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Product solubility control in cellooligosaccharide production by coupled cellobiose and cellodextrin phosphorylase

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Abstract

Soluble cellodextrins (linear β-1,4-D-gluco-oligosaccharides) have interesting applications as ingredients for human and animal nutrition. Their bottom-up synthesis from glucose is promising for bulk production, but to ensure a completely water-soluble product via degree of polymerization (DP) control (DP ≤ 6) is challenging. Here, we show biocatalytic production of cellodextrins with DP centered at 3 to 6 (~96 wt.% of total product) using coupled cellobiose and cellodextrin phosphorylase. The cascade reaction, wherein glucose was elongated sequentially from α-D-glucose 1-phosphate (αGlc1-*P*), required optimization and control at two main points. First, kinetic and thermodynamic restrictions upon αGlc1-*P* utilization (200 mM; 45°C, pH 7.0) were effectively overcome (53% → ≥90% conversion after 10 hrs of reaction) by in situ removal of the phosphate released via precipitation with Mg^{2+}. Second, the product DP was controlled by the molar ratio of glucose/αGlc1-*P* (~0.25; 50 mM glucose) used in the reaction. In optimized conversion, soluble cellodextrins in a total product concentration of 36 g/L were obtained through efficient utilization of the substrates used (glucose: 98%; αGlc1-*P*: ~80%) after 1 hr of reaction. We also showed that, by keeping the glucose concentration low (i.e., 1–10 mM; 200 mM αGlc1-*P*), the reaction was shifted completely towards insoluble product formation (DP ~9–10). In summary, this study provides the basis for an efficient and product DP-controlled biocatalytic synthesis of cellodextrins from expedient substrates.

KEYWORDS
cascade reaction, cellodextrin, degree of polymerization and solubility control, in situ product removal, phosphorylase

1 | INTRODUCTION

Cellodextrins are linear β-1,4-D-gluco-oligosaccharides. Naturally, they are comprised in cellulose polysaccharides chains. At a degree of polymerization (DP) of about 8 to 10, cellodextrins are hardly soluble in water. Shorter, soluble cellodextrins have emerging applications in human and animal nutrition. They are not digested by humans and represent useful dietary fibers (Flint, Bayer, Rincon, Lamed, & White, 2008). In livestock animals (e.g., poultry, cattle), cellodextrins are interesting feed ingredients showing potential prebiotic and health-promoting properties (Sybesma, Kort, & Lee, 2015). Cellodextrins are potential bulking

Abbreviations: αGlc1-*P*, α-D-glucose 1-phosphate; CbP, cellobiose phosphorylase (EC 2.4.1.20); CdP, cellodextrin phosphorylase (EC 2.4.1.49); DP, degree of polymerization; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose.; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

agents (Akpinar & Penner, 2008; Patel & Goyal, 2011). Applied as such or in chemically derivatized form, they could be used as cosmetics additives (Viravau & Degoule, 2013). Despite these various promising properties cellodextrins are not broadly available. Industrial processes for their bulk production are lacking.

One way to produce cellodextrins is through chemical (e.g., acid-catalyzed) or enzymatic hydrolysis of cellulose. Although attractive in principle, there are important concerns. For chemical production, the yield is moderate (≤68%; Billès, Coma, Peruch, & Grelier, 2017). Enzymatic hydrolysis gives a mixture of cellodextrins. However, cellulosic substrates are recalcitrant to enzymatic attack (Zhang & Lynd, 2004). The requirement to purify the enzymes used (e.g., endo-cellulase) from other activities (e.g., β-glucosidase) presents an additional difficulty (Yamasaki, Ibuki, & Isaka, 2012). Furthermore, isolation of the cellodextrins from hydrolysis mixtures necessitates significant efforts in the downstream processing (Zhang & Lynd, 2003). A promising approach that overcomes the technological hurdles of cellulose conversion is to produce cellodextrins through bottom-up synthesis. Since chemical synthesis involves multistep procedures, enzymatic routes are generally preferred (Billès et al., 2017).

Using hydrolytic enzyme (cellulase) in an aqueous-organic solvent, cellodextrins were synthesized to an average DP of 22 (Kobayashi, Kashiwa, Kawasaki, & Shoda, 1991). β-D-Cellobiosyl fluoride was used as the substrate. While elegant as a method, limitations on applicability for the large-scale synthesis arise. The glycosyl fluoride must be synthesized chemically. Spontaneous and enzyme-catalyzed hydrolyses of β-D-cellobiosyl fluoride are competing reactions to the cellobiosyl transfer in the synthetic process. This diminishes the product yield based on donor substrate. A non-hydrolytic enzyme (e.g., glycoside phosphorylase) that is additionally able to utilize an expedient glucosyl donor substrate would therefore represent an interesting biocatalytic system for synthesis (Desmet & Soetaert, 2012; Luley-Goedl & Nidetzky, 2010; Nakai, Kitaoka, Svensson, & Ohtsubo, 2013; Pergolizzi, Kuhaudomlarp, Kalita, & Field, 2017; Ubiparip, Beerens, Franceus, Vercauteren, & Desmet, 2018).

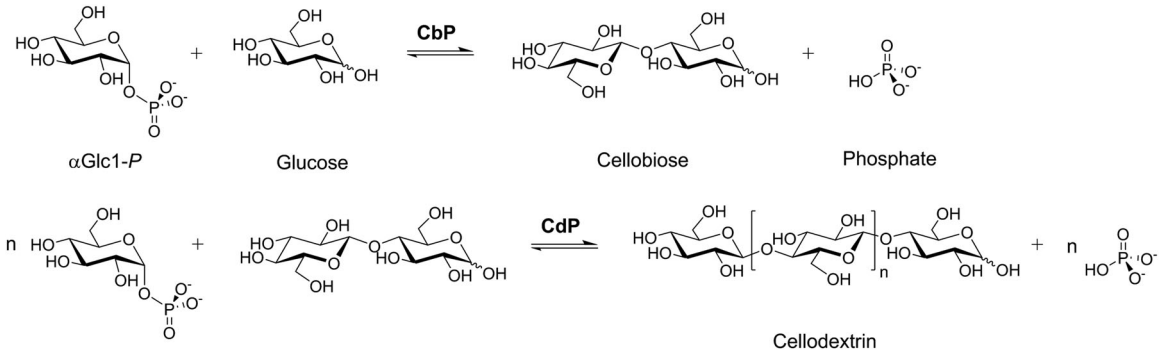
α-D-Glucose 1-phosphate (αGlc1-*P*) is a natural glucosyl donor that is promising for use in cellodextrin synthesis. Cellobiose phosphorylase (CbP; EC 2.4.1.20) catalyzes the synthesis of cellobiose from glucose. Cellodextrin phosphorylase (CdP; EC 2.4.1.49) elongates cellobiose to form cellodextrins whose DP range, hence also solubility, depends on the enzyme and the reaction conditions used (Hiraishi et al., 2009; Nakai et al., 2010; Petrovic, Kok, Woortman, Ciric, & Loos, 2015). Synthesis of cellobiose by CbP has been demonstrated (Kitaoka, Sasaki, & Taniguchi, 1992; Suzuki, Kaneda, Nakai, Kitaoka, & Taniguchi, 2009) and an industrial process for large-scale production is under implementation at Pfeifer & Langen GmbH & Co. KG (Koch, Hässler, & Kipping, 2016). In this process, αGlc1-*P* is prepared from sucrose using sucrose phosphorylase in the presence of phosphate. Parallel cascades using two phosphorylases, one producing αGlc1-*P* donor and the other producing the glucoside of interest, have been used to synthesize various disaccharides (e.g., α,α-trehalose, laminaribiose) as well as oligo- and polysaccharides (e.g., amylose, β-glucan; for reviews, see Luley-Goedl & Nidetzky, 2010; Nakai et al., 2013; Pergolizzi et al., 2017).

An innovative application of CbP and CdP for cellodextrin production is a linear cascade reaction starting from glucose, as shown in Scheme 1. Glucose is more expedient than cellobiose as the substrate for building up cellodextrins. We show here the biocatalytic production of soluble cellodextrins with DP centered at 3 to 6 using coupled CbP and CdP. In situ product removal of the phosphate released was key to overcome kinetic and thermodynamic restrictions of the overall conversion. Reaction engineering was essential to achieve DP control in the product and thus to specify its solubility/insolubility by design. In summary, this study provides the basis for an efficient and product DP-controlled biocatalytic synthesis of cellodextrins from expedient substrates. It supports the use of enzyme cascades in synthesis (for a general review, see Sperl & Sieber, 2018).

2 | MATERIALS AND METHODS

2.1 | Materials

Unless stated, chemicals were of the highest purity available from Sigma-Aldrich (Vienna, Austria) or Carl Roth (Karlsruhe, Germany).



SCHEME 1 Linear reaction cascade of cellobiose and cellodextrin phosphorylase for the synthesis of cellodextrins from glucose and αGlc1-*P*. For soluble cellodextrins (DP ≤ 6), *n* is 1 to 4

Reagent-grade cellodextrin standards with DP 2 to 6 were from CarboSynth (Compton, Berkshire, UK).

2.2 | Enzyme preparation and activity assays

The CbP from *Cellulomonas uda* (*Cu*CbP; GenBank identifier AAQ20920.1) and the CdP from *Clostridium stercorarium* (*Cs*CdP; GenBank identifier AAC45511.1) were prepared according to literature (Nidetzky, Griessler, Schwarz, & Splechtna, 2004; Tran, Desmet, De Groeve, & Soetaert, 2011). The putative CdP from *Clostridium cellulosi* (*Cc*CdP; GenBank identifier CDZ24361.1; Figures S1 and S2) was expressed in *Escherichia coli* BL21 using a self-constructed plasmid vector (pC21e1) harboring the codon-optimized gene (GenScript Biotech Corp., Piscataway, NJ) under control of a P*tac*I promoter (see the Figures S3 and S4). Enzymes were produced with N-terminal His-tag. The *E. coli* strains were grown in LB-medium (0.1 mg/ml ampicillin) at 37°C. Expression was done overnight at 25°C using isopropyl β-D-1-thiogalactopyranoside for induction (*Cu*CbP: 0.1 mM; *Cs*CdP: 0.01 mM; *Cc*CdP: 1.0 mM). Cells were harvested (5,000 rpm, 4°C, 20 min), suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.5) and disrupted by ultrasonication with a Sonic Dismembrator Model 505 (Fisher Scientific, Vienna, Austria) using the following protocol: 6 min in total, alternating 2 s pulse on/4 s pulse off at 60% amplitude. The cell extract was recovered (15,000 rpm, 4°C, 20 min) and the enzymes were purified using pre-packed (1.6 × 2.5 cm; 5 ml) HisTrap FF crude columns (GE Healthcare Europe, Vienna, Austria) on an ÄKTA prime plus system (GE Healthcare Europe). His-tagged proteins were eluted with imidazole (0.01–0.3 M). The purified proteins were desalted using Vivaspin Turbo 30 kDa cut-off concentrator tubes (Sartorius Stedim, Vienna, Austria) and MES buffer (100 mM, pH 7.0). Enzyme purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure S5). Protein concentration was determined with Roti-Quant reagent (Carl Roth) using BSA as standard.

The activity of *Cu*CbP was determined in the direction of cellobiose synthesis (Nidetzky et al., 2004; 50 mM glucose; 50 mM αGlc1-*P*; pH 7.0, 45 °C). The activity of *Cc*CdP was determined in the direction of cellodextrin synthesis (50 mM cellobiose; 50 mM αGlc1-*P*; pH 7.0, 45 °C). Phosphate release from αGlc1-*P* was measured using the colorimetric assay of Saheki, Takeda, and Shimazu (1985). One unit (U) of activity is the enzyme amount producing 1 μmol phosphate/min under the conditions used. For *Cu*CbP, the temperature profile of activity was determined at pH 6.5 between 30 and 70 °C using increments of 10°C. The pH optimum was determined at 45°C between 4.5 and 8.0 using increments of 0.5 pH units. For *Cc*CdP, the temperature profile of activity was determined at pH 7.0 between 35 and 70°C using increments of 5°C. The pH optimum was determined at 55°C between 4.0 and 8.5 using increments of 0.5 pH units.

2.3 | Synthesis of cellodextrins

All reactions were carried out at 45°C using an agitation rate of 300 rpm on a ThermoMixer C (Eppendorf, Vienna, Austria). A total volume of 0.5 ml was used. The reaction mixture contained 200 mM αGlc1-*P*, 50 mM glucose, 5 mM dithiothreitol, 0.5 or 1.5 U purified *Cu*CbP, and 0.5 U purified *Cc*CdP in MES buffer (100 mM, pH 7.0). Magnesium chloride ($MgCl_2$) was optionally added at 50, 100, or 200 mM. Samples were taken from the reactions at certain times and used for further analyses.

For product synthesis at controlled DP, reactions were carried out as described above but using a variable glucose concentration between 1 and 150 mM. In addition, 200 mM $MgCl_2$ were used. The enzyme concentration was 3 U/ml for *Cu*CbP and 1 U/ml for *Cc*CdP. To optimize the DP distribution in soluble cellodextrin product, reactions were performed at 50 mM glucose and 200 mM αGlc1-*P* using different *Cu*CbP/*Cc*CdP ratios. *Cu*CbP was constant at 3 U/ml and *Cc*CdP was varied at 1, 2, or 3 U/ml.

Mass- or mole-based yields of the soluble and insoluble cellodextrins released are expressed on the glucose or αGlc1-*P* added to the reaction, as indicated in text.

2.4 | Analytics

Reaction samples were centrifuged (15,000 rpm, 5 min). The solid was used for insoluble product analysis (see later). The supernatant was heated (95°C, 5 min) to inactivate the enzyme and centrifuged again.

2.4.1 | Soluble cellodextrins

The cellodextrins were analyzed by thin-layer chromatography (TLC). A mobile phase of ethyl acetate, acetic acid, and water (50:25:25, by volume) was used. Staining was with thymol reagent (thymol/ethanol/H_2SO_4, 0.5:95:5, w/v/v) at 95°C. The cellodextrins were additionally analyzed by high-performance liquid chromatography (HPLC) on a Hitachi LaChrom HPLC system (Merck, Darmstadt, Germany) using a Luna 5 μm NH_2 column (100 Å, 250 × 4.6 mm; Phenomenex, Aschaffenburg, Germany) operated at 40°C. Acetonitrile-water (67.5:32.5, by volume) was used as eluent at a flow rate of 1.5 ml/min. Cellobiose was analyzed by HPLC using an Aminex HPX-87H Column (300 × 7.8 mm; Bio-Rad Laboratories, Vienna, Austria) operated at 60°C. Sulfuric acid (5 mM) was used as eluent at a flow rate of 0.5 ml/min. Refractive index detection was used to quantitate cellodextrins and cellobiose. Calibration was with authentic standards (CarboSynth). αGlc1-*P* was determined enzymatically using a continuous coupled enzyme assay (Eis & Nidetzky, 1999).

2.4.2 | Insoluble cellodextrins

To recover insoluble cellodextrins from reactions containing $MgCl_2$, the precipitated magnesium phosphate was dissolved in 100 mM

MES (pH 4.5). The insoluble cellulose was neither dissolved nor hydrolyzed under these conditions. Therefore, the pellet of the original sample was washed several times until phosphate was no longer released into the supernatant. Thus prepared insoluble cellodextrins were washed with water, lyophilized, weighed, and dissolved in 4% (w/w) NaOD-D_2O to a concentration of 10 mg/ml. To determine their DP, insoluble cellodextrins were analyzed by nuclear magnetic resonance (NMR). ^{1}H-NMR spectra were recorded on a Varian Inova 500 NMR Spectrometer (Agilent Technologies, Santa Clara, CA) applying 32 scan cycles. All ^{1}H-NMR spectra were analyzed using the MestReNova program (mestrelab.com). The average DP of the cellodextrins was calculated with the relationship, $DP = \frac{H_\alpha + H_\beta + H_1}{H_\alpha + H_\beta}$, where H_α and H_β are the integrals of the reducing-end α-anomeric and β-anomeric proton signals. H_1 is the sum integral of the other C1 proton signals in the cellodextrin molecules.

3 | RESULTS AND DISCUSSION

3.1 | Cellodextrin synthesis using coupled CbP and CdP

The *Cc*CdP is a new enzyme identified from this study (Figures S1 and S2). It was functionally expressed in *E. coli* BL21. When assayed with cellobiose as acceptor substrate, the purified enzyme had a specific activity of 14 U/mg (pH 7.0; 55 °C). The *Cc*CdP showed only weak activity with glucose, about 0.5% of that with cellobiose. Functional assignment as CdP, originally made from the sequence (Figure S1), was thus confirmed for *Cc*CdP. Biochemical data for *Cu*CbP, *Cs*CdP (Tran et al., 2011) and *Cc*CdP (Figure 1) suggest suitable conditions for a linear cascade reaction to produce cellodextrins from glucose using coupled CbP and CdP: pH 7.0 and 45°C. *Cu*CbP is much less thermo-active/stable than *Cs*CdP, as shown in Figure 1a. This makes it difficult to establish the combined application of *Cu*CbP and *Cs*CdP under optimum conditions for both enzymes. In contrast to *Cs*CdP (Tran et al., 2011) and other (hyper)thermostable CdPs (from *C. thermocellum*; Arai, Tanaka, & Kawaguchi, 1994; *Thermosipho africanus*; Wu et al., 2017; and *Ruminococcus albus*; Sawano, Saburi, Hamura, Matsui, & Mori, 2013), *Cc*CdP offers a temperature profile of activity well compatible with *Cu*CbP activity at an optimal temperature of 45°C (Figure 1a). Reaction thermodynamic analysis with eQuilibrator (Flamholz, Noor, Bar-Even, & Milo, 2012) shows that cellobiose synthesis from αGlc1-*P* and glucose is largely independent of pH in the range of 6.0 to 9.0. This validated an operational pH of 7.0 for cellodextrin synthesis, as suggested from the pH-activity profiles in Figure 1b.

Thus, in preliminary experiments, we performed the transfer reaction (200 mM αGlc1-*P*; 50 mM glucose) of *Cc*CdP (1 U/ml) in the presence of *Cu*CbP (1 or 3 U/ml). Figure 2a shows representative time courses of αGlc1-*P* conversion. Note: we verified that the donor substrate (αGlc1-*P*) conversion was a suitable reporter of the enzymatic synthesis. There is close mass balance between utilization of αGlc1-*P* and the production of cellobiose/cellodextrins. The result implies that hydrolysis of αGlc1-*P* does not occur. Absence of hydrolytic activity towards their donor substrate is a particular advantage of these glycoside phosphorylases (CbP, CdP) for glycoside synthesis (e.g., Suzuki et al., 2009; for reviews, see Luley-Goedl & Nidetzky, 2010; Pergolizzi et al., 2017). When 200 mM αGlc1-*P* and 50 mM glucose were offered, the enzymatic reaction gradually approached an apparent equilibrium at about 55% conversion of the initial αGlc1-*P* concentration. Glucose was largely depleted at this point, but still detectable, as shown in Figure 2b. Using *Cu*CbP activity in 3-fold excess over *Cc*CdP activity (1 U/ml) was

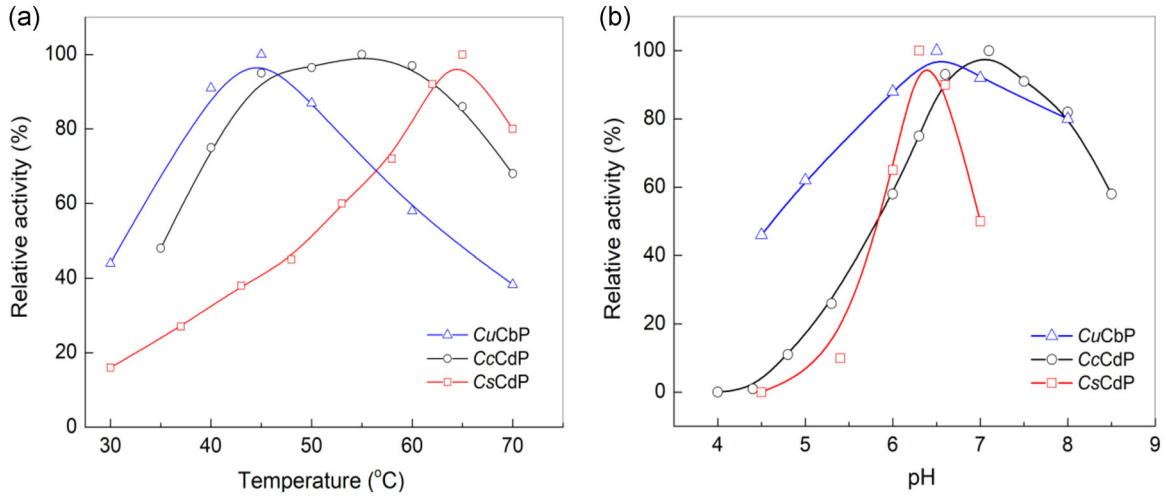


FIGURE 1 Temperature (a) and pH (b) profiles of activity of *Cu*CbP (blue), *Cc*CdP (black) and *Cs*CdP (red). Data for *Cu*CbP and *Cc*CdP are from this study, the data of *Cs*CdP is from literature (Tran et al., 2011). The temperature profiles of *Cc*CdP and *Cu*CbP, *Cs*CdP are from pH 7.0 and 6.5, respectively. The pH profiles of *Cc*CdP and *Cu*CbP, *Cs*CdP are from 55°C and 45°C, respectively. For *Cu*CbP, the pH profile was determined here in 50 mM buffer (pH 4.5–5.0 acetate; pH 6.0–7.0 MES; pH 8.0 HEPES). For *Cc*CdP the pH profile was determined here in 50 mM buffer (pH 4.0–6.0 citrate; pH 6.0–7.0, MES; pH 7.5–8.5 HEPES). All activities are for the synthesis direction using αGlc1-*P* and glucose (*Cu*CbP) or cellobiose (*Cs*CdP, *Cc*CdP) as substrates in saturating concentrations. The activities are normalized with the maximum activity given as 100%. αGlc1-P, α-D-glucose 1-phosphate; CbP, cellobiose phosphorylase; CdP, cellodextrin phosphorylase [Color figure can be viewed at wileyonlinelibrary.com]

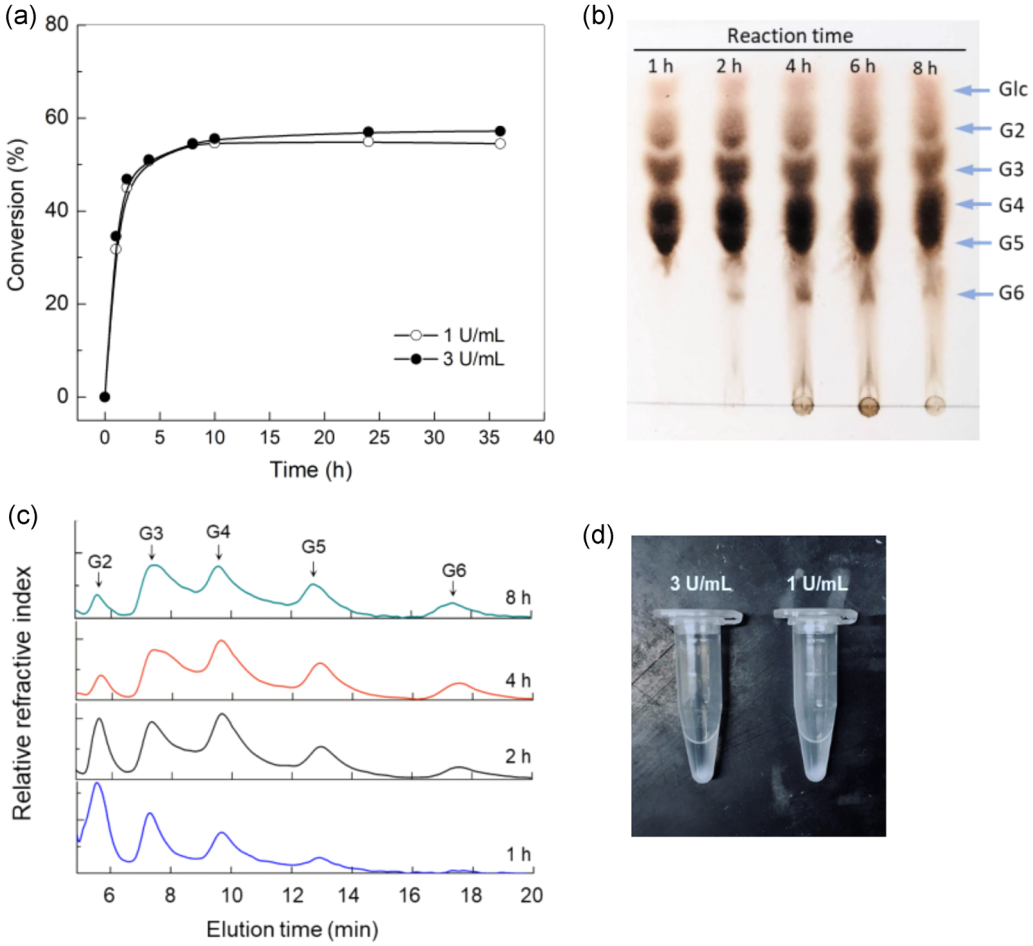


FIGURE 2 Reaction time course analysis for cellodextrin synthesis by coupled *Cu*CbP and *Cc*CdP at 45°C and pH 7.0. (a) Conversion of αGlc1-*P* in reactions that contained 200 mM αGlc1-*P*, 50 mM glucose, 1 (○) or 3 (●) U/ml *Cu*CbP and 1 U/ml *Cc*CdP. TLC (b) and HPLC (c) analysis of the reaction mixture from the 3 U/ml *Cu*CbP reaction. (d) Insoluble cellodextrins as visible precipitate after 10 hrs of reaction with 1 or 3 U/ml *Cu*CbP. αGlc1-P, α-D-glucose 1-phosphate; CbP, cellobiose phosphorylase; CdP, cellodextrin phosphorylase; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography [Color figure can be viewed at wileyonlinelibrary.com]

without effect on reaction time course, indicating that *Cc*CdP was the rate-determining enzyme under the conditions used. The reaction time course was characterized by a fast initial phase (≤2 hrs) of product formation in which about 90% of the total phosphate was released. The apparent equilibrium was then approached more slowly within 10 hrs.

Product analysis by TLC and HPLC (Figure 2b,c) revealed that soluble cellodextrins of DP 2 to 6 were formed. As shown in Figure 4b, the initial reaction for 1 hr gave soluble cellodextrins at a concentration of 19 g/L, with a product distribution of G2 (17 wt.%), G3 (44 wt.%) and G4 (33 wt.%). The maximum soluble cellodextrin concentration (26 g/L) was obtained after 4 hrs of reaction. At this point, the oligosaccharides were composed of G2 (7.8 wt.%), G3 (23 wt.%), G4 (36 wt.%), G5 (24 wt.%), and G6 (9.1 wt.%). We additionally found that the relative portion of soluble cellodextrins in total product gradually decreased after 6 hrs. Insoluble cellodextrins, present after 10 hrs of reaction, are shown in Figure 2d. The amount of insoluble product accounted for about 9.2 mol% of the initial glucose used, corresponding to 4 wt.% of the total cellodextrins (i.e., soluble and insoluble; ~26 g/L) formed.

The results in Figure 2a imply that shifting the equilibrium of the coupled enzyme reaction would be essential to improve the efficiency of cellodextrin synthesis. Previously, α-glucose 1-fluoride was applied as donor substrate of the phosphorylases to push the enzymatic conversion, glucose to cellobiose or cellobiose to cellodextrins. Both CbP (Nidetzky et al., 2004) and CdP (Nakai et al., 2010) are able to use α-glucose 1-fluoride as donor substrate in place of αGlc1-*P*. Release of fluoride is less readily reversible than the release of phosphate. However, while effective in changing the equilibrium position of the phosphorylase reactions, α-glucose 1-fluoride is less attractive than αGlc1-*P* for the bulk cellodextrin production. In situ product removal was therefore considered as an alternative "thermodynamic pull" strategy to be applied to the biocatalytic synthesis.

3.2 | In situ product removal

Previous studies (Kadokawa, Shimohigoshi, Yamashita, & Yamamoto, 2015; Suzuki et al., 2009) demonstrated the removal of phosphate from phosphorylase reactions as an insoluble magnesium ammonium

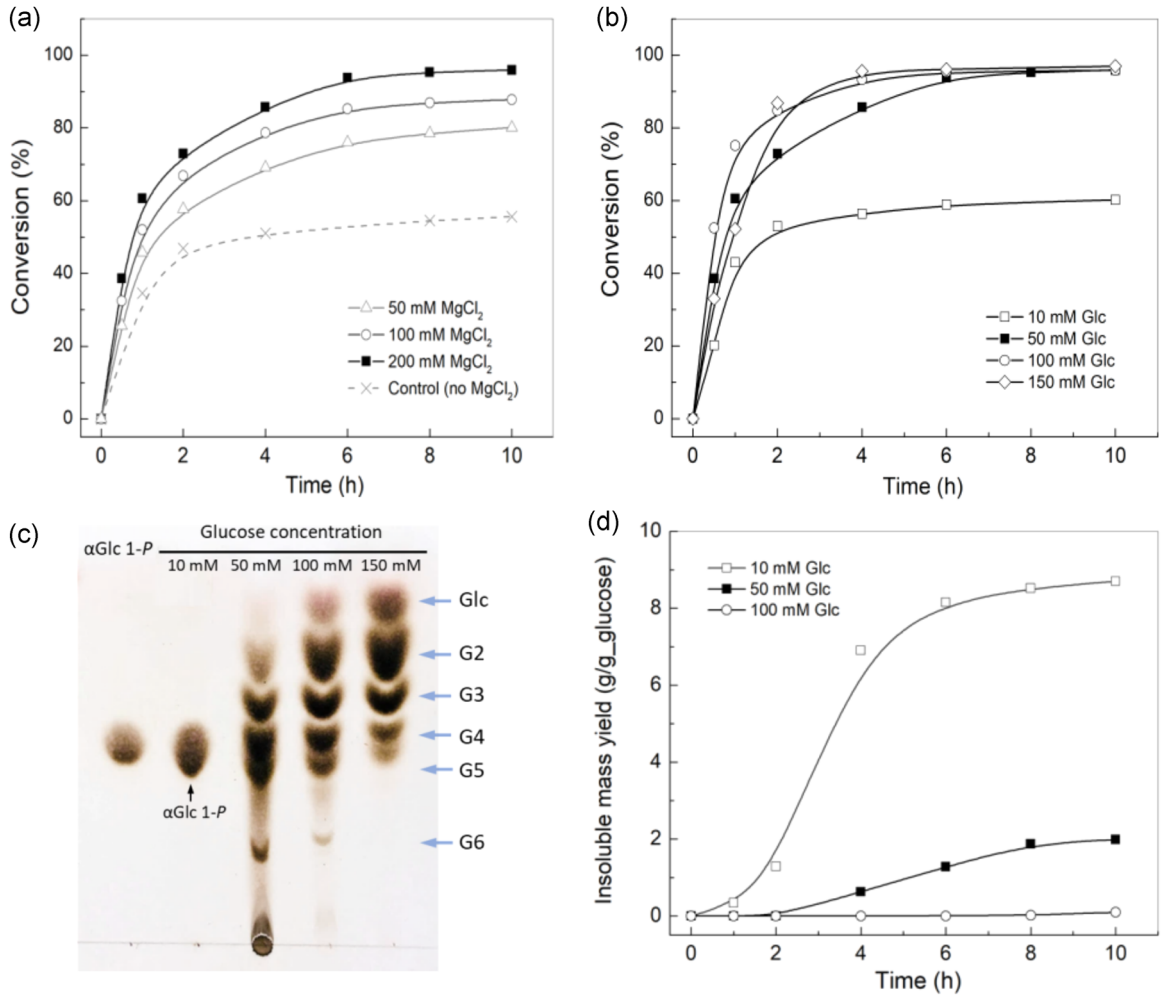


FIGURE 3 Cellodextrin synthesis using in situ phosphate removal. (a) Effect of $MgCl_2$ concentration on the conversion of αGlc1-*P* in reactions that contained 200 mM αGlc1-*P*, 50 mM glucose, 3 U/ml *Cu*CbP and 1 U/ml *Cc*CdP. (b) Effect of the glucose concentration on the conversion of αGlc1-*P* in reactions that contained 200 mM αGlc1-*P*, 200 mM $MgCl_2$, 3 U/ml *Cu*CbP, and 1 U/ml *Cc*CdP. (c) TLC analysis of the varied glucose reactions at apparent reaction equilibrium. (d) Insoluble cellodextrin formation at different glucose concentrations over time. The insoluble mass yield is calculated as mass insoluble cellodextrins/mass glucose used. αGlc1-P, α-D-glucose 1-phosphate; CbP, cellobiose phosphorylase; CdP, cellodextrin phosphorylase; TLC, thin-layer chromatography [Color figure can be viewed at wileyonlinelibrary.com]

salt. At pH 8.6, added magnesium proved effective in driving the glycosyl phosphate conversion to a higher yield. We adapted this earlier concept of reaction engineering and found that it was also useful at a pH of 7.0 (used here for cellodextrin synthesis). $MgCl_2$ was used in the current study. We verified that αGlc1-*P* did not precipitate in the presence of $MgCl_2$. We compare in Figure 3a the coupled enzymatic reactions in the absence and presence of 50, 100, and 200 mM $MgCl_2$. The conversion of αGlc1-*P* (200 mM) was faster (two-fold at ~1 hr) and gave higher yield (53% → 96%) when 200 mM $MgCl_2$ was added as compared with the control that did not contain $MgCl_2$. As shown in Figure S6, approximately 92% of the phosphate released from αGlc1-*P* was successfully removed from the solution by Mg^{2+} precipitation. In addition, to assess the effect of phosphate removal on cellodextrin productivity, we compared the enzymatic reactions (3 U/ml *Cu*CbP, 1 U/ml *Cc*CdP, 200 mM αGlc1-*P*, and 50 mM glucose) performed in the absence and presence of 200 mM $MgCl_2$ after 1 hr of reaction (both reaching ~60% of maximum αGlc1-*P* conversion; Figure 3a). Using the data from Figure 4b, we show that the reaction with Mg^{2+} yielded a 1.6-fold higher productivity (30 g L^{-1} hr^{-1}) than the control reaction without Mg^{2+} (19 g L^{-1} hr^{-1}). Moreover, the effect of $MgCl_2$ was dependent upon the concentration used. Only 80 to 86% of the αGlc1-*P* (200 mM) was converted in the reaction when $MgCl_2$ was added at 50 or 100 mM. Phosphate started to accumulate once Mg^{2+} had been depleted from solution due to precipitation (Figure S6). We thus show that in situ removal of phosphate was effective not only in overcoming the thermodynamic limitation on the enzymatic synthesis but also in removing kinetic restriction on the αGlc1-*P* utilization. The distribution of DP in soluble cellodextrins was not changed upon the addition of $MgCl_2$ (Figure 4b). In particular, the relative abundance of cellobiose was not altered. Tentatively, therefore, we ascribe the effect of phosphate removal on the overall conversion rate to the

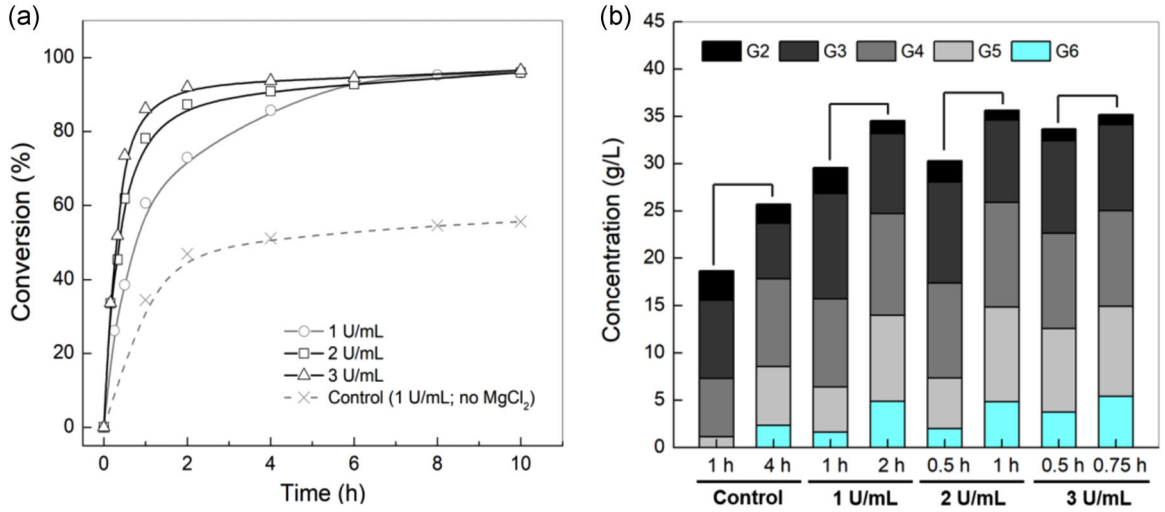


FIGURE 4 Reaction time course in αGlc1-*P* conversion (a) and product composition (b) at different *Cc*CdP loadings in reactions that contained 200 mM αGlc1-*P*, 50 mM glucose, 200 mM $MgCl_2$ (except for the control), and 3 U/ml *Cu*CbP. The activity of *Cc*CdP was varied between 1 (○), 2 (□), and 3 (Δ) U/ml. αGlc1-P, α-D-glucose 1-phosphate; CbP, cellobiose phosphorylase; CdP, cellodextrin phosphorylase [Color figure can be viewed at wileyonlinelibrary.com]

mitigation of phosphate inhibition on the cellobiose-forming enzyme, *Cu*CbP.

3.3 | DP control in cellodextrin product

Earlier studies used CdP for repetitive β-glycosylation of cellobiose and analyzed the DP of the cellodextrins thus formed (Petrovic et al., 2015). The cellodextrins got longer (average DP 7.1 → 13.7) as the cellobiose (primer) concentration (constant molar ratio cellobiose/αGlc1-*P* 1:20) was decreased (10 mM → 0.2 mM). To establish conditions for the synthesis of soluble cellodextrins by the coupled *Cu*CbP and *Cc*CdP, we varied the ratio of glucose/αGlc1-*P* and monitored the oligosaccharide formation over time. Unlike previous works that focused exclusively on the structural properties of the cellodextrin products obtained (Hiraishi et al., 2009; Petrovic et al., 2015), we here sought to develop the basis for an efficient biocatalytic process. Besides control of DP for product solubility, the cellodextrin yield, the final product concentration, and the productivity were important parameters to be additionally considered. We show in Figure 3b the time courses of αGlc1-*P* conversion (200 mM; $MgCl_2$ added at 200 mM, 3 U/ml *Cu*CbP, 1 U/ml *Cc*CdP) when glucose was offered in the range 10 to 150 mM. In Figure 3c, we show a TLC analysis of the corresponding product mixtures for reactions at apparent reaction equilibrium. Two prominent effects of the glucose concentration were revealed. At the lowest glucose concentration of 10 mM, the maximum conversion of αGlc1-*P* was limited to about 59% by the acceptor substrate (glucose) available in solution (Figure 3b). At the time when the αGlc1-*P* utilization effectively stalled (6–10 hrs), neither glucose nor any other soluble cellodextrin (G2–G6) was detectable by TLC (Figure 3c). At higher glucose concentrations, the αGlc1-*P* utilization was complete (≥95%) after approximately 10 hrs of reaction. The initial reaction rate increased dependent on the glucose concentration and reached saturation at around 150 mM. The prominent DP of the cellodextrin products decreased from greater than 6 (insoluble) at 10 mM glucose to 2-3 at 150 mM glucose (Figure 3c). We show in Figure 3d that the solubility of cellodextrin products was not only dependent on the glucose/αGlc1-*P* ratio, but also on the reaction time. Using 10 mM glucose, insoluble cellodextrins were produced from the beginning of the reaction and accumulated to an insoluble mass yield of 8.7 g/g glucose after 10 hrs. Under the condition of 50 mM glucose, the cellodextrins were soluble within the initial 2 hrs of reaction. Insoluble cellodextrins were gradually produced afterwards and accumulated to an insoluble mass yield of 2 g/g glucose after 10 hrs of reaction (Figure 3d). Using 100 mM glucose, however, the cellodextrins formed were soluble throughout. Compared with the 50 mM glucose reaction, a substantial amount of G2 (~25 mol.%) was present in the total cellodextrin product mixture (~43 g/L) after 10 hrs of reaction when 100 mM glucose were used (Figure 3c). In conclusion, product DP in cellodextrin synthesis from glucose is mainly determined by the molar ratio of acceptor (glucose) and donor substrate (αGlc1-*P*). Reaction progress (or time) is another important factor. Considering both, the product solubility can be conveniently controlled in a biocatalytic production.

3.4 | Optimized conversion for synthesis of soluble cellodextrins

Because cellobiose is already on its way to a commercial product (Koch et al., 2016), we considered the production of cellodextrins with DP between three and six. Besides avoiding insoluble product formation, another task for reaction engineering was to minimize the cellobiose content in the final product mixture. Results in Figure 3c led to the suggestion that a glucose concentration of around 50 mM should be used. At this glucose concentration, cellodextrins of mainly DP 3 to 6

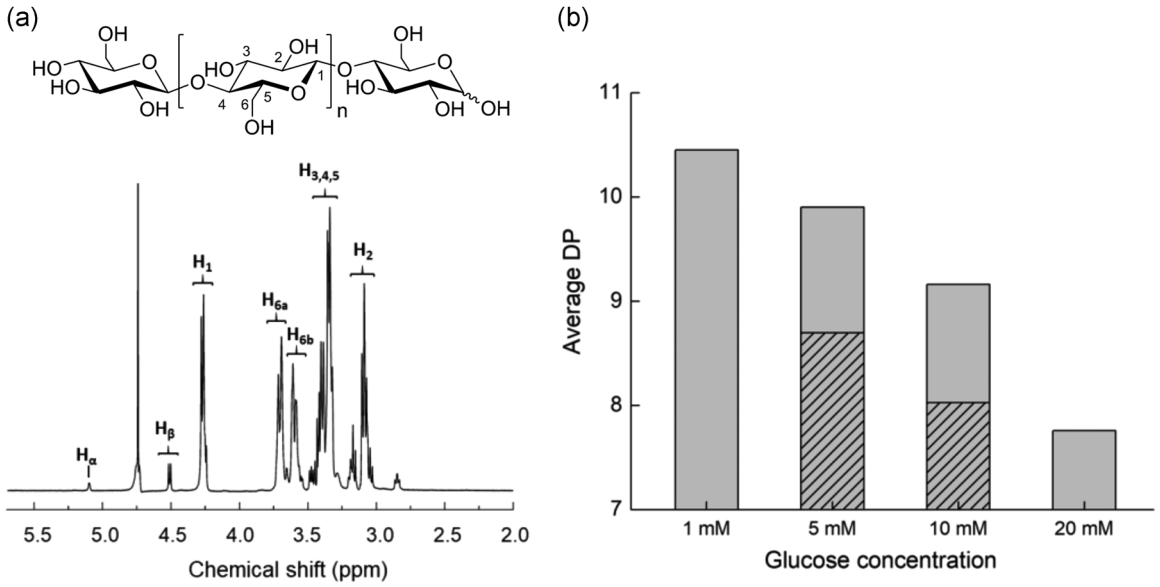


FIGURE 5 Characterization of insoluble cellodextrins by ^{1}H-NMR. (a) NMR spectrum with signals assigned to the positions indicated. H_α and H_β are for the anomeric proton. (b) Average DP in different insoluble cellodextrins prepared using varied glucose concentration under otherwise identical conditions (200 mM αGlc1-*P*, 200 mM $MgCl_2$, 3 U/ml *Cu*CbP, 1 U/ml *Cc*CdP). The hatched bars in 5 and 10 mM present the reaction without $MgCl_2$ addition. αGlc1-P, α-D-glucose 1-phosphate; CbP, cellobiose phosphorylase; CdP, cellodextrin phosphorylase; DP, degree of polymerization; NMR, nuclear magnetic resonance

were formed and the cellobiose content in the product mixture was low (≤4 wt.%). Experiments performed with 50 mM glucose under standard conditions (200 mM of αGlc1-*P* and $MgCl_2$; 3 U/ml *Cu*CbP and 1 U/ml *Cc*CdP) gave soluble cellodextrins at a concentration of 35 g/L obtained after 2 hrs of reaction (Figure 4b). However, as shown in Figure S7 and indicated in Section 3.3, the reaction time was an important factor of product solubility. Precipitation started shortly after 2 hrs (Figure 3d and Figure S7) when 70 to 80% of the initial αGlc1-*P* was converted. A substantial loss of the cellodextrins formed as an insoluble product (34 mol.% calculated from soluble mole ratio of 66 mol.%) was observed at 6 hrs of reaction. To shorten the time required for donor substrate conversion, we doubled the volumetric activity of the rate-limiting enzyme *Cc*CdP. Under these conditions (3 U/ml *Cu*CbP, 2 U/ml *Cc*CdP), indeed, about 78% of αGlc1-*P* were converted within 1 hr (Figure 4a) and the cellodextrin products formed were completely soluble, as shown in Figure S7B. Figure 4b shows that the soluble cellodextrins were obtained at 32 g/L and comprised mainly G3 (38 wt.%) and G4 (32 wt.%) after 0.5 hr of reaction. The content of G5 and G6 increased in relative abundance dependent upon the reaction time. After 1 hr of reaction, soluble cellodextrins were produced at 36 g/L. They comprised G3 (25 wt.%), G4 (31 wt.%), G5 (28 wt.%), and G6 (14 wt.%). The molar yield of the cellodextrin products was 78% and 98% based on the αGlc1-*P* and glucose added to the reaction, respectively. By way of comparison, the control reaction that did not involve in situ removal of phosphate gave 22 g/L of soluble cellodextrins after 1 hr in a molar yield of just approximately 35% on the αGlc1-*P* added (Figure 4). This two-fold enhancement in yield may be ascribed to both the enhanced utilization of αGlc1-*P* and the rigorous control of product solubility during the synthesis. The cellodextrin product from the reaction without phosphate removal comprised mainly G2 – G4. In addition, we showed that further increased *Cc*CdP activity (3 U/ml) gave soluble cellodextrins at a similar concentration of approximately 35 g/L already after 0.5 to 0.75 hr of reaction (3 U/ml *Cu*CbP, 3 U/ml *Cc*CdP). The cellodextrins were composed of mainly G3 to G6 (~96 wt.%), as shown in Figure 4b. However, due to the faster reaction at the elevated *Cc*CdP activity, the operational window for product solubility control was narrowed down to just a few minutes. It was, therefore, less suitable for practical application in the synthesis.

Use of glucose instead of cellobiose for cellodextrin production is attractive first and foremost because of lower substrate costs. In addition, glucose is much better soluble than cellobiose (i.e., 909 g/L compared with 120 g/L; Huebner, Ladisch, & Tsao, 1978). To examine whether the single-step *Cc*CdP reaction starting from cellobiose would give a different product composition, we performed the conversion identically as in the *Cu*CbP-*Cc*CdP reaction using 50 mM cellobiose instead of glucose. A slight decrease in the αGlc1-*P* conversion was observed for the single-enzyme *Cc*CdP reaction (Figure S8) as compared with the two-enzyme *Cu*CbP-*Cc*CdP reaction. Using 2 U/ml *Cc*CdP individually, soluble cellodextrins were released at a concentration of 31 g/L after 0.5 hr of reaction. The product distribution (G3, 28 wt.%; G4, 33 wt.%; G5, 25 wt.%; G6, 7.6 wt.%) was similar as in the two-enzyme reaction (3 U/ml *Cu*CbP, 2 U/ml *Cc*CdP) for 0.5 to 1 hr (Figure 4b).

3.5 | Shift towards a completely insoluble cellodextrin product

Considering a large amount of precipitated product formed in the 10 mM glucose reaction (Figure 3d), we asked whether the conversion of αGlc1-*P* could be driven completely towards insoluble cellodextrin

production. A series of reactions were performed (200 mM of each αGlc1-*P* and $MgCl_2$; 3 U/ml *Cu*CbP, 1 U/ml *Cc*CdP) that used glucose concentrations in the range of 1 to 20 mM. TLC analysis of the supernatants after 10 hrs of reaction showed no soluble cellodextrins (G2–G6) and no glucose remaining (data not shown). The αGlc1-*P* utilization was however incomplete and decreased from 59 to 7.4% as the initial glucose concentration decreased from 10 mM to 1 mM. The insoluble mass yield from reactions with 5 and 10 mM glucose was 9.1 g/g glucose and 8.7 g/g glucose, respectively. The insoluble cellodextrins were recovered and analyzed by ^{1}H-NMR. A representative ^{1}H-NMR spectrum is shown in Figure 5a. According to literature (Isogai, 1997), signals at around 5.12 and 4.53 ppm are assigned to the α- and β-anomeric proton at the reducing end of the cellodextrin, respectively. The signals at around 4.28 ppm are assigned to internal anomeric protons. The average DP of the insoluble cellodextrins was calculated from the integrals of these proton signals (Petrovic et al., 2015). We show in Figure 5b that the average DP increased from 7.8 to 10.4 when initial glucose concentration decreased from 20 to 1 mM. The overall trend is as expected because lower product concentrations reduce the likelihood of cellodextrin precipitation triggered by interchain hydrogen bond interaction (Nishiyama, Langan, & Chanzy, 2003). The average DP values fit well with the literature describing oligosaccharides formation by CdP from cellobiose (Petrovic et al., 2015) and other acceptors (e.g., glucosides, sophorose, and laminaribiose; Adharis, Petrović, Özdamar, Woortman, & Loos, 2018; Hiraishi et al., 2009; Nakai et al., 2010; Yataka, Sawada, & Serizawa, 2015). Interestingly, we found that the average DPs were higher when the synthesis was performed in the presence of Mg^{2+}. For example, 5 and 10 mM glucose reactions gave average DPs of 9.9 and 9.2 in the presence of Mg^{2+}, respectively, whereas the corresponding DPs in the absence of Mg^{2+} were 8.6 and 8.0 (hatched bars in Figure 5b). Further research is necessary to explain the effect of Mg^{2+} on insoluble cellodextrin formation. Note that the magnesium phosphate precipitate was conveniently removed from insoluble cellodextrin by dissolving it at slightly acidic conditions (pH 4.5). Pure insoluble cellodextrins were obtained thus.

4 | CONCLUSIONS

Summarizing, we present here a new CdP from *Clostridium cellulosi* and show the application of the enzyme in a linear cascade reaction with *Cu*CbP for the synthesis of soluble cellodextrins from glucose and αGlc1-*P*. In situ removal of phosphate by precipitation with Mg^{2+} was key to overcome thermodynamic restrictions on the conversion of αGlc1-*P*. It also mitigated enzyme inhibition by phosphate. The *Cc*CdP elongated the cellobiose formed by *Cu*CbP until the cellodextrin products reached a DP (≤6) limiting for solubility. The sequential build-up of cellodextrins implied that the product DP was controlled kinetically. Besides reaction progress adjustable by the time or enzyme activity, therefore, the molar ratio glucose/αGlc1-*P* was the main variable affecting the DP. When 200 mM αGlc1-*P* was used, the production of soluble cellodextrins necessitated this ratio to be 0.25 or greater. Using a molar ratio of 0.05 or smaller, only insoluble cellodextrins were obtained. Overall, optimized reaction (200 mM of αGlc1-*P* and $MgCl_2$, 50 mM glucose, 3 U/ml *Cu*CbP, 2 U/ml *Cc*CdP) gave completely soluble cellodextrins with DP centered at 3 to 6 (~96 wt.%). A final product concentration of 36 g/L was obtained at short reaction times (~1.0 hr) with a conversion yield of 78% and 98% based on αGlc1-*P* and glucose, respectively. This study, therefore, provides the basis for an efficient and product DP-controlled biocatalytic synthesis of cellodextrins from expedient substrates.

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DATA AVAILABILITY

Data obtained in the current study are available from DOI 10.5281/zenodo.1436337.

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Additional supporting information may be found online in the Supporting Information section.

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