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Effect of fatty acid chain length on initial reaction rates and regioselectivity of lipase-catalysed esterification of disaccharides

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Abstract

In a reaction medium mixture of 9:11 *t*-BuOH and pyridine (v/v) the effect of fatty acid chain length (C-4–C-12) on *C. antarctica* lipase B (Novozym 435, EC 3.1.1.3) catalysed esterification was studied. α and β maltose 6′-*O*-acyl esters in an anomeric molar ratio of 1.0:1.1 were synthesised independently of the chain length, but the initial specific reaction rate increased with decreasing chain length of the acyl donor. The product yield followed the same trend with a lauryl ester yield of 1.1% (mol/mol) and a butyl ester yield of 27.6% (mol/mol) after 24 h of reaction. With sucrose as the acyl acceptor the 6′-*O*-acyl and 6-*O*-acyl monoesters were formed with fatty acids of chain length C-4 and C-10 while the 6′,6-*O*-acyl diester was formed only with butanoic acid (C-4:0) as acyl donor. The 6′-*O*-acyl and 6-*O*-acyl monoesters and the 6′,6-*O*-acyl diester of butanoic acid were produced in a molar ratio of 1.0:0.5:0.2 and with decanoic acid (C-10:0) the 6′-*O*-acyl and 6-*O*-acyl monoesters were formed in the ratio of 1.0:0.3. The highest initial reaction rate and yield were obtained with the shortest chain length of the acyl donor. Initial reaction rates and ester yields were affected by the solubility of the disaccharide, with higher reaction rates and yields with maltose than with sucrose, while no formation of esters were observed with either cellobiose or lactose as acyl acceptors.

Keywords: Lipase; Disaccharides; Fatty acids; Solvents; Esterification

1. Introduction

Carbohydrate esters are an interesting group of compounds with surfactant and emulsifying properties. They are biodegradable, non-toxic and can be produced from renewable resources (fatty acids and native carbohydrates). The specific properties of the ester, including the hydrophilic:lipophilic balance (HLB value) is controlled by the type of acyl group, the degree of substitution and the degree of polymerisation of the carbohydrate. In fact, a broad range of functional properties can be obtained with different types of esters, with the hydrophilic, carbohydrate part being important to the HLB value. In contrast to conventional available food emulsifiers it is possible to produce carbohydrate fatty acid esters that covers a wide range of HLB values (from 2 to 18).[1]

Synthesis of carbohydrate esters can be catalysed by hydrolytic enzymes (EC 3) in a reversed hydrolysis reaction provided that the water activity is controlled and kept sufficiently low. In particular, lipases and proteases are being applied for the regioselective acylation of mono and disaccharides. Enzyme-catalysed acylation has been performed in solvent based systems by transesterification using vinyl laurate as acyl donor[2] and by reversed hydrolysis with native carbohydrates and fatty acids as substrates[3] which also has been reported for solvent free systems.[4] Compared to conventional chemical catalysis the enzyme catalysis can be performed at milder process conditions and with higher specificity of the reaction.

The activity and specificity of hydrolases in organic solvents is highly influenced by the nature of the solvent.[5] Previous research has tried to identify a general

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relationship between the ability of enzymes to catalyse reactions in organic solvents and the physico-chemical properties of the solvents. The parameters investigated have included the dielectric constant, the dipole moment, the water solubility and miscibility and hydrophobicity (log P). Consistent correlations have been obtained with the solvent hydrophobicity and it has been shown that enzymes for example lipases generally have higher activities at high log P values.[6–8] A hydrophobic solvent is however not necessarily a good choice for achieving optimal rates and degrees of conversion, depending on the solubility of the hydrophilic substrate.[9] Therefore optimal reaction conditions are a compromise between enzyme activity and substrate solubility, for which investigation of the effect of solvent mixtures and medium engineering is required. In the present study a mixed reaction medium, favouring the solubility of carbohydrate, was used to study the effect of fatty acid chain length on a lipase-catalysed esterification of native disaccharides using an immobilised preparation of *C. antartica* lipase B. Reactions were characterised with regard to the products obtained, the yields and reaction rates.

2. Results and discussion

For all fatty acids investigated (C-4–C-12) the immobilised preparation of *C. antarctica* lipase B (Novozym 435) catalysed the formation of the corresponding 6′-*O*-acyl monoester with maltose as acyl acceptor (Fig. 1). Independently of the acyl donor chain length α and β maltose 6′-*O*-acyl esters were synthesised in the anomeric molar ratio of 1.0:1.1. The identification of the presence of mono acylated maltose ester by mass spectroscopy was confirmed by ^{13}C NMR spectra of the purified compound as maltose 6′-*O*-acyl esters (Table 1). Using the same biocatalyst in refluxing *t*-BuOH, Woudenberg-van Oosterom et al.[10] obtained exclusively the 6′-*O*-acyl maltose monoester in a transesterification reaction with maltose and ethyl butanoate. With a *Humicola lanuginosa* lipase[10] immobilised on Celite the predominant product obtained was 6′-*O*-acyl maltose monoester using maltose and different vinyl esters in a transesterification reaction in 4:1 *t*-amyl alcohol/Me_2SO (v/v), less than 1% (unit not given) of the maltose was converted into diesters. The three investigations show that the 6′-*O*-acyl maltose monoester is the only—or predominant—product formed even under different reaction conditions.

With maltose as acyl acceptor the initial specific reaction rate increased with decreasing chain length of the acyl donor from 0.06 μmol min^{-1} g $biocatalyst^{-1}$ for lauric acid (C-12:0) to 1.24 μmol min^{-1} g $biocatalyst^{-1}$ for butanoic acid (C-4:0). The product yield followed the same trend with a lauryl ester yield of 1.1% (mol/mol) and a butyl ester yield of 27.6% (mol/mol) after 24 h of reaction (see Table 2). Yields of all esters increased with incubation time with the longer chain esters showing the highest relative increases. With sucrose as the acyl acceptor both 6′-*O*-acyl and 6-*O*-acyl monoesters were formed with butanoic acid (C-4:0) and decanoic acid (C-10:0) fatty acid, while 6′,6-*O*-acyl diester was only formed with butanoic acid (C-4:0) as the acyl donor (Fig. 1). The 6′-*O*-acyl and 6-*O*-acyl monoesters and the 6′,6-*O*-acyl diester were produced in the molar ratio of 1.0:0.5:0.2 with butanoic acid, while the 6′-*O*-acyl and 6-*O*-acyl monoesters were

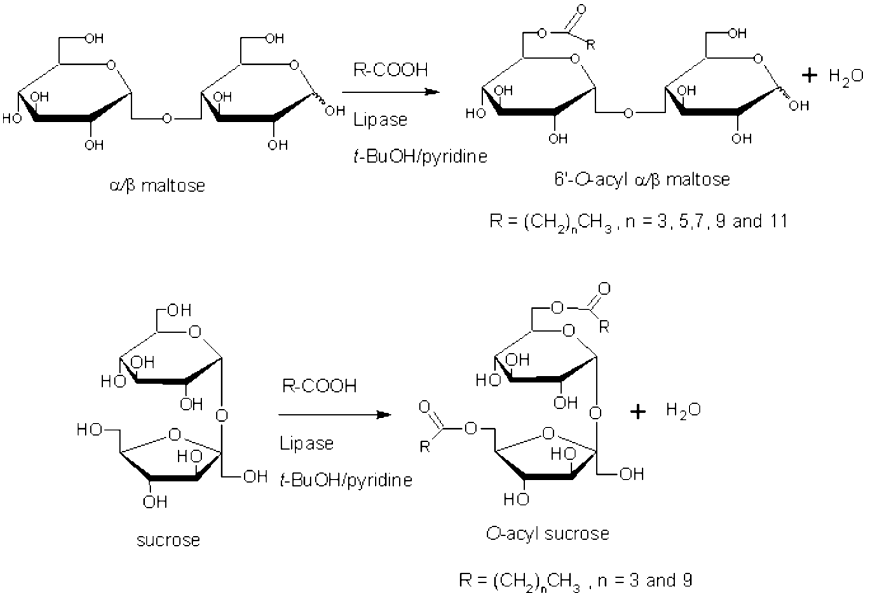

Fig. 1. Lipase-catalysed esterification reactions of maltose and sucrose with fatty acid.

Table 1
Chemical shifts (δ) of maltose and its esters in pyridine-d_5 at 303 K

	α′,α Maltose in Me_2SO-d_6	6′-*O*-Butanoyl-α′,α maltose in Me_2SO-d_6	α′,α Maltose in pyridine-d_5	6′-*O*-Decanoyl α′,α maltose in pyridine-d_5
H-1′	5.03	4.99	5.98	5.91
H-2′	3.25	3.25	4.21	4.21
H-3′	3.41	3.40	4.64	4.60
H-4′	3.10	3.05	4.21	4.05
H-5′	3.51	3.70	4.61	4.70
H-6′a	3.64	4.02	4.37	4.80
H-6′b	3.64	4.29	4.56	5.06
H-1	4.94	4.92	5.86	5.87
H-2	3.21	3.19	4.19	4.19
H-3	3.72	3.69	4.90	4.89
H-4	3.33	3.25	4.40	4.33
H-5	3.67	–	4.68	4.70
H-6a	3.64	3.56	4.55	4.53
H-6b	3.64	3.66	4.47	4.51
	α′,β Maltose in Me_2SO-d_6	6′-*O*-Butanoyl-α′,β maltose in Me_2SO-d_6	α′,β Maltose in pyridine-d_5	6′-*O*-Decanoyl α′,β maltose in pyridine-d_5
H-1′	5.01	4.96	5.94	5.87
H-2′	3.25	3.26	4.20	4.19
H-3′	3.42	3.41	4.63	4.60
H-4′	3.10	3.06	4.21	4.05
H-5′	3.53	3.72	4.61	4.69
H-6′a	3.48	4.02	4.38	4.80
H-6′b	3.64	4.30	4.57	5.08
H-1	4.33	4.30	5.28	5.30
H-2	2.98	2.98	4.13	4.13
H-3	3.41	3.38	4.39	4.37
H-4	3.33	3.25	3.87	–
H-5	3.22	3.20	3.87	3.90
H-6a	3.71	3.73	4.50	4.45
H-6b	3.58	3.51	4.50	4.57

Table 2
Initial specific reaction rates and yields of the *C. antarctica* lipase B-catalysed synthesis of 6′-*O*-acyl maltose esters as effected by acyl donor chain length and incubation time[a]

Acyl donor chain length	Initial specific reaction rate (μmol/min/g biocatalyst)	Yield% (mol/mol)		
		24 h	48 h	144 h
C-4	1.24	27.6	34.7	40.1
C-6	0.67	13.4	18.9	28.9
C-8	0.33	4.1	8.0	12.8
C-10	0.13	3.4	4.7	9.7
C-12	0.06	1.1	1.7	3.6

[a] Yields are given as mole ester per mole carbohydrate substrate.

formed in a ratio of 1.0:0.3 with decanoic acid. MS spectra revealed the presence of a mono and a diesterified sucrose, which was confirmed by the ^{13}C NMR spectra (see Table 3). Using *H. lanuginosa* lipase immobilised on Celite (25 mg mL^{-1}) in a reaction medium containing 4:1 *t*-amyl alcohol/Me_2SO (v/v) in a transesterification reaction with sucrose and vinyl fatty acid esters Ferrer et al.[11] observed the formation of a

monoester 6-*O*-acyl sucrose and two diesters, 6,1′-di-*O*-acyl sucrose and 6,6′-di-*O*-acyl sucrose. With 0.03 M sucrose and 0.06 M vinyl laurate a 31% conversion of sucrose to monoester and less than 5% to diester was achieved in 24 h. Woudenberg–van Oosterom et al.[10] using the same biocatalyst as in the present investigation found that sucrose was acylated with ethyl butanoate as the acyl donor equally at the 6 or 6′ position, when *t*-BuOH was used as solvent. With refluxing *t*-BuOH at 82 °C they obtained an 18% conversion in 24 h of crystalline sucrose to its monoester instead of 1% when using *t*-BuOH at 40 °C. They observed that ester formation was lower with crystalline sugar as the acyl acceptor than with amorphous sucrose suggesting that the solubility of the sugars is critical to ester formation (18% conversion of crystalline sugar as compared to 40% of amorphous sugar in 24 h).

In the present investigation both the reaction rates and ester yields were lower when sucrose was used as the acyl acceptor than when maltose was used. No sugar esters were formed with butanoic acid and lauric acid as acyl donors and either cellobiose or lactose as acyl acceptors. Table 4 shows that maltose was considerably more soluble in 9:11 *t*-BuOH:pyridine (v/v) than sucrose, lactose and cellobiose. Lactose and cellobiose were the least soluble. In the absence of pyridine, lactose and cellobiose showed no solubility in *t*-BuOH. Thus, the more carbohydrate is solubilised, the higher the probability of ester formation. Increasing the polarity of the solvent will increase the solubility of the

Table 3
Chemical shifts (δ) of sucrose and its esters in pyridine-d_5 at 303 K

Compound	Sucrose	6′-*O*-Butanoyl-sucrose	6-*O*-Butanoyl-sucrose	6,6′-*O*-Dibutanoyl-sucrose	6-*O*-Decanoyl-sucrose	6′-*O*-Decanoyl-sucrose
Atom						
C-1	93.9	93.7	93.7	93.3	93.5	93.5
C-2	73.8	73.8	73.6	73.4	73.4	73.7
C-3	75.5	75.5	75.2	75.1	75.1	75.3
C-4	72.3	72.5	71.9	72.1	71.8	72.2
C-5	75.3	75.0	72.2	71.9	71.9	74.9
C-6	62.9	63.0	64.7	65.2	64.5	62.9
H-1	6.25	6.22	6.19	6.20	6.20	6.26
H-2	4.21	4.21	4.19	4.20	4.20	4.23
H-3	4.70	4.71	4.68	4.68	4.70	4.74
H-4	4.25	4.22	4.07	4.00	4.09	4.24
H-5	4.80	4.80	4.86	4.89	4.88	4.83
H-6a	4.37	4.36	4.76	4.67	4.81	4.40
H-6b	4.55	4.54	4.98	5.05	5.00	4.57
C-1′	65.3	64.7	65.1	64.5	64.9	64.6
C-2′	–	–	–	–	–	–
C-3′	80.4	79.7	–	79.4	–	79.5
C-4′	76.0	77.1	76.2	76.8	76.0	76.8
C-5′	84.9	81.2	84.8	81.0	84.6	81.0
C-6′	63.3	66.9	63.8	66.8	63.7	66.8
H-1′a	4.36	–	4.34	4.34	4.35	4.39
H-1′b	4.40	4.37	4.39	4.37	4.39	4.40
H-3′	5.04	5.02	5.04	5.03	5.04	5.06
H-4′	5.09	4.98	5.01	4.94	5.03	5.02
H-5′	4.54	4.64	4.59	4.67	4.60	4.69
H-6′a	4.32	4.99	4.38	–	4.40	5.04
H-6′b	4.37	5.02	4.47	5.02	4.48	5.08

Table 4
Solubility of lactose, cellobiose, maltose and sucrose in *t*-BuOH and 9:11 *t*-BuOH:pyridine (v/v) at 45 °C

Solvent	Lactose (mM)	Cellobiose (mM)	Maltose (mM)	Sucrose (mM)
t-BuOH	–	–	12 ± 1	4 ± 0.2
t-BuOH:pyridine	18 ± 2	2 ± 0.2	215 ± 12	27 ± 3

Table 5
Initial specific reaction rates and yields of total *O*-acyl sucrose esters from the *C. antarctica* lipase B-catalysed synthesis using C-4 or C-10 as acyl donors[a]

Acyl donor chain length	Initial specific reaction rate (μmol/min/g biocatalyst)	Yield % (mol/mol)		
		24 h	48 h	144 h
C-4	0.12	2.6	4.1	8.5
C-10	0.02	0.4	0.8	2.0

[a] Reactions were carried out for up to 144 h. Yields are given as mole ester per mole carbohydrate substrate.

carbohydrate, but will also increase the susceptibility of the enzyme to denaturation.

An increase in initial specific reaction rate with decrease in chain length of the acyl donor was also observed in the formation of sucrose esters with either butanoic acid (C-4:0) or decanoic acid (C-10:0). The initial reaction rates of total ester formation were 0.12 and 0.02 μmol min^{-1} g $biocatalyst^{-1}$ for butanoic and decanoic acid respectively, and the yield of sucrose ester with butanoic acid was higher than with decanoic acid (see Table 5). The same effect has been shown with protease-catalysed transesterification reactions where the reaction rate decreased with increasing chain length of the acyl donor and the size of the carbohydrate acyl acceptor.[12,13]

C. antarctica lipase has high activity for short and medium chain fatty acids and decreasing activity for long chain fatty acids reflecting the structure of the enzyme's binding site, which is an elliptical, steep funnel of 9.5×4.5 Å.[14] Substrate specificity is reflected in the shape of the binding site. When the fatty acid chain length or the size of the carbohydrate is increased steric conflicts possibly occur reducing the probability of product formation. Other structural elements also play a role in mediating chain length specificity, like the hydroxyl-binding site and the lid. *C. antarctica* lipase is one of the few lipases studied, which is able to use underivatised fatty acids to esterify sugars. Other lipases and proteases require vinyl or isopropenyl activated fatty acid derivatives for transesterification reactions to occur.[12,13,15] Reactions with vinyl or isopropenyl esters occur at much higher rates than underivatised fatty acids.[15] In the present investigation *C. antarctica* lipase accepted underivatised donors and catalysed 3.6–40.1% conversion of maltose to the corresponding ester in 144 h, depending on the fatty acid chain length. Both with sucrose and maltose esters continued to be formed for at least 144 h, but the rate of ester formation decreased after approximately 60 h. The yields obtained are comparable with the results of Cao et al.[16] using polypropylene immobilised *C. antarctica* lipase with maltose or sucrose and palmitate as substrates in *t*-BuOH at 60 °C. With regard to conversion of sucrose, addition of pyridine to the solvent allows a reduction in temperature (from 60 to 45 °C) with no reduction in specific reaction rates.

3. Experimental

Synthesis of carbohydrate fatty acid esters.—The enzyme reactions were carried out in 25 mL glass-stoppered Erlenmeyer flasks in 5 mL solutions of 9:11 *t*-BuOH:pyridine (v/v) which contained 30 mM fatty acid and 100 mg of either maltose, lactose, sucrose or cellobiose. 0.5 g molecular sieves (3 Å) were added to remove the water from the reaction mixture, which was equilibrated at 45 °C on a rotary shaker table (250 rpm) for 24 h. The reaction was then initiated by adding 50 mg of immobilised Novozym 435. The reaction mixture was incubated for 148 h (45 °C, 250 rpm). Aliquots of 50 μL reaction medium were transferred at timed intervals to precooled (−20 °C) glass vials and stored at −20 °C for later analysis. All reactions were carried out in duplicate or triplicate.

Thin-layer chromatography.—For analytical TLC, 20 μL samples were applied as spots to Silica gel 60 plates of 0.25 mm thickness (E. Merck, Germany). Esters were separated using mobile phase consisting of 35:10:4:1 chloroform:MeOH:HOAc:water (v/v). The sample analytes were visualised with 1:1 MeOH:2 M H_2SO_4 (v/v) sprayed on to the plate and developed at 140 °C for 10 min.[3]

Gas chromatography.—A gas chromatograph equipped with a 10 m WCOT fused silica column (Chrompack, Knebel, Denmark), a split/splitless injection port and an FID detector was used. Before injection, the sample components were derivatised. The reaction medium was evaporated at 45 °C in vacuum for 10 min in a vacuum centrifuge (Heto, Denmark) equipped with a −110 °C cool trap, 100 μL pyridine containing 3.3 mg/mL octyl-β-D-glucopyranoside as internal standard, 80 μL BSTFA and 40 μL Me_3SI, was added and the samples were incubated at 70 °C for 30 min. Pyridine was then removed in a vacuum centrifuge at 45 °C, pressure <1 mbar for 5 min and 1 mL *n*-heptane was added. Aliquots (0.5 μL) were injected using the splitless injection mode. The temperature

profile of the analysis was initiated at 90 °C for 1 min, then from 90 to 310 °C at a rate of 30 °C min^{-1} and finally 310 °C for 15 min.

Isolation of the maltose and sucrose esters.—The reaction mixture was filtered through glass wool to remove the immobilised enzyme and molecular sieves. The unused carbohydrate and its corresponding esters were precipitated by using three times the volumes of hexane to dissolve the unreacted fatty acid and centrifuged at 4300*g*. The precipitate was washed again with hexane and recentrifuged. This procedure was repeated three times. The precipitate containing unreacted carbohydrates and their corresponding esters was dried, dissolved in 100 L of pyridine and subjected to preparative TLC using plates of 0.5 mm thickness and the same mobile phase as described for the analytical TLC. Product bands were identified, scraped off the plates, transferred to microcolumns and eluted with MeOH. Products showed distinctly as a white turbid solution. The purified esters were analysed by NMR and MS.

Nuclear magnetic resonance.—NMR studies were carried out on a BRUKER DRX600 spectrometer equipped with a TXI (H/C/N) xyz-grad probe. Saturated solutions of the samples in pyridine-d_5 (except for butanoyl-maltose, where Me_2SO-d_6 was used) were prepared and analysed at 303 K by means of 2QF-COSY, TOCSY (120 ms mixing time) and $^{13}C-^{1}H$-HSQC spectra. Both ^{1}H and ^{13}C NMR spectra have been referenced by the solvent signals: 7.22 ppm (^{1}H)/123.87 ppm (^{13}C) for pyridine, 2.5 ppm (^{1}H)/39.51 ppm (^{13}C) for DMSO. In some cases DEPT135 spectra were recorded in addition. The pyranose systems in maltose were assigned by help of a ROESY experiment. Table 1 gives the NMR peak assignments for maltose and its corresponding esters and Table 3 gives the NMR peak assignments for sucrose and its corresponding esters.

Mass spectrometry.—Mass spectrometry was performed on a Bruker Esquire LC–MS iontrap. Solid samples were extracted with either 100% MeCN or 100% MeOH. The samples were centrifuged for 15 min at 14,000*g* and the supernatant collected. The supernatant was diluted 10, 100 and 1000 times in MeOH. Samples were injected by a syringe pump into the ESI-interface starting with the highest dilution. If no satisfactory signal was obtained the next dilution was selected. Total ion current was controlled by the software and an average of ten spectra were collected for each sample spectra.

Solubility studies.—Saturated solutions of the different sugars were prepared in *t*-BuOH and 9:11 *t*-BuOH:pyridine (v/v) and kept on a rotary shaker at 250 rpm at 45 °C. Aliquots of the supernatants were then dried and weighed to give the concentration of the dissolved sugars. Experiments were carried out in triplicates and quadruplicates.

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