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Enzymatic Modification of Cellulose Using *Leuconostoc mesenteroides* B-742CBM Dextransucrase

KIM, DOMAN[1,2,5]*, YOUNG-MIN KIM[3], MI-RAN PARK[3], HWA-JA RYU[4], DON-HEE PARK[1], AND JOHN F. ROBYT[6]

[1]*Department of Biochemical Engineering,* [2]*The Research Institute for Catalysis,* [3]*Department of Biomedical Engineering*
[4]*Department of Fine Chemical Engineering, Chonnam National University, Kwang-Ju 500-757, Korea*
[5]*Research Center for New Bio-Materials in Agriculture, Seoul National University, Suwon 441-744, Korea*
[6]*Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011, USA*

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Abstract In addition to catalyzing the synthesis of dextran from sucrose as a primary reaction, dextransucrase also catalyzes the transfer of glucose from sucrose to other carbohydrates that are present or are added to the reaction digest. We have 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 *Leuconostoc mesenteroides* B-742CBM. The final reaction products were composed of soluble- and insoluble-glucans. The yields of soluble- and insoluble-glucans were theoretically 21% ±2.2 and 68% ±5.1, respectively. The remainder of the reaction products was recovered as a mixture of oligosaccharides that could not be precipitated by 67% (v/v) ethanol. Treating the modified glucans with endo-dextranase and/or cellulase, oligosaccharides were produced that were not formed from the hydrolysis of native cellulose or B-742CBM dextran. The modification of the cellulose was confirmed by methylation and acid hydrolysis of the soluble- and insoluble-glucan. Both (1→4) and (1→6) glycosidic linkages were found in both of the glucans.

Key word: Acceptor reaction, enzymatic modification, cellulase, cellulose, dextranase, dextransucrase, glucosyl transfer, *Leuconostoc mesenteroides*

The primary reaction for glucansucrases is the conversion of sucrose into polysaccharide (glucan) according to the following reaction [13]:

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

*Corresponding author
Phone: 82-62-530-1844; Fax: 82-62-530-1849;
E-mail: dmkim@pasteur.chonnam.ac.kr

The reaction is essentially irreversible and the main products are high molecular weight ($1-10\times10^8$ Da) dextran and D-fructose. Leucrose (5-*O*-α-D-glucopyranosyl-D-fructopyranose) arises as a minor product from the acceptor reaction of D-fructose [1]. 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 added to the reaction mixture. 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 [12]. They include monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Not all of the acceptors, however, react to the same degree. When the acceptor is a monosaccharide or disaccharide, series of oligosaccharide acceptor-products are usually produced [12]. In fact, there are two classes of acceptors, those that give a homologous series of oligosaccharides, each one differing from the other by one glucose residue, and those acceptors that only form a single acceptor-product containing one glucose residue more than the acceptor. All of the glucans synthesized by glucansucrases are branched [3]. The type of branch linkage and the degree of branching varies for different glucansucrases. The principal branch linkage found is α-(1→3), although α-(1→2) and α-(1→4) branch linkages have also been observed for different glucansucrases [3]. Robyt and Taniguchi [11] found that the synthesizing or elongating enzyme is also responsible for forming branch linkages, and separate branching enzymes are not required. The mechanism for forming branch linkages is by an acceptor-reaction in which a dextran chain itself acts as an acceptor and either D-glucose or the dextran chain being synthesized is transferred to an acceptor dextran chain, forming a branch linkage between D-glucose or the acceptor dextran

chain. Lee *et al.* [8, 9] showed that *Leuc. mesenteroides* B-742CB dextransucrase could modify various starch granules or gelatinized starches and pullulan. The resulting modified starches were more resistant to be hydrolyzed by α-amylase, isoamylase, pullulanase, and endo-dextranase than the unmodified starches [8]. After modification of granule starch with B-742CB dextransucrase, the modified starch becomes solubilized. The soluble modified starch had D-glucose added to the hydroxyl groups at positions C3, C4, and C6. Kim and Robyt obtained *L. mesenteroides* mutants from several strains (e.g., B-512FM, B-742, B-1142, B-1299, and B-1355) that were constitutive for specific glucansucrases. 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 [4-6]. 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, were unsuccessful. These failures might have been due to not using the right dextransucrase (the dextransucrase was not specified in the patent). In 1964, it may not yet have being recognized that different *Leuc. mesenteroides* strains elaborated different kinds of glucansucrases with different specificities. In this paper, we report the successful modification of cellulose using sucrose and *Leuc. mesenteroides* B-742CBM dextransucrase that has been further developed from B-742CB as a hyper-producer of dextransucrase.

MATERIALS AND METHODS

Carbohydrates and Reagents

Cellobiose was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Cellotriose, maltodextrins, isomaltodextrins were available in 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.

Isolation of *Leuconostoc mesenteroides* B-742CBM

A constitutive and dextransucrase hyper-producing mutant was isolated after mutation using ethyl methane sulfonate (EMS) as previously described [4, 5].

Enzymes

Dextransucrases were prepared by culturing *Leuc. mesenteroides* B-742CBM and B-512FMCM in glucose medium as described by Kim and Robyt [4-6]. α-Amylase, isoamylase, pullulanase, dextranase, and α-cellulase were purchased from Sigma Chemical Co.

Modification Reaction

α-Cellulose (5 g) was suspended in 250 ml of buffer (20 mM Sodium-acetate, pH 5.2) and dextransucrase (200 IU/reaction digest) was added. One IU of dexransucrase indicates the release of 1 μmole of fructose from sucrose per min at pH 5.2 and 28°C. The reaction was initiated by the addition of 100 ml of 100 mM sucrose at the rate of 0.2 ml per min by continuously pumping into the digest. After the addition of the sucrose solution, the reaction was continued for an additional 30 min with stirring. After reaction, the insoluble material in the digest was collected by centrifugation, washed with water (3-times), and titurated with acetone and ethanol. The supernatants from the centrifugation were treated with 2 volumes of ethanol to precipitate any polysaccharide that had become soluble during the modification reaction. These were also titurated with acetone and ethanol. The polysaccharides thus obtained were used in further reactions with enzymes and characterized as described in the following. Modified cellulose (0.5%, w/v) was dissolved or suspended in citrate-phosphate buffer (20 mM, pH 5.5 for dextranase and pH 6.0 for cellulase, containing 0.02% sodium azide) and incubated with dextranase (0.5 U/ml) or cellulase (1.0 U/ml) for 24 h at 37°C. The carbohydrate compositions of the digests were analyzed by three ascents on Whatman K5 plates (18.5 cm path) using 4/10/3 of nitromethane/1-propanol/water [8].

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 analyzed for the methylated products using TLC [10]. The methylation analysis products were separated by two ascents on Whatman K6 plates (18.5 cm path) 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 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 carried out by scanning the plate with a Bio-Rad Imaging Densitometer, model GS-670 (Bio-Rad Laboratories, Hercules, CA, U.S.A).

Acceptor-Reaction Digests

Dextransucrase 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. The reaction was conducted for 6 h at 28°C to consume the sucrose completly.

RESULTS AND DISCUSSION

The modification of cellulose with B-742CMB dextransucrase gave 21% ± 2.2 soluble- and 68% ± 5.1 insoluble-polysaccharides. The theoretical percent yielded was based on the sum of the weight of the cellulose plus 0.48 ×g of sucrose added to the reaction digest.

Figure 1 shows the hydrolysis products of modified cellulose after treatment of cellulase and/or dextranase.

The hydrolytic enzymes did not produce any products from B-742CBM dextran. The cellulase hydrolysis of insoluble-cellulose produced only D-glucose in trace amount. However, many kinds of hydolysis products were produced from the reaction of the enzymes with the modified soluble-glucan. In particular, several oligosaccharides were produced by the reaction with cellulase. This means that a portion of the soluble-glucan is composed of the β-(1→4) cellulose-like structure. The soluble, modified cellulose, thus, has both β-(1→4) linkages as well as α-(1→6) linkages.

We have reacted cellobiose and cellotriose with sucrose and B-742CMB dextransucrase (Fig. 2). Only one acceptor product was produced from the cellobiose acceptor reaction. Interestingly, the efficiency of acceptor reaction was increased as the size of the acceptor increased. In the case of B-512FMCM dextransucrase, the portion of cellobiose acceptor product was 25.5% of total carbohydrate (excluding fructose and unreacted cellobiose) and that of cellotriose was 69.4%. Several oligosaccharides were also produced as acceptor products. With the B-742CBM dextransucrase reaction, only one acceptor product was produced and the portion of cellobiose acceptor product was 41.9% of the total carbohydrate (excluding fructose and unreacted cellobiose), and that of cellotriose was 70.6%. Fu and Robyt showed that *Leuc. mesenteroides* B-512FM dextransucrase could transfer D-glucose from sucrose to maltodextrins, and maltotriose to maltooctaose [1]. The percentage of 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 maltoheptaose [2]. This suggests a possibility that higher molecular glucan chains might react with specific dextransucrases, yielding single glucose residues attached by an α-(1→6) linkage to the glucan chain [9]. This might be a possibility for the cellulose modification by B-742CBM dextransucrase.

To confirm the modification of cellulose in the soluble-glucan, methylation and acid hydrolysis analyses were performed (Fig. 3 and Table 1).

Figure 3 (lane 3) shows the methylation analysis of dextran synthesized by a constitutive dextransucrase elaborated by *Leuc. mesenteroides* B-742CBM. This glucan shows a high degree of branching, as evidenced by the large amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-glucose, indicating that it is highly (1→3) branched. Lane 4 shows the methylation analysis of native cellulose, which gives only 2,3,6-tri-*O*-methyl-D-glucose showing only the presence of (1→4) linked glucose residues. Lane

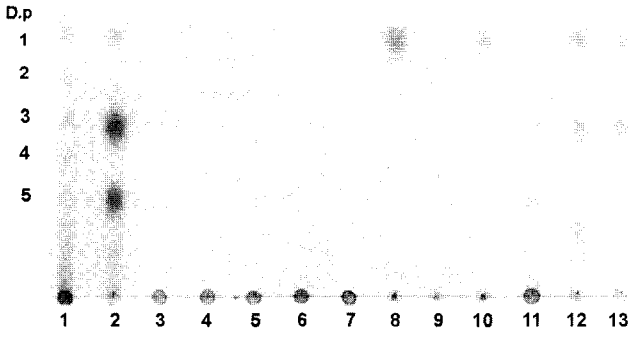

Fig. 1. TLC separation of cellulase and/or dextranase hydrolysis products of B-742CBM dextran and soluble glucan prepared from dextransucrase modification of cellulose.

Lanes 1 and 2 are standards of maltodextrins and isomaltodextrins, respectively. Lanes 3 to 7 show the hydrolysis products of B-742CBM dextran by α-amylase, isoamylase, pullulanase, dextranase, and α-cellulase, respectively. Lanes 8 to 10 show the cellulose hydolysis products by α-cellulase, dextranase, and a mixture of both enzymes. Lanes 11 to 13 show the α-cellulase, dextranase, and the mixture of both enzymes hydrolysis products from the soluble glucan obtained from the modification reaction.

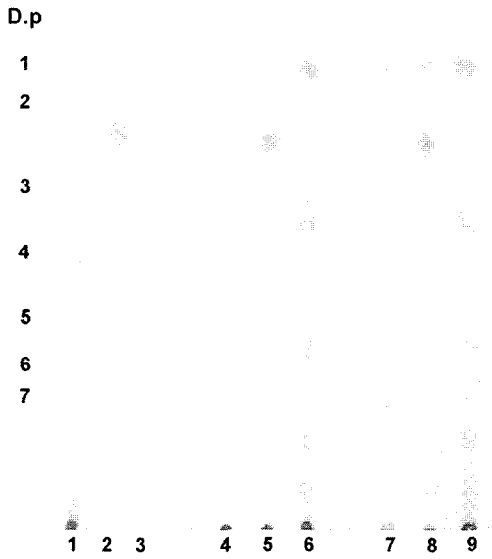

Fig. 2. The acceptor reaction products of *Leuconostoc mesenteroides* B-512FMCM and B-742CBM dextransucrase using cellobiose and cellotriose.

Lane 1, standards of maltodextrins; lanes 2 and 3, standards of cellobiose and cellotriose, respectively; lanes 4-6, the reaction products of B-512FMCM dextransucrase; lanes 7-9, the reaction products of B-742CBM dextransucrase. Lanes 4 and 7 show the reaction digests of dextransucrase only with sucrose. Lanes 5 and 8 show the acceptor reaction digests of dextransucrase with cellobiose and sucrose. Lanes 6 and 9 show the acceptor reaction digests of dextransucrase with cellotriose and sucrose.

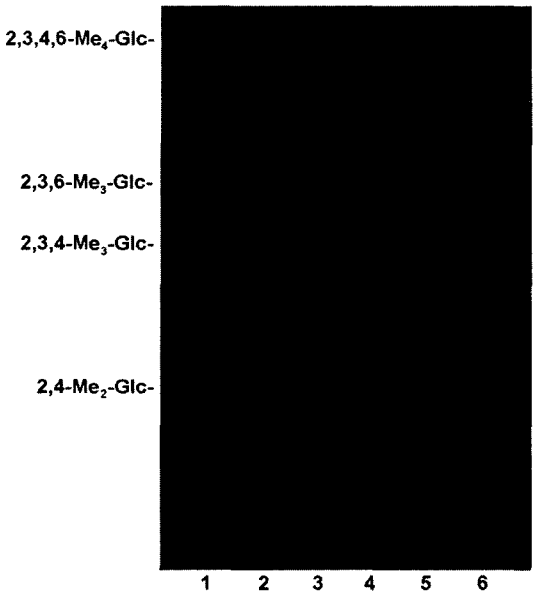

Fig. 3. TLC separation of *O*-methylated-D-glucoses obtained from the methylation analysis of soluble- and insoluble-glucans. Lane 1, the methylation analysis of maltotriose; lane 2, the methylation analysis of gentiobiose; lane 3, the methylation analysis of B-742CBM dextran; lane 4, the methylation analysis of α-cellulose; lane 5, the methylation analysis of the modified soluble-glucan; lane 6, the methylation analysis of the modified insoluble-glucan.

Table 1. Proportions of the methylated D-glucose in the acid hydrolysis products of the methylated cellobiose, B-742CBM dextran, modified soluble- and insoluble-glucans.

	% of methylated glucose			
	Cellobiose	B-742CBM Dextran	Soluble Glucan	Insoluble Glucan
2,3,4,6-Me_4-Glc	50.6	8.2	13.6	4.3
2,3,6-Me_3-Glc	49.4	-	22.7	58.4
2,4,6-Me_3-Glc	-	12.5	6.0	-
2,3,4-Me_3-Glc	-	64.2	42.6	26.4
2,4/2,3-Me_2-Glc	-	15.1	15.1	10.9

5 shows the methylation analysis of the soluble glucan (prepared from B-742CBM dextransucrase modification). 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 linkage (1→6) is dominant. This glucan is also highly branched through α-(1→3) linkage, as shown by the formation of 2,3,4,6-tetra-*O*-methyl-D-glucose from the non-reducing ends of the branched chains. It is quite interesting that there is a (1→4) linkage that does not exist in B-742CBM dextran. The (1→4) linkage can be found in cellulose that is not soluble in water. Thus, this result demonstrates that the cellulose has been modified by dextransucrase to give a soluble product. Lane 6 shows the methylation analysis of insoluble-glucan (obtained after B-742CBM dextransucrase modification). It contains primarily (1→4) linkages as evidenced by the formation of large amounts of 2,3,6-tri-*O*-methyl-D-glucose, but also (1→6) linkages as evidenced by the formation of large amounts of 2,3,4-tri-*O*-methyl-D-glucose. These results indicate that there are dextran-like structures in the insoluble glucan as well as cellulose-like structures. Thus, native cellulose was modified by dextransucrase and sucrose, in a manner similar to the formation of the soluble-glucan. However, the modification is less, as shown by the formation of smaller amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose. Since there are still significant amounts of (1→6) linkages, it is possible that instead of high numbers of branching points, there are longer branch dextran chains.

In summary, a new process has been developed for the modification of insoluble cellulose with a specific dextransucrase from *L. mesenteroides* B-742CBM and sucrose to give two new glucans with their structures and characteristics different from native cellulose. The optimization of the process is in progress to give higher yields of the soluble-glucan.

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