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Preparation and enzymatic behavior of surfactant-enveloped enzymes for glycosynthesis in nonaqueous aprotic media

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ABSTRACT

Surfactant-enveloped enzymes (SEEs) were prepared from pure cellulases, cellobiohydrolase I and endoglucanase I (Cel7A and Cel7B, respectively), *via* simply freeze-drying water-in-oil emulsions, wherein the aqueous phase containing each cellulase was stabilized with the nonionic surfactant, dioleyl-*N*-D-glucona-L-glutamate. The enzymatic tolerance of SEEs to various nonaqueous solvents was investigated, aiming at a novel synthetic approach in biocatalytic glycoengineering. SEE-Cel7A preserved *ca.* 67% of the original activity after 3 h incubation in lithium chloride (LiCl)/dimethylacetamide (DMAc) that is a good solvent for carbohydrates but completely deactivates intact enzymes. This excellent enzymatic durability depended on the preparation conditions of SEEs, *e.g.* pH and salt species of the aqueous phase during SEE preparation. SEE-Cel7A or SEE-Cel7B was applied as a biocatalyst to synthesize cellulose, a sugar polymer which is insoluble in common solvents but dissolves in LiCl/DMAc. Both SEEs could catalyze the direct dehydration of cellobiose without any activation of the anomeric carbon, a property that is indispensable for conventional chemo-enzymatic synthesis. The SEE-Cel7A provided short-chain cellulose with the degree of polymerization (DP) *ca.* 20, and longer-chain cellulose with DP *ca.* 60 was preferentially obtained by the SEE-Cel7B, possibly through preferential reverse hydrolysis instead of inherent hydrolysis. Nonaqueous SEE-mediated biocatalysis using inexpensive glycohydrolases and sugars that do not need to be chemically modified beforehand would have potentially wide applications in glycoengineering.

1. Introduction

Hydrolytic enzymes have attracted much attention as a powerful tool for carrying out catalytic reactions in synthetic chemistry and manufacturing *via* their condensation reactions (reverse hydrolysis). In particular, proteases and lipases have been successfully applied in various practical processes [1–4]. Carbohydrates play a prominent role in all biological systems [5,6], and thus *in vitro* glycosynthesis has attracted wide interest from academic and industrial circles. Substantial research into enzymatic carbohydrate synthesis has been reported with regard to the lab-scale use of transglycosidases and glycosynthases [7–9]. Such intracellular enzymes are difficult to use in large quantity because they are found in minute amounts *in vivo* and require sugar nucleotides as donors due to very strict substrate specificity [10].

Cellulases are typical extracellular glycohydrolases and mediate the catalytic hydrolysis of oligo- and polysaccharides composed of β-1,4-linked D-glucose under mild aqueous conditions [11,12]. Commercial applications of cellulases have been adopted in food, textile and papermaking industries [13], as well as in biosaccharification and bioethanol production [14,15]. Enzymatic reaction of cellulase at a cellulose crystallite/water interface has been investigated, aiming at efficient saccharification [16]. The fungus *Trichoderma reesei* (*T. reesei*) is well known for its outstanding ability to produce a complete set of enzymes that can hydrolyze cellulose to component sugars. *T. reesei* secretes a wide range of cellulases, at least two cellobiohydrolases (CBHs; Cel6A and Cel7A) and five endoglucanases (EGs; Cel5A, Cel7B, Cel12A, Cel45A and Cel61A) [17,18]. CBHs contain the active sites in tunnel-shaped

Abbreviations: CBH, cellobiohydrolase; $2C_{18}\Delta^9$GE, dioleyl-*N*-D-glucona-L-glutamate; 3-D, three-dimensional; DMAc, dimethylacetamide; DP, degree of polymerization; EG, endoglucanase; GH7, glycoside hydrolase family 7; LiCl, lithium chloride; NMR, nuclear magnetic resonance; *p*NPL, *p*-nitrophenyl-β-D-lactopyranoside; SEC, size exclusion chromatography; SEE, surfactant-enveloped enzyme; SEE-Cel7A, surfactant-enveloped pure cellulase Cel7A; SEE-Cel7B, surfactant-enveloped pure cellulase Cel7B; *T. reesei*, *Trichoderma reesei*; *T. viride*, *Trichoderma viride*; W/O, water-in-oil.

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pockets, whereas the catalytic regions of EGs are located in open substrate-binding clefts. Such structural variations bring about the difference in catalytic features, *e.g.* processive and non-processive hydrolysis for CBHs and EGs, respectively [19,20].

Cellulase-catalyzed reverse hydrolysis for glycosynthesis has been actively investigated in aqueous and aqueous-organic miscible systems. Ogata et al. [21] have reported the enzymatic synthesis of pentyl-β-lactoside from lactose (a second substrate of cellulase) and pentanol in acetate buffer. However, the yield was only 1.1% even under extremely high concentrations (>1 M) of reactants with a long reaction time (*ca.* 6 days), and there were no oligosaccharides formed *via* a cellulase-mediated condensation reaction, possibly due to low reverse hydrolysis efficiency in the water phase and the inevitable hydrolysis of the products. Kobayashi et al. [22] successfully synthesized pure cellulose from β-D-cellobiosyl fluoride, with a high yield (>70%), using cellulase in an acetonitrile/acetate system to prevent as much undesirable hydrolysis of the products as possible. However, a synthetic product, *i.e.* cellulose, was insoluble in the semi-aqueous media, resulting in the formation of short-chain cellulose (the degree of polymerization (DP) < 22). As well, the fluoridation of the anomeric carbon at the reducing end of cellobiose, which was also carried out by organic synthesis approaches, was required; such chemical design of precursor sugars is generally indispensable for conventional enzymatic glycosynthesis, *i.e.* called chemo-enzymatic synthesis [23–25].

In our previous study, complete enzymatic synthesis of cellulose with more than DP 100 from cellobiose without any activation of the anomeric carbon was first achieved in a nonaqueous lithium chloride (LiCl)/dimethylacetamide (DMAc) system, well-studied solvent for dissolving solid-state cellulose but causes inevitable inactivation of enzymes [22,26]. A novel biocatalyst active even in aprotic organic media, a surfactant-enveloped enzyme (SEE), was successfully prepared by enveloping crude cellulase with a specific nonionic surfactant, dioleyl-*N*-D-glucona-L-glutamate ($2C_{18}\Delta^9$GE) [27]. Direct glyco-modification of solid-state cellulose surfaces; regenerated-cellulose film [28] and cellulose filter paper [29], and one-step lactosylation of hydrophobic alcohols [30] were also achieved using SEEs in nonaqueous aprotic media. The nonaqueous SEE-mediated biocatalysis is expected to provide a promising approach for a facile glycosynthesis in various organic media although further improvement is still required.

In this study, the SEEs were prepared separately from two types of pure cellulases, CBH I (Cel7A, processive type) and EG I (Cel7B, non-processive type) to further advance nonaqueous biocatalysis as a novel synthetic approach in glycoengineering. Enzymatic tolerance to various organic solvents was estimated by the residual hydrolytic activity assay after solvent-exposure treatment. SEE-Cel7A or SEE-Cel7B was utilized in nonchemo-enzymatic cellulose synthesis in nonaqueous 8% (w/v) LiCl/DMAc, and the characteristic differences in the yields and chain lengths of synthesized cellulose was compared. Reverse hydrolysis in the SEE-mediated biocatalysis is also discussed with regard to the supramolecular structures of their catalytic sites.

2. Materials and methods

2.1. Materials

Cel7A and Cel7B were purified by chromatographic separation from Celluclast® (crude cellulase mixtures from *T. reesei*, Novozymes Japan, Co. Ltd.) with the yields of *ca.* 600 mg and *ca.* 50 mg, respectively (Supporting Information). Nonionic surfactant $2C_{18}\Delta^9$GE was synthesized according to Goto's method [27]. The water used in this study was purified with a Milli-Q system (Millipore, Co. Ltd.). *p*-Nitrophenyl-β-D-lactopyranoside (*p*NPL), cellobiose and other chemicals were of reagent grade and used without further purification (Sigma–Aldrich, Co. Ltd.).

2.2. SEE preparation

Each purified cellulase (10 nmol) was added to 1 mL of 50 mM aqueous medium: disodium hydrogen phosphate solution (pH 9.0), sodium carbonate buffer (pH 9.0), sodium hydrogen phosphate buffer (pH 7.0), sodium acetate buffer (pH 4.8), sodium hydrogen phosphate solution (pH 4.2) or salt-free deionized water (pH *ca.* 7.0). The cellulase solution was mixed with 2 mL of $2C_{18}\Delta^9$GE/toluene with a molar ratio of cellulase/$2C_{18}\Delta^9$GE: 1/600–1/3600, followed by vigorous homogenization for 3 min (room temperature, 15,000 rpm). The cloudy water-in-oil (W/O) emulsion was immediately frozen with liquid nitrogen, and then freeze-dried for 24 h. The SEEs were obtained as a white powder, called SEE-Cel7A and SEE-Cel7B. The preparation outline is illustrated in Scheme 1.

2.3. Enzymatic tolerance assay

As-prepared SEE (enzyme content: 10 nmol) was suspended in 1 mL of each organic solvent: *n*-hexane, chloroform, acetonitrile, DMAc, 8% (w/v) LiCl/DMAc or methanol, and then incubated with gentle stirring at 40 °C for 3 and 6 h. The treated suspension was centrifuged at 3000 × *g* for 30 min to collect the SEEs. The SEE fractions were re-dispersed in 900 μL of sodium acetate buffer (500 mM, pH 4.8), and subsequently 100 μL of 10 mM *p*NPL solution was added to start the hydrolytic reaction. After incubation at 40 °C for 20 min, the reaction was quenched by adding 100 μL of triethylamine, and the amount of *p*-nitrophenol liberated was calculated from the variation in the absorbance at 405 nm using a U-3000 spectrophotometer (HITACHI, Co. Ltd.). The sample preparation and enzymatic tolerance assay were repeated at least three times as independent experiments.

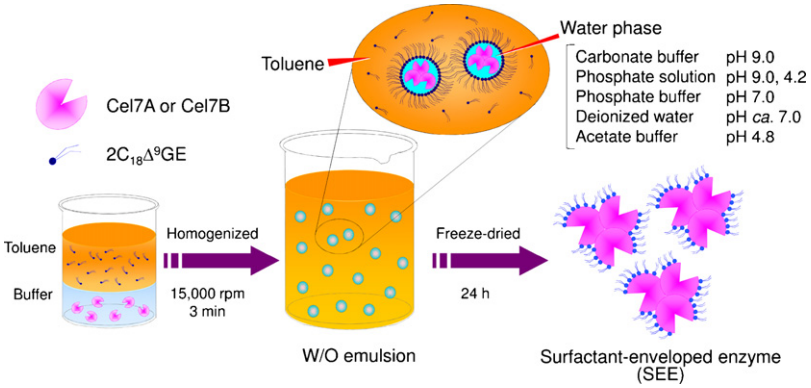
2.4. Cellulose synthesis and analysis

Powder-state SEE (preparation conditions: entry 3 and 8 for Cel7A and Cel7B, respectively, in Table 3) was poured into 1.5 mL of DMAc containing cellobiose (300 or 600 mM) and 8.0 wt% LiCl, then the condensation reaction proceeded at 40 °C for 12 h (Scheme 2). The reaction mixture was centrifuged at 3000 × *g* for 30 min to remove SEE. The supernatant was evaporated until the volume was decreased by *ca.* 80%, and the product was deposited with methanol. The precipitate was thoroughly washed with methanol and chloroform to remove LiCl, DMAc and $2C_{18}\Delta^9$GE, followed by freeze-drying. Subsequently, water-soluble and water-insoluble portions (oligomers (DP < 6) and the longer-chain fraction, respectively) were separated in water (Milli-Q). Size exclusion chromatography (SEC) was carried out to characterize the oligomers on a TOSOH SEC instrument equipped with KS-801 and KS-802 columns (Shodex Co. Ltd.); mobile phase, ultrapure water; temperature, 40 °C; flow rate, 1.0 mL min^{-1}; detector, refractive index. The average DPs of the water-insoluble products were determined by viscometric analysis according to TAPPI Test Methods T230. Nuclear magnetic resonance (NMR) analysis was performed in deuterium oxide on a JEOL JMN-AL400 FT-NMR spectrometer (399.65 MHz).

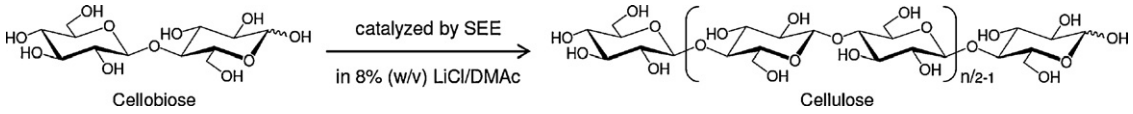
3. Results and discussion

3.1. Enzymatic tolerance of SEEs to various organic solvents

Sufficient enzymatic tolerance of SEEs in organic media is an essential factor for achieving effective nonaqueous glycosynthe-



Scheme 1. Schematic illustration of the preparation outline of SEEs. Cellulase solution was mixed with $2C_{18}\Delta^9$GE/toluene with a molar ratio of cellulase/$2C_{18}\Delta^9$GE: 1/600–1/3600, followed by vigorous homogenization for 3 min. The cloudy water-in-oil (W/O) emulsion was immediately frozen with liquid nitrogen, and then freeze-dried for 24 h. The SEEs were obtained as a white powder, called SEE-Cel7A and SEE-Cel7B.



Scheme 2. Enzymatic synthesis of cellulose from cellobiose in a nonaqueous 8% (w/v) LiCl/DMAc using either SEE-Cel7A or SEE-Cel7B.

sis. However, a direct assay for the enzymatic polymerization of sugars is virtually difficult due to the divergent formation of polysaccharides with various DPs as the condensation reaction proceeds. Thus, in this study the enzymatic tolerance of SEEs to various organic solvents was estimated from the results of residual hydrolytic activity measurements after solvent-exposure treatments using SEE-Cel7A, which was enough in quantity secured for the assay. Table 1 lists the residual enzymatic activity in terms of the hydrolysis rate of *p*NPL of SEE-Cel7A treated with each organic solvent for 3 h. Enveloping Cel7A enzyme with $2C_{18}\Delta^9$GE surfactant interfered with its enzymatic activity; however the SEE-Cel7A demonstrated higher relative residual activity, *i.e.* high tolerance after solvent-exposure, than that of intact Cel7A without $2C_{18}\Delta^9$GE under all conditions. Highly polar organic solvents had negative influences on the residual activity of SEEs as compared with other solvents with low or no polarity such as chloroform or *n*-hexane, respectively. Nevertheless, interestingly the SEE-Cel7A treated with DMAc (log Pow = −0.796) preserved *ca.* 66% of the original SEE-Cel7A activity, whereas the polar solvents acetonitrile and methanol (log Pow = −0.38 and −0.82, respectively) drastically decreased the hydrolytic activity (residual activity: *ca.* 13% and *ca.* 24%, respectively). The recovery percentage of the tested SEEs from methanol or acetonitrile by centrifugation reached up to 95% by weight, being a negligibly small loss for the considerable reduction of the residual enzymatic activities. The comparison of the entries 7–10 provided interesting trends for SEEs. In the absence of surfactants, the deleterious effect of LiCl on the residual enzymatic activity was observed (entries 8 and 10), while such negative effect was perfectly settled by surfactant-wrapping of enzymes (entries 7 and 9). LiCl makes a complex with DMAc and strongly coordinated with unpaired electrons, *e.g.* hydroxyl groups of cellulose for the complete dissolution. It was presumed that the hydrophobic alkenyl chains of $2C_{18}\Delta^9$GE prevented such undesirable interaction with the surfaces of hydrophilic, naked Cel7A. Some researchers have attempted to correlate the enzymatic functions with the properties of organic solvents, such as dielectric constant, hydrogen bond donor/acceptor ability, polarity index and log Pow [31]. However, Valivety et al. [32] have reported the difficulties in determining a single parameter for solvent polarity that can predict the enzyme activity in organic media. In this work, the solvent toler-

Table 1
Residual enzymatic activity of SEE-Cel7A after 3 h incubation in various organic solvents; the water phase of the W/O emulsions during SEE-Cel7A preparation: 50 mM disodium hydrogen phosphate solution. Percentage values in parentheses show the relative residual activity against the original one (entry 14).

Entry	Solvent	$2C_{18}\Delta^9$GE	Residual enzymatic activity (nM min^{-1} (μmol of enzyme)$^{-1}$)	Relative residual activity (%)	log Pow
1	*n*-	+	565	72.0	3.900
2	Hexane	–	1447	(18.2)	
3	Chloroform	+	695	88.7	1.970
4		–	1618	(20.4)	
5	Acetonitrile	+	101	13.0	−0.380
6		–	480	(6.0)	
7	DMAc	+	516	65.8	−0.796
8		–	297	(3.7)	
9	8%	+	523	66.8	–
10	LiCl/DMAc	–	21	(0.3)	
11	Methanol	+	184	23.6	−0.820
12		–	327	(4.1)	
13	Deionized	+	784	100.0	–
14	water	–	7934	(100.0)	

ance behavior of SEEs showed similar trends as reported, *i.e.* SEEs were negatively influenced on the enzymatic tolerance by polar organic solvents as compared to other solvents with low or no polarity. Although a definite correlation was not possible, such high durability of SEEs in DMAc and LiCl/DMAc should prove promising in glycoengineering.

It is often difficult to compare enzyme activities among various cellulases due to the differences in the substrates for each enzyme. In our previous study, the SEE was prepared from crude cellulase (the ratio of protein/$2C_{18}\Delta^9$GE: 2/1 by weight) [26], and thus the accurate estimation for enzymatic activities of SEEs with various molar ratios of enzyme/$2C_{18}\Delta^9$GE involved technical difficulties. In this study, SEEs were prepared from only pure cellulase, and thus it became possible that the molar ratios of cellulase/$2C_{18}\Delta^9$GE were investigated.

Cel7A and Cel7B, which are both classified in glycoside hydrolase family 7 (GH7) and can hydrolyze *p*NPL as a common substrate, were used after complete purification. The SEEs are collected by simple centrifugation and can be subjected to the assay in an aqueous system. This enabled detailed examination of the enzymatic characteristics of SEEs based on the enzymatic activity per 1 mole of enzyme. However, the assay is limited by the fact that a low estimate of the hydrolytic activity of SEEs is determined as compared with intact cellulases without solvent-exposure for the following reasons: (i) poor dispersion of SEEs in aqueous systems, and (ii) the lower accessibility of *p*NPL molecule to the substrate-binding site.

In this enzymatic tolerance assay, it was revealed that the SEE-Cel7A treated with DMAc or with 8% (w/v) LiCl/DMAc had high activity up to *ca.* 70% of the original SEE-Cel7A, even though intact Cel7A was completely deactivated by the solvent resulting in a loss in enzymatic function. Next, the factors influencing the enzymatic tolerance of SEE-Cel7A and SEE-Cel7B to 8% (w/v) LiCl/DMAc was investigated with regard to the preparation conditions of SEEs.

3.2. Effects of aqueous phase pH and salt species in SEE preparation on enzymatic tolerance to 8% (w/v) LiCl/DMAc

In general, enzymatic activity in an aqueous phase strongly depends on the pH values and salt factors such as concentration and species. In our previous studies, the aqueous pHs of W/O emulsions during SEE preparation significantly influenced the apparent efficiency of polymerization and glyco-modification, although such enzymatic reactions proceeded in a nonaqueous system [26,28,29]. The crude cellulases used contained various types of enzymes having different catalytic behaviors [19,20], and thus their application became complicated for elucidating solvent tolerance and the catalytic mechanism of SEEs. Therefore, in the present study, surfactant-enveloped pure cellulase Cel7A (SEE-Cel7A) was prepared at different pH values and salt species in the aqueous phase of W/O emulsions to investigate the influences of these factors on the enzymatic tolerance to 8% (w/v) LiCl/DMAc.

Table 2 shows the hydrolytic activities of SEE-Cel7As, which were prepared at various pHs and salt species, after 3 h incubation in 8% (w/v) LiCl/DMAc. Although the same amounts of pure enzyme Cel7A were subjected to the SEE preparation, residual enzymatic activities were remarkably different. The SEE-Cel7A prepared using phosphate solution at pH 9.0 possessed the highest hydrolytic activity, *ca.* 67% of the original activity. Aqueous phases of W/O emulsions at higher pHs apparently brought about higher residual activities, and sodium phosphate was superior to other salts even outside the range of buffering effects. In our previous study, crude SEEs prepared with a phosphate solution at pH 9.0 have proven effective even beyond the limit of its buffering capacity [26], although the optimal pH of cellulase used is 4.5–5.0 in an aqueous hydrolysis reaction. For the reverse hydrolytic reaction, similar phenomena have been reported for glycosylation with chitinase in an aqueous medium [23,25]. In the present study, SEE-Cel7A with phosphate solution at pH 9.0 showed the highest residual activity after solvent-exposure, as similar as reported for the enzymatic synthesis of long-chain cellulose [26]. The mechanisms of pH-memory [1] and salt-mediated protection [33] of enzymes in organic media are still unknown; however phosphate solution at pH 9.0 was effective for enhancing the enzymatic tolerance of SEE-Cel7A in 8% (w/v) LiCl/DMAc.

Highly resistant SEEs in an aprotic organic medium with a high LiCl salt concentration were successfully designed, and in the following section two types of SEEs prepared from either Cel7A or Cel7B with disodium hydrogen phosphate were subjected to nonaqueous biocatalysis in 8% (w/v) LiCl/DMAc for direct enzymatic polymerization of cellobiose without any activation of the anomeric carbon by chemical pretreatment. Chain lengths and yields of as-synthesized cellulose are discussed with regard to the differences in the three-dimensional (3-D) structures of Cel7A and Cel7B.

3.3. Nonaqueous biocatalytic behaviors of SEE-Ce17A and SEE-Cel7B in cellulose synthesis

Cel7A and Cel7B are classified in the same GH family 7 from their amino acid sequences, but exhibit quite different catalytic behaviors, presumably due to the 3-D structures of their catalytic domains [19,20]. Cel7A has a 50 Å-long tunnel-shaped catalytic site with many substrate-binding subsites, whereas Cel7B possesses the active region in an open cleft [17,34]. It is also known that Cel7B has much higher enzymatic activity than Cel7A [35–37]; in this study *p*NPL hydrolytic activities of intact Cel7A and Cel7B were measured to be 7.9 and 27.8 $\mu M\,min^{-1}$ (μmol of enzyme)$^{-1}$, respectively, as shown in Table 3.

Table 3 compares the residual enzymatic activity of SEE-Cel7A and SEE-Cel7B exposed to 8% (w/v) LiCl/DMAc for 0, 3 and 6 h, with regard to the amount of surfactant ($2C_{18}\Delta^9$GE) versus each enzyme. In the case of SEE-Cel7A, the concentration of $2C_{18}\Delta^9$GE/toluene in the SEE preparation remarkably affected the enzymatic tolerance, resulting in better residual activity at a concentration of 5.0 mg/mL of $2C_{18}\Delta^9$GE/toluene (entry 3). SEE-Cel7B was not as dependent on the concentration of $2C_{18}\Delta^9$GE/toluene. In both cases, surfactant $2C_{18}\Delta^9$GE reduced the original activity, possibly due to the inhibition of substrate-binding, however the enzymatic tolerance differed depending on the type of enzyme. These results implied that the open, cleft-type active domain of Cel7B, a non-processive enzyme, could interact with substrates while the tunnel-shaped catalytic site of Cel7A, a processive-type enzyme, was easily influenced by enveloping the surface of the enzyme with surfactant. In terms of practicality, Cel7A can be obtained with ease in large quantities from fungi and thus, is a major candidate as a biocatalyst for nonaqueous glycosynthesis. Therefore, a controllable factor such as the amount of surfactant for influencing the SEE efficiency is of great significance. These reaction conditions may be applied to other enzymes having a similar 3-D structure and processive-type catalytic reaction, *e.g.* chitinase.

Either SEE-Cel7A or SEE-Cel7B was subjected to non-chemo-enzymatic, *i.e.* completely enzymatic, cellulose synthesis in nonaqueous 8% (w/v) LiCl/DMAc. In both cases, synthetic products had only a β-1,4-glycosidic linkage, from NMR results of the samples (Supporting Information). Thus, direct, enzymatic cellulose synthesis from cellobiose without any activation of the anomeric carbon, which is indispensable for conventional chemo-enzymatic synthesis, was possible using SEEs prepared from pure cellulases in a nonaqueous system, similar to our previously published results [26,28–30]. On the other hand, there were characteristic differences found in the conversion and chain length, depending on the type of enzymes utilized. In the case of SEE-Cel7A, water-insoluble cellulose with average DP *ca.* 20, which was determined by visco-

Table 2
Effects of salt species and pH values in the water phase of W/O emulsions on the hydrolytic activity of SEE-Cel7A after 3 h incubation in 8% (w/v) LiCl/DMAc.

Entry	Water phase	pH of the water phase	Buffering effect	Residual enzymatic activity (nM min^{-1} (μmol of enzyme)$^{-1}$)	Relative residual activity (% against entry 13 in Table 1)
1	Carbonate	9.0	+	477	60.8
2	Phosphate	9.0	–	523	66.8
3	Phosphate	7.0	+	472	60.3
4	Water	7.0	–	197	25.1
5	Acetate	4.8	+	193	24.6
6	Phosphate	4.2	–	388	49.5

Table 3
Residual hydrolytic activity of SEE-Cel7A and SEE-Cel7B after 0, 3 and 6 h incubation in 8% (w/v) LiCl/DMAc. Percentage values in parentheses show the relative residual activity against the original activity of respective untreated SEEs.

Entry	Enzyme	Concentration of enzyme (μM)	$2C_{18}\Delta^9$GE (mg/mL)	Enzymatic activity (nM min^{-1} (μmol of enzyme)$^{-1}$) Incubation time in 8% LiCl/DMAc (h)					
				0		3		6	
1	Cel7A	10	0.0	7934	–	0	–	–	–
2		10	2.5	288	(100)	14	(5.0)	11	(3.8)
3		10	5.0	784	(100)	523	(66.8)	169	(21.5)
4		10	10.0	246	(100)	26	(10.4)	19	(7.7)
5	Cel7B	10	0.0	27,796	–	0	–	–	–
6		10	5.0	485	(100)	87	(18.0)	29	(6.0)
7		10	10.0	399	(100)	80	(20.1)	44	(11.0)
8		10	15.0	185	(100)	40	(21.4)	26	(14.0)

metric analysis, was synthesized in *ca*. 15% yield. For SEE-Cel7B, longer-chain cellulose with DP *ca*. 60 and lower yield of *ca*. 3% was obtained. SEC analysis revealed the formation of cello-oligomers with DPs ranging from 3 to 5 (total conversion: less than 2%), whereas only quite small peaks were found in the SEC profile of the SEE-Cel7B product (Fig. 1), indicating low yields of water-soluble cello-oligomers. There are few reports about glycosynthesis using purified enzymes; Hayashi et al. [38] have reported the contribution of three pure processive-type enzymes (*T. viride* cellulases) to the transglycosylation of *p*NP-β-D-cellobioside in an acetonitrile/water co-solvent system. One of them (Exo I) could produce *p*NP-β-D-cellotetraoside at *ca*. 1.5% conversion. In a water-containing system, long-chain cellulose was not obtained, even though the anomeric carbon of cellobiose was activated by the *p*NP substituent. Nevertheless, SEE-Cel7A significantly produced a water-insoluble cellulose portion from cellobiose without any chemical modifications at *ca*. 15% yield (10-fold). According to the report by Hayashi et al. [35], the residual activity of Exo I after incubation in an acetonitrile/water mixture was regarded as being almost identical to that of the original crude cellulase, and thus the transcellobiosylation property was possibly attributed to that of the processive enzyme component. In this study, Cel7A, a processive-type enzyme, provided shorter chain polymers (DP *ca*. 20) with higher conversion (*ca*. 15%) than Cel7B, a non-processive enzyme. In our previous work, the SEE prepared from crude cellulases, *i.e.* the mixture of Cel7A and Cel7B, gave cellulose with DP > 100 and *ca*. 5% yield [26]. Therefore, it was reasonably presumed in this case that SEE-Cel7A first catalyzed the condensation between cellobiose and then SEE-Cel7B promoted the chain elongation using *in situ* synthesized short-chain cellulose. Such role-sharing is very unique and may have great potential for the functional design of glyco-materials in nonaqueous SEE-mediated biocatalysis.

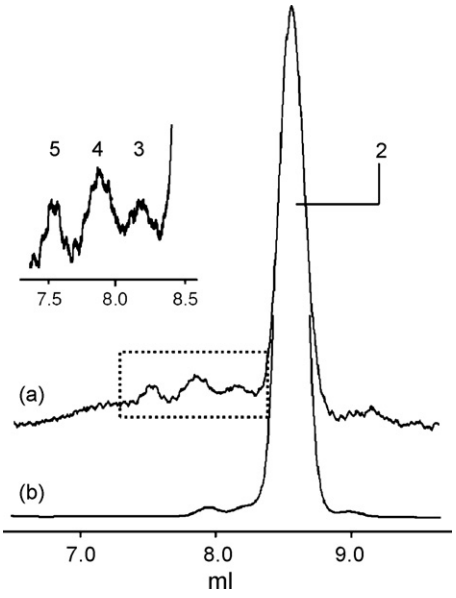


Fig. 1. SEC elution patterns of the water-soluble portions of products synthesized from 300 mM cellobiose using (a) SEE-Cel7A or (b) SEE-Cel7B. The symbol number 2, 3, 4 and 5 correspond to cellobiose, cellotriose, cellotetraose and cellopentaose, respectively.

4. Conclusions

Two types of SEEs were prepared from pure cellulases, CBH I (Cel7A) or EG I (Cel7B). Enzymatic tolerance to nonaqueous solvents depended on the preparation conditions of SEEs and, in particular, SEE-Cel7A treated with 8% LiCl/DMAc preserved *ca*. 67% of the original SEE-Cel7A activity. Both SEEs catalyzed the direct dehydration of cellobiose without tedious chemical pretreatment and synthesized cellulose. The SEE-Cel7A preferentially provided short-chain cellulose with DP *ca*. 20, and longer-chain cellulose with DP *ca*. 60 was obtained using the SEE-Cel7B, the products reflective of each enzyme's inherent hydrolytic behavior. Nonaqueous SEE-mediated biocatalysis using inexpensive glycohydrolases would have wide applications in glycosynthesis and glycoengineering applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.08.010.

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