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Modification of *Acetobacter xylinum* Bacterial Cellulose Using Dextransucrase and Alternansucrase

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Abstract In addition to catalyzing the synthesis of glucan from sucrose as a primary reaction, glucansucrase also catalyzes the transfer of glucose from sucrose to other carbohydrates that are present or are added to the reaction digest. Using dextransucrase and alternansucrase, prepared from *Leuconostoc mesenteroides* B-742CBM and B-1355C, respectively, we modified the bacterial cellulose in *Acetobacter xylinum* ATCC10821 culture, and then produced a characteristic cellulose that is soluble and has a new structure. There were also some partially modified insoluble cellulose and oligosaccharides in the modification culture. After methylation and following acid hydrolysis of both the soluble and insoluble glucans, there were (1→4) as well as (1→6) and (1→3) glycosidic linkages in the soluble glucan.

Key words: Acceptor reaction, enzymatic modification, bacterial cellulose, dextransucrase, alternansucrase, glucansucrase, *Leuconostoc mesenteroides*

The primary reaction for glucansucrases is to convert sucrose into glucan according to the following reaction:

$$n\ \text{Sucrose} \rightarrow (\text{Glucose})_{n-m-w} + (n-m)\ \text{Fructose} + m\ \text{Leucrose} + w\ \text{Glucose}$$

The reaction is essentially irreversible and the main products have the high molecular weight ($1-10\times10^8$ Da) of dextran and fructose. Kim and Robyt have obtained *L. mesenteroides* mutants from several strains (e.g., B-512FM, B-742, B-1142, B-1299, and B-1355) that are constitutive for specific glucansucrases [6-9]. Unlike the parental strain, the mutants do not require sucrose in the growth media for glucansucrase elaboration and hence the enzyme preparations from these mutants are devoid of glucan [2, 7, 9]. In addition to catalyzing the synthesis of glucans from sucrose, glucansucrases can also catalyze the transfer of glucose from sucrose to other carbohydrates that are present or have been added to the reaction digest. The added carbohydrates are called acceptors and the reaction is called an acceptor-reaction. The acceptor specificity is quite broad with different carbohydrate acceptors being recognized [17]. They include monosaccharides, disaccharides, oligosaccharides, and polysaccharides [6, 11, 12]. When the acceptor is a monosaccharide or a disaccharide, there is usually a series of oligosaccharide acceptor products produced [17]. All of the glucans synthesized by glucansucrases are branched. The type of branch linkage and the degree of branching (% branching) varies among the different glucansucrases. The principal branch linkage found is α-(1→3) in dextran, although α-(1→2) and α-(1→4) branch linkages have also been observed [5]. Robyt and Taniguchi found a mechanism for forming branch linkages by an acceptor-reaction in which a glucan chain itself acted as an acceptor and the chain being synthesized was transferred to an acceptor chain, forming a branch linkage between the two chains [16]. Kim *et al.* showed that *L. mesenteroides* B-742CB(M) dextransucrase could modify various starch granules or gelatinized starches, and pullulan [11, 12]. Modified starches were more resistant to the hydrolysis by α-amylase, isoamylase, pullulanase, and endo-dextranase than those to native starch. After modification of granule starch with B-742CB dextransucrase, a soluble modified starch was produced. The products were modified by the addition of glucose to positions C3, C4, and C6 of free hydroxyl groups of glucose residues in the starch. Recently, we have also synthesized new glucans having new structures and new characteristics, by transferring D-glucose of sucrose to α-cellulose and by using the constitutive dextransucrase obtained from *L. mesenteroides* B-742CBM. The final reaction products were composed of soluble and insoluble glucans. There were both (1→4) and (1→6) glycosidic

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linkages in both of the glucans [10]. In 1886, Brown first reported that the pellicle of *Acetobacter xylinum* was composed of pure cellulose [1]. Its structural features and mechanical properties were unique and differed considerably from plant cellulose, and bacterial cellulose was expected to be used widely in various industries [15]. In a patent issued in 1964 [14], it was proposed that cellulose and other polysaccharides could be modified using dextransucrase and sucrose. Attempts to obtain these products by other investigators, however, have resulted in failure. These failures might have been due to not using the right dextransucrase (the dextransucrase was not specified in the patent). In 1964, it might not have been recognized that different *L. mesenteroides* strains elaborated different kinds of glucansucrases with different specificities. In this paper, we report the modification of bacterial cellulose using sucrose and *L. mesenteroides* B-742CBM dextransucrase or B-1355C alternansucrase in *A. xylinum* culture. This modification method produces a new bacterial cellulose that has a unique structure and probably new property.

MATERIALS AND METHODS

Carbohydrates and Reagents

Cellobiose was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cellotriose, maltodextrins, and isomaltodextrins were available at the Laboratory of Carbohydrate Chemistry and Enzymology at Iowa State University, Ames, IA, U.S.A. Whatman K6 thin-layer chromatography (TLC) plates were purchased from Fisher Scientific, U.S.A. All other chemicals were of reagent grade and commercially available.

Enzymes

Dextransucrase and alternansucrase were prepared from *L. mesenteroides* B-742CBM and B-1355C, respectively, with glucose, as described by Kim and Robyt [7, 8, 10].

Culture Conditions and Modification Procedure

A. xylinum ATCC10821 was cultivated in HS (Hestrin and Schramm) medium [4]: 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) bacto peptone, 0.3% (w/v) sodium phosphate (dibasic), and 0.1% (w/v) citrate in 20 mM Na-acetate buffer (pH 5.2). Twenty ml of seed culture grown for 42 h at 28°C was inoculated into 400 ml of medium in a 1-l flask. It was then cultured for 6 days at 28°C. Twenty-four h our after inoculation, glucansucrase (200 IU/reaction digest) was added to the culture. One IU of dextransucrase indicates the release of 1 μmol of fructose from sucrose per min at pH 5.2 and 28°C. The reaction was initiated by the addition of 100 mM sucrose to the culture. A total of 400 ml was gradually added at the rate of 0.067 ml per min. After addition of sucrose was complete, the reaction was allowed to proceed for an additional 30 min with stirring. After reaction, any insoluble material in the digest was collected by centrifugation, washed with water (3-times), and triturated with acetone and ethanol. The supernatants after centrifugation were treated with 2 volumes of ethanol to precipitate any polysaccharide that became soluble during the modification reaction. The precipitates were also titurated with acetone and ethanol. These polysaccharides were used in the methylation analysis.

Methylation Analysis

To determine the position of the substitution, the modified cellulose was methylated using the Hakomori reagent, followed by acid hydrolysis with 2 M trifluoroacetic acid and analysis of the methylated products using TLC [11, 13]. The methylation analysis products were separated by two ascents on Whatman K6 plates using 3:9:1 CH_3CN/$CHCl_3$/CH_3OH, followed by development of the plate by dipping into 0.3% *N*-(1-naphthyl)-ethylenediamine and 5% sulfuric acid in methanol and by heating at 120°C for 10 min. 2,3,4,6-tetra-*O*-methyl-D-glucose was obtained from Sigma Chemical Co., 2,3,6-tri-*O*-methyl-D-glucose by methylating cyclomaltohexaose, and 2,3,4-tri-*O*-methyl-D-glucose by methylating B-512F dextran (Sigma Chemical Co.). The quantitative determination of the *O*-methylated sugars directly on the TLC plate was achieved by scanning the plate with a Bio-Rad Imaging Densitometer, model GS-670 (Bio-Rad Laboratories, Hercules, CA).

Acceptor-Reaction Digests

Dextransucrase and alternansucrase acceptor reaction digests contained 100 mM sucrose, 100 mM cellobiose or cellotriose, 20 mM sodium acetate buffer (pH 5.2), 2 mM calcium chloride, 0.01% sodium azide, and 120 mIU of dextransucrase and alternansucrase, respectively. The reaction was conducted for 6 h at 28°C for complete consumption of the sucrose.

RESULTS AND DISCUSSION

We have reacted cellobiose or cellotriose with sucrose, B-742CBM dextransucrase, and B-1355C alternansucrase (Table 1). Only one acceptor product was produced from the cellobiose acceptor reaction as reported by other dextransucrases [17]. Interestingly, the efficiency of the acceptor reaction increased for cellotriose. In the case of B-742CBM dextransucrase reaction, the portion of cellobiose acceptor product was 26.7% of the total carbohydrate (excluding fructose and unreacted cellobiose) and that of cellotriose was 53.1%. B-1355C alternansucrase also showed similar acceptor reaction efficiency: the portion of cellobiose acceptor product was 20.1% of the total carbohydrate (excluding fructose and unreacted cellobiose) and that of cellotriose was 55.6%. In addition, several kinds of

Table 1. The relative percentage of acceptor reaction products and glucan formed by *Leuconostoc mesenteroides* B-742CBM dextransucrase and B-1355C alternansucrase using cellobiose and cellotriose.

Acceptor	Enzyme	Relative % of acceptor products and polymer				
		CB1	CT1	CT2	CT3	Polymer
Cellobiose	B-742CBM Dextransucrase	26.7				73.3
	B-1355C Alternansucrase	20.1				79.9
Cellotriose	B-742CBM Dextransucrase		25.3	17.4	10.4	46.9
	B-1355C Alternansucrase		39.6	16.0		44.4

Dextransucrase and alternansucrase acceptor reaction digests contained 100 mM sucrose, 100 mM cellobiose or cellotriose, 20 mM sodium acetate buffer (pH 5.2), 2 mM calcium chloride, 0.01% sodium azide, and 120 mIU of dextransucrase and alternansucrase, respectively. The reaction was conducted for 6 h at 28°C to completely consume sucrose.

CB1, 2'-α-D-glucopyranosyl cellobiose. CT1 to CT3, cellotriose connected by glucose, isomaltose, and isomaltotriose, respectively.

oligosaccharides were also produced as acceptor products. Fu and Robyt [3] showed that *L. mesenteroides* B-512FM dextransucrase could transfer glucose from sucrose to maltodextrins; maltotriose to maltooctaose. The efficiency of the acceptor reaction decreased exponentially as the size of the maltodextrin increased. Similar results were obtained for *Streptococcus mutans* dextransucrase and mutansucrase with the exception that the reaction percentage decreased with maltopentaose and then started to increase again with maltohexaose and heptaose, suggesting the possibility that some glucans used higher molecular glucan chains as acceptors giving glucose residues attached by α-(1→6) linkage to the glucan chain [11, 12]. This might give the possibility to modify for bacterial cellulose by B-742CBM dextransucrase or B-1355C alternansucrase.

To confirm the existence of cellulose materials in a soluble glucan, methylation and subsequent acid hydrolysis analyses were performed. Figure 1 (lane 3) shows the methylation analysis of dextran synthesized by a constitutive dextransucrase elaborated by *L. mesenteroides* B-742CBM. This glucan shows a high degree of branching, as evidenced by 2,4-di-methyl-D-glucose (39.2%) and 2,3,4,6-tetra-*O*-methyl-D-glucose (4.7%), indicating that the major branching linkages in this dextran is (1→3). Lane 4 shows the methylation analysis of bacterial cellulose. It shows only a (1→4) linked glucose residue. Lane 5 shows the methylation analysis of the soluble glucan (prepared from B-742CBM dextransucrase modification of bacterial cellulose). The glucan has (1→3), (1→4), and (1→6) glycosidic linkages in the main chains, as shown by the formation of 2,4,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3,4-tri-*O*-methyl-D-glucose, respectively. The latter is the predominant tri-*O*-methyl-D-glucose residue, indicating that the (1→6) linkage is dominant. This glucan is also highly branched through a (1→3) linkage, as shown by the formation of 2,4-di-*O*-methyl-D-glucose (20.2%) from the branched D-glucose residue and 2,3,4,6-tetra-*O*-methyl-D-glucose (18.8%) from the non-reducing ends of the branched chains. It is of interest that there is (1→4) linkage in soluble glucan that does not exist in B-742CBM

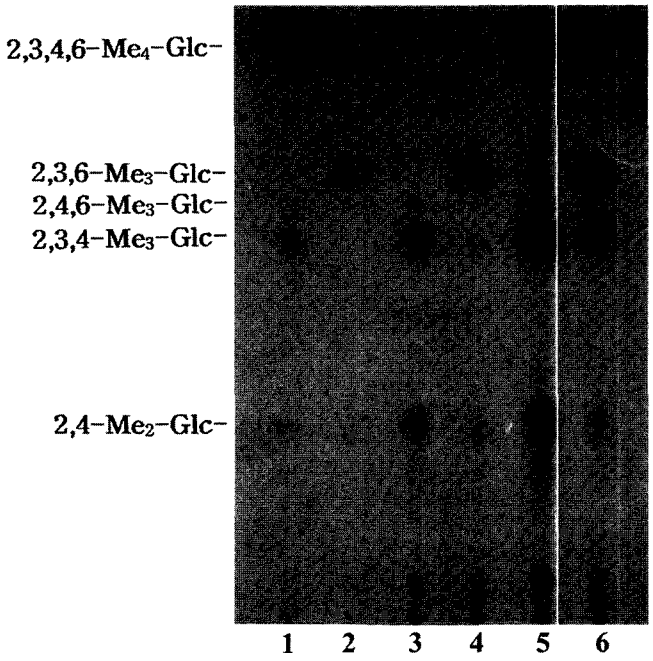

Fig. 1. TLC separation of *O*-methylated-D-glucoses obtained from the methylation analysis of B-742CBM modified soluble- and insoluble-glucans.

Lane 1, the methylation analysis of gentiobiose; Lane 2, the methylation analysis of maltotriose; Lane 3, the methylation analysis of B-742CBM dextran; Lane 4, the methylation analysis of bacterial cellulose; Lane 5, the methylation analysis of the modified soluble-glucan; Lane 6, the methylation analysis of the modified insoluble-glucan.

dextran. The (1→4) linkage can be found in bacterial cellulose that is not soluble in water.

Thus, this result indicates that the insoluble bacterial cellulose has been modified by dextransucrase and solubilized. Lane 6 shows the methylation analysis of insoluble glucan (prepared after B-742CBM dextransucrase modification of bacterial cellulose). It contains primarily the (1→4) linkage as evidenced by the formation of large amounts of 2,3,6-tri-*O*-methyl-D-glucose (46.8%), but also the (1→6) linkage as shown by the formation of large amounts of 2,3,4-tri-*O*-methyl-D-glucose (31.3%). These

Table 2. Proportions of the methylated D-glucose in the acid hydrolysis products of the methylated bacterial cellulose, B-1355C glucan, modified soluble and insoluble glucans.

	% of methylated glucose			
	Bacterial Cellulose	B-1355C Glucan	Soluble Glucan	Insoluble Glucan
2,3,4,6-Me_4-Glc	14.6	18.9	20.3	6.3
2,3,6-Me_3-Glc	61.4	-	2.5	35.3
2,4,6-Me_3-Glc	-	13.7	11.0	-
2,3,4-Me_3-Glc	-	48.2	44.4	30.1
2,4-Me_2-Glc	-	19.2	17.5	3.1
2,3-Me_2-Glc	24.0	-	4.3	25.2

results indicate that there are dextran-like structures in the insoluble glucan as well as cellulose-like materials. Thus, *A. xylinum* bacterial cellulose was modified by dextransucrase and sucrose, in a manner similar to the formation of the soluble glucan. However, the modification was less, as shown by the formation of smaller amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose (8.4%). Since there were still significant amounts of (1→6) linkages, it was possible that, instead of high numbers of branching points, there were longer branched dextran chains.

Table 2 shows proportions of the methylated D-glucose in the acid hydrolysis products of the soluble and insoluble glucan (prepared from B-1355C alternansucrase modification of bacterial cellulose). The soluble glucan contains (1→3), (1→4), and (1→6) glycosidic linkages in the main chains, as shown by the formation of 2,4,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3,4-tri-*O*-methyl-D-glucose, respectively. The latter is the predominant tri-*O*-methyl-D-glucose residue (44.4%), indicating that the (1→6) linkage is dominant. It is also quite interesting to note that there is a (1→4) linkage (2.5%) that does not exist in B-1355C alternan. Thus, this result indicates that the insoluble bacterial cellulose is also modified by alternansucrase (or glucansucrase) and solubilized.

The insoluble glucan contains primarily (1→4) linkages as evidenced by the formation of 2,3,6-tri-*O*-methyl-D-glucose (35.3%) and also (1→6) linkage as shown by the formation of large amounts of 2,3,4-tri-*O*-methyl-D-glucose (30.1%). These results indicate that there are dextran-like structures in insoluble glucan as well as cellulose-like structure. However, the modification is less, as shown by the formation of smaller amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose (6.3%). Since there are still significant amounts of (1→6) linkages, it is possible that, instead of high numbers of branching points, there are longer branched chains. Generally, most glucansucrases transfer glucose to other carbohydrates with the linkage of α-(1→6) [10, 11]. Thus, bacterial cellulose was modified by alternansucrase and sucrose, in a manner similar to the modification of bacterial cellulose by B-742CBM dextransucrase.

In summary, we have developed a new process for the modification of growing insoluble bacterial cellulose with specific glucansucrases and sucrose to produce modified bacterial cellulose from native bacterial cellulose, which have possibly different structures and characteristics. The optimization of the process for higher yields of the soluble glucan is in progress.

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