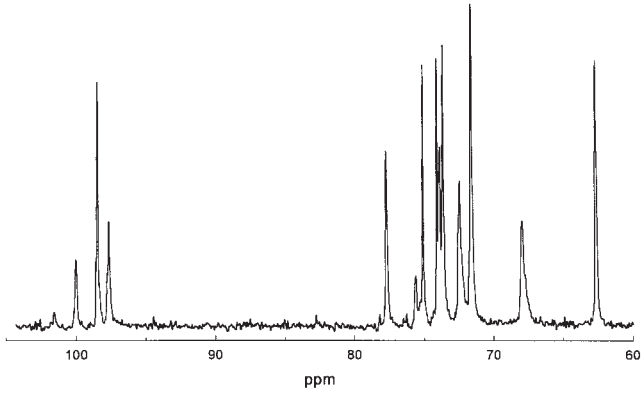

Figure 3 Proton-decoupled ^{13}C-NMR spectrum of fraction R1510-S glucan.

R1510-S to yield glucose and a slightly lower molecular-weight polysaccharide fraction (R1510-S′). Once the $\alpha(1 \rightarrow 2)$-linked glucosyl branches were removed, the remaining polysaccharide was completely hydrolyzed to small oligosaccharides, indicating a typical dextran-like structure for R1510-S′. Methylation analysis of fraction R1510-S′ shows it to contain approximately 80% of $\alpha(1 \rightarrow 6)$-linked glucosyl residues, with the remainder

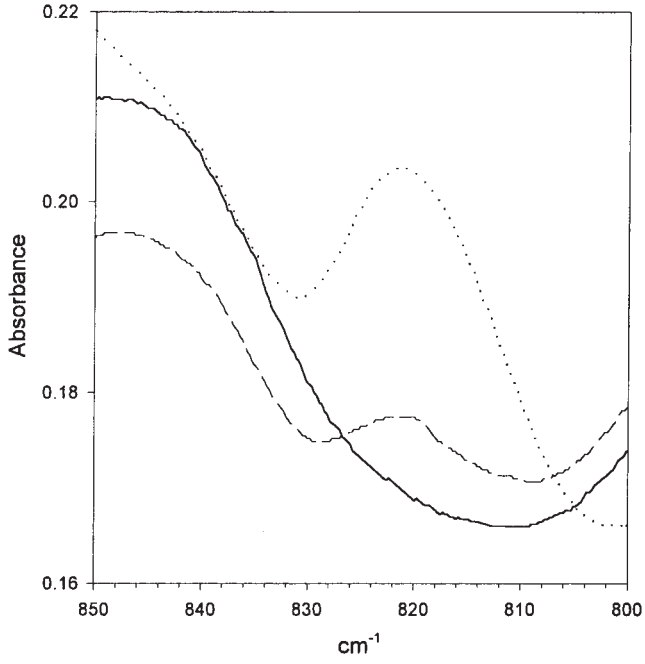

Figure 4 Portion of FTIR spectra showing the presence or absence of absorbance bands at 820 cm^{-1} for *L. mesenteroides* NRRL B-523 insoluble dextran (.....), R1510-R (– – –) and R1510-I (———) insoluble glucan fractions.

being $\alpha(1 \rightarrow 2)$ and $\alpha(1 \rightarrow 3)$ branch linkages, to give about 10% branching. All fractions contained at least some α(1–3)-linked residues in linear, non-branched residues. Whereas the original R1510-I contained 12% of such linkage types, the insoluble residue after endodextranase treatment (R1510-R) contained 50%. Analysis of fraction R1510-R by FTIR spectroscopy revealed an absorbance band at 820 cm^{-1}, which is diagnostic for linear sequences of $\alpha(1 \rightarrow 3)$ linkages. It is clear that removal of the dextran-like fragments leaves behind a fragment with a much higher proportion of linear $\alpha(1 \rightarrow 3)$-linked glucosyl residues. This insoluble, dextranase-resistant fragment appears to be similar to the insoluble glucans from streptococci and *L. mesenteroides* strain NRRL B-523. These insoluble glucans are believed to be non-uniform in structure, containing portions rich in $\alpha(1 \rightarrow 3)$-linked sequences and portions rich in $\alpha(1 \rightarrow 6)$-linked sequences. Such a structure could, for example, arise from the simultaneous action of two or more enzymes, which act together to form graft copolymers [6].

Recently, numerous mutants of *L. mesenteroides* have been described which contain altered proportions of various glucans and their respective glucansucrases [9,14,20]. In general, these mutants differ from the wild types by lacking one or more of the glucansucrases, by expressing one or more of the enzymes constitutively, or by altered levels of expression. In only one previous instance has the appearance of a new polysaccharide been noted. Mukerjea *et al* [16] described the presence of a dextran containing $\alpha(1 \rightarrow 2)$ branches in a mutant of *L. mesenteroides* NRRL B-742, a strain not previously known to make such a dextran.

Our present work describes some of the structural features of a new insoluble dextran from a mutant of *L. mesen-*

teroides NRRL B-1355. The presence of contiguous $\alpha(1 \rightarrow 3)$ linkages and $\alpha(1 \rightarrow 2)$ branches has not previously been described for any glucan derived from strain B-1355. The fraction L dextran from *L. mesenteroides* NRRL B-1355 is a relatively difficult-to-dissolve fraction of mainly $\alpha(1 \rightarrow 6)$-linked dextran, with a very low degree of $\alpha(1 \rightarrow 3)$ branching [8]. Nearly-linear dextrans of this sort are often poorly soluble and gel-like. However, the glucan produced by mutant R1510 is particulate and does not disperse into a gel-like state. The evidence presented here can only be explained by a nonuniform structure for R1510-I glucan. It apparently contains domains rich in $\alpha(1 \rightarrow 6)$-linked d-glucosyl residues, with a high proportion of single d-glucosyl units linked through $\alpha(1 \rightarrow 2)$ branch linkages. These domains contain a nonuniform distribution of such branch linkages, which allows for the hydrolysis by endodextranase. The more densely branched dextranase-resistant domains are approximately 300 glucosyl residues in size. Other domains in R1510-I are rich in linear sequences of $\alpha(1 \rightarrow 3)$-linked d-glucosyl residues and also contain some $\alpha(1 \rightarrow 2)$ branching. These domains are responsible for the insoluble nature of R1510-I. Lesser amounts of $\alpha(1 \rightarrow 3)$ branch points also occur throughout the molecules. The presence of distinct domains suggests the possibility of graft copolymer formation similar to that described by Hare *et al* [6] for streptococcal mutans. The presence of distinct domains with such a high degree of structural complexity makes it difficult to explain the biosynthesis of R1510-I in terms of a single enzyme. However, the structural features of R1510-I reported here [$\alpha(1 \rightarrow 2)$ linkages and contiguous linear $\alpha(1 \rightarrow 3)$ linkages] do not support a conclusion that alternansucrase had a role in its formation.

Continued study of this and other mutants may lead to a better understanding of the mechanism of biosynthesis of dextrans and the underlying genetics of glucansucrases.

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