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Characterization of Oligocellulose Synthesized by Reverse Phosphorolysis Using Different Cellodextrin Phosphorylases

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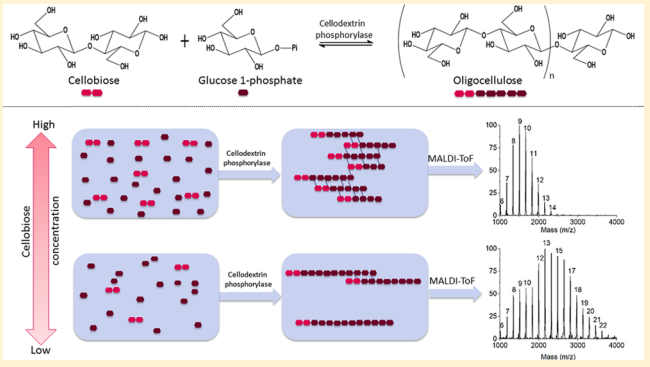
Supporting Information

ABSTRACT: Much progress was made in the straightforward and eco-friendly enzymatic synthesis of shorter cellulose chains (oligocellulose). Here, we report the determination of a molar mass distribution of the oligocellulose synthesized from cellobiose (CB) and α-glucose 1-phosphate by reverse phosphorolysis, using enzymes cellodextrin phosphorylase from *Clostridium stercorarium* or *Clostridium thermocellum* as catalyst. The oligocellulose molar mass distribution was analyzed using three different methods: ^{1}H NMR spectroscopy, matrix assisted laser desorption/ionization−time-of-flight mass spectrometry (MALDI-ToF MS) and size exclusion chromatography (SEC). The molar mass distribution of the synthesized oligocellulose was only dependent on the concentration of cellobiose used in the reaction. Data obtained from MALDI-ToF MS and SEC were almost identical and showed that oligocellulose synthesized using 10 mM CB has an average degree of polymerization (DP_n) of ∼7, while a DP_n of ∼14 was achieved when 0.2 mM CB was used in the reaction. Because of solvent limitation in SEC analysis, MALDI-ToF MS was shown to be the technique of choice for accurate, easy and fast oligocellulose molar mass distribution determination.



Extensive work of cellulose synthesis on the laboratory scale has been carried out over the past 30 years.[1] Cellulose synthesis using organic chemistry pathways is a challenging process and faces many difficulties concerning regio- and stereoselectivity. In this way, cellulose with a degree of polymerization (DP) of around 20 was synthesized.[2,3] To overcome these problems in the synthesis of shorter cellulose chains (oligocellulose) enzyme catalyzed synthesis was shown to be the approach of choice.[4−6] In the first approaches of enzymatic cellulose synthesis, enzyme cellulase was used as catalyst in the transglycosylation reaction of β-cellobiosyl fluoride. Using this method cellulose with a DP_n of around 22 was synthesized.[7,8] Kitaoka et al. reported the synthesis of cellulose with DP over 100 from cellobiose with a cellulase/surfactant complex in a nonaqueous LiCl/DMAc medium.[9]

Previously it was shown that the enzyme cellodextrin phosphorylase (CdP) can also be utilized for the synthesis of oligocellulose in aqueous solution via reverse phosphorolysis.[10−12] CdP belongs to the inverting glycoside hydrolase family 94 (GH94) and it reversibly catalyzes the conversion of cellodextrins to α-D-glucose 1-phosphate (G1P) and cellodextrins with reduced chain length.[13,14] Using appropriate conditions the reaction can be shifted from degradation toward synthesis of cellulose. In this approach natural substrates were used: G1P as a Glc donor and Glc or cellobiose (CB) as a Glc acceptor. However, it should be emphasized that Glc was shown to be a very poor acceptor for CdP, and was used as an acceptor only in the synthesis of crystalline oligocellulose.[11,14] Oligocellulose synthesis with CdP as a catalyst has an advantage over the other biosynthetic approaches, since natural substrates are used and the synthesis reaction is performed in a nontoxic environment. Besides the straightforward and eco-friendly synthesis, it is also of great importance to find a good analytical approach for the characterization of such obtained oligocellulose products. Several techniques can be used for the determination of the oligocellulose molar mass distribution, such as ^{1}H NMR, size-exclusion chromatography (SEC), or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS).[15] The average DP of low molar mass cellulose can be determined via ^{1}H NMR, as reported before.[16] SEC is probably the most commonly used method for the analysis and characterization of poly- and oligosaccharides.[17−20] However, in the case of cellulose, reliability of the results achieved by SEC is still under question.[21] MALDI-ToF MS was also shown to be a very powerful tool for characterization of molecular structures of oligosaccharides.[20,22−24]

In the present study, characterization of the oligocelluloses synthesized using CB and G1P as substrates, and cellodextrin phosphorylases from *Clostridium stercorarium* (CsCdP) or *Clostridium thermocellum* (CtCdP), respectively, as catalysts is

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presented. ^{1}H NMR, SEC, and MALDI-ToF MS were employed to extract information on molar mass distributions of the synthesized oligocelluloses. We compared the results obtained with each of the methods as well as the results when CdP from two different bacterial sources were used for the oligocellulose synthesis. Furthermore, we investigated the influence of the primer (cellobiose) concentration on the molar mass distribution of the synthesized oligocelluloses.

EXPERIMENTAL SECTION

Materials. α-D-Glucose 1-phosphate, cellobiose, and poly(ethylene glycol)bis(amine), M_w = 3000 Da, were purchased from Sigma-Aldrich. Maltoheptaose was obtained after the acid catalyzed hydrolysis of β-cyclodextrin in a yield of 10%. Residual β-cyclodextrin was removed as a *p*-xylene/β-cyclodextrin complex, as previously described in details.[25]

Expression, Purification, and Assay of CsCdP and CtCdP. Expression of CsCdP and CtCdP was performed as described before.[14,26] Briefly, *Escherichia coli* BL21 RIL harboring plasmid pTrc99a-CsCdP and *E. coli* BL21-Gold(DE3) harboring plasmid pET28a-CtCdP cultures were grown in Luria−Bertani medium, containing 0.5 mg/mL ampicillin at 37 °C, and 75 μg/mL kanamycin at 22.5 °C, respectively, with continuous shaking at 200 rpm. Enzyme expression was induced with 0.01 mM IPTG and 0.1 mM IPTG, for the CsCdP and CtCdP *E. coli* strains, respectively, when A_{600} reached 0.65. The cells were harvested by centrifugation after 4 h of expression at 37 °C for *E. coli* harboring CsCdP, or overnight expression at 22.5 °C for *E. coli* harboring CtCdP. Harvested cells were resuspended in 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.5.

Resuspended cells were disrupted using French press, 25 kpsi pressure was applied. Subsequently the crude extracts were ultracentrifuged at 40.000 rpm and 4 °C for 45 min. Afterward the supernatants were then applied on a Ni-NTA gravity-flow column (Qiagen). The His-tagged CsCDP and CtCdP were eluted in stepwise manner with a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 100, 200, or 300 mM imidazole, respectively. Subsequently, fractions containing CsCdP or CtCdP were applied on 50 MWCO concentrators (Millipore) and buffer was exchanged for 100 mM MES buffer, pH 6.4 for CsCdP sample, and 100 mM HEPES buffer, pH 7.5 for CtCdP sample. The purity of the enzymes was checked by 4−20% SDS-PAGE, using the SeeBlue Pre-Stained Standard (Invitrogen) as the molecular weight standard.

Activity tests were performed with 200 mM G1P-disodium salt and 10 mM cellobiose, in 100 mM MES buffer, pH 6.5 at 45 °C for CsCdP, and in 100 mM HEPES buffer, pH 7.5 at 45 °C for CtCdP. The final pH of the reaction mixture was 6.6 and 7.6 performed in 100 mM MES and 100 mM HEPES buffer, respectively. The samples were inactivated for 5 min at 95 °C prior to determination of the amount of orthophosphate by modified Fiske and Subbarow method.[27,28] One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate in 1 min under these conditions.

In Vitro Synthesis of Oligocellulose Using CsCdP or CtCdP. The purified CsCdP (0.1 IU) or CtCdP (0.1 IU) were incubated for 72 h at 45 °C in 100 mM MES buffer, pH 6.4 or 100 mM HEPES buffer, pH 7.5, respectively, containing 200, 100, 20, or 4 mM G1P-disodium salt and 10, 5, 1, or 0.2 mM CB, respectively. The final pH was in the range of 6.4−6.5 and 7.5−7.6 for the reaction mixtures performed in 100 mM MES and 100 mM HEPES buffer, respectively. After incubation, the reaction products were collected by centrifuging at 16.000 g for 10 min and subsequently washed with distilled water followed by centrifugation several times. Washed products were frozen in liquid nitrogen and freeze-dried overnight. The temperature of the cold trap was −45 °C and the pressure was 1 Pa.

Instruments. The ^{1}H NMR spectra were recorded on a 400 MHz Varian VXR apparatus applying 128 scan cycles and using 4% (w/w) NaOD−D_2O as the solvent. The concentrations of the samples were 15 mg/mL.

All ^{1}H NMR spectra were analyzed using MestReNova program (mestrelab.com). Baseline correction (Bernstein polynomial fit, order 3), automatic phase correction, smoothing (moving average filter, span 2) and calibration using solvent as an internal standard were applied to all spectra. Subsequently integration was applied.

The average DP was calculated using the next equation

$$DP_n = \frac{(H\alpha + H\beta + H1)}{(H\alpha + H\beta)} \quad (1)$$

where Hα, Hβ, and H1 represents integrals of alpha-anomeric, beta-anomeric, and proton on C1 position in oligocellulose, respectively. The number-average molecular weight ($\bar{M}_n$) was calculated via equation

$$\bar{M}_n = DP_n M_o + 18 \quad (2)$$

where M_o is the molecular weight of dehydrated Glc (in cellulose).

The MALDI-ToF spectra were recorded on a Voyager DE-PRO MALDI-TOF instrument (Applied Biosystems). Aqueous suspension of product (0.5 μL), concentration 2.5 mg/mL was spotted on a MALDI target and mixed immediately with 1 μL of 10 mg/mL 2,5-dihydroxybenzoic acid (Sigma-Aldrich) in 50% acetonitrile/0.1% (v/v) trifluoroacetic acid. Low laser intensity was applied on all the samples. Spectra were calibrated externally using standard peptides with molecular weights of 2351.6, 3354.5, and 5734.5 Da. Noise filter (correlation factor of 0.9) and Gaussian smoothing (filter width of 19 points) were applied to all spectra using Data Explorer (TM) software (Applied Biosystems).

$\bar{M}_n$ and weight-average molecular weight values were calculated using the next equations

$$\bar{M}_n = \frac{\Sigma_i(N_i M_i)}{\Sigma_i N_i} \quad (3)$$

$$\bar{M}_w = \frac{\Sigma_i(N_i M_i^2)}{\Sigma_i(N_i M_i)} \quad (4)$$

where N_i represents area below the peak of the ith oligocellulose species and M_i represent the molar mass of that species. PDI then was calculated using the equation

$$PDI = \frac{\bar{M}_w}{\bar{M}_n} \quad (5)$$

SEC of the oligocelluloses, using DMSO with 0.05 M LiBr as eluent, was performed on an Agilent Technologies 1260 Infinity from PSS (Mainz, Germany) consisting of an isocratic pump, auto sampler without temperature regulation, an online degasser, an inline 0.2 μm filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz), and multiangle laser light scattering detector (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS,

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Mainz) was used for data processing. The samples were injected with a flow rate of 0.5 mL/min into a PFG guard-column and three PFG SEC columns 100, 300, and 4000, purchased from PSS. The columns were held at 80 °C and the detectors were held at 60 °C (Visco) and 45 °C (RI). A standard pullulan kit (PSS, Mainz, Germany) with molecular weights from 342 to 805 000 Da was used to generate a universal calibration curve. The values obtained in this work are the $\bar{M}_w$, $\bar{M}_n$, and PDI.

Oligocellulose samples were dissolved in DMSO with 0.05 M LiBr (HPLC grade, ≥ 99.7%) at a concentration of 2 g/L and mixed overnight at 45 °C with 600 rpm. Samples were filtered through 0.45 μm filters after shaking. Standards were dissolved in the same eluent at room temperature at 2 g/L concentration.

RESULTS AND DISCUSSION

Synthesis and Analysis of Oligocellulose by ^{1}H NMR, MALDI-ToF, and SEC. CsCdP and CtCdP enzymes were chosen for oligocellulose synthesis as their biochemical background has already been studied quite extensively.[10,14,26,29,30] In addition these two enzymes are far away from each other on the phylogenetic tree of GH94 family.[14] They share only ∼25% amino acid sequence identity as calculated using Kalign web server for multiple sequence alignment.[31] If the molar mass distributions of the synthesized oligocellulose are dependent on the different cellodextrin phosphorylases, these two enzymes would be a good choice to prove that.

Oligocellulose was synthesized from CB and G1P, the natural substrates for the CdP enzymes, and two reactions were set. In the first reaction catalyst was CsCdP enzyme and in the second CtCdP enzyme. The reactions catalyzed by these enzymes are reversible, therefore the ratio of G1P (200 mM) compared to CB (10 mM) was kept high enough to ensure a shift of the equilibrium of the reaction toward oligocellulose synthesis. As the reactions proceeded, a white precipitation of the product was observed in both reactions.

We have separated insoluble and soluble fractions of the reactions. The insoluble fractions were first washed several times with water. Then the insoluble and soluble fractions were freeze-dried, dissolved in 4% (w/w) NaOD−D_2O and subsequently analyzed by ^{1}H NMR.

^{1}H NMR spectra of the insoluble and the soluble fractions revealed different structures in these two parts. ^{1}H NMR spectra of the insoluble fraction from both reactions, catalyzed by CsCdP or CtCdP, did not differ and showed signals corresponding to those ascribed to protons of oligocellulose.[11] ^{1}H NMR spectra of the soluble fractions were also the same when CsCdP or CtCdP were used as catalysts. They showed proton signals that correspond only to those of G1P (Figure 1). During the ^{1}H NMR analysis of the soluble fraction the presence of protons ascribed to cellobiose or oligocellulose were not detected, indicating that all glucose acceptor (CB) was elongated to the insoluble oligocellulose. Analysis of the synthesized oligocellulose present in the insoluble fraction was based on comparison of the integrals Hα (δ = 5.21), Hβ (δ = 4.63), and H1 (δ = 4.38) protons. Integrals for oligocellulose synthesized using CsCdP are Hα = 1, Hβ = 2.96, and H1 = 34.51, and for oligocellulose synthesized using CtCdP are Hα = 1, Hβ = 3.25, and H1 = 35.79. DP_n of the products was calculated using eq 1, and $\bar{M}_n$ was calculated from eq 2. The calculated DP_n was 9.7 and 9.4 for oligocellulose synthesized using CsCdP and CtCdP, respectively, while $\bar{M}_n$ was 1591 and 1544 g/mol oligocellulose synthesized using CsCdP and CtCdP, respectively.

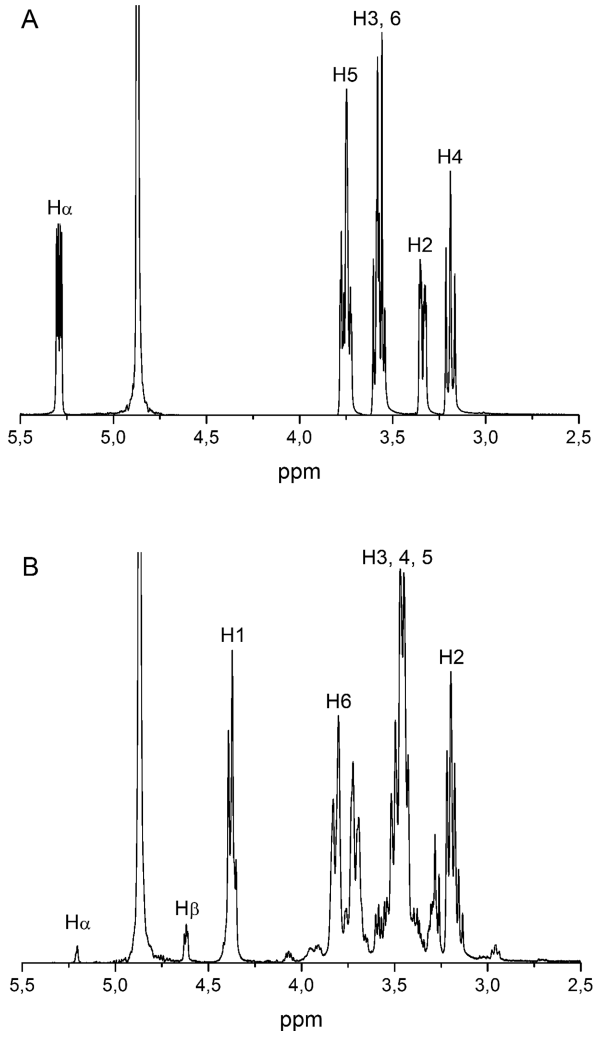

Figure 1. ^{1}H NMR spectra of soluble (A) and insoluble (B) fractions of the synthesis reaction when CtCdP was used as a catalyst. The symbols H1−H6 indicate the signals for C1−C6 protons, respectively, in oligocellulose and α-D-glucose 1-phosphate. Hα and Hβ indicate signals for the α-anomeric and β-anomeric protons, respectively.

Aqueous suspensions of the insoluble fraction of the reactions with CsCdP or CtCdP as catalysts were made in concentration of 2.5 mg/mL. Samples prepared on this way were directly spotted on the MALDI-ToF target and analyzed. In Figure 2 are shown MALDI-ToF MS spectra of the insoluble fraction of the reactions. In both spectra, peaks with a peak-to-peak mass difference of 162.2 Da (one glucosyl unit) were observed. The masses range from 851.8 to 1500 Da corresponding to individual oligocellulose containing 5 to 9 Glc units. The number of Glc units in an individual oligocellulose chain was calculated using the following formula [n × 162.2 (dehydrated Glc) + 18 (reducing end) + 23 (Na^+)], where n represents the number of Glc units in an individual oligocellulose chain. Determination of the molar mass distribution of the synthesized products is based on the assumption that the area below individual oligocellulose peaks in the MALDI-ToF MS spectrum is proportional to its molar ratio in the mixture. On the basis of this assumption, the molar ratio was calculated for each individual oligocellulose

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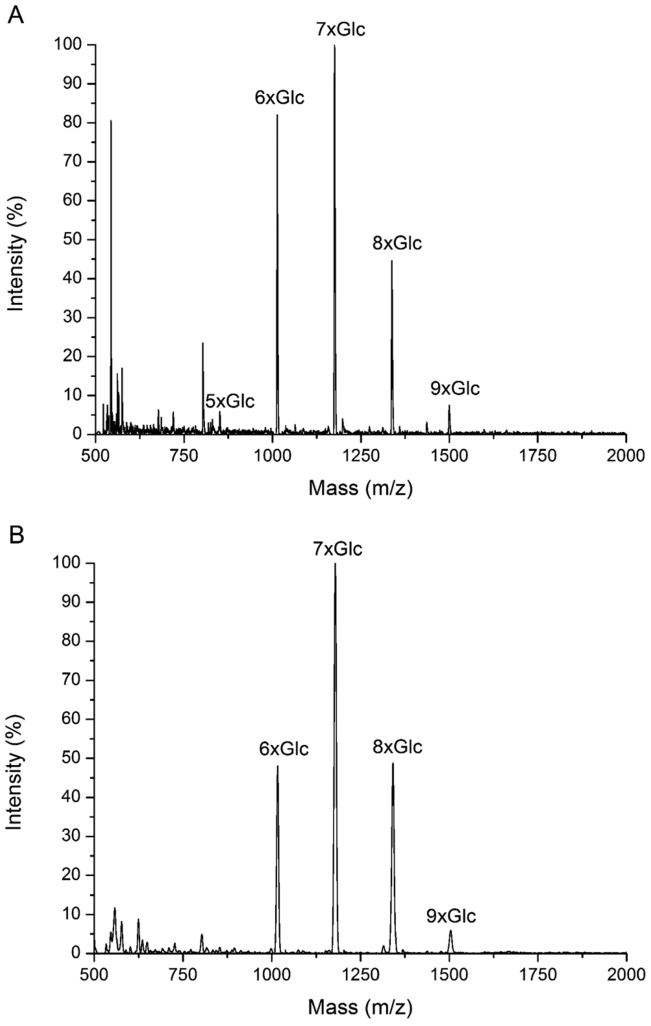

Figure 2. MALDI-ToF spectra of insoluble fractions of the reactions when CsCdP (A) or CtCdP (B) was used as catalyst. Individual oligocellulose with peak-to-peak mass difference of 162.15 Da are indicated (in terms of Glc units).

synthesized using CsCdP or CtCdP. When the molar ratio (represents N_i in the eqs 3 and 4) and the molecular weight (represents M_i in the eqs 3 and 4) for individual oligocellulose is known, then $\bar{M}_n$, $\bar{M}_w$, and PDI of the synthesized oligocellulose were calculated using the eqs 3, 4, and 5, respectively. Values determined by MALDI-ToF MS differ significantly from the ^{1}H NMR results and were almost identical for oligocellulose synthesized using CsCdP or CtCdP. For CsCdP synthesized oligocellulose DP_n was calculated to be 6.9, while $\bar{M}_n$ = 1136 g/mol, $\bar{M}_w$ = 1151 g/mol and PDI = 1.01, whereas for CtCdP synthesized oligocellulose DP_n was calculated to be 7.1, and $\bar{M}_n$ = 1165 g/mol, $\bar{M}_w$ = 1178 g/mol, and PDI = 1.01.

Using MALDI-ToF MS we could not detect any cellobiose or oligocellulose in the soluble part of both reactions.

It is well-known that numerous inter- and intramolecular hydrogen bonds in cellulose result in the difficulty to dissolve it in aqueous and most common organic solvents.[1,32] Several aqueous and nonaqueous cellulose solvents were developed in the past, such as 10% NaOH, *N*,*N*-dimethylacetamide/LiBr, or DMSO/tryethylamine/SO_2,[33] as well as recently some ionic liquids.[32] However, we dissolved the synthesized oligocelluloses (2 mg/mL) in pure DMSO or DMSO/H_2O (w/w 75:25), while precipitation was not observed until the DMSO/H_2O ratio was 50:50 (w/w) (Figure 3). Solubility of oligocellulose with DP_n of ∼8 in pure DMSO was also mentioned before.[10] The oligocelluloses dissolved in DMSO were then analyzed by SEC with multi detection. In the Figure 4 the elution profiles of the synthesized oligocelluloses from both reactions are shown. The synthesized oligocelluloses were detected by viscometer and refractometer. The molecular mass was too low to be detected by the light scattering detector. Pullulan with molecular weights from 342 to 805 000 Da were used as standards. The exact refractive increment index d*n*/d*c* is not known for cellulose. Therefore, we used the advantage of knowing exact injected mass of the samples to calculate molar mass distribution.[34] Calculations were performed using universal calibration with data from both viscometer and refractometer. Conventional calibration was performed when only data from the refractometer were used in the calculation (Supporting Figure 2.). Results for $\bar{M}_n$, $\bar{M}_w$, DP_n, and PDI obtained from SEC for the oligocellulose, synthesized when CsCdP or CtCdP was used as catalyst, are presented in Table 1. Additionally the soluble fractions from both reactions were dissolved in water and also analyzed by SEC, however no masses above 400 Da were detected.

Values for $\bar{M}_n$, $\bar{M}_w$, DP_n, and PDI of the synthesized oligocellulose obtained by ^{1}H NMR, MALDI-ToF, and SEC are summarized in Table 2. The results obtained by MALDI-ToF and SEC were not fully congruent with the ^{1}H NMR results. While MALDI-ToF and SEC results differ among each other for less than 10%, they differ from ^{1}H NMR results for 30−60%. Because of the low intensity of the peaks belonging to the anomeric protons in ^{1}H NMR spectroscopy, significant errors can be made with peaks integrations and consequently calculations of DP_n. The longer cellulose chains are the ratio

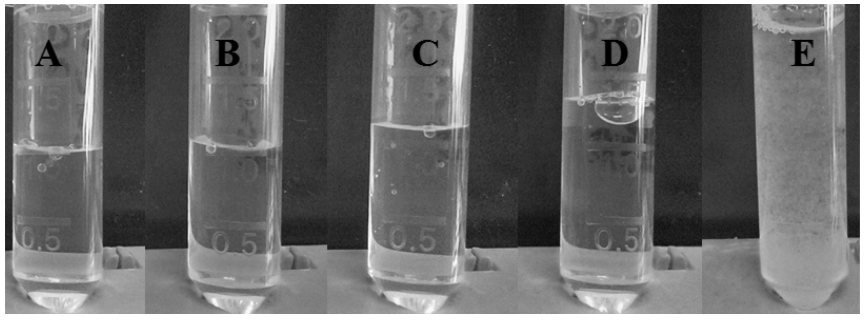

Figure 3. Solubility of oligocellulose in DMSO/H_2O system. Ten mg/mL of oligocellulose $DP_n \approx 7$ in DMSO (A), 5% H_2O in DMSO (B), 10% H_2O in DMSO (C), 25% H_2O in DMSO (D), and DMSO/H_2O (w/w 50:50) (E).

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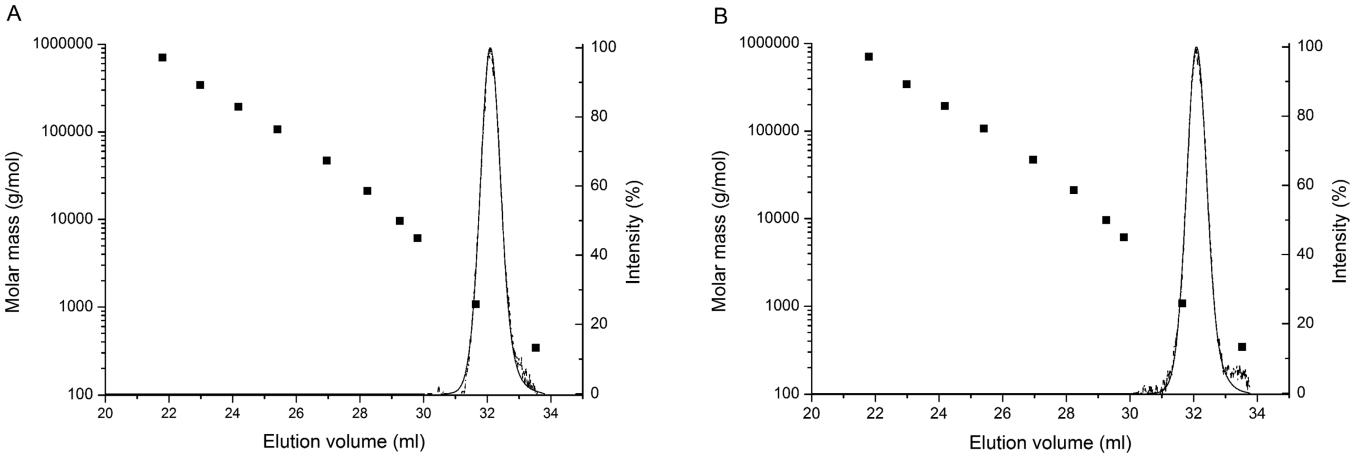

Figure 4. Elution profiles of the insoluble fractions of the reactions when CsCdP (A) or CtCdP (B) was used as a catalyst. Products were detected by viscometer (solid line) and refractometer (dashed line). Pullulan standards are also shown indicating their molar mass and elution volume (squares).

Table 1. $\bar{M}_n$, $\bar{M}_w$, DP_n, and PDI Values of Synthesized Oligocellulose Using 10 mM CB Determined by SEC[a]

	$\bar{M}_n$ (g/mol)	$\bar{M}_w$ (g/mol)	DP_n	PDI
CsCdP, universal cal.	1000	1098	6.1	1.1
CsCdP, conventional cal.	1140	1207	6.9	1.06
CtCdP, universal cal.	1022	1108	6.2	1.08
CtCdP, conventional cal.	1158	1217	7	1.05

[a]Values are obtained using universal calibration and conventional calibration.

between anomeric peaks and H1 peak becomes higher, and therefore errors made during anomeric peaks integrations bigger.

Results obtained from ^{1}H NMR confirmed that the insoluble fraction from both synthesis reactions corresponds only to oligocellulose. DP_n of ∼7 was determined by MALDI-ToF MS and SEC. MALDI-ToF MS data revealed that insoluble fractions of both reactions consist mostly of the individual oligocellulose having DP of 6, 7, or 8 (Figure 2). It means that as soon as cellohexaose or celloheptaose are produced precipitation is initiated and subsequently they are hardly or not at all accessible for further elongation. This indicates that both enzymes although far away from each other on the phylogenetic tree of GH94 family and sharing only ∼25% amino acid sequence identity have the same enzymatic mechanism in which occurs a complete dissociation between the enzyme and the product after each addition of a glucose moiety.

Additionally the effect of the primer concentration (CB) on the molar mass distribution of the synthesized oligocellulose was also investigated. We characterized oligocellulose synthesized from 0.2, 1, or 5 mM CB and 4, 20, or 100 mM G1P respectively, using CtCdP enzyme as catalyst. The CB: G1P ratio at the beginning of all three reactions was 1:20 to ensure oligocellulose synthesis direction. As the reactions proceeded in all three solutions a white precipitation of the oligocellulose product was observed. After the reactions the insoluble fractions were separated, washed and freeze-dried.

Products from all three reactions were analyzed by MALDI-ToF MS. In spectra from all three reactions, peaks with peak-to-peak mass difference of 162 Da were observed. Figure 5 shows that the mass range differs in the reactions, and increases with the decrease of primer concentration used in the reaction. The highest mass range was observed in the reaction with 0.2 mM CB, from 1014 to 3770 Da, corresponding to oligocellulose DP from 6 to 23. The lowest mass range was observed in the reaction with 5 mM CB, from 1014 to 2311 Da. We used MALDI-TOF MS data to calculate $\bar{M}_n$, $\bar{M}_w$, and PDI of the products (Figure 6 and Table 3). All three values increase as lower primer concentration is used in the reaction. Lower primer concentration results in the lower concentration of the synthesized oligocellulose, and therefore reduced chances for creation of intermolecular hydrogen bond interactions and less intense precipitation. This effect enables partial solubility of individual oligocellulose with DP > 7 and its further elongation. This can explain why a decrease in CB concentration from 10 mM to 0.2 mM doubles the $\bar{M}_n$ and the $\bar{M}_w$ values of the synthesized oligocellulose.

The issue of the higher mass polymer fragmentation during MALDI-ToF measurements was reported before.[35,36] To examine possible fragmentation of the oligocellulose we recorded MALDI-ToF spectra of 2.5 mg/mL aqueous solution of maltoheptaose and 2.5 mg/mL aqueous suspension of

Table 2. Molar Mass Distribution of the Synthesized Oligocellulose Determined by ^{1}H NMR Spectroscopy, MALDI-ToF, and SEC[a]

	CsCdP					CtCdP			
	$\bar{M}_n$ (g/mol)	$\bar{M}_w$ (g/mol)	DP_n	PDI		$\bar{M}_n$ (g/mol)	$\bar{M}_w$ (g/mol)	DP_n	PDI
^{1}H NMR	1591		9.7		^{1}H NMR	1544		9.4	
MALDI-ToF	1136	1151	6.9	1.01	MALDI-ToF	1165	1179	7.1	1.01
SEC, universal cal.	1000	1098	6.1	1.01	SEC-universal cal.	1022	1108	6.2	1.08
SEC, conventional cal.	1140	1207	6.9	1.06	SEC-conventional cal.	1158	1217	7.1	1.05

[a]Data are presented for oligocellulose synthesized using 10 mM CB when CsCdP (left) or CtCdP (right) was used as catalyst.

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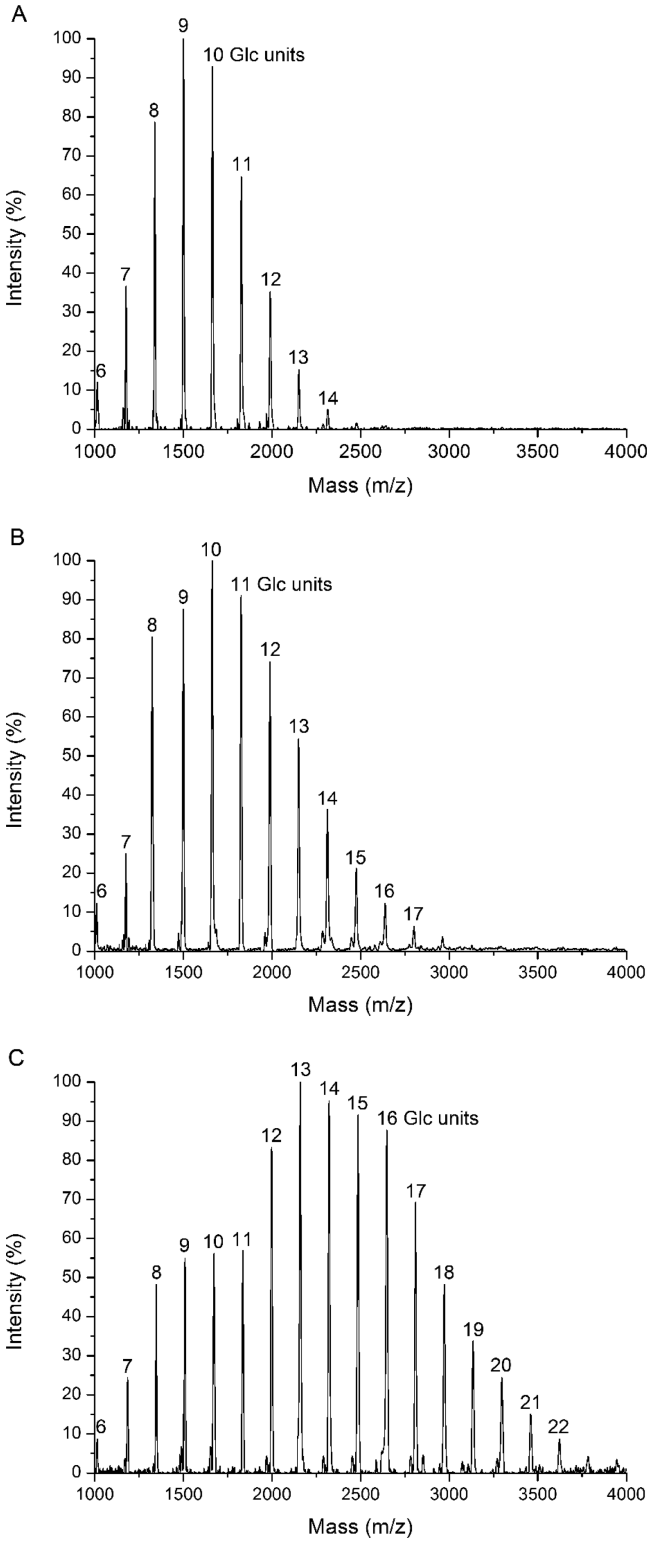

Figure 5. MALDI-ToF spectra of the synthesized oligocellulose using 5 (A), 1 (B), and 0.2 mM (C) CB when CtCdP was used as a catalyst. Individual oligocellulose with peak-to-peak mass difference of 162.15 Da are indicated (in terms of Glc units).

commercially available poly(ethylene glycol) bis(amine), Mw = 3.000 Da (Sigma-Aldrich), using the same instrument parameters as for oligocellulose measurements. Maltoheptaose was chosen as a standard as it differs from oligocellulose only in the configuration of the glycosidic bonds. In the MALDI-ToF

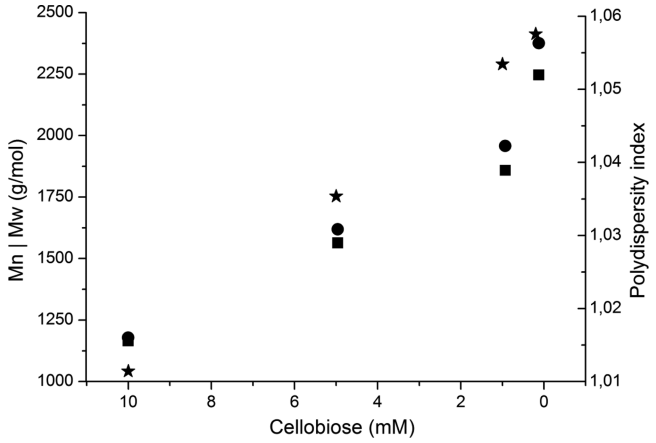

Figure 6. $\bar{M}_n$ (squares), $\bar{M}_w$ (circles), and PDI (stars) of the synthesized oligocelluloses versus CB concentration used in the synthesis reactions with CtCdP as a catalyst. The values were determined by MALDI-ToF MS.

Table 3. Molar Mass Distribution of the Oligocellulose Synthesized Using Different CB Concentrations[a]

CB concentration (mM)	$\bar{M}_n$ (g/mol)	$\bar{M}_w$ (g/mol)	DP_n	PDI
10	1165	1179	7.1	1.01
5	1564	1619	9.5	1.03
1	1859	1958	11.4	1.05
0.2	2246	2376	13.7	1.06

[a]Values are determined by MALDI-ToF MS.

spectrum of maltoheptaose only one peak with the mass of 1176.5 Da is observed (Figure 7). The observed peak

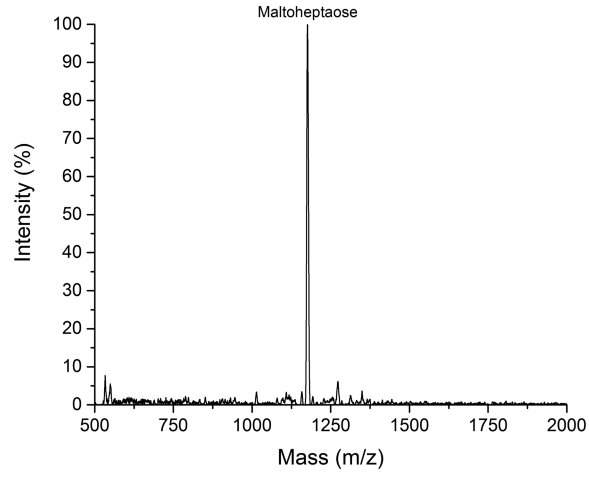

Figure 7. MALDI-ToF spectrum of maltoheptaose obtained after the acid catalyzed hydrolysis of β-cyclodextrin.

corresponds to the maltoheptaose molecule charged with Na^+ (7 × 162.2 (dehydrated Glc) + 18 (reducing end) + 23 (Na^+)). No other significant peaks on lower masses were detected suggesting absence of oligosaccharide fragmentation during MALDI-ToF measurements using the described parameters. Poly(ethylene glycol) bis(amine) contains C−C and C−O bonds, closely resembling to cellulose, and has an average molecular weight that is in the mass range of our studied cellulose oligomers. Furthermore, it appears as a suspension in aqueous solvent system used for MALDI-ToF measurements,

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similarly to the studied cellulose oligomers. As we can see from the MALDI-ToF spectrum of poly(ethylene glycol) bis(amine) (Supporting Figure 3), peaks with peak to peak mass difference of 44 Da appears around 3 kDa area. No relevant peaks on lower masses are detectable which indicates that there is no significant fragmentation of the oligomers during measurements. The observed results also indicate that the type of a mixture (solution or suspension) does not play a major role in the MALDI-ToF measurements. According to the results of these experiments we concluded that no significant fragmentation of oligocellulose happens during MALDI-ToF measurements and calculated molar mass distributions for oligocellulose are relevant.

^{1}H NMR spectra of the oligocellulose synthesized using 0.2, 1, or 5 mM CB showed only signals corresponding to those ascribed to the protons of oligocellulose. However, $\bar{M}_n$, $\bar{M}_w$, and DP_n values of the products, calculated via ^{1}H NMR differs more significantly from the values obtained by MALDI-ToF MS or SEC, as the products have a higher molecular mass. Thus, DP_n of the product obtained from the reaction with 0.2 mM CB, calculated from ^{1}H NMR is ∼20.5.

The product of the reaction with 0.2 mM CB was insoluble in DMSO with 0.05 M LiBr even when heated up to 80 °C and therefore was not analyzed by SEC. Products from the other two reactions were soluble in DMSO with 0.05 M LiBr at room temperature and analyzed by SEC. The molar mass distribution values were very similar to those determined by MADLI-ToF MS, $\bar{M}_n$ was 1416 and 1664 Da for the oligocellulose synthesized using 5 and 1 mM CB, respectively. PDI of both products was higher than the one calculated for oligocellulose synthesized with 10 mM CB, and in a very good agreement with MALDI-ToF MS data.

CONCLUSIONS

The present work deals with precise determination of the molar mass distributions of shorter celluloses and oligocelluloses. The oligocelluloses were synthesized by reverse phosphorolysis using the enzymes cellodextrin phosphorylase from *Clostridium stercorarium* or *Clostridium thermocellum* as catalyst. The three techniques that have been used in this study were shown to give different information on $\bar{M}_n$, $\bar{M}_w$, DP_n, and PDI values of synthesized oligocellulose. ^{1}H NMR spectroscopy was shown to be the least reliable method for determination of the oligocellulose molar mass distribution. According to the results presented in this work, the higher the oligocellulose molar mass is, the less accurate the determination by ^{1}H NMR is. $\bar{M}_n$, $\bar{M}_w$, DP_n, and PDI values of the synthesized oligocellulose determined by SEC and MALDI-ToF MS analyses were almost identical, proving that both of methods can be used for precise determination of the oligocellulose molar mass distribution. However, one obvious limitation of the SEC analysis is the absence of the appropriate solvent that can dissolve higher molecular mass oligocelluloses. On the other hand, we showed that using MALDI-ToF MS oligocellulose with an average molecular weight of at least 3−4 kDa ($DP_n \approx 25$) can be analyzed even as aqueous suspension, which makes sample preparations very simple and straightforward. That brings MADLI-ToF MS to the most optimal technique for the determination of the molar mass distribution of shorter celluloses. Furthermore, only a minute amount of the analyte that is required for an analysis is another advantage of this technique.

The products synthesized in the presence of the different primer concentration were shown to differ from each other with respect to their molar mass distribution. Using lower concentration of CB, it is possible to double the $\bar{M}_n$ and the $\bar{M}_w$ values of the synthesized oligocellulose.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01098.

Figures showing 4−20% SDS-PAGE of purified CtCdP and CsCdP, molar mass distribution of oligocellulose synthesized using CsCdP or CtCdP, and MALDI-ToF spectrum of commercially available poly(ethylene glycol) bis(amine) (PDF)

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Notes

The authors declare no competing financial interest.

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