Characterization of cyclodextrin glycosyltransferases (CGTases) and their application for synthesis of alkyl glycosides with oligomeric head group

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ABSTRACT

Cyclodextrin glycosyltransferases (CGTases) from Paenibacillus macerans, Thermoanaerobacter sp. ATCC 53627, Bacillus steatorrhophilus and a Carboxydocella sp. (phylogenetically identified from genomic DNA) were characterized with respect to their catalytic activity in different reactions, with emphasis on reactions useful for the elongation of the carbohydrate group of alkyl glycosides. All CGTases had activities between 95 and 115 U/mg in the coupling reaction between α-cyclodextrin (α-CD) as glucosyl donor and β-dodecyl maltoside as glucosyl acceptor, but differed very much in the competing hydrolysis of α-CD. The α-CD hydrolysis activity ranged from 0.13 U/mg for P. macerans CGTase to 10.5 U/mg for the Carboxydocella sp. (CspCGT13). Furthermore, the disproportionation activity was much lower for the Paenibacillus CGTase compared to the other CGTases, and consequently this enzyme produced the highest yield of the primary coupling product β-dodecyl maltooctaoside, which is a valuable surfactant. For production of a polydisperse alkyl glycoside product, disproportionation reactions are useful and the other three CGTases of the current study are efficient catalysts. The newly discovered Carboxydocella sp. (CspCGT13) CGTase has the special feature to produce more of products with even longer carbohydrate groups than the primary coupling product.

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1. Introduction

Cyclodextrin glucosyl transferases (CGTases) (EC 2.4.1.19) are produced by a wide range of bacteria, particularly Bacillus strains, and their main use is as catalysts in the production of cyclodextrins from starch [1,2]. However, these enzymes can catalyze also other reactions of practical interest. Important examples include disproportionation reactions in which part of the donor substrate is transferred to the acceptor substrate and coupling reactions in which a cyclodextrin is cleaved and the resulting maltooligosaccharide is transferred to an acceptor substrate [1,3]. In addition, the CGTases can catalyze hydrolysis reactions, but this activity is usually quite low. Coupling and disproportionation reactions have been used to glycosylate a wide variety of substances to make new products. These modifications have been carried out to achieve increased solubility, stability, biodegradability, improved antitumor and insecticidal activity and decreased cytotoxicity [4]. Due to the instability of vitamin C, various glycosylated derivatives have been developed to improve stability without any change in biological activity [5]. Likewise, hydroquinone glycosides, which have wide application in the cosmetic industry, have been produced by transglycosylation reactions catalyzed by CGTase from Thermoanaerobacter sp. ATCC 53627 to improve the stability and thereby the efficacy of hydroquinone [6]. Furthermore, glycosylation using Bacillus macerans (B. macerans) CGTase was used to increase the solubility of piceid, which in turn made its absorption more efficient [7]. Using a similar glycosylation strategy, the pharmacological activity of steroidal saponins was improved [8]. The CGTase-catalyzed glycosylation reactions is a practically useful alternative to Nature's normal glycosylation reactions involving glycosyl transferases, which has the major drawback in that they require sugar nucleotides or sugar phosphates as glycosyl donors [9].

Alkyl glycosides are non-ionic surfactants used for diverse applications, such as in protein science, personal care and food products [10]. However, the acid catalyzed Fischer process, used to prepare the commercially available surfactants of this type, generates products having an average of only 1–1.5 glucose residues. Likewise, enzymatic preparation of alkyl glycosides is mainly limited to

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products containing one monosaccharide residue [11–14]. Elongation of the carbohydrate part of alkyl glycosides is of particular interest, since it has been shown for other non-ionic surfactants that elongation of the hydrophilic group makes the surfactant milder to cells and tissues [15]. It has been shown that quite efficient coupling between α-cyclodextrin and alkyl glycosides can be achieved by CGTases, and this opens up the possibility to synthesize a whole range of highly interesting surfactants with elongated carbohydrate groups [16,17]. For some applications it might be beneficial to use a surfactant with exactly specified length of the carbohydrate part, and in this case it is important that the coupling reaction dominates over the disproportionation reaction. In other applications it might be advantageous to use a polydisperse surfactant containing a wide range of alkyl glycosides differing in the number of glucose residues, and in this case an enzyme efficient in disproportionation reactions is advantageous for the synthesis. It is thus of interest to identify both CGTases which catalyze primarily coupling reactions and for CGTases efficient in disproportionation reactions. So far, little information is available concerning the reactivity of CGTases in coupling and disproportionation reactions involving alkyl glycosides as acceptor substrates.

In the current study, a comparison was made between different CGTases with respect to their activity in different reactions, with special emphasis on reactions useful for the elongation of alkyl glycosides. The comparison included the commercially available CGTases from Paenibacillus macerans (here abbreviated Pm CGTase; the organism was previously called B. macerans), Bacillus steatothermophilus (Bs CGTase) and Thermoaeroabacter sp. ATCC 53627 (Toruzyme® 3.0L, Ts CGTase) as well as a newly discovered CGTase from Carboxydocella sp. (CspCGT13).

2. Materials and methods

2.1. Materials

Pm CGTase was purchased from Amano Enzyme Europe Ltd. (Milton Keynes, UK), Ts CGTase (Toruzyme® 3.0L) from Novozymes ( Bagsvaerd, Denmark), Bs CGTase from US Biological (Massachusetts, US) and Succharomyces cerevisiae α-glucosidase Type 1 from Sigma–Aldrich. The components for Luria–Bertani medium were purchased from Duchefa Biochemie (Haarlem, The Netherlands). α-Cyclodextrin (α-CD) was purchased from Wacker Chemie AG (Germany), β-dodecyl maltoside (β-DDM) from Antrace Inc. (Maumee, US), methyl-α-o-glucopyranoside (α-MG) from Sigma–Aldrich (US) and 4, 6-O-benzylidene p-nitrophenyl α-α-n-maltoheptaoside (BPNPG7) from Megazyme ( Wicklow, Ireland). HPLC grade solvents were from VWR International (Stockholm, Sweden).

2.2. Cloning and expression of CGTase from Carboxydocella sp. (CspCGT13)

A PCR based screening method was adopted for isolation of cyclodextrin glycosyl transferase (CGT) encoding sequences. The source was genomic DNA from isolates of thermophilic bacteria. The screening method consisted of two forward and four reverse primers for conserved amino acid sequences of region I and II, specific for cyclodextrin glycosyl transfersases of GH family 13 [18]. The primers were designed according to the CODEHOP strategy as previously described [19,20]. The CspCGT13 (GenBank accession number KJ806554) gene was isolated from Carboxydocella strain MAT3016 (16S rRNA sequence, GenBank accession number KJ806553). The retrieval of the 16S rDNA and CspCGT13 genes from genomic DNA is described by Ara et al. [21]. Further cultivation of the bacterial strain has not succeeded. The CGTase gene (CspCGT13) was expressed with the C-terminal His-tag in Escherichia coli strain BL21 DE3 (Novagen) at 37 °C in Erlenmeyer flasks using Luria–Bertani (LB) medium containing 100 μg/mL ampicillin and induced with 10% L-rhamnose at OD620 of 0.6. After 19 h of induction, the cells were harvested by centrifugation at 6000 × g at 4 °C for 20 min.

2.3. Purification of CGTase from Carboxydocella sp. (CspCGT13)

The cell pellet was dissolved in binding buffer (20 mM Tris–HCl, 750 mM NaCl, 20 mM imidazole pH 7.5) and the cells were disrupted by ultrasonication 3 times for 3 min, each time at 60% amplitude, using a 14-mm titanium probe (UP400S, Dr. Hielscher, Stuttgart, Germany). The supernatant was separated from the insoluble cellular fraction by centrifugation at 12000 × g for 20 min. The purification was performed by immobilized metal ion affinity chromatography using an ÄKTA prime system (Amersham Biosciences, Uppsala, Sweden). The column used for purification was a 5 mL His-Trap FF crude column (GE Healthcare, Sweden), containing 0.1 M copper (II) sulfate. Initially, the column was equilibrated with binding buffer and later the supernatant was loaded onto the column. The unbound proteins were washed off with binding buffer until the absorbance reached a steady baseline. Elution of the protein was achieved with a linear gradient of 20–250 mM imidazole (in 20 mM Tris–HCl, 0.75 mM NaCl, pH 7.5). The fractions containing protein were pooled and analyzed by SDS–PAGE [22]. After SDS–PAGE analysis, protein fractions were dialyzed against 20 mM sodium citrate buffer, pH 6.0, using a 3000 Da molecular mass cut-off dialysis membrane ( Spectrum Laboratories, Rancho Dominguez, CA), and stored at −20 °C until use. The presence of the active form was confirmed using a microtiter plate-based DNS-activity assay using wheat starch (Sigma–Aldrich) as substrate.

2.4. Enzyme assays

All CGTase catalyzed reactions were carried out at 60 °C with shaking at 700 rpm (HLC-Thermo shaker, Model MKR 13, Boven- den, Germany) with substrate solutions prepared in 10 mM sodium citrate buffer (pH 5.2) containing 2 mM CaCl2.

2.4.1. Coupling activity

The coupling activity was determined using 50 mM of α-MG as acceptor substrate and 10 mM of α-CD as donor with 1 mL reaction volume. The reaction was started by adding an appropriate amount of enzyme (1–5 μg/mL). Incubation was done for different time periods, before termination of the reaction by dilution with 3 mM NaOH. The consumption of α-CD was measured by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD, see below). The concentration of α-CD was determined by using a 5-point calibration curve (4–20 μM). One unit of coupling activity was defined as the amount of enzyme coupling 1 μmol of α-CD to α-MG per min.

2.4.2. Disproportionation activity

Disproportionation activity was measured by following a published method [23] with some modifications. The reaction mixture contained 2.5 mM of BPNPG7, a donor substrate blocked at the nonreducing end with a benzylidine group and at the reducing end with a p-nitrophenyl group and 10 mM maltose as acceptor. The reaction mixture with an appropriate amount of CGTase (0.62–1.25 μg/mL) was incubated at 60 °C and 50 μL aliquots were removed after different time periods (2, 5, 8 and 11 min). The reaction was stopped by adding 10 μL of 1.2 M HCl and incubation at 60 °C for 5 min, followed by neutralization of the mixture with 10 μL of 1.2 M NaOH. To neutralized samples, 100 μL (0.5 U) of α-glucosidase Type 1 in 800 mM potassium phosphate (pH 7.0) was added and incubated for 1 h at 37 °C to liberate p-nitrophenol. The
pH of the samples was raised to above 8 by adding 0.83 ml of 1 M sodium carbonate and the absorbance was measured at 401 nm. The concentration of p-nitrophenol released was determined using a 7-point calibration curve (0.005–0.1 mM). One unit of disproportionation activity was defined as the amount of enzyme converting of 1 μmol of BPNP G7 per min.

2.4.3. Hydrolysis activity

Hydrolysis activity at 60 °C was determined using 200 mM of α-CD and 10 μg/ml of CGTase. Samples were taken after 1 h and diluted in milli-Q water for analysis by HPAEC-PAD of different hydrolysis products: maltohexose (G6), maltopentose (G5), maltotetraose (G4), maltotriose (G3) and maltose (G2). The concentrations were determined, using 5-point calibration curves (4–20 μM). One unit of hydrolysis activity was defined as the amount of enzyme releasing 1 μmol of linear oligosaccharides per min.

2.4.4. Measurement of protein concentration

Protein concentrations were determined using the Bradford reagent (Sigma–Aldrich, St. Louis, US) with bovine serum albumin (BSA) as the protein standard.

2.5. Dodecyl-β-D-maltotetraoside (β-DDMO) synthesis

All synthesis reactions were carried out at 1 ml scale in 4.5 ml septum capped glass vials using a thermo shaker (HLC-Thermo shaker, Model MKR 13, Bovenden, Germany) set at 60 °C and 700 rpm. All substrate solutions contained α-CD, β-DDMO and 2 mM CaCl2 in 10 mM Na-citrate buffer (pH 5.2) unless otherwise stated. 50 μl aliquots of the reaction mixture were removed and mixed with 950 μl of 3 mM NaOH to stop reaction prior to analysis by high performance liquid chromatography with charged aerosol detector (HPLC-CAD).

The effect of different buffers (sodium citrate, sodium acetate, citrate phosphate and succinate buffer, 10 mM, pH 5.2) on the synthesis of β-DDMO was studied using 50 mM β-DDM and 200 mM α-CD as substrates and 5 μg/ml of Pm CGTase as catalyst.

2.6. Preparative synthesis and purification of β-DDMO

Preparative synthesis was carried out using 50 mM β-DDM, 200 mM α-CD, 5 μg/ml Pm CGTase, in a total reaction volume of 40 ml in a 250 ml round bottomed flask with magnetic stirring. The reaction temperature was maintained at 60 °C using an oil bath (Heidolph MR 2002, Heidolph Instruments GmbH & Co., Germany). After 25 min of incubation time, the reaction was terminated by boiling for 10 min. After cooling the reaction mixture to room temperature, 80 ml of 20% methanol was added. The diluted reaction mixture (preheated at 60 °C to form a clear solution) was loaded on a C18 reverse phase flash chromatography column preconditioned with 20% methanol. Elution was carried out using a stepwise gradient (20–75% methanol) to elute unreacted α-CD/β-DDM and the primary coupling product (β-DDMO). At 60 and 65% methanol, 5 ml fractions were collected. The purity of fractions containing mainly β-DDMO was checked by HPLC and satisfactory fractions were pooled. The solvent was evaporated using a rotary evaporator (Buchi RE 111 Rotavapour, Switzerland). The final product was suspended in milli-Q water and then lyophilized. The product was used for making standard curves for the HPLC-CAD analysis described below.

2.7. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis

Consumption of α-CD in the coupling activity assay and formation of different hydrolysis products were analyzed by HPAEC-PAD (ICS-5000, Dionex, Sunnyvale, CA, USA) using a Dionex CarloPac™ PA 200 column (3 mm × 250 mm, 8.5 μm) and a Dionex CarloPac™ PA 200 guard column (3 mm × 50 mm, 8.5 μm). Elution was carried out at flow rate of 0.5 ml/min using 50% milli-Q water (A) and a gradient of 400 mM Na-acetate (B) and 200 mM of NaOH (C). The proportion of (B) was increased from 0 to 20% in 30 min and the proportion of (C) was decreased from 50 to 30%. The column was equilibrated for 5 min with 50% each of (A) and (C) after each run. Standards of α-CD, maltotetraose, maltopentose, maltotriose, maltotriose and maltose (Sigma–Aldrich) were used to identify hydrolysis products. Samples were diluted in milli-Q water (to keep the final concentrations of samples below 30 μM) before analysis. The injection volume was 10 μl.

2.8. High-performance liquid chromatography with charged aerosol detection (HPLC-CAD)

Alkyl glycosides were analyzed using a Dionex HPLC system (Thermo Scientific Dionex Ultimate® 3000) provided with a C18-RP column (Acclaim™ RS LC 120 C18, 2.2 μm 120 Å, 2.1 mm × 150 mm, Thermo Scientific) connected to a charged aerosol detector (CAD) operating at 30 °C with a nebulizer gas flow of 2.5 L/min and an operating gas pressure of 35 psi (2.4 bar). Elution was carried out using a linear gradient of acetonitrile (A) and 0.1% (v/v) acetic acid in milli-Q water (B) at a flow rate of 0.4 ml/min and an injection volume of 5 μl. The proportion of eluent (A) was increased from 35 to 70% in 20 min and after that the proportion of (A) was returned to 35% in 1 min and was kept steady for 4 min before start of the next run. Standard curves of β-DDMO, β-DDM and β-dodecyl maltotriose were prepared under identical operating conditions.

3. Results and discussion

3.1. Characterization of CGTases

The His-tagged CspCGT13 was overexpressed in E. coli and after one-step purification by IMAC the enzyme was estimated to have a purity of 80–90% (Fig. 1). According to SDS-PAGE, the molecular mass of the protein band was 75 kDa. The three commercially available CGTases had lower purity (Fig. 1), but in order to facilitate comparisons with literature data obtained with those enzymes, no purification was attempted.

The main aim of this study was to evaluate the coupling activity of CGTases, because this reaction is very useful in surfactant synthesis. The coupling reaction is probably the least investigated of the CGTase catalyzed reactions, and when studied the aim has often been to reduce its rate. Successful decrease of both coupling and hydrolysis activity was achieved by directed evolution of Thermoanaerobacterium CGTase (Ta CGTase), while the desired cyclization activity was much less affected [24]. The coupling activity of the CGTases studied here was measured in a model reaction with α-CD as glycolysol donor and α-MG as acceptor. The reaction rates were determined by quantification of α-CD, using HPAEC-PAD. The standard curve is shown as Fig. S1 in Supplementary data. CspCGT13 expressed the highest coupling activity followed by Ts CGTase, whereas Pm CGTase and Bs CGTase had considerably lower activity (Table 1). In addition to the quantification of the coupling activity, the HPAEC-PAD assay also provided an overview of the reaction products. Pm CGTase catalyzed the formation of one main product, which was expected to be the primary coupling
product methyl α-maltoheptaoside (Fig. 2). In addition, a few minor peaks were observed and tentatively identified as other methyl α-maltooligosaccharides formed in disproportionation reactions. The disproportionation products were formed in large amounts in the reactions catalyzed by the other three CGTases (Fig. 2).

Supplementary Fig. S1 related to this article can be found, in the online version, at doi:10.1016/j.procbio.2015.02.016.

For the measurement of disproportionation activity, BPNPG7 was used as donor substrate and maltose as acceptor. Pm CGTase expressed the lowest disproportionation activity, while the other three CGTases had similar activities in this assay (Table 1). The ratio between coupling and disproportionation activities in the model reactions was the highest for Pm CGTase, which thus is a promising candidate when the aim is to carry out coupling reactions with minimal interference from disproportionation reactions, for example in the synthesis of a primary coupling product, such as β-dodecyl maltooctaoside (β-DDMO).

### Table 1

<table>
<thead>
<tr>
<th>CGTase source</th>
<th>Coupling activity* (U mg⁻¹)</th>
<th>Disproportionation activity* (U mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. macerans</td>
<td>203 ± 16</td>
<td>25.1 ± 5.0</td>
</tr>
<tr>
<td>Thermoanaerobacter sp.</td>
<td>349 ± 10</td>
<td>91.7 ± 4.0</td>
</tr>
<tr>
<td>ATCC 53627</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxydоcella sp.</td>
<td>457 ± 12</td>
<td>119.8 ± 1.7</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>193 ± 14</td>
<td>117.4 ± 2.7</td>
</tr>
</tbody>
</table>

*50 mM α-MG (acceptor) and 10 mM α-CD (donor), enzyme concentration (1–5 µg/mL).

### 3.2. Finding reaction conditions for coupling of α-CD and β-DDM

Preliminary optimization of the reaction conditions for the coupling reaction between α-CD and β-DDM was carried out using Pm CGTase as catalyst. The major product was the primary coupling product β-DDMO. The optimal pH for the reaction has been determined previously to 5.2 [16]. No significant differences were observed between reactions in different buffers having this pH value (results not shown), and 10 mM citrate buffer containing 2 mM calcium chloride was chosen for the rest of the study. The rate in the coupling reaction can be expected to increase with increasing concentration of the substrates, but at too high substrate concentrations precipitation can occur [25]. Increasing the α-CD concentration from 100 to 200 mM at a β-DDM concentration of 50 mM caused a considerable increase in reaction rate, while further increase to 300 mM α-CD caused negligible effects on the initial reaction rate and only a slight improvement of the yield of coupling product in the later stages of the reaction (Fig. 3), which is in agreement with a previous study [16]. Concentrations of 200 mM α-CD and 50 mM β-DDM were chosen for further studies.

### 3.3. Activity of CGTases in coupling reactions and in competing α-CD hydrolysis

The initial rates in the coupling reaction between α-CD and β-DDM were between 95 and 115 U/mg for the four CGTases (Table 2). The coupling activities found in the current study depended on the acceptor substrate used, with higher rates observed with α-MG (Table 1) than with β-DDM (Table 2). In a previous study, maltose was shown to be the best acceptor [26]. The alkyl chain makes β-DDM into a worse acceptor than maltose. A major reason is probably that β-DDM forms micelles (the critical micelle concentration is 0.17 mM) and complexes with α-CD, and these aggregated forms of the substrate can be expected to be less efficient due to steric hindrance [25,27].

A potential side reaction in the coupling between α-CD and β-DDM is the hydrolysis of α-CD. The activity of the CGTases in this reaction was measured separately at an α-CD concentration of 200 mM, and large differences between the enzymes were observed (Table 2). The Pm CGTase expressed almost two orders of magnitude lower hydrolysis activity than the CspCGT13 and about one order of magnitude lower activity than Ts CGTase. In measurements of hydrolysis activities of related CGTases using starch as substrate it was found that Ts CGTase had about 18 times higher hydrolysis activity compared to the Bacillus circulans strain 251 CGTase, while the cyclization and disproportionation activities differed with less than a factor of 2 [28]. Also on starch, CspCGT13 expresses relatively high hydrolysis activity: 13.1 U/mg [21]. Low hydrolysis activity might be a common feature of Bacillus/Paenibacillus CGTases, although Bs CGTase expressed relatively high hydrolysis activity on α-CD.

The pattern of products formed in the hydrolysis of α-CD was studied as well, and the results after 1 h reaction time are...
Fig. 2. HPAEC-PAD chromatograms showing product profiles in coupling reactions between α-MG (50 mM) and α-CD (10 mM) catalyzed by different CGTases (5 μg/mL). Panel (a) Thermoanaerobacter sp. ATCC 53627 (black), P. macerans (red); Panel (b) Carboxydocella sp. (CspCGT13) (black), B. stearothermophilus (blue); peak 1–4: disproportionation products, peak 5: residual α-CD, peak 6: primary coupling product. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shown in Table 3. In the reaction catalyzed by Pm CGTase, only maltohexaose was detected. In the other cases further hydrolysis and/or disproportionation reactions caused the formation of shorter oligosaccharides. In the cases of Ts and Bs CGTases, maltose and maltotriose were detected and in the case of CspCGT13 all saccharides between maltose and maltohexaose were formed. It can be assumed that maltohexaose was the primary hydrolysis product in all cases and that disproportionation or further hydrolysis caused the formation of the shorter oligosaccharides. The observation that only maltohexaose was formed by Pm CGTase correlates well with the low hydrolysis and disproportionation activities of this enzyme.

<table>
<thead>
<tr>
<th>CGTase source</th>
<th>Product composition (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maltohexaose</td>
</tr>
<tr>
<td>P. macerans</td>
<td>100</td>
</tr>
<tr>
<td>Thermoanaerobacter sp. ATCC 53627</td>
<td>45</td>
</tr>
<tr>
<td>Carboxydocella sp.</td>
<td>37</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>82</td>
</tr>
</tbody>
</table>

* n.d (not detected).

Table 4
Product profiles of different CGTases in the reaction between 50 mM β-DDM and 200 mM α-CD at the reaction time giving the maximal concentration of the primary coupling product (β-DDMO).

<table>
<thead>
<tr>
<th>CGTase</th>
<th>Residual substrate β-DDM (mM)</th>
<th>Disproportionation products (mM)</th>
<th>1° Coupling product β-DDMO (mM)</th>
<th>2° Coupling product (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. macerans</td>
<td>24</td>
<td>6.4</td>
<td>15</td>
<td>2.5</td>
</tr>
<tr>
<td>Carboxydocella sp.</td>
<td>13</td>
<td>25</td>
<td>7</td>
<td>0.7</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>26</td>
<td>13</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>Thermoanaerobacter sp. ATCC 53627</td>
<td>20</td>
<td>22</td>
<td>6</td>
<td>0.4</td>
</tr>
</tbody>
</table>
3.4. Optimization of production of the primary coupling product

Once the primary coupling product is formed by the CGTase, it can be further converted in disproportionation, hydrolysis and secondary coupling reactions. In order to get a maximal yield of the primary coupling product it is thus necessary to stop the reaction at a suitable time. The time course of the *Pm* CGTase catalyzed reactions starting with α-CD and β-DDM was studied using different enzyme concentrations (Fig. 4). As expected, the time needed to reach the maximum decreased with increasing enzyme concentration and the maximal β-DDMO concentration was similar in most cases. However, at the highest enzyme concentration a lower maximal yield of primary coupling product was obtained. It thus seems that the primary coupling reaction to some extent was limited by other factors than the enzyme concentration, while the reactions consuming the primary coupling product were clearly favored by the increased enzyme concentration.

![Graph](image)

The CGTases were compared with respect to their ability to produce β-DDMO from β-DDM. *Pm* CGTase produced the highest maximal β-DDMO concentration, followed by *Bs*, *CspCGT13* and *Ts* CGTases (Table 4). When the maximal β-DDMO concentration was reached, the remaining substrate concentration was between 13 and 26 mM, and all the product mixtures contained below 3 mM of the secondary coupling product. Relatively high concentrations of disproportionation products were detected, especially for the *CspCGT13* and *Ts* CGTases. The product profiles are shown as reversed phase HPLC-CAD chromatograms in Fig. 5. The standard curve is shown as Fig. S2 in Supplementary data. It is worth pointing out that considerable amounts of dodecyl glucopyranoside were detected in all cases except with the *Pm* CGTase. Longer dodecyl
oligosaccharides were observed mainly in the reaction mixture obtained with CspCGT13.

Supplementary Fig. S2 related to this article can be found, in the online version, at doi:10.1016/j.procbio.2015.02.016.

The ratio between the coupling and disproportionation activity is of special importance when aiming at the preparation of the primary coupling product using a CGTase catalyzed conversion. Among the enzymes evaluated in the current study, Pm CGTase was clearly the best one with a ratio of about 8 of the activities listed in Table 1. In agreement with this, Pm CGTase also produced the highest ratio of coupling to disproportionation products in Table 4. Comparison with literature data on coupling/disproportionation ratios is somewhat difficult because of differences concerning reaction conditions and sometimes also substrates. However, it is obvious that a coupling/disproportionation ratio of 8 is unusually high. The thoroughly studied B. circulans strain 251 CGTase had a ratio of about 0.2 when α-CD was used as donor and 0.3 when β-CD was used [3], while Ta CGTase had a coupling activity with α-CD of 1.3 times the disproportionation activity [22]. It thus seems that the Pm CGTase is unusually well suited for coupling reactions involving α-CD.

The maximal β-DMMO yield obtained with the Pm CGTase was 30%. This yield is mainly dependent on the specificity of the enzyme and the substrate concentrations used. β-DMMO competes with α-CD as donor substrate and the substrate ratio α-CD/β-DMM is thus a key parameter. In this study a ratio of 4 was used. This moderate excess of α-CD is attractive for practical applications, but using a ratio of 40, it was possible to increase the yield to about 80% [16]. The ideal way to reach a high yield at a moderate excess of α-CD is to use an enzyme with an even more pronounced preference for the coupling reaction compared to disproportionation. The Pm CGTase is the best one of those included in the present study, but it seems worthwhile to search for even better enzymes.

All CGTase-catalyzed reactions in this study were carried out at 60 ◦C to avoid problems due to thermal enzyme inactivation. The Pm CGTase has been reported to have a half life of about 132 and 18 min at 60 and 70 ◦C respectively [29], while the other enzymes are more thermostable (data not shown). Working at higher temperature than 60 ◦C can be advantageous, especially since it can make it possible to work at higher substrate concentrations, without problems due to aggregation of substrates and products.

4. Conclusion

In conclusion, Pm CGTase was shown to be the most advantageous catalyst for coupling of α-CD and β-DMM, with minimal interference from disproportionation reactions. Another beneficial feature of this enzyme was its unusually low hydrolytic activity, which also promoted high yields of coupling product. If instead a broad product distribution is desired, disproportionation reactions are useful and the other three CGTases of the current study are efficient catalysts. CspCGT13 has the special feature to produce more of products longer than the primary coupling product.

Acknowledgements

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