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Regioselective monoacylation of cyclomaltoheptaose at the C-2 secondary hydroxyl groups by the alkaline protease from *Bacillus subtilis* in nonaqueous media

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Abstract—Transesterification of cyclomaltoheptaose (β-CD) with divinyl butanedioate, divinyl hexanedioate, and divinyl decanedioate, catalyzed by the alkaline protease from *Bacillus subtilis* in anhydrous DMF for 5 days, furnished the corresponding vinyl-β-CD derivatives. The products were characterized by ESI-MS, ^{1}H NMR, ^{13}C NMR, IR, and DSC. The results indicated the products to be monosubstituted esters, with monoacylation occurring at the C-2 secondary hydroxyl groups of β-CD. The regioselectivity of the monoacylation as catalyzed by alkaline protease was not affected by the chain length of the acyl donor.

Keywords: Cyclomaltoheptaose (β-CD); Protease; Regioselectivity; Secondary acylation; Transesterification

1. Introduction

Cyclodextrins are truncated, cone-shaped cyclic oligosaccharides, composed of 6 (in α-), 7 (in β-), 8 (in γ-) or more α-(1→4)-linked glucoses. All the secondary hydroxyl groups at the 2 and 3 positions of the glucose units are on one side of the torus, and all the primary hydroxyl groups at the 6 position are on the other side. Because of their hydrophobic cavity, cyclodextrins have gained prominence in recent years and have been widely applied in many fields, for example, in the formation of stable complexes,[1] as drug carriers[2] for protecting enzymes from denaturation, increasing their activities and stereoselectivity in biotechnology,[3–5] and for separation and adsorption of chiral materials.[6] CD derivatives containing vinyl groups are important monomers for preparing linear polymers that have the many hydrophobic cavities of CDs, thus broadening the applicability of these polymers. Because of the similar reactivity of the hydroxyl groups, it is difficult to effect selective monosubstitution of CDs, and multistep group transfer strategy based on protection/deprotection is required.[7] Tong et al.[8] and Rao et al.[9] utilized basic catalysis to prepare monosubstituted β-CD derivatives directly, but the reaction conditions were complex and required precise pH control. As compared with conventional chemical catalysis, enzyme catalysis allows higher selectivity under milder and simpler process conditions. Several papers have described the synthesis of vinyl sugar esters by enzyme catalysis and the polymerization of corresponding monomers.[10,11] We know of no research on the use of enzyme catalysis for synthesis of vinyl-containing β-CD monoesters.[12]

In our previous work, we synthesized vinyl glucose esters,[13] vinyl sucrose esters,[14] vinyl lactose esters,[15] and their polymers by using highly activated divinyl dicarboxylates. In this research, the alkaline protease from *Bacillus subtilis* was used to catalyze the transesterification of three divinyl dicarboxylates with β-CD, to afford predominantly monosubstituted β-CD esters

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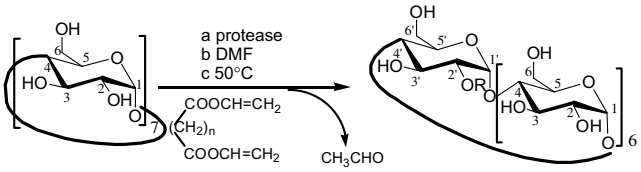
containing vinyl groups. The vinyl groups of these β-CD esters are useful polymerizable moieties to produce pendant β-CDs polymers having side chains of different lengths.

2. Results and discussion

Proteases have received increasing attention for specific and regioselectively acylation in organic solvents. Efficient transesterification onto CD of three highly activated divinyl dicarboxylates was catalyzed by protease in DMF at 50 °C (Fig. 1). Because the acyl donors were in excess, no bridged molecules were observed; 80% of the esters obtained were monosubstituted (**1**, **2**, **3**), and 20% were di- and tri-substituted esters (the MS of multisubstituted sugar esters are not shown). The position of the substituent in compounds **1**, **2**, and **3** was verified by ^{1}H NMR and ^{13}C NMR (Table 1).



1. R: $CO(CH_2)_2COOCH{=}CH_2$ Mono-(2-*O*-vinylbutanedioyl)-βCD
2. R: $CO(CH_2)_4COOCH{=}CH_2$ Mono-(2-*O*-vinylhexanedioyl)-βCD
3. R: $CO(CH_2)_8COOCH{=}CH_2$ Mono-(2-*O*-vinyldecanedioyl)-βCD

Figure 1. Enzymatic synthesis of β-CD monoesters.

2.1. Monosubstituted β-CD esters formed by protease catalysis

The degree of substitution (DS) for CD derivatives gives the average number of substituted groups per cyclodextrin molecule. The various methods for determining DS in cyclodextrins include elemental analysis, MS, and ^{1}H and ^{13}C NMR spectroscopy. Because of the highly functionalized and symmetrical nature of CDs,[16,17] most CD derivatives prepared by conventional chemical catalysis are multisubstituted and involve different positional isomers. In a report of lipase-catalyzed esterification of β-CD,[12] as another approach for preparing CD derivatives, the DS of the CD derivatives was from 0.38 to 6.78 and the products were multisubstituted mixtures. It is thus concluded that selective monosubstitution is not easy.

In this work, the DS of compounds **1**, **2**, and **3** was determined by ^{1}H NMR and ESI-MS. Monosubstitution of all three products was proved in ^{1}H NMR by the ratio of proton integral for –OCH=CH_2 groups and the glucose units of β-CD, and was also confirmed by negative ESI-MS. The MS of **3** is shown as a representative example in Figure 2. Molecular ions $[M+2H_2O–H]^-$ at m/z 1295.4, 1323.3, and 1379.4 corresponded to the molecular weights of **1**, **2**, and **3**, respectively. DSC gave reasonable evidence for H_2O in $[M+2H_2O–H]^-$. In the DSC diagram, the endothermic peak below 100 °C corresponded to the loss of water from the compounds, indicating that there might be some water molecules

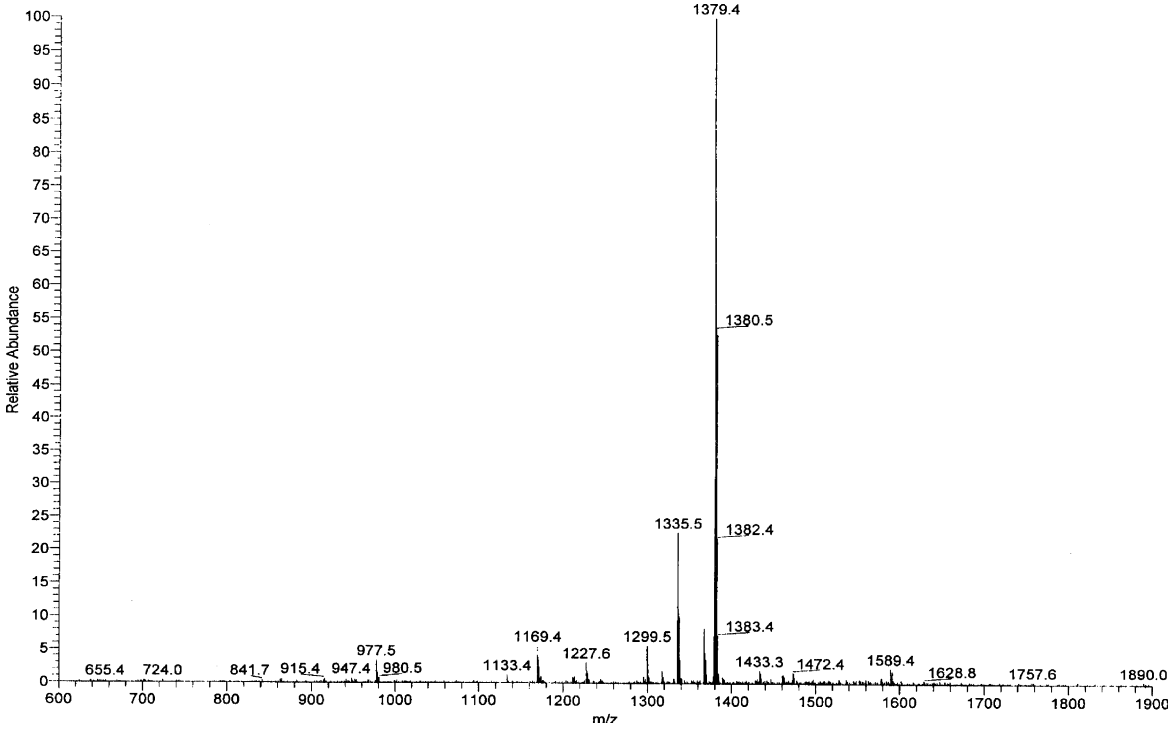


Figure 2. MS of mono-(2-*O*-vinyldecanedioyl)-β-CD (**3**).

inside the CD cavity or integrated in the crystal structure.[18] Similar MS analysis indicating H_2O inclusion was reported by Caccia et al.[19] The MS analysis was in good agreement with that of ^1H NMR.

2.2. Regioselective acylation of β-CD at the C-2 secondary hydroxyl groups as catalyzed by protease

The regioselectivity was confirmed by ^{13}C NMR as demonstrated in Table 1. Based on the general strategy described by Yoshimoto et al.[20] acylation of a hydroxyl group would cause a downfield shift of the *O*-acylated carbon ($^*CH_2OCOR$), while the adjacent carbon ($^*CCH_2OCOR$) would move upfield in ^{13}C NMR. Analysis of the ^{13}C NMR spectra of **1**, **2**, and **3** revealed that esterification of the CD occurred at the C-2 secondary hydroxyl position. In the ^{13}C NMR spectrum of **1**, a small peak around 73.78 ppm corresponded to the downfield shifted C-2′, and another small peak around 98.83 ppm corresponded to the upfield shifted C-1′, strong peaks around 72.43 and 102 ppm were signals of C-2 and C-1 located on the nonsubstituted glucose units, thus resembling the downfield shift of C-2 and the upfield shift of C-1 in 2-monotosyl-β-CD[21] and 2-monobenzoyl-β-CD **4**,[22] as listed in Table 1. Furthermore, as compared with downfield shift of C-6 substituted β-CD derivatives (e.g., 6-*O*-monobenzoyl-β-CD **5** listed in Table 1),[8] no similar change was observed in compounds **1**, **2**, and **3**. The evidence therefore suggests that the substitution had most probably occurred at C-2 secondary hydroxyl position.

The ^1H NMR spectra also provided substitutional information on the 2 position. In the ^1H NMR spectrum of **1**, the C-1 proton of one of the glucose residues appeared downfield as a separate doublet (δ 5.05 ppm) from the remaining six C-1 protons (multiplet δ 4.89 ppm), and a similar pattern was also observed in **2** and **3**. This pattern was caused by the modification of

Table 1. Chemical shifts of ^{13}C NMR of β-CD and monosubstituted derivatives

Carbon atom	β-CD D_2O	**1** Me_2SO-d_6+D_2O	**2** D_2O	**3** D_2O	**4**[a] Me_2SO-d_6	**5**[b] Me_2SO-d_6
C-1	102.58	102.34	102.37	102.43	101.98	102.69
		102.28	102.27	102.27		102.07
		102.11	102.19	102.20		102.01
		101.85	102.04	101.98		101.65
			101.84	101.77		
C-1′		98.83	98.67	99.29	98.29	
C-2	72.67	72.43	72.62	72.41	72.63	72.49
						72.28
C-2′		73.78	73.52	73.80	73.89	72.13
						72.02
C-3	73.89	73.43	73.36	73.73	72.99	73.28
		73.33		73.69		73.13
				73.60		
C-3′		69.80	70.29	70.75	69.62	73.09
						73.03
						72.94
C-4	81.94	81.94	81.47	81.41	81.64	82.48
C-4′		81.76	81.37	81.36	80.99	81.67
		81.68	81.12	81.07		81.60
		81.30	80.99	80.96		81.57
			80.35	80.77		81.34
C-5	72.89	72.38	72.13	72.26	72.05	C-2
C-5′		72.71	72.07	72.20	71.12	69.09
C-6	61.17	60.71	60.70	60.65	59.94	59.98
		60.35	60.47	60.31		59.72
		60.01		60.26		59.68
C-6′						64.26
C=O		172.14	175.10	175.59	165.92	165.70
		170.67	172.73	172.67		
$-OCH=CH_2$		141.53	141.20	141.37		
$-OCH=CH_2$		99.08	99.74	99.65		
$(CH_2)_n$		28.89	33.69	34.06		
			33.53	33.67		
			24.04	28.58		
			23.87	28.35		
				28.22		

[a] **4**,2-*O*-Monobenzoyl-β-CD, referred to by Hao et al.[22]
[b] **5**,6-*O*-Monobenzoyl-β-CD, referred to by Tong et al.[8]

the secondary hydroxyl group on C-2.[9] However, in the spectra of 6-*O*-(2-hydroxypropyl)-β-CD[9] and 6-*O*-sulfoalkyl-β-CD,[16] all seven C-1 protons appeared at 4.85 ppm as one doublet, and no downfield separation occurred. Thus, the ^{1}H NMR result was consistent with that of ^{13}C NMR.

β-CD has multiple hydroxyl groups on C-2, C-3, and C-6 of the glucopyranose unit. Among these three kinds of hydroxyl groups, the secondary ones on C-3 are the least reactive, attributable to hydrogen bonding between the protons of the 3-hydroxyl groups and the oxygen atoms of the C-2 hydroxyl groups.[16] The hydroxyl groups on C-6 are the most reactive toward electrophilic reagents, because they are primary and the most basic ($pK_a = 15$–16). The secondary hydroxyl groups on C-2 are the most acidic with pK_a of 12.1.[23] Thus, under normal circumstances, an electrophilic reagent attacks the 6 position. However, in the transesterification catalyzed by the alkaline protease from *B. subtilis*, the secondary hydroxyl groups on C-2 of CD are acylated preferentially over the primary hydroxyl groups.

Although the three acyl donors used in this paper had different chain lengths, the position acylated on β-CD was the same. This implies that the regioselectivity of the alkaline protease from *B. subtilis* is not affected by the chain length of the acyl donors.

In conclusion, three polymerizable vinyl β-CD monoesters having different carbon chain lengths were synthesized by the alkaline protease from *B. subtilis* in DMF at 50 °C. The regioselectivity of the enzyme was not affected by the chain length of the acyl donor. In the transesterification of CD with divinyl butanedioate, divinyl hexanedioate, and divinyl decanedioate, the enzyme showed high regioselectivity, 80% of the products obtained were the 2-*O*-substituted β-CD monoesters, along with 20% of multiesters. Further research concerning the regioselectivity of the enzyme and preparation polymers of different side-chain length containing β-CDs are in progress.

3. Experimental

3.1. Materials

The alkaline protease from *B. subtilis* (EC 3. 4. 21. 62) was purchased from Wuxi Enzyme Co. Ltd (Wuxi, PR China). β-CD was recrystallized twice in water and dried at 110 °C for 2 h. DMF was dried over 3 Å molecular sieves for 24 h before use. Divinyl butanedioate, divinyl hexanedioate, and divinyl decanedioate were produced and purified as described in the patent literature.[24] Butanedioic acid, hexanedioic acid, decanedioic acid, and vinyl acetate and all other chemicals were of the highest purity commercially available.

3.2. Analytical methods

Infrared spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. The position of acylation in the enzymatically prepared β-CD esters was established by ^{1}H NMR and ^{13}C NMR (Bruker DMX 500). Solvents used were Me_2SO-d_6 and D_2O with Me_4Si as an internal reference. Chemical shifts were given on the δ scale. Mass spectra were obtained on a Bruker Esquire-LC instrument for electro-spray (ESI-MS) measurements (solvent: methanol; negative mode). DSC was carried out using a STA 409 workstation. The size of samples used in the DSC analysis varied between 5.9 and 7.2 mg. A nitrogen atmosphere was used with the DSC cell and samples were always heated from 30 to 550 °C with a heating rate of 10 °C/min. The progress of reactions was monitored by TLC with 8:5:2 (v/v) EtOAc–MeOH–H_2O as eluent. The spots were developed by spraying with concd H_2SO_4 followed by heating.

3.3. Transesterification reactions

β-CD (1.32 mmol, 1.5 g) was dissolved in DMF (30 mL) containing 5.28 mmol divinyl butanedioate (0.9 g), divinyl hexanedioate (1.04 g), or divinyl decanedioate (1.34 g), respectively. The reaction was initiated by adding the alkaline protease from *B. subtilis* (30 mg/mL) and the suspension was shaken at 250 rev/min for 5 days at 50 °C. Formation of the sugar ester was confirmed by TLC. The reaction was terminated by filtering off the enzyme. Divinyl dicarboxylates were washed off by *n*-hexane. The DMF was evaporated under reduced pressure. The crude products, **1**, **2**, and **3**, composed of multisubstituted sugar esters, monosubstituted sugar esters, unreacted CD and minor solvent, weighed 1.42, 1.44, and 1.47 g, respectively. The crude products were isolated by chromatography on silica gel with gradient elusion by 8:5:1 (v/v) EtOAc–MeOH–H_2O to give compounds **1**, **2**, and **3**. The process of extraction and separation was monitored by TLC.

3.3.1. Mono-(2-*O*-vinylbutanedioyl)-β-CD (1). White powder (yield: 4.8%); R_f 0.21; IR (KBr): ν 3398 (OH), 1742 (C=O), 1647 (C=C); ^{1}H NMR (Me_2SO-d_6+D_2O): δ 7.21 (dd, 1H, J 6.28, 13.95 Hz, –OCH=), 5.05 (d, 1H, J 3.0 Hz, H-1′), 4.89 (m, 6H, H-1), 4.70 (d, 1H, J 4.83 Hz, CH_2=), 4.41 (d, 1H, J 15.0 Hz, CH_2=), 3.92 (m, 2H), 3.65–3.27 (br m, 40H, H-2, 3, 4, 5, 6), 2.69 (m, 4H, –CH_2–CH_2– of butanedioyl part); ^{13}C NMR (Me_2SO-d_6+D_2O) was shown in Table 1 (**1**); ESI-MS (m/z): 1295 $(M_1+2H_2O–H)^-$, M_1 corresponding exactly to the molecular weight of mono-(2-*O*-vinylbutanedioyl)-β-CD.

3.3.2. Mono-(2-*O*-vinylhexanedioyl)-β-CD (2). White powder (yield: 10.2%); R_f 0.29; IR (KBr): ν 3394 (OH),

1735 (C=O), 1647 (C=C); ^{1}H NMR (D_2O): δ 7.06 (dd, 1H, J 5.5, 13.8 Hz, –OCH=), 5.18 (d, 1H, J 3.5 Hz, H-1′), 4.98 (m, 6H, H-1), 4.83 (d, 1H, J 15.0 Hz, CH_2=), 4.04 (t, 1H), 3.82–3.49 (br m, 41H, H-2, 3, 4, 5, 6), 2.44 (m, 4H, –CH_2–COOCH=CH_2, –CH_2–COO–β-CD), 1.64 (m, 4H, other 2CH_2 of hexanedioyl part); ^{13}C NMR (D_2O) is shown in Table 1 (**2**); ESI-MS (m/z): 1323 (M_2+2H_2O–H)$^-$, M_2 corresponding exactly to the molecular weight of mono-(2-*O*-vinylhexanedioyl)-β-CD.

3.3.3. Mono-(2-*O*-vinyldecanedioyl)-β-CD (3). White powder (yield: 12.6%); R_f 0.35; IR (KBr): v 3386 (OH), 1735 (C=O), 1647 (C=C); ^{1}H NMR (D_2O): δ 7.08 (dd, 1H, J 6.0, 13.9 Hz, –OCH=), 5.18 (d, 1H, J 3.2 Hz, H-1′), 4.96 (m, 6H, H-1), 4.84 (d, 1H, J 15.0 Hz, CH_2=), 3.96 (m, 2H), 3.83–3.47 (br m, 40H, H-2, 3, 4, 5, 6), 2.46 (m, 2H, –CH_2–COOCH=CH_2), 2.30 (t, 2H, J 6.5 Hz, –CH_2–COO–β-CD), 1.61, 1.52, 1.25 (m, 12H, other CH_2 decanedioyl part); ^{13}C NMR (D_2O) is shown in Table 1 (**3**); ESI-MS (m/z): 1379 (M_3+2H_2O–H)$^-$, M_3 corresponding exactly to the molecular weight of mono-(2-*O*-vinyldecanedioyl)-β-CD.

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