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Two-step enzymatic synthesis of maltooligosaccharide esters

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Abstract

Glucose and maltose esters were synthesised in organic media by employing a lipase (E.C. 3.1.1.3) from *Candida antarctica*. In a second reaction step, a transglycosylation catalysed by a cyclodextrin glycosyltransferase (E.C. 2.4.1.19) from either *Paenibacillus* sp. F8 or *Bacillus* sp. strain no. 169 (DSM 2518) extended the degree of polymerisation (DP) of the carbohydrate moieties of the carbohydrate esters. The donor substrates used were either a cyclodextrin, a maltooligosaccharide or starch. The highest rate of low DP maltooligosaccharide ester formation was obtained when starch was used as glycosyl donor and caproyl maltose as glycosyl acceptor. The structures of two of the products were identified by ^{1}H and ^{13}C NMR and MALDI-TOF MS as capronate monoesters of maltotriose and maltotetraose, with the ester bond at C-6 of the second glucose unit from the reducing end.

Keywords: Maltooligosaccharide esters; Lipase; Organic media; Cyclodextrin glycosyltransferase; Transglycosylation

1. Introduction

Due to their amphiphilic, emulsifying and bioactive properties there is growing interest in the application of carbohydrate fatty acid esters both as biodegradable detergents [1–3] and as fine chemicals for cosmetic [4], food [5,6] and pharmaceutical applications [7–11]. Carbohydrate esters can be synthesised in high yields by lipase-catalysed reversed hydrolysis reactions in organic media [12–15]. Due to the specificity of the enzymes, it is often possible to synthesise specific isomers of carbohydrate esters, especially monoesters. These products are difficult to obtain by conventional organic synthesis, where usually polyesters are formed [6]. Specific regioisomers of a carbohydrate ester can be produced using different enzymes and it has also been shown that the regio- and stereospecificity of the hydrolases can be altered by changing the solvent composition [16–18].

Only a few reports are available on the esterification of carbohydrates with a degree

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of polymerisation (DP) of above two [19–21]. This may be due to their low solubility in most organic solvents and/or to the substrate specificity of the enzyme. *C. antarctica* lipase is able to catalyse the synthesis of glucose and maltose monoesters in *tert*-butanol while maltotriose esters cannot be synthesised [22,23]. A subtilisin-catalysed synthesis of 6^{III}-*O*-butyryl maltotriose in pyridine has been reported [19].

Synthesis reactions with two enzymatic reaction steps offer new possibilities for obtaining specific carbohydrate esters [4,7,14,24–26]. For instance, Carrea and co-workers obtained regioisomerically pure 1-*O*-acyl fructose by the combined action of subtilisin in organic medium and an α-glucosidase in aqueous medium [24]. This isomer could not be produced in a pure form by a hydrolase-catalysed acylation of fructose in organic media [3,27].

Cyclodextrin glycosyltransferases (CGTases, E.C. 2.4.1.19) are capable of inter- and intramolecular glycosyl transfers as well as hydrolysis reactions using α-(1→4)-glucans (e.g., amylose) as donor substrate. Since hydrolysis reactions are disfavoured by CGTases [28], the glycosyl transfer reactions are dominating. The intramolecular glycosyl transfer reactions leads to the formation of a wide range of cyclic α-(1→4)-glucans, such as the α-, β-, and γ-cyclodextrins [28,29]. Since the enzyme can utilise a wide range of carbohydrates as acceptor substrates, we investigated if carbohydrate esters can also be accepted as substrates.

In this report we describe a method for synthesis of maltooligosaccharide esters by a combination of a lipase-catalysed acylation of glucose or maltose in organic media and a CGTase-catalysed transglycosylation in aqueous media.

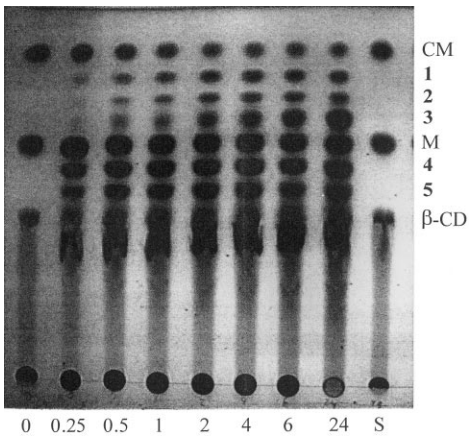

Fig. 1. TLC analysis of reaction products obtained by incubating *Paenibacillus* sp. F8 CGTase with 2% (w/v) β-cyclodextrin and 0.5% CM in 50 mM Tris pH 8.0 containing 2 mM $CaCl_2$, 0.02% NaN_3, at 40 °C for various periods of time (h); **1**–**5**, products **1**–**5**; S, standards; CM, 6′-*O*-caproyl maltose; M, maltose; β-CD, β-cyclodextrin.

Table 1
Reaction products **1**–**3** synthesised by incubation of *Paenibacillus* sp. F8 CGTase with β-cyclodextrin and CM and isolated by preparative TLC

Product	R_f value	Compound
1	0.82	6^{II}-*O*-Caproyl maltotriose [a]
2	0.76	6^{II}-*O*-Caproyl maltotetraose [a]
3	0.70	Caproyl maltopentaose [b]

[a] Structure obtained by MS and NMR analysis.
[b] Based on chromatographic analysis.

2. Results and discussion

6-*O*-Caproyl glucose (CG) and 6′-*O*-caproyl maltose (C6-(O-6)-α-D-Glc*p*-(1→4)-D-Glc*p*) (CM) were synthesised in *tert*-butanol from capronic acid and either glucose or maltose, using immobilised *C. antarctica* lipase (Novozym SP435) as catalyst [22]. TLC analysis of the reaction mixture obtained by incubating *Paenibacillus* sp. F8 CGTase with β-cyclodextrin and CM revealed the formation of three products (**1**–**3**) with R_f values between those of CM and maltose (Fig. 1 and Table 1). Their R_f values indicated that they were more hydrophobic than the maltooligosaccharides. Two further products (**4** and **5**) with R_f values corresponding to maltotriose ($R_f = 0.58$) and maltotetraose ($R_f = 0.51$) could also be detected. Products **1**–**3** were isolated by preparative TLC. GC analysis of **1** and HPAEC-PAD analysis of **1**–**3** also indicated the presence of capronate esters of maltotriose, maltotetraose and maltopentaose, respectively. The analysis showed that **1** and **2** were not contaminated with other carbohydrates, while **3** also contained glucose. MALDI-TOF mass spectrometry of **1** and **2** indicated that they were capronate ester of maltotriose ($MW = 602.6$ g mol^{-1}) and

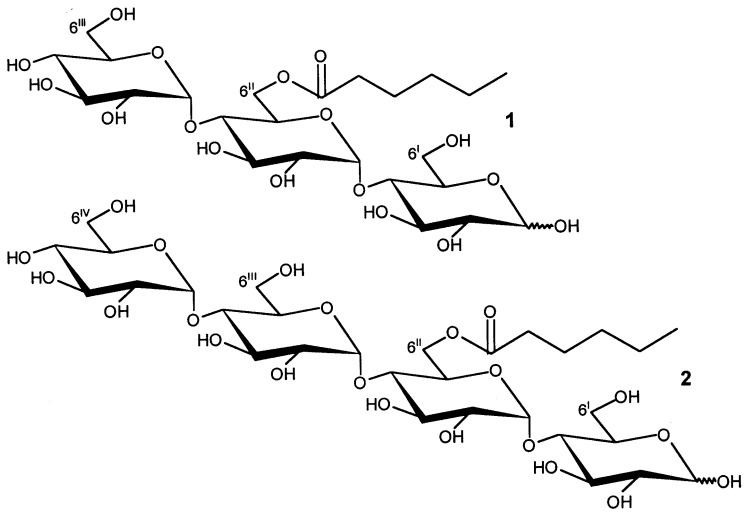

Fig. 2. Structure of the synthesised 6^{II}-*O*-caproyl maltotriose (**1**) and 6^{II}-*O*-caproyl maltotetraose (**2**).

maltotetetraose (MW = 764.3), respectively. Both were observed as their sodium adducts.

The ^{1}H and ^{13}C spectra of **1** and **2** were assigned by using two-dimensional experiments (^{1}H COSY, TOCSY, NOESY and ^{1}H–^{13}C-HSQC). The compounds were thereby identified as maltotriose and maltotetraose esters with the capronate group at C-6 of the glucose unit next to the reducing residue, i.e., 6^{II}-*O*-maltotriose and 6^{II}-*O*-maltotetraose, respectively (Fig. 2). This could be determined from the assignment of the spin system of glucose residues and especially from the fact that the H-6 protons of the second glucose residue from the reducing end of both products were shifted down field. Likewise, a clear effect was observed from the ^{13}C signals of C-5 and C-6 on the second glucose units from the reducing end which were shifted up and down field, respectively, relative to the underivatised glucose residues. The sequence of the residues could be determined from NOE contacts. ^{1}H and ^{13}C chemical shifts of **1** and **2** are listed in Table 2.

Products with identical R_f values to those obtained with β-cyclodextrin were obtained using either starch, α-cyclodextrin, γ-cyclodextrin, maltohexaose or maltoheptaose as donor substrate. However, the formation rates of the products were different. The highest rate of caproyl maltotriose formation (29 μmol min^{-1} g^{-1}) was found with starch as donor substrate, followed by β-cyclodextrin (11 μmol min^{-1} g^{-1}). The product formation rates obtained with the maltooligosaccharides as donor substrates were lower by a factor of about ten than with the cyclodextrins.

With CG as acceptor substrate, a product with the same R_f value as CM was observed in addition to the products shown in Fig. 1. The formation rates of the maltooligosaccharides with low DP were reduced by approximately 50% compared to the reactions using CM as acceptor.

It was also investigated if it was possible to synthesise maltooligosaccharide esters with longer acyl chains. 6-*O*-myristyl glucose and 6′-*O*-myristyl maltose were prepared using the *C. antarctica* lipase and employed in a glycosyl transfer reaction catalysed by the CGTase from *Paenibacillus* sp. F8 with starch or β-cyclodextrin as donor substrates. With myristyl maltose as acceptor substrate, TLC analysis indicated the formation of myristyl maltooligosaccharides in the same pattern as obtained with the caproyl maltooligosaccharides. However, the yields of myristyl maltooligosaccharides were significantly lower than the corresponding yields of caproyl maltooligosaccharides. No oligosaccharide esters could be detected using myristyl glucose as acceptor substrate which was possibly due to the low solubility of myristyl glucose in the aqueous reaction medium used. Similar results were obtained with a CGTase preparation from *Bacillus* sp. strain 169 (DSM 2518). This indicates that the glycosyl transfer reactions observed were not restricted to a specific type of CGTase.

Table 2
^{1}H-chemical shifts, coupling constants in Hz (given in parenthesis) and ^{13}C-chemical shifts of **1** [α-D-Glc*p*-(1→4)-(C6-(O-6))-α-D-Glc*p*-(1→4)-D-Glc*p*] and **2** [α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)(C6-(O-6))-α-D-Glc*p*-(1→4)-D-Glc*p*]

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
1							
α-Glc-(1→4)	5.319 (3.8)	3.536 (10)	3.646 (9.5)	3.403 (9.5)	3.632	3.799	3.737
C6-(O-6)	2.404 (7.4)	1.590	1.266	1.271	0.832 (6.5)		
α-Glc-(1→4)	5.353 (3.8)	3.589 (10)	3.928 (10)	3.638 (9)	4.011 (3, 4.8)	4.446 (12.2)	4.202
α-Glc	5.183 (3.8)	3.522 (10)	3.923 (9)	3.584 (9)	3.891	3.812	3.745
β-Glc	4.608 (8)	3.226 (9)	3.723 (9)	3.589 (9)	3.538 (4, 6)	3.854 (12)	3.704
2							
α-Glc-(1→4)	5.331 (3.8)	3.538 (10)	3.650 (9.5)	3.382 (9.5)	3.669	3.802	3.728
α-Glc-(1→4)	5.338 (3.8)	3.576 (10)	3.917 (9)	3.644 (9)	3.727	3.764	3.770
C6-(O-6)	2.403 (7.4)	1.585	1.265	1.273	0.833		
α-Glc-(1→4)	5.361 (3.8)	3.587 (10)	3.924 (10)	3.645 (9)	4.001 (3, 4.8)	4.419 (12.2)	4.221
α-Glc	5.185 (3.8)	3.519 (10)	3.925 (9)	3.590 (9)	3.895	3.806	3.743
β-Glc	4.608 (8)	3.229 (9)	3.726 (9)	3.594 (9)	3.544 (4, 6)	3.857 (12)	3.707
	C-1	C-2	C-3	C-4	C-5	C-6	
1							
α-Glc-(1→4)	101.3	72.7	73.7	70.1	73.7	61.6	
C6-(O-6)	35.6	24.8	22.5	22.5	14.0		
α-Glc-(1→4)	100.6	72.4	74.2	78.5	70.0	64.4	
α-Glc	92.9	72.3	74.2	78.3	71.0	61.5	
β-Glc	96.8	75.0	77.2	78.3	75.5	61.7	
2							
α-Glc-(1→4)	101.0	72.6	73.9	70.3	73.7	61.4	
α-Glc-(1→4)	101.0	72.5	74.2	78.1	72.3	61.1	
C6-(O-6)	34.6	24.5	22.6	22.6	14.1		
α-Glc-(1→4)	100.5	72.5	74.2	78.1	70.0	64.4	
α-Glc	93.0	75.0	77.2	78.1	75.5	61.7	
β-Glc	96.8	75.0	77.2	78.1	75.5	61.7	

The above results show that glucose and maltose esters with the ester bond at C-6 and C-6′, respectively, can be used as glycosyl acceptors in a CGTase catalysed transglycosylation. Based on the mechanism for transglycosylation reactions by Mosi et al. [30] the following scheme for the synthesis of maltooligosaccharide esters is proposed for the case of 6^{II}-*O*-caproyl maltotriose using either starch or β-cyclodextrin as donor substrate and 6′-*O*-caproyl maltose as acceptor (Fig. 3). The inter- and intramolecular glycosyl transfer reactions as well as the hydrolysis of both cyclic and linear α-(1→4)-glucans by CGTases proceeds via a double-displacement mechanism where the intermediate is covalently bound to the enzyme [30]. With starch

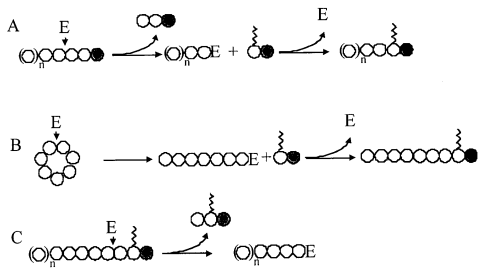

Fig. 3. Proposed pathways of the formation of 6^{II}-*O*-caproyl maltotriose from CM and α-(1→4)-glucans catalysed by CGTase. (○) Non-reducing glucose unit. (●) Reducing glucose unit. (▼) Position of enzyme attack. (A) Intermolecular glycosyl transfer attaching CM to a linear α-(1→4)-glucan. (B) Intermolecular glycosyl transfer opening a cyclic α-(1→4)-glucan (β-CD) and attaching CM. (C) Deglycosylation forming 6^{II}-*O*-acyl maltotriose and enzyme–substrate complex which can participate in a glycosylation step according to step (A) or (B). *n* can be a positive integer up to the DP of starch. CM: 6′-*O*-caproyl maltose.

as donor substrate, CGTases are capable of attacking α-(1→4)-glucans in a random fashion, resulting in the formation of a large variety of maltooligosaccharide–enzyme intermediates [29] (Fig. 3(A)). It is likely that a variety of maltooligosaccharides esters with higher DP are formed initially and act as substrate for several subsequent glycosyl transfer reactions. This results in the accumulation of caproyl maltotriose and other caproyl maltooligosaccharides with a low DP (Fig. 3(C)). With β-cyclodextrin as donor substrate, only the maltoheptaose–enzyme intermediate can be formed. If CM is the acceptor, this results in the formation of caproyl maltononaose (Fig. 3(B)). Therefore, a second enzymatic attack must have taken place to form the 6^{II}-*O*-caproyl maltotriose and other caproyl maltooligosaccharides of low DP (Fig. 3(C)). Following the reaction mechanism shown above, the products formed with CM as glycosyl acceptor should have the ester group at C-6 of the second glucose unit from the reducing end. This has been confirmed by the structural analysis of both **1** and **2**.

According to the reaction mechanism above it is likely that the caproyl maltooligosaccharides formed with CG as glycosyl acceptor have the ester group at C-6 of the reducing glucose unit. However, this has not yet been confirmed by structural analysis of reaction products.

The results show that novel maltooligosaccharide esters can be synthesised by a combination of an esterification catalysed by a lipase in organic media and a glycosylation catalysed by a CGTase in aqueous media.

3. Experimental

Chemicals and enzymes.—Cyclodextrins were from Wacker Chemie (Burghausen, Germany). Immobilised lipases from *C. antarctica* (Novozym 435) was a kind gift of Novo Nordisk (Bagsvaerd, Denmark). A purified CGTase preparation from *Paenibacillus* sp. F8 [31] and a crude CGTase preparation from *Bacillus* sp. strain 169 (DSM 2518) [32] was obtained as described previously. All chemicals were of analytical grade or purer.

Lipase-catalysed synthesis of glucose and maltose esters.—6-*O*-Caproyl glucose was prepared in *tert*-butanol using a lipase from *C. antarctica* [22]. 6′-*O*-Caproyl maltose was prepared by a similar procedure according to Woudenberg-van Oosterom et al. [23]. The structure of these compounds analysed by GC confirmed that regioisomerically pure compounds were formed. The incubation times were 24 h with the glucose and 72 h with the maltose preparation. The esters were isolated together with the unreacted carbohydrates by evaporation of about 80% of the *tert*-butanol followed by the addition of 5 volumes of hexane to precipitate the carbohydrates and esters. The precipitate was washed twice with hexane to remove remaining fatty acids and finally dried in vacuum. Preparations containing a mixture of 6-*O*-caproyl glucose (CG) and glucose (47:3 mol/mol; 24:1 w/w), and a mixture 6′-*O*-caproyl maltose (CM) and maltose (16:11 mol/mol; 31:19 w/w) were obtained and used without further purification. Myristyl monoesters of glucose and maltose were prepared accordingly, except that the maltose preparation was incubated for 96 h. The yields were similar to the ones obtained with CG and CM.

CGTase-catalysed synthesis of maltooligosaccharide esters.—Glycosyltransferase activity of the CGTases was employed using starch or β-cyclodextrin as donor substrate and either the CG or CM preparations as acceptor substrates. Soluble starch (2% v/w) or β-cyclodextrin (2% v/w) were mixed with either 100 mg of the CM preparation or 50 mg of the CG preparation (corresponding to 0.14 mmol CM and 0.32 mmol CG respectively) in 5 mL of a 50 mM Tris pH 8 buffer containing 2 mM $CaCl_2$ and 0.02% sodium azide. The reaction was started by adding 1 unit of the CGTase solution (a unit is defined as the amount of CGTase which produces 1 μmol of β-cyclodextrin min^{-1} from soluble starch at 50 °C and pH 7). The mixtures were incubated at 40 °C with gentle stirring for 2 h. At regular time intervals, aliquots (100 μL) were transferred to Eppendorf tubes preheated to 110 °C to stop the reaction. Controls contained a boiled enzyme solution. All samples were stored at −20 °C until further analysis.

Analytical TLC and determination of reaction rates.—Analytical thin layer chromatography (TLC) was carried out on precoated 20×20 cm glass plates with a silica gel 60 layer (0.25 mm thickness). The samples taken at different time intervals were centrifuged (14,000 rpm, 1 min) and 5 μL aliquots were applied as spots on the plate which was then developed in a 3:1:1:1 (v/v) mobile phase of EtOH–$CHCl_3$–HOAc–water. Sample components were visualised by dipping the plate in a 4:1 (v/v) mixture of EtOH–H_2SO_4 and heating at 140 °C for 8 min. For the analysis of reactions with CM as glycosyl donor, standards of maltose and CM were run in parallel on all plates. The spot intensities were measured using a gel reader system (Gel Doc 1000, Bio-Rad). Standard curves (spot intensities as a function of μg of substance applied) for CM and maltose indicated identical response factors (within 10%). It was assumed that products with R_f values between the ones for maltose and CM would show a similar response factor. For the reactions with CG as glycosyl donor, response factors were determined similarly by using standards of CG and glucose. Reaction rates (in μmol min^{-1} g^{-1} enzyme) were calculated based on the response factors and the calculated molecular weights.

All experiments were carried out in duplicate and no more than 5% difference in the determined R_f values, and 10% difference in the determined reaction rates were observed.

Preparative TLC.—Preparative TLC of the reaction products obtained after 48 h of incubation of CM with starch and *Paenibacillus* sp. F8 CGTase was carried out on precoated 20×20 cm glass plates with a silica gel 60 layer (0.5 mm thickness). Centrifuged reaction mixture (0.5 mL) was applied to the TLC plate which was then developed in a 3:1:1:1 (v/v) mobile phase of EtOH–$CHCl_3$–HOAc–water. An approximately 1 cm wide band in the middle of the plate was cut out and visualised as described above for analytical TLC. Three bands containing different products with R_f values between those of maltose and CM were selected. The silica containing the bands was scraped off the plate, placed in a 10 mL syringe with a plug of glass wool in the bottom. The silica was washed with hexane and the products were eluted with MeOH. All samples were subjected to HPAEC-PAD and GC analysis.

HPAEC-PAD.—High-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD) analysis of maltooligosaccharides was carried out as described previously [31]. Since ester bonds were hydrolysed upon analysis due to the high pH used, the detection of maltotriose, maltotetraose and maltopentaose indicated the presence of the corresponding capronate esters in **1**–**3**. HPAEC-PAD analysis of **3** indicated the presence of significant amounts of glucose apart from the caproyl maltopentaose expected. It was therefore not attempted to obtain structural data of this compound.

GC analysis.—Gas chromatographic (GC) analysis was carried out according to Degn et al. [22] with modifications in the temperature programming. Before injection, the sample components were silylated with a mixture of bis(trimethylsilyl)trifluoroacetamid and trimethylsilylimidazol. Aliquots (1 μL) were injected using splitless injection. Initial temperature was 90 °C for 1 min, the temperature was then increased to 310 °C at a rate of 30 °C min^{-1} and then kept at 310 °C for 15 min. Sample components were detected by a flame ionisation detector. Maltooligosaccharides and maltooligosaccharide esters with a DP of 4 and above were not possible to detect by this method. GC analysis of **1** showed a compound with a longer retention time than maltotriose indicating the presence of a maltotriose ester.

MALDI-TOF MS.—Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) was carried out on a Bruker REFLEX apparatus, using a matrix of α-cyano-4-cinnamic acid.

NMR.—Nuclear magnetic resonance (NMR) spectroscopy of product **1** [α-D-Glc*p*-(1→4)-(C6-(O-6)-α-D-Glc*p*-(1→4)-D-Glc*p*] and **2** [α-D-Glc*p*(1→4)-α-D-Glc*p*(1→4)(C6-(O-6))-α-D-Glc*p*-(1→4)-D-Glc*p*] was carried out using a Bruker DRX600 spectrometer operating at 600.13 MHz for ^{1}H and 150.1 MHz for ^{13}C. Samples of approximately 1 mg were prepared in 0.6 mL D_2O and spectra were

obtained at 27 °C. Two-dimensional spectra (COSY, TOCSY, NOESY, $^{1}H-^{13}C$ HSQC) were obtained using standard Bruker pulse sequences. Chemical shifts are given relative to acetone, 2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C.

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