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Enzymatic Synthesis of Oligosaccharides, Alkyl and Terpenyl Glucosides, by Recombinant *Escherichia coli*-Expressed *Pichia etchellsii* β-Glucosidase II

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Abstract

The biosynthetic activity of yeast *Pichia etchellsii* β-glucosidase II (*Bgl*II) expressed in recombinant *Escherichia coli* was utilized for synthesis of cellooligosaccharides, alkyl and terpene glucosides. Cellooligosaccharides with a degree of polymerization of 3 and greater were resolved by thin-layer chromatography (TLC) using an ethyl acetate:1-propanol:2-propanol:water (8:5:1:1) solvent system followed by visualization with 0.2% naphtho-resorcinol reagent. Using 2*M* cellobiose and 15 IU of partially purified *Bgl*II, 57 mmol/L of oligosaccharides (comprising mostly cellotriose and cello-pentaose) was synthesized in 16 h. Similarly, alkyl glucosides with chain lengths from 6 to 10 carbons were synthesized and products extracted to near purity by ethyl acetate extraction. The same extraction method was employed to separate, to near purity, various monoterpenyl (nerol, geraniol, citronellol) glucosides. A reliable and simple method for separation of cellooligosac-charides using a combination of Bio-Gel P-2 gel filtration and charcoal celite adsorption chromatography was developed. The cellooligosaccharides were separated to purity as confirmed by TLC. The enzyme was among the very few that could synthesize a wide variety of glycoconjugates.

Index Entries: β-Glucosidase; *Pichia etchellsii*; cellooligosaccharides; alkyl glucosides; monoterpenyl glucosides.

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Introduction

β-D-Glucoside glucohydrolases or β-glucosidases (EC 3.2.1.21), members of the glycosyl hydrolase families, catalyze the transfer of the glycosyl group between oxygen nucleophiles. Such a transfer reaction results in hydrolysis of the β(1→4)-glucosidic bond shared among monosaccharides, and monosaccharides and amino, terpene, or alkyl groups releasing the corresponding sugar, amino, terpene, or alkyl moieties. Like many other hydrolytic enzymes, changes in reaction environment lead to display of biosynthetic activities by these enzymes. Such changes include a high substrate concentration, a decrease in water activity, and an introduction of organic solvent in the reaction environment. It is proposed that synthesis occurs by reversal of hydrolysis (under thermodynamic control) or by transglycosylation approach (under kinetic control) *(1)*. The first method requires long incubation conditions required for equilibrium. In the transglycosylation method, synthesis is achieved in shorter time periods. Both approaches have been used for synthesis of oligosaccharides using microbial β-glucosidases such as *Aspergillus foetidus (2)*, *Aspergillus niger (3)*, *Fusarium oxysporum (4)*, *Trichoderma pseudokoningii (5)*, and plant β-glucosidase from sesame *(6)*. β-Glucosidases have also been used for synthesis of alkyl glucosides with alkyl chain lengths varying from one to eight carbons using almond β-glucosidase; the alcohols act as acceptors of the glycosyl group, and glucose has been the saccharide donor in reverse hydrolysis mode (for a review, *see* ref. *7*) except in one study *(8)* in which methylglucoside was also used as saccharide donor.

Interest in the synthesis of oligosaccharides and other glycoconjugates has arisen owing to a variety of functions exhibited by these molecules. Many of the oligosaccharides act as cell-surface receptors, are important functional constituents in animal feed, and are artificial sweeteners (for a review, *see* ref. *7*). The alkyl glucosides are nonionic surfactants that can be used as detergents, as food surfactants, and in the pharmaceutical sector. Their role in solubilization, reconstitution of biologic membranes, and preparation of lipid vesicles has been reported *(9)*. The enzymatic synthesis of these compounds is preferred over chemical routes because few nonspecific byproducts are formed and synthesis involves fewer steps.

The separation of oligosaccharides synthesized in enzymatic reactions remains a challenging problem owing to the neutral and nonionizing nature of these molecules. No methods based on electrical properties can be employed for separation because carbohydrates (with the exception of sialic acid) do not ionize unless a high-pH buffer (>12.0) is employed. Their polar nature also limits the utility of micellar reagents such as sodium dodecyl sulfate. Ionization under mild conditions has been achieved by complexing with borate or by chemical modification, such as reductive amination *(10)*. In addition to the problem of adding charge, the carbohydrates exhibit complex regio- and stereoisomerism, which significantly

complicates the separation process. In spite of these limitations, a variety of methods have been tried for analysis and separation of oligosaccharides, such as adsorption chromatography on graphitized carbon columns *(11)*; high-performance liquid chromatography (HPLC) *(12)*; gel filtration *(13–15)*, affinity- *(16)*, and ion-exchange chromatography *(17–19)*; and capillary electrophoresis *(20)*. Each procedure has a unique set of problems associated with the method employed.

We have been investigating the biosynthetic capacity of β-glucosidase enzymes for synthesis of oligosaccharides and other glucosides. Native β-glucosidase BGLI, purified from the cell wall of yeast *Pichia etchellsii (21)*, and *P. etchellsii Bgl*I and *Bgl*II, expressed from recombinant *Escherichia coli (22,23)*, have been used for synthesis of alkyl glucosides and oligosaccharides, respectively. *Bgl*II also catalyzed the synthesis of β-1-*N*-acetamido-D-glucopyranose β(1→6) and β(1→3) isomers, which is a glycosylasparagine mimic *(24)*. These disaccharides are important fragments of phytoalexin-elicitor oligosaccharides involved in plant defense mechanisms and serve as communication sites between host plants and fungal pathogens *(25)*.

From our continuing studies on biosynthetic activities exhibited by *Bgl*II, we report here on the synthesis of cellooligosaccharides, alkyl and monoterpene glucosides. We also describe the combination of gel filtration and charcoal celite adsorption chromatographic techniques for achieving effective separation of various cellooligosaccharides.

Materials and Methods

Chemicals

Cellobiose; cellotriose; cellotetraose; cellopentaose; *p*-nitrophenyl-β-D-glucopyranoside (pNPG); *p*-nitrophenol (pNP); hexyl-, heptyl-, octyl-, and decylglucosides; hexanol; heptanol; octanol; decanol; citronellol; nerol; and geraniol were purchased from Sigma (St. Louis, MO). Precoated silica gel 60 F_{254} thin-layer chromatography (TLC) plates were obtained from Merck. All other chemicals were of analytical grade and the highest purity and were obtained locally.

Microbial Strains and Growth Conditions

The recombinant *E. coli* pBG22:JM109, constructed as described previously *(26)*, was the source of *P. etchellsii Bgl*II enzyme. The cells were maintained as glycerol stocks at –70°C. Single well-separated colonies on Luria-agar medium containing 100 μg/mL of ampicillin were used for inoculating 250 mL of Luria broth supplemented with 100 μg/mL of ampicillin in four parallel 1-L flasks. Cells were grown at 37°C for 18 h with shaking at 250 rpm.

Localization of Bgl*II in Recombinant Cells*

Biosynthetic reactions with *Bgl*II were performed using either partially purified enzyme or the purified enzyme (obtained from the peri-

plasmic space). For this, the broth was obtained by growing the recombinant pBG22:JM109 for 18 h. The cells were harvested by centrifuging at 8000*g* for 10 min at 4°C. The cell-free broth served as the extracellular fraction. The pellet was subjected to osmotic shock treatment according to Nossal and Heppel *(27)* with some modifications. The cell pellet was suspended in half-culture volume of buffer A (0.85% NaCl in 10 m*M* Tris-Cl buffer, pH 8.5) and mixed well. The resulting cell suspension was centrifuged at 8000*g* for 10 min at 4°C, and the pellet was again suspended in half-culture volume of buffer B (20% sucrose in 30 m*M* Tris-Cl buffer, pH 8.5). EDTA was then added to a final concentration of 40 m*M*. The cell suspension was mixed gently for 10 min at room temperature, and the cells were pelleted at 8000*g* for 20 min at 4°C followed by suspension in one-tenth culture volume of ice-cold milli-Q water and mixed in an ice-water bath for 30 min. The cell suspension was centrifuged at 10,000*g* for 30 min. The periplasmic fluid was released in the supernatant. The cell pellet, thoroughly washed in 50 m*M* sodium phosphate buffer, pH 7.0, was suspended in one-tenth culture volume of the same buffer and sonicated at an amplitude of 10 μ for 10 min with intermittent cooling in the presence of 1 m*M* phenylmethylsulfonyl fluoride. The cell-free extract obtained after centrifuging at 15,000*g* for 20 min served as the intracellular fraction. The pellet obtained represented the cell-bound fraction. β-Glucosidase activity was measured in all four (culture broth, osmotic fluid, intracellular, and cell-bound) fractions. The enzyme was partially purified from the periplasmic fraction by 40–80% fractional ammonium sulfate precipitation, and the specific activity of this enzyme preparation was 0.29 IU/mg of protein. This preparation was used for synthesis of oligosaccharides. The enzyme was also purified to homogeneity from the periplasmic fraction, as described elsewhere *(28)*, to a specific activity of 2.77 IU/mg of protein. The purified enzyme was used for synthesis of various alkyl and monoterpene glucosides.

Synthesis of Oligosaccharides, Alkyl and Terpenyl Glucosides

Synthesis of oligosaccharides was performed in a 5-mL reaction mixture containing 15 IU of partially purified enzyme with 2000 m*M* cellobiose in 50 m*M* phosphate buffer (pH 7.0) at 45°C. Dimethylsulfoxide (DMSO) was added to 25% (v/v) to drive the reaction in the biosynthetic mode, as demonstrated previously *(23)*. The reaction was terminated after 16 h by heating the vials at 100°C for 1 min. The samples were centrifuged to remove the precipitated proteins, and a 15-μL aliquot was loaded onto TLC plates (as described in the next section) for qualitative analysis of oligosaccharides synthesized. The centrifuged samples were also passed through a 0.45-μm filter and saccharides quantified by HPLC as described previously *(23)* using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and 1.25 m*M* H_2SO_4 as eluent. The column temperature was maintained at 50°C. The standards were used and eluted under similar conditions.

The synthesis of alkyl and terpenyl glucosides was performed in a 1.5-mL reaction mixture containing 1.5 IU of purified enzyme, 80 m*M* primary alcohols (hexanol, heptanol, octanol, or decanol) or monoterpenols (nerol, geraniol, citronellol), 20% (v/v) DMSO, and 12% (350 m*M*) cellobiose or 12% (667 m*M*) glucose in 50 m*M* sodium phosphate buffer, pH 7.0. DMSO was added to solubilize long-chain alcohols and to provide a monophasic reaction environment. The reaction mixtures were incubated at 37°C for 20 h. The glucoside products were extracted from the reaction mixture using ethyl acetate. For this, 20% (v/v) ethyl acetate was added to the reaction vials and the contents were mixed on a vortex. The vials were allowed to stand at room temperature for 10 min followed by centrifuging at 8000*g* for 5 min. Ethyl acetate containing the extracted glucosides formed the upper clear layer, which was drawn out carefully. The solvent was next evaporated off (air-dried), and methanol:water (7:3 [v/v]) was added to the vial to dissolve the extracted glucosides. The products were analyzed by TLC as described next.

TLC Analysis of Oligosaccharides, Alkyl and Terpene Glucosides

Ascending TLC was performed for qualitative determination and separation of oligosaccharide products. An aliquot (15 µL) of biosynthetic reaction mixture (passed through charcoal celite chromatography to remove excess cellobiose from the reaction mixture) was spotted on precoated silica gel 60 F_{254} TLC plates along with the standards glucose, cellobiose, laminaribiose, sophorose, gentiobiose, cellotriose, cellotetraose, and cellopentaose. The plates were run in three types of solvents to choose the most suitable one for effective separation and to determine maximum resolution at lowest sugar concentrations. Solvent 1 was ethyl acetate:1-propanol:2-propanol:water in a ratio of 8:5:1:1 (v/v), solvent 2 was chloroform:methanol:0.2% $CaCl_2$ in water in a ratio of 4:4:1 (v/v), and solvent 3 was ethyl acetate:isopropanol:water:pyridine in a ratio of 26:14:7:20 (v/v). After running, the plates were dried at 80–90°C for 5 min. The spots were visualized either by (1) spraying with 0.2% naphthoresorcinol reagent in H_2SO_4:ethanol (5:95 [v/v]) solution followed by heating at 110°C for 10 min to develop the spots for solvents 1 and 2, or by (2) spraying with diphenylamine reagent containing aniline:diphenylamine:orthophosphoric acid (85%) in a ratio of 5:5:1 (v/v) followed by heating at 110°C for 5 min for solvent 3.

For qualitative determination of alkyl and terpenyl glucosides, 20 µL of ethyl acetate–extracted products was spotted on silica gel plates, along with the heat-killed enzyme control and standard alkyl glucosides, namely, hexyl-, heptyl-, octyl-, and decylglucosides. Ascending TLC was performed using ethyl acetate:2-propanol:distilled water in a ratio of 40:30:10 (v/v). After running, the plates were dried as already described, and separated products were visualized by spraying with 0.2% naphthoresorcinol reagent followed by heating at 110°C for 10 min *(29)*.

Separation of Cellooligosaccharides

The choice of separating matrix and column dimensions was determined from running cellulose hydrolysate containing mixtures of oligosaccharides of different degrees of polymerization (DPs). Acid hydrolysis of Avicel cellulose (10 g) was performed by the addition of 100 mL of concentrated HCl. The mixture was stirred and allowed to stand at room temperature for 3 min. Ice-cold fuming HCl (100 mL) was added and the mixture stirred again. The resulting solution was then warmed and allowed to stand at room temperature for 3 h. To this 600 mL of ice-cold water was added. The pH was brought to neutral by adding 210 g of $NaHCO_3$. The gelatinous material was removed by centrifugation and the clear supernatant was collected for analysis. Total reducing sugars was estimated by the orcinol-H_2SO_4 method *(30)*. About 15 mg of total sugars was loaded onto Sephadex G-15 and Bio-Gel P-2 columns (55 × 1.25 cm) in separate experiments. Elution was performed with Milli-Q water at a flow rate of 15 mL/h. Fractions of 500 µL were collected for analysis. For elution of the products synthesized by enzyme, gel filtration chromatography was performed on a Bio-Gel P-2 (Pharmacia) column (55 × 1.25 cm). The contents of the biosynthetic reaction (5 mL) were loaded onto the column, and elution was performed with Milli-Q water at a flow rate of 15 mL/h. Samples (500 µL) were collected and aliquots (20 µL) of alternate fractions loaded onto TLC plates and analyzed for the products. Second-stage charcoal celite chromatography was performed on pooled fractions (as described under Results and Discussion) after first-stage gel filtration chromatography. A 5 × 1 cm glass column was packed with charcoal and celite 535 (1:1), mixed, and stirred in water. Fines were removed by filtration. Elution was done by a step gradient of ethanol from 0 to 20% (v/v) as optimized for individual sugars in separate experiments. Fractions (27–47) from first-stage chromatography were loaded onto the charcoal celite column and eluted with 7% ethanol. Similarly, for separation of higher oligosaccharides (DP > 3), fractions (18–21) were loaded onto the charcoal celite column and eluted with 15% ethanol. The eluted fractions were analyzed for purity on TLC plates.

Analytical Methods

β-Glucosidase activity was routinely assayed using pNPG as the substrate *(31)*. One international unit of enzyme activity was defined as the amount of enzyme required to release 1 µ*M* of pNP/min. The protein was estimated according to Lowry *(32)*. Total sugars were estimated by the orcinol-H_2SO_4 method using 0.2% orcinol in 70% (v/v) H_2SO_4 *(30)*.

Results and Discussion

Localization of β*-Glucosidase in Recombinant* E. coli *pBG22:JM109*

Enzyme localization studies were performed to determine the fraction containing maximum activity of the enzyme. Table 1 lists the distribution

Table 1
Distribution of *Bgl*II in Subcellular Fractions of *E. coli* pBG22:JM109

Cell fraction	Enzyme activity (mU/mL)	Total volume (mL)	Total activity (mU)[a]
Periplasmic fluid	90.0	90.0	8100.0 (97)[a]
Cell-free extract	45.0	5.0	225.0 (2.7)
Cell-bound fraction	23.0	0.6	13.8 (0.3)
Total			8338.8 (100)

[a]Numbers in parentheses indicate the percent of total enzyme in that fraction.

of β-glucosidase in various subcellular fractions. The maximum enzyme activity, 90 mU/mL, was localized in the periplasmic fraction. This fraction was used for obtaining partially purified enzyme with (40–80%) ammonium sulfate fractionation and also the pure enzyme. Such localization suggested recognition of the leader sequence of *Bgl*II enzyme by *E. coli*. Whereas most β-glucosidases of bacterial origin have been reported to be excreted to the periplasmic space in *E. coli*, such as BgxA of *Erwinia chrysanthemii (33)* and BglA of *Erwinia herbicola (34)*, no yeast enzyme has been reported to be targeted to the periplasmic space. Such processing of yeast enzyme makes this system a good model for studying the transport of yeast proteins in Gram-negative bacteria.

Synthesis of Oligosaccharides

The synthesis of oligosaccharides was performed using partially purified enzyme prepared from periplasmic extract. In our earlier studies on the effect of temperature on oligosaccharide yield, 45°C was found to be the best among the temperatures of 37, 45, and 55°C; hence, 45°C was used in the following studies. Analysis of an aliquot (20 µL) by TLC (data not shown) indicated the presence of high amounts of cellobiose, which masked the detection of other synthesized oligosaccharides. Accordingly, excess cellobiose was removed by passing the reaction mixture over a charcoal celite column, and excess glucose and cellobiose were removed using 100 mL each of water and 5% (v/v) ethanol in step gradient mode, respectively. This was found to selectively remove glucose and excess cellobiose. The remaining oligosaccharides were eluted from the column with a linear gradient of 7–15% (v/v) ethanol. The samples were pooled, concentrated by lyophilization, and an aliquot (12 µL) was loaded onto TLC plates along with the standards. Of the three solvent systems employed, the best separation was achieved with solvent 1 (ethyl acetate:1-propanol:2-propanol:water in a ratio of 8:5:1:1 [v/v]). The amount of water in the solvent was also varied to vary partitioning coefficients of various sugars, but it had an adverse effect on separation. The results of TLC analysis of oligosaccharides are shown in Fig. 1. As shown, the synthesis of gentiobiose, β(1→6) disaccharide of glucose, as well as a number of other oligosaccharides of higher DPs was observed. Analysis of the reaction products with HPLC

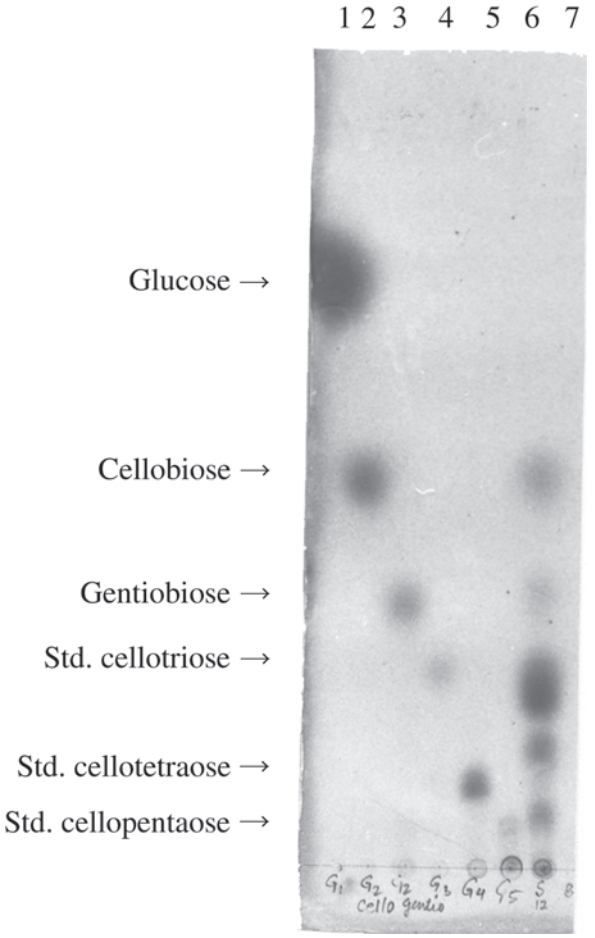

Fig. 1. TLC analysis of reaction products obtained with cellobiose as initial substrate using ethyl acetate:1-propanol:2-propanol:water solvent in a ratio of 8:5:1:1 (v/v). Glucose and excess cellobiose were previously removed by passage over a charcoal celite column. *Lane 1*, glucose; *lane 2*, cellobiose; *lane 3*, gentiobiose; *lane 4*, cellobiose; *lane 5*, cellotetraose; *lane 6*, cellopentaose; *lane 7*, 12 µL of reaction products.

gave an oligosaccharide concentration of 57.3 mmol/L. About 50% of the initial cellobiose was left unutilized.

Reports have appeared on the synthesis of oligosaccharides by other microbial systems but these have been up to a chain length of only three *(35)*. In this regard, the ability of *Bgl*II to synthesize higher oligosaccharides (DP > 3) is noteworthy. The function of this enzyme in the native *P. etchellsii* is not known. Since many of the low molecular weight oligosaccharides act as cellulase inducers *(36)*, it is possible that association of this yeast with cellulose degraders in nature could be of advantage for induction of cellulases. In a similar fashion, sophorose and its isomers have been reported to induce synthesis of cellulase in *Aspergillus terreus (37)* and *Trichoderma* sp. *(38)*.

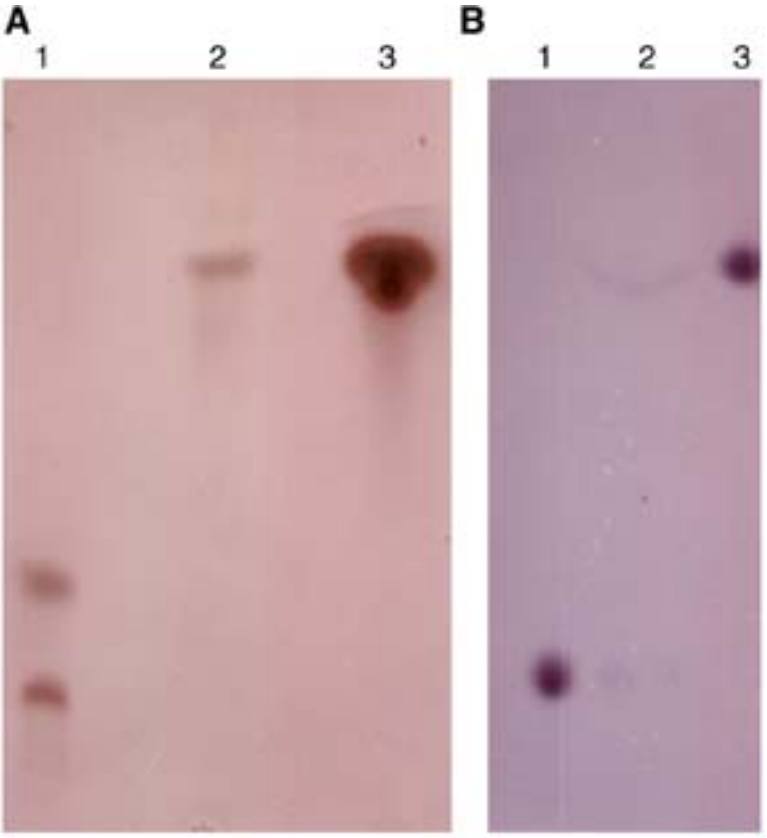

Fig. 2. **(A)** TLC analysis of ethyl acetate-extracted alkyl glucoside using ethyl acetate:2-propanol:distilled water solvent system in a ratio of 40:30:10 (v/v). *Lane 1*, standard glucose and cellobiose; *lane 2*, ethyl acetate-extracted octylglucoside from reaction mixture; *lane 3*, standard octylglucoside dissolved in ethyl acetate. **(B)** TLC analysis of ethyl acetate-extracted alkyl glucosides using same solvent system as in (A). *Lane 1*, standard cellobiose; *lane 2*, ethyl acetate-extracted decylglucoside from reaction mixture; *lane 3*, standard decylglucoside dissolved in ethyl acetate.

Synthesis of Alkyl and Terpenyl Glucosides

The synthesis of various alkyl glucosides, such as hexyl-, heptyl-, octyl-, and decylglucosides, was performed using purified *Bgl*II. The results of octyl- and decylglucoside synthesis are shown in Fig. 2A and 2B, respectively. The ethyl acetate-extracted octylglucoside appeared as a clear band on the TLC plate (Fig. 2A, *lane 2*) as identified from migration of standard octyl-β-D-glucoside (Fig. 2A, *lane 3*). Similarly, decylglucoside was identified (Fig. 2B, *lane 2*) from the migration of the standard decyl-β-D-glucoside (Fig. 2B, *lane 3*). Synthesis of hexyl- and octylglucosides was also performed using glucose as an acceptor, but low yields were obtained (data not shown). Extraction of these glucosides with ethyl acetate led to their concentration and purification and eliminated largely unreacted cellobiose from the

reaction mixture. This indicated the feasibility of using this enzyme for biosynthesis of higher-chain alkyl glucosides, which are otherwise difficult to synthesize. Synthesis of alkyl glucosides having up to eight carbons (i.e., octylglucosides) has been reported, primarily using almond enzyme in reverse hydrolysis mode (for a review, *see* refs. *7*). Thus, the synthesis of higher alkyl glucosides (i.e., decylglucoside) reported in our study using cellobiose in the transglycosylation approach is novel.

Biosynthesis of various monoterpenyl glucosides (namely, neryl, geranyl, and citronellylglucosides) was also carried out with cellobiose, and the products were resolved by TLC (data not shown). No standards were commercially available for these compounds and, therefore, their exact identification was difficult. However, the R_f values (0.85) were in agreement with the values reported for these compounds by Gunata et al. *(29)*. Interestingly, the synthesis of decyl- and monoterpenyl glucosides was negligible when glucose was used as the glucosyl donor.

Separation of Oligosaccharides

In an attempt to develop a simple method for separation of oligosaccharides synthesized through transglycosylation activity of β-glucosidase, Sephadex G-50 and Bio-Gel P-2 were evaluated with cellulose hydrolysates prepared by hydrolyzing Avicel cellulose. About 15 mg of reducing sugars was loaded onto these columns for separation. Bio-Gel P-2 was found to give better resolution of the cellooligosaccharides. Accordingly, the enzyme-catalyzed synthesis reaction mixture was loaded onto a Bio-Gel P-2 column (55 × 1.25 cm) and elution performed with Milli-Q water at a flow rate of 15 mL/h. The eluted fractions were analyzed by TLC, and the results are shown in Fig. 3. As observed, the earlier samples (13–25) eluting from the column contained higher oligosaccharides (DP > 3), and the latter samples also contained cellobiose and glucose, which appeared from fraction 77 onward. In an attempt to separate these higher oligosaccharides, samples (27–47) were pooled and loaded onto a freshly packed charcoal celite column and eluted with a step gradient of 7% ethanol. At this ethanol concentration, only G3 eluted from the column, based on our standardized protocols. This was verified by pooling the fractions and loading on TLC. The eluted sample showed up as a single spot (Fig. 4, *lane 1*) and was confirmed to be pure when matched against the standard cellotriose (Fig. 4, *lane 2*). Fractions (18–21) from Bio-Gel P-2 (Fig. 3) were also loaded onto a celite column and eluted with 15% ethanol, but in this case cellooligosaccharides higher than DP 3 coeluted. The mixture of these sugars (obtained from charcoal celite with 15% ethanol elution) was again loaded onto a Bio-Gel P-2 column. The eluted fractions were analyzed by TLC and the results are shown in Fig. 5. As clearly seen, the components of higher DP were separated and tentatively assigned as being higher oligomers. Samples were scraped off the TLC plates, dissolved in the solvent, again run to check purity, and found to be pure. Although the exact linkage between the different saccharide units could not be established, except for

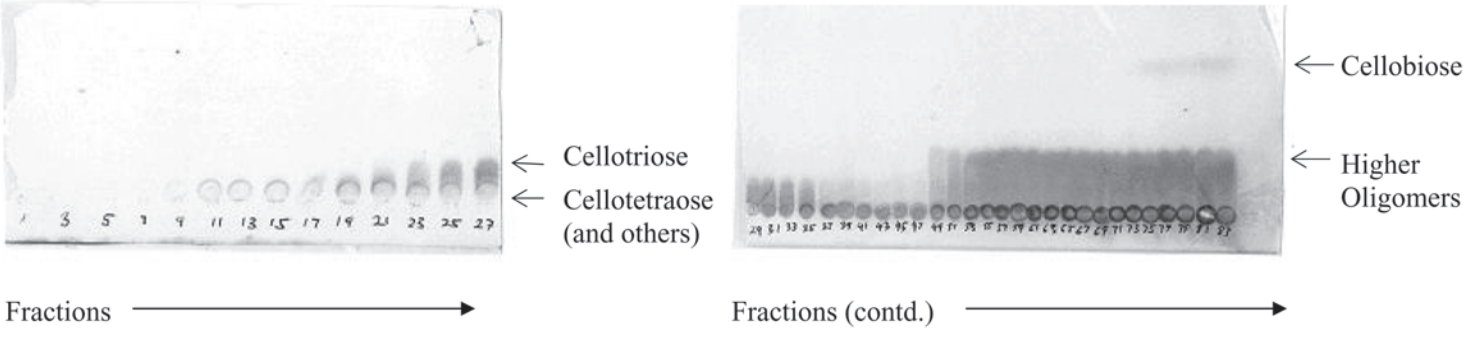

Fig. 3. TLC analysis of enzymatically synthesized oligosaccharides that were separated on Bio-Gel P-2 column. Fractions (as indicated) were collected, and a sample aliquot (12 µL) from the fractions indicated by the numbers given below was analyzed. Separation and detection were done as described for Fig. 1.

Fig. 4. TLC analysis of cellotriose eluted from charcoal celite column with step gradient of 7% ethanol. *Lane 1*, cellotriose from column; *lane 2*, standard cellotriose.

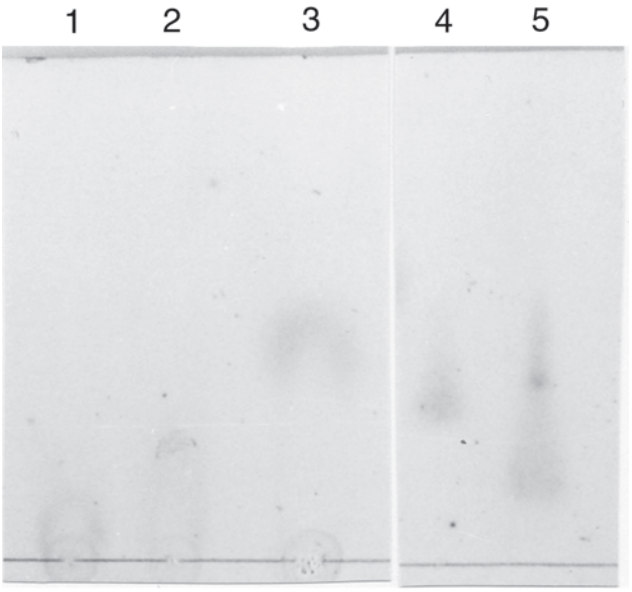

Fig. 5. TLC analysis of higher oligosaccharides eluted from charcoal celite followed by Bio-Gel P-2 gel filtration chromatography, *Lane 1*, eluted oligosaccharide (dp ?); *lane 2*, eluted cellopentaose; *lane 3*, eluted purified oligomer (dp ?); *lane 4*, standard cellotetraose; *lane 5*, standard cellopentaose.

cellotriose, the results suggested that the compounds of higher DP had linkages other than the β(1→4) type.

The work presented herein indicates the feasibility of synthesizing such cellooligosaccharide derivatives of higher chain length using an enzymatic method. A number of good chemical approaches have been reported, but these require blocking and deblocking of the hydroxyl groups of α-D-glucose derivative for regioselective construction of β-(1→4)-glycosidic bonds. In addition, complete stereocontrol of the bond formation has not been achieved. The use of enzymes in such reactions leads to biosynthesis of selected cellooligosaccharides owing to the regiospecificity of the enzymes. The combination of gel filtration and charcoal celite chromatography in a simple two-step method resulted in separation to homogeneity of the synthesized compounds.

Conclusion

The enzymatic synthesis of cellooligosaccharides, alkyl and terpene glucosides was demonstrated in this work. The cellooligosaccharides were separated to homogeneity as shown by TLC analysis, through a combination of gel filtration and partitioning matrix such as charcoal celite. The method allowed a good stepwise elution of sugars from the column. As the polymerization increased, the differences in the physical properties of the neutral oligosaccharides continued to decrease, and, thus, other methods such as derivatization to impart some different property such as charge or affinity can be employed to ease separation. The structure of these oligosaccharides can be determined by nuclear magnetic resonance, which will help in understanding the kind of linkages and transfer reactions, whether kinetically or equilibrium controlled, catalyzed by the enzyme. The ability of the enzyme to synthesize a number of oligosaccharides makes this enzyme very useful for both commercial and academic purposes. This is one of the very few enzyme systems for which synthesis of such a wide variety of glycoconjugates has been reported.

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