Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 16 (2005) 615–622

Thermolysin catalyses the synthesis of cyclodextrin esters in DMSO

Ninfa Rangel Pedersen,[a] Janni Brogaard Kristensen,[a,b,†] Guy Bauw,[a] Bart Jan Ravoo,[b,‡] Raphael Darcy,[b] Kim Lambertsen Larsen[a] and Lars Haastrup Pedersen[a,*]

[a]*Department of Life Sciences, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark*
[b]*Centre for Synthesis and Chemical Biology of the Conway Institute, Department of Chemistry, National University of Ireland, University College Dublin, Belfield, Dublin 4, Ireland*

Received 10 September 2004; accepted 7 December 2004

Abstract—Fatty acid esters of cyclodextrins (CDs) were synthesised in a one-step reaction with native CDs as acyl acceptors and vinyl-activated fatty acid esters as acyl donors. Immobilised preparations of thermolysin, subtilisin, the alkaline protease AL-89 and *Candida antarctica* lipase B were investigated for their catalytic properties regarding transesterification in solvents of increasing hydrophilicity. The synthesis of cyclodextrin fatty acid esters was proved to be catalysed enzymatically by thermolysin in DMSO. The obtained products were analysed by TLC and their structures characterised by NMR, MS and FTIR spectroscopy. With vinyl decanoate as acyl donor β-CD was esterified at all seven glucose C-2 positions resulting in heptakis(2-*O*-decanoyl)-β-cyclodextrin as the major product. With vinyl butyrate, substitution occurred at all the C-2 and partially at the C-3 or C-6 positions resulting in an average degree of substitution of nine. Between 20% and 25% (w/w) of the acyl donor was converted to esters in 20 h corresponding to an estimated total conversion of the acyl acceptor in the case of maltosyl-β-CD. In the subtilisin and AL-89 catalysed reactions, product formation was simultaneously catalysed non-enzymatically by inorganic buffer salts in aprotic, hydrophilic solvents and with the lipase no products were formed in any of the solvents investigated.

1. Introduction

Cyclodextrins (CDs) are natural, cyclic maltodextrins produced from starch by the action of 4-α-glucanotransferases. CDs with a degree of polymerisation from six to several hundreds have been discovered[1] with the most important being α-, β- and γ-CD composed of six, seven and eight α-D-(1→4) glucopyranoside moieties, respectively. The structures of these small CDs are viewed as hollow, truncated cones where the C-6 primary alcohols crown the narrow rim while the wider rim is crowned by the secondary alcohols at positions C-2 and C-3 (Fig. 1). This arrangement results in a polar, hydrophilic outer surface and a relatively apolar, hydrophobic cavity making CDs capable of forming inclusion complexes with hydrophobic guest molecules. This property is particularly of interest for controlling the administration and release of drugs and biomedical molecules with poor water solubility. Inclusion complexation can alter the physicochemical properties of both the drug and the cyclodextrin molecule, and has been used to stabilise, solubilise and decrease the volatility of drug molecules.[2]

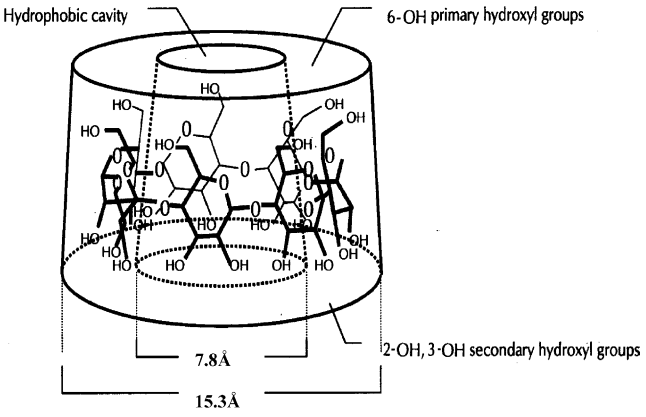

Figure 1. Chemical structure and conformation of β-cyclodextrin.

* Corresponding author. Tel.: +45 9635 8497; e-mail: lhp@bio.aau.dk
† Present address: BioCentrum-DTU, Technical University of Denmark, DK-2800 Lyngby, Denmark.
‡ Present address: Laboratory of Supramolecular Chemistry and Technology, University of Twente, PO Box 217, 7500 AE Enschede, The Netherlands.

0957-4166/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2004.12.009

However native α- and β-CDs are known to be parenterally unsafe due to their nephrotoxicity and can extract cholesterol and phospholipids from cell membranes,[2] furthermore the high external hydrophilicity of CDs reduces the affinity to biological membranes.[3] Although the hydrophilic CDs can enhance drug absorption, hydrophobic CD derivatives may have broader applicability and could serve as novel slow-release carriers of water-soluble complexes with drugs.[4] Therefore, since the late 1970's research into chemical derivatisation of CDs has increased, in order to provide molecules which circumvent the shortcomings of natural CDs. Substitution of any hydroxyl group by small alkyl or hydroxyalkyl ether groups often results in an increased solubility of the derivative and its complexes.[5,6] Thus amphiphilic cyclodextrin derivatives have been engineered to form nanoparticles and vesicles with liposome-like properties[7] showing specific binding affinity towards lectin proteins[8] and DNA[9] in aqueous environments. α-, β- and γ-CDs having 18, 21 and 24 hydroxyl groups, are readily modified chemically and since the hydroxyl groups are nucleophilic in nature the initial reaction which determines the regioselectivity and degree of substitution, is an electrophilic attack. The hydroxyl groups at the C-6 position are the most basic and often the most nucleophilic, those at the C-2 are the most acidic and those at the C-3 are the least accessible. The reactivity of the hydroxyl groups is influenced by the reaction conditions. Furthermore, the hydrophobic cavity of the CDs often interacts with the substitution reagent to direct the substitution to an unexpected hydroxyl group; therefore regioselective derivatisation of CDs using conventional organic synthesis is not an easy task and often involves elaborate protection and deprotection steps.[5,10,11]

Enzyme-catalysed synthesis of carbohydrate esters has proven very efficient in terms of regioselective substitution of unprotected, native mono- and disaccharides and both lipases and proteases have been found to catalyse these reactions in non-aqueous reaction media.[12–17] Aprotic, hydrophilic solvents such as DMSO are very good solvents for carbohydrates in which proteases have proven to be active whereas lipases are not.[12,14,15] Thus acetylation of CDs has been achieved with a serine protease, proteinase N in DMSO[18] while a similar protease, subtilisin in reversed micelles was employed to catalyse esterification of CDs in a hydrophobic solvent.[19]

Herein, the catalytic properties of one lipase and three proteases are investigated with respect to acylation of CDs by a transesterification reaction using vinyl fatty acid esters in organic solvents of increasing hydrophilicity—and furthermore the effect of fatty acid chain length on the regioselective properties of thermolysin is studied.

2. Results

2.1. Thermolysin-catalysed reactions

Thermolysin catalysed the formation of β-CD esters using vinyl esters of butyrate, decanoate and laurate, respectively, as acyl donors in DMSO. In the corresponding controls containing pH-adjusted immobilisation matrix without enzyme, product formation was not observed (Fig. 2). NMR analysis of the products from the reaction with vinyl decanoate showed that β-CD was esterified exclusively at the glucose C-2 positions. The chemical shifts measured by ^{1}H NMR are shown in Table 1. For the native β-CD the proton signals of the hydroxyl groups were observed at $\delta = 4.42$ ppm for the 6-OH primary alcohol and for the secondary alcohols at $\delta = 5.68$ (2-OH) and $\delta = 5.64$ (3-OH). No signals were observed in this region in the corresponding esters. The OH groups of the β-CD esters were assigned in the ^{1}H NMR spectra between $\delta = 4.5$–4.7 ppm with signals disappearing when small amounts of D_2O were added to the d_6-DMSO. Regarding the ester acyl chain, signals for the terminal methyl group occurred at $\delta = 0.85$ ppm and for the secondary alkyl groups at $\delta = 1.53$ (C-3) and $\delta = 2.30$ (C-2). From the chemical shifts obtained in the ^{1}H–^{13}C HSQC NMR spectra of the products it could be concluded that substitution at 2-OH had occurred with both decanoate and butyrate. Additionally partial substitution at 3-OH or 6-OH with butyrate was indicated (see Fig. 3).

The degree of substitution was estimated from ^{1}H NMR spectra calculating the ratio between the integrated signals from the H-1 and the terminating methyl group of the acyl chain, and furthermore by comparing the signals of the OH groups in the absence and presence of D_2O. This way the average degree of substitution of the β-CD decanoate esters was estimated as 7 and as 8.8–9.0 for the β-CD butyrate esters.

MALDI-MS analysis revealed a mixture of β-CD decanoate esters with 6–9 substitutions of which the hepta substituted ester gave the highest signal intensity. With vinyl butyrate a mixture of β-CD butyrate esters of 6–11 substitutions was obtained with the octa and nano substituted esters showing the highest intensities (see Fig. 4).

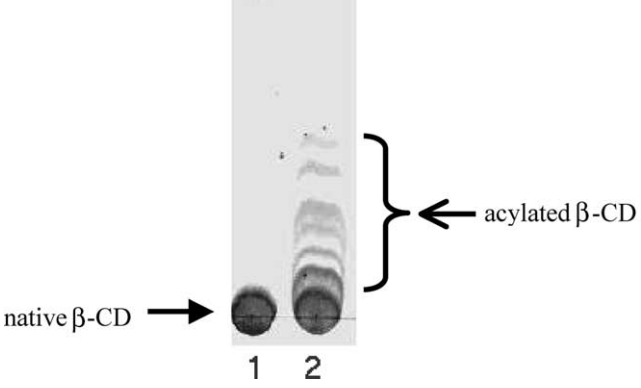

Figure 2. TLC of β-cyclodextrin and its laurate esters. Ester synthesis catalysed by thermolysin in DMSO. Mobile phase: $CHCl_3$/MeOH/AcOH/H_2O (70:20:8:2, v/v/v/v). (1) Reaction with Celite Na-MOPS control. (2) Reaction with immobilised thermolysin.

Table 1. NMR signal assignment of β-cyclodextrin and its butyrate and decanoate esters

Position	β-CD		β-CD-butyrate ester		β-CD-decanoate ester	
	δ (^{1}H, ppm)	δ (^{13}C, ppm)	δ (^{1}H, ppm)	δ (^{13}C, ppm)	δ (^{1}H, ppm)	δ (^{13}C, ppm)
1	4.82 (d) J_{1-2} = 3.5	103.1	5.08 (d) J_{1-2} = 3.2	97.5	5.06 (dd) J_{1-2} = 3.6	97.8
2	3.29 (m) J_{2-3} = 9.3	74.3	4.44 (t) J = 9	73.2	4.44 (d) J_{2-3} = 6.9	73.2
3	3.64 (m) J = 9.0	73.6	3.6–3.8 (m)	69.5	3.78 (m)	69.6
4	3.34 (m) J = 9.0	82.7	3.46 (t)	80.1	3.46 (t) J = 9	80.4
5	3.59 (m)	73.2	3.6–3.8 (m)	71.9	3.60 (m)	71.9
6	3.64 (m)	61.2	3.6–3.8 (m)	60.1	3.60 (m)	60.0
6′	3.64 (m)		3.6–3.8 (m)		3.74 (m)	
OH-2	5.68 (d)		—		—	
OH-3	5.64 (d)		4.5–4.7 (m)		4.5–4.6 (s/d)	
OH-6	4.42 (t)		4.5–4.7 (m)		4.5–4.6 (s/d)	
Acyl chain						
C1				174		174
C2: –CH_2–			2.31 (m)	35.4	2.30 (m)	33.6
C3: –CH_2–			1.55 (m)	17.8	1.53 (m)	24.4
C4–C9: –CH_2–			—		1.23 (m)	31.5/29.0/22.3
Terminal –CH_3			0.87 (t)	13.6	0.85 (t)	14.0

Ester synthesis was catalysed by thermolysin in DMSO. Samples of β-CD, β-CD-butyrate and decanoate esters were analysed in d_6-DMSO. The coupling constants, J, are given in Hz. Number of peaks in brackets: s: singlet, d: duplet, t: triplet, dd: doublet of doublets; m: multiplet.

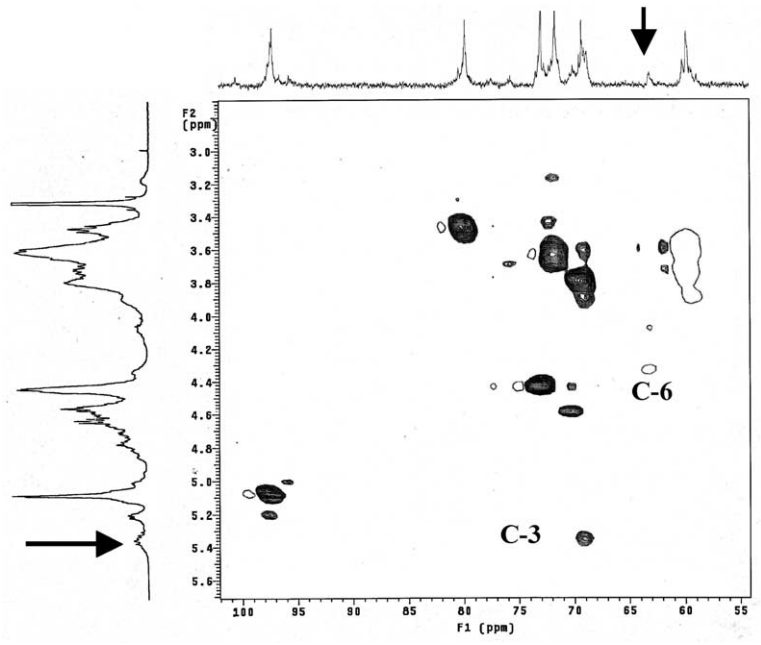

Figure 3. ^{1}H–^{13}C HSQC NMR spectra of a mixture of β-cyclodextrin butyrate esters. Correlation peaks of the acylated H/C-3 and H/C-6 are indicated.

Prolonged reaction did not change the product profiles of the decanoate and butyrate esters of β-CD as evaluated by TLC.

Thermolysin furthermore catalysed the synthesis of α-, β-, γ- and maltosyl-β-CD esters with vinyl laurate as the acyl donor in DMSO and DMF, respectively, whereas no product formation was observed in pyridine and *t*-BuOH. MS analysis showed that CD esters with varying degrees of substitution were obtained, depending on the type of CD, when vinyl laurate was used as acyl donor in DMSO (Table 2). Thus α-CD had 1–4, β-CD had 1–7 (see also Fig. 5), γ-CD had 1–5 and maltosyl-β-CD had 2–5 substitutions. FTIR analysis of the native CDs and their laurate esters showed a distinct signal in the spectrum at 1733 cm^{-1}, indicating the presence of an ester carbonyl group. All fatty acid esters of α-, β-, γ- and maltosyl-β-CD displayed spectra similar to the one shown in Figure 6. The bands at 3377 and 1646 cm^{-1} were from OH groups in the glucose moieties

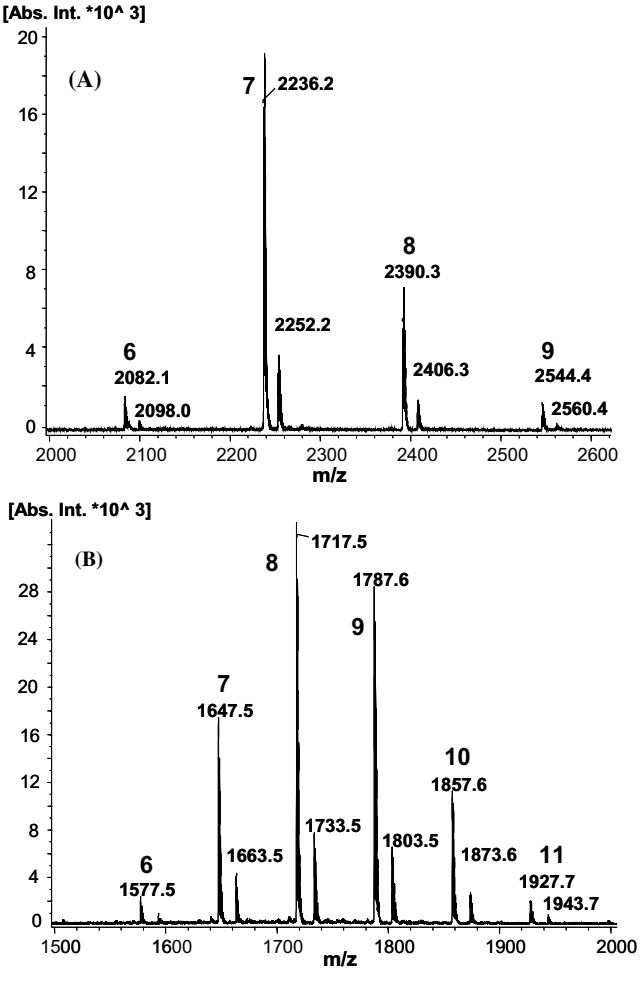

Figure 4. MALDI-MS spectra of β-CD decanoate esters (A) and β-CD butyrate esters (B). Masses of Na adducts (first series) and K adducts (second series) are shown with numbers indicating the degree of substitution.

while peaks at 2923 and 2854 cm^{-1} were interpreted as CH vibrations in both the glucose moieties and in the fatty acyl chain. In the fingerprint region of 1500–800 cm^{-1} the signals derived from a variety of mainly CH-, COC- and OH-vibrations from both CD and the acyl chain.

After 20 h the degree of conversion of acyl donor obtained in DMSO with maltosyl-β-CD as the acyl acceptor was 25%. With α-, β- and γ-CD, respectively, a 20% conversion of acyl donor was obtained after 20 h.

2.2. Serine hydrolase-catalysed reactions

Vinyl laurate was the acyl donor and α-, β-, γ- or maltosyl-β-CD were acyl acceptors in reactions catalysed by either subtilisin or the alkaline protease, AL-89 where CD esters were synthesised in DMSO and DMF, respectively, as detected by TLC (Fig. 7) and confirmed by MS and FTIR. The corresponding control reactions with pH-adjusted immobilisation matrix in the absence of enzyme also showed formation of CD esters, but the matrix alone did not catalyse ester formation (Fig. 7). With pyridine or *t*-BuOH as solvent no product formation was observed in the presence of enzyme, and in the control reactions. With *Candida antarctica* lipase B (CALB) no CD ester synthesis was observed in any of the solvents used.

3. Discussion

3.1. Thermolysin-catalysed reactions

Immobilised thermolysin catalysed the transesterification of cyclodextrins with vinyl esters of butyrate, decanoate or laurate in DMSO. The corresponding control reactions in the absence of enzyme did not result in ester formation, proving that the esterification was catalysed enzymatically by thermolysin. Synthesis of sucrose laurate using vinyl laurate as acyl donor has previously been shown to be catalysed by inorganic salts in the absence of enzyme.[14,20] However in the present reaction an organic buffer was used. With both β-CD-decanoate and butyrate esters it was shown that the C-2 position of all seven glucose moieties was the primary target of substitution while with β-CD-butyrate esters two additional substitutions at the C-3 or the C-6 position were shown, indicating that the regioselectivity of thermolysin is directed primarily towards the C-2 position of glucose. In a similar reaction in DMSO with sucrose as acyl acceptor thermolysin showed the same regioselectivity towards the C-2 position of the glucose moiety in sucrose producing 2-*O*-lauroyl-sucrose.[12] Xiao et al.[21] achieved regioselective monoacylation of β-CD at the C-2 position with divinyl esters of butanedioate, hexanedioate and decanedioate as acyl donors, respec-

Table 2. Predicted and detected molecular masses of cyclodextrin laurate esters

Number of substitutions	α-CD ester (*m/z*)		β-CD ester (*m/z*)		γ-CD ester (*m/z*)		Maltosyl-β-CD ester (*m/z*)	
	Predicted	Detected	Predicted	Detected	Predicted	Detected	Predicted	Detected
1	1176.3	1177.6	1339.3	1339.5	1502.0	1501.4		
2	1358.6	1359.7	1521.6	1521.7	1683.6	1683.7	1845.9	1846.4
3	1541.0	1541.8	1704.0	1704.0	1866.0	1865.9	2028.3	2028.0
4	1723.3	1724.0	1886.3	1886.0	2048.3	2049.0	2210.6	2211.0
5			2068.7	2069.1	2230.6	2231.9	2392.9	2393.6
6			2250.9	2251.2				
7			2433.2	2433.3				

Ester synthesis was catalysed by thermolysin in DMSO. Some of the esters are visible as Na adducts. Masses were detected by ESI-MS.

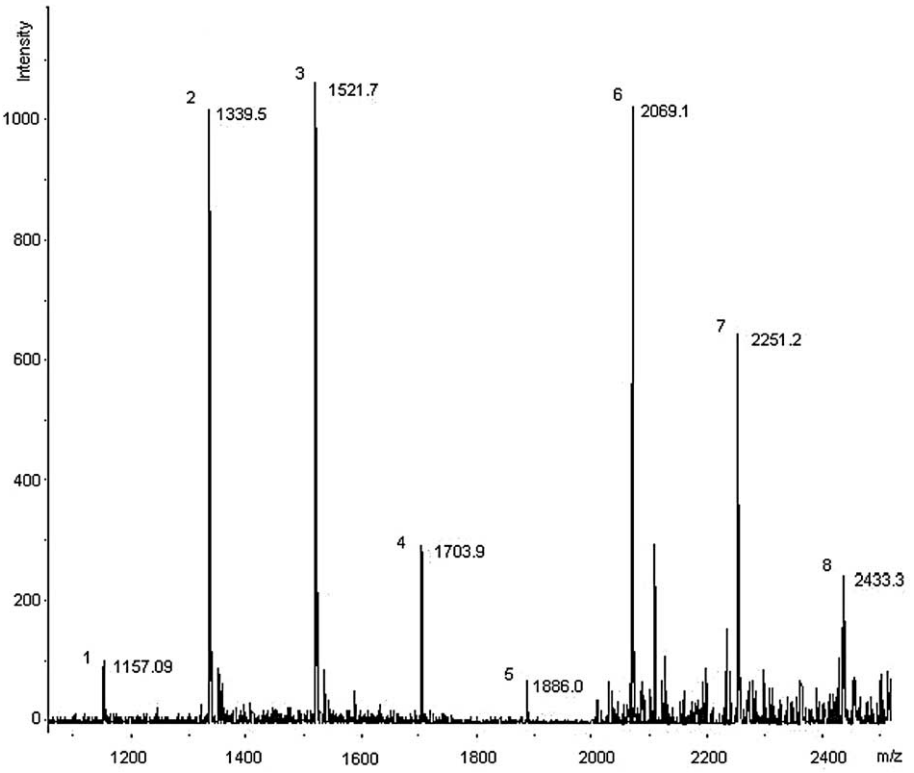

Figure 5. Electrospray mass spectra (ESI-MS) of β-cyclodextrin and its laurate esters. Peak no 1: native β-CD, peak nos. 2, 3, 4, 5, 6, 7 and 8: β-CD laurate esters with 1, 2, 3, 4, 5, 6 and 7 substitutions, respectively. Na adducts of β-CD esters with one to seven substitutions are shown; ester synthesis was catalysed by thermolysin in DMSO. The figure is composed of two overlaid zoom scans of the same sample. Intensities are not necessarily a representation of concentrations.

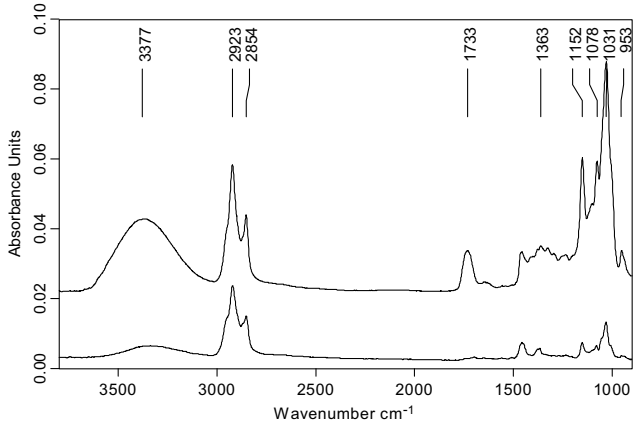

Figure 6. ATR-FTIR spectra of native α-cyclodextrin (lower spectrum) and its laurate esters, DS 1–4 (upper spectrum). Ester synthesis was catalysed by thermolysin in DMSO. Carbonyl ester absorption is visible at 1733 cm^{-1}; note that the baseline of the upper spectrum is shifted 0.02 AU up to facilitate comparison.

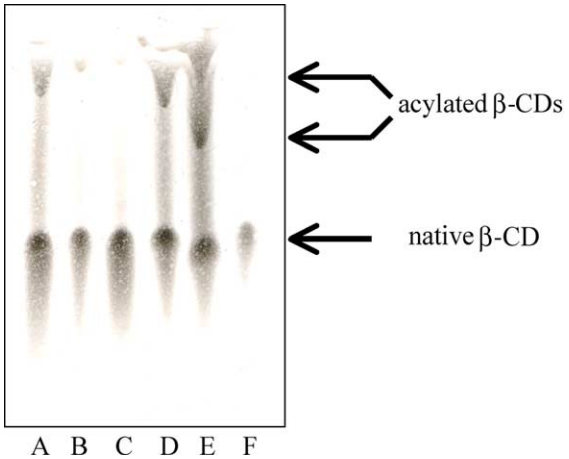

Figure 7. TLC of β-cyclodextrin and its laurate esters obtained in reactions catalysed by immobilised proteases AL-89 and subtilisin in DMSO. AL-89: crude preparation of alkaline protease from *Bacillus pseudofirmus*; subtilisin: commercial preparation from *B. subtilis*. Mobile phase: CH_3CN/H_2O/liquid NH_3 (6:3:1, v/v/v). Samples contained reaction mixtures of (A) immobilised AL-89; (B) Celite buffer control with 10 mM sodium carbonate buffer at pH 10; (C) Celite only; (D) Immobilised subtilisin; (E) Celite buffer control with sodium phosphate buffer at pH 7.5; (F) Substrates only.

tively, in DMF using an alkaline protease from *Bacillus subtilis*. The water content and method of pH equilibration of the enzyme prior to initiation of the reaction was not reported. The position of substitution was verified by ^1H and ^{13}C NMR in d_6-DMSO and D_2O. Eighty percent of the esters obtained were monosubstituted while the remaining 20% were di- and tri-esters as estimated by electrospray (ESI) MS of the purified products. In the present study the degree of substitution was estimated from ^1H NMR spectra and the average degree of substitution of the β-CD decanoate esters was

estimated as 7 and 8.8–9.0 for the β-CD butyrate esters. Although MALDI-MS does not directly provide quantitative information simply as the function of the signal intensity[22] these findings were coherent with the qualitative results of the MALDI-MS analyses. With an average degree of substitution of seven heptakis(2-*O*-decanoyl)-β-cyclodextrin was identified as one of the major products. Furthermore the substitution pattern of β-CD esters proved to be dependent on the chain length of the acyl donor, as shown in Tables 1 and 2. Thus with vinyl laurate a mixture of β-CD esters with up to seven substitutions were obtained, while an average of seven and nine substitutions was obtained with vinyl decanoate and vinyl butyrate, respectively. This was in contrast to Xiao et al. who independently of the chain length of the divinyl acyl donor obtained monoacylated β-CD as the major product.[21] As the acyl donors and the enzymes used in the two processes were different, there could be several reasons to the different substitution patterns achieved, where physical restrictions of the active site and binding of the substrates could be essential factors.

By conventional chemical syntheses, Dubes et al. obtained a 20–95% yield of *O*-2 and *O*-3-acylated heptakis(6-*O*-*tert*-butyldimethylsilyl)-β-CDs at 70 °C in 48 h with hexanoyl chloride and acetic anhydride as the acyl donors in dry pyridine.[23] In the present study a 25% conversion of the vinyl laurate was obtained in 20 h corresponding to an estimated total conversion of maltosyl-β-CD and an average degree of substitution of 3.6 of the resulting product. This was coherent with the ESI-MS analysis showing a mixture of maltosyl-β-CD esters of 2–5 substitutions. In the reactions with α, β and γ-CD where laurate esters of 4, 7 and 5 substitutions were obtained with a conversion of the vinyl laurate of 20%. Hence the corresponding yields of esters based on CD conversion was estimated as ranging between 30% and 80%.

3.2. Ester synthesis with serine hydrolases and inorganic buffers

Inorganic buffers catalysed the formation of cyclodextrin laurate esters in the absence of enzyme in DMSO and DMF. Similar observations were reported regarding synthesis of sucrose laurate esters.[14,20] Disodium hydrogen phosphate in low concentration has been shown to catalyse regioselectively the acetylation of starch and β-cyclodextrin at the 2-position in DMSO at 40 °C with vinyl acetate as acetyl donor, and sodium carbonate has been proven to catalyse the same reaction with starch as the acetyl acceptor.[18,24] Increasing the temperature or the concentration of inorganic salt and acetyl donor will however lead to acetylation of the C-6 and C-3 positions as well.[24]

By the use of ^{1}H NMR Akkara et al.[19] found that the protease subtilisin (from *B. subtilis*), ion paired with dioctyl sulfosuccinate, catalysed the acylation of cyclodextrins at positions C-2 and C-3 with vinyl fatty acid esters as acyl donors in isooctane. Proteinase N (from *B. subtilis*) has been shown to catalyse the regioselective acetylation of β-cyclodextrin at the C-2-position by a transesterification with vinyl acetate at 39 °C for 70 h in both DMSO and DMF resulting in yields of approximately 84% (mol/mol CD).[18]

Although hydrophilic aprotic solvents are efficient in dissolving CDs, no CD ester synthesis was observed in any of the solvents investigated with CALB. Lipases have been shown to be inactive in concentrations in DMSO higher than 30% (v/v)[25,26] and therefore their inability to catalyse CD ester formation seems to be caused by enzyme inactivation. Pyridine has been shown to reduce lipase activity significantly and *t*-BuOH is a relatively poor solvent for native carbohydrates.[25] Furthermore the substrate binding pocket of CALB has the form of an elliptical, steep funnel of 9.5×4.5 Å[27] and exhibits a high degree of substrate selectivity regarding the bulkiness of the substrate.[13,27,28] Therefore it could be expected that physical restrictions of the active site prevents or limits accommodation of the relatively large and rigid CDs, in which the approximate height of the cone is 7.8 Å and the diameter of the wider rim with the secondary alcohols is 13.7, 15.3 and 16.9 Å for α-, β- and γ-CD, respectively. *Rhizomucor miehei* lipase (Chirazyme), on the other hand, has a hydrophopic crevice-like, binding site located near the protein surface with a length of 22 Å.[27] This may be an essential factor in explaining that this lipase catalyses the synthesis of organic acid esters of β-cyclodextrin and of two derivatives heptakis-(2,6-dimethyl-*O*)-β-cyclodextrin (DM-β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) in *n*-heptane at 50 °C. In 96 h β-CD esters were obtained with a substitution in moles per mole of 6.78 of acetate, 0.38 of propionate and 2.43 of *i*-butyrate, with NMR analysis showing that substitution at the C-2-position could not be ruled out. The DM-β-CD was substituted with 2.5 mol acetate per mole at the available 3-positions whereas HP-β-CD had available 2-, 3- and 6-positions and was substituted with 2 mol acetate/mole.[29]

4. Conclusion

We have developed a one-step enzyme-catalysed reaction for the esterification of native cyclodextrins using thermolysin in DMSO. The regioselectivity of thermolysin was directed primarily towards the C-2 position. Unprotected β-CD was regioselectively acylated at the C-2 position or at the C-2 position and additionally, partly at the C-3 or C-6 positions depending on the fatty acid chain length of the acyl donor. Hence a mixture of (2-*O*-decanoyl)-β-CD esters with an average degree of substitution of seven was obtained and heptakis(2-*O*-decanoyl)-β-cyclodextrin was identified as one of the major products. Furthermore a mixture of β-CD-butyrate with an average degree of substitution of nine was obtained. Using vinyl laurate as acyl donor the degree of substitution was shown to be dependent on the type of native cyclodextrin. A 25% degree of conversion of the acyl donor was achieved in 20 h corresponding to an estimated total conversion of the acyl acceptor, maltosyl-β-CD.

5. Experimental

5.1. Chemicals

α-, β- and γ-CD were obtained from Wacker Chemicals, GmbH Burghauser (Germany). 6-*O*-maltosyl-β-CD (maltosyl-β-CD) was from Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan). Vinyl laurate (98.5% purity), and vinyl butyrate (98% purity) were from Fluka Chemie AG, (Buch, Switzerland). Vinyl decanoate (95% purity) was from Sigma–Aldrich Chemie GmbH, Steinheim, (Germany). Dimethylsulfoxide (DMSO), *tert*-butanol (*t*-BuOH), pyridine, *N*,*N*-dimethyl formamide (DMF), *iso*propanol (*i*PrOH), methanol (MeOH) and Celite 545, were from Sigma, St. Louis (USA). All chemicals used were of analytical grade.

5.2. Enzymes

Candida antarctica lipase B (CALB) (E.C. 3.1.1.3), an immobilised preparation denoted Novozym 435 and subtilisin (E.C. 3.4.21.62), an endopeptidase from *Bacillus subtilis* were kind gifts from Novozymes, Bagsværd, Denmark; thermolysin (E.C. 3.4.24.27), a thermostable extracellular metalloendopeptidase from *Bacillus thermoproteolyticus* was obtained from Calbiochem (USA) and a crude preparation of the alkaline serine protease, AL-89 from *Bacillus pseudofirmus* was produced according to Pedersen et al.[14]

5.3. Immobilisation of enzymes

Lyophilised AL-89 (30 mg crude preparation) was dissolved in 1.0 mL 10 mM sodium carbonate buffer, pH 10. Subtilisin (30.0 mg) was dissolved in 1.0 mL 10 mM sodium phosphate buffer, pH 7.5. Thermolysin (30.0 mg) was dissolved in 1.0 mL 50 mM 3-morpholinopropanesulfonic acid (Na-MOPS) buffer at pH 7.5. The dissolved enzyme preparations were mixed thoroughly with 1 g of acid-washed Celite and vacuum dried as described previously.[12] As controls, 1.0 mL of each buffer was thoroughly mixed with 1.0 g of acid-washed Celite and vacuum dried in the same way as the immobilised enzyme. The controls will be referred to as Celite buffer controls.

5.4. Enzymatic ester synthesis

Reactions were started by adding 100 mg/mL CALB, 150 mg/mL of immobilised proteases (either AL-89, subtilisin and thermolysin) or Celite buffer controls to 1.04 M vinyl laurate and 100 mg/mL of CD resulting in concentrations of 0.10, 0.09, 0.08 and 0.07 M of α-, β-, γ- and maltosyl-β-CD, respectively. The solvents investigated were DMSO, DMF, pyridine and *t*-BuOH, respectively. Reaction volume was 1.0 mL and reaction time was 20 h. Reactions were carried out in 25 mL Schott bottles in a heating block (Telemodule 40TC, Germany) with magnetic stirring at 250 rpm and 45 °C. Under the same conditions 0.09 M β-CD and 1.04 M vinyl ester of respectively butyric, decanoic and lauric acid were incubated with thermolysin in DMSO for 20 h in a reaction volume of 1 mL and for 73 h in a reaction volume of 10 mL. All solvents were stored over 0.3 nm molecular sieves (Merck, Germany). At termination the enzyme reaction mixture was centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge to remove immobilised enzyme. Before spectroscopic and spectrometric analysis, 35% (v/v) methanol was added to the supernatant until all the CD esters were precipitated and the precipitate was dried under vacuum prior to further analyses.

5.5. TLC analysis

Analytical TLC was performed using silica gel 60 plates (Merck, Germany) according to Pedersen et al.[13] and Jindrich et al.[30]

5.6. HPLC analysis

To quantify the degree of conversion the concentration of the reactants (acyl donor and acceptor) was measured by reversed-phase HPLC with RI detection on termination of the reaction. For determination of reactant concentration a standard curve of each reactant was prepared. Analysis was performed in triplicate using a C18 column (Waters C18, 5 μm column, 4.6 × 250 mm HPLC cartridge, Ireland) with a refractive index detector Knauer (Germany). Eluent was MeOH/water, 95:5 (v/v) at a flow rate of 1 mL min^{-1} CD esters were eluted by MeOH/EtOH, 80:20 (v/v).

5.7. MS analysis

For analysis of laurate esters 1 mg of dried CD ester was dissolved in a 1 mL mixture of MeOH/*i*PrOH, 1:1 (v/v) and centrifuged, transferring the supernatant to an eppendorf tube. After repeating the centrifugation procedure thrice, the supernatant was diluted 10×, 100× and 1000× with MeOH/*i*PrOH, 1:1 (v/v) and analysed by electrospray injection mass spectrometry (ESI-MS), starting with the lowest concentration, on an Esquire-LC Mass Spectrometer (Bruker, Germany). Injection speed was 100 μL/h.

Analysis of β-CD esters of butyric and decanoic acid was performed by MALDI-TOF MS on a Reflex III (Bruker, Germany). Samples were applied to nitrocellulose-coated target plates according to Kussman et al.[31] Coating solution was a 1:4 (v/v) mixture of 0.1% (v/v) nitrocellulose in acetone and saturated CCA (α-cyano-4-hydroxy cinnamic acid) acetone solution. The coating solution (0.25 μL) was applied and allowed to dry. CD esters were solubilised in either MeOH or *i*PrOH and mixed 1:1 (v/v) with 0.1% (v/v) trifluoroacetic acid in 33% CH_3CN saturated with CCA and 1 μL was applied to the target plate.

5.8. FTIR analysis

CD ester was qualitatively analysed by mid-infrared spectroscopy on an IFS 66V/S FTIR spectrometer with a Deuterium TriGlycine Sulfate detector (Bruker, Germany). The sampling unit was a 45° ZnSe attenuated total reflectance (ATR) crystal with six reflections on

the sample surface having surface area of 7 cm^2 (1 cm × 7 cm). Measurements were performed by evaporating 10 μL of approximately 1 $mg\,mL^{-1}$ dried native CD or CD ester dissolved in MeOH/*i*PrOH, 1:1 (v/v) directly on the ZnSe crystal surface. Each spectrum was the average of 64 measurements at a resolution of 4 cm^{-1} and scanner velocity of 10 kHz. All spectra were recorded at 30.0 ± 0.5 °C with the clean ATR crystal as background.

5.9. NMR analysis

NMR analyses of buryrate and decanoate cyclodextrin esters were carried out on 300 and 500 MHz Varian Unity INOVA spectrometers from Varian Inc., Paolo Alto, CA, USA, using Varian VNMR software. Samples were dissolved in DMSO-d_6 or $CDCl_3$ (deuterated chloroform) and spectra were recorded at 30 °C. All spectra were recorded using standard pulse sequences, instrument settings, and procedures, ^{13}C–^{1}H HSQC spectra were recorded at 500 MHz.

Acknowledgements

We would like to acknowledge Dr. Reinhard Wimmer, Dr. Torben Snabe, Dr. Jeppe Emmersen, Dr. Amare Gessesse, Dr. Antonino Maszzaglia, Ph.D. student Finn L. Aachmann and Mrs. Geraldine Fitzpatrick for technical assistance and useful discussions.

References

1. Larsen, K. L. *J. Inclusion Phenom. Macrocycl. Chem.* **2002**, *43*, 1–13.
2. Stella, V. J.; Rajewski, R. A. *Pharm. Res.* **1997**, *14*(5), 556–567.
3. Duchêne, D.; Pochel, G.; Wouessidjewe, D. *Adv. Drug Del. Rev.* **1999**, *36*(1), 29–40.
4. Hirayama, F.; Uekama, K. *Adv. Drug Del. Rev.* **1999**, *36*, 125–141.
5. Szejtli, J. *Chem. Rev.* **1998**, *98*, 1743–1753.
6. Mazzaglia, A.; Ravoo, B. J.; Darcy, R.; Gambadauro, P.; Mallamace, F. *Langmuir* **2002**, *18*, 1945–1948.
7. Ravoo, B. J.; Darcy, R. *Angew. Chem., Int. Ed.* **2000**, *39*, 4324–4326.
8. Mazzaglia, A.; Forde, D.; Garozzo, D.; Malvagna, P.; Ravoo, B. J.; Darcy, R. *Org. Biol. Chem.* **2004**, *2*, 957–960.
9. Cryan, S. A.; Holohan, A.; Donohue, R.; Darcy, R.; O'Driscoll, C. M. *Eur. J. Pharm. Sci.* **2004**, *21*, 625–633.
10. Frömming, K. H.; Szejtli, J. *Cyclodextrins in Pharmacy*; Kluyver Academic: The Netherlands, 1994.
11. Khan, A. R.; Forgo, P.; Stine, K. J.; D'souza, V. T. *Chem. Rev.* **1998**, *98*, 1977–1996.
12. Pedersen, N. R.; Halling, P. J.; Pedersen, L. H.; Wimmer, R.; Matthiesen, R.; Veltman, O. R. *FEBS Lett.* **2002**, *519*, 181–184.
13. Pedersen, N. R.; Wimmer, R.; Emmersen, J.; Degn, P.; Pedersen, L. H. *Carbohydr. Res.* **2002**, *337*, 1179–1184.
14. Pedersen, N. R.; Wimmer, R.; Matthiesen, R.; Pedersen, L. H.; Gessesse, A. *Tetrahedron: Asymmetry* **2003**, *14*, 667–673.
15. Potier, P.; Bouchu, A.; Gagnaire, J.; Queneau, Y. *Tetrahedron: Asymmetry* **2001**, *12*, 2409–2419.
16. Therisod, M.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3977–3981.
17. Koeller, K. M.; Wong, C.-H. *Chem. Rev.* **2000**, *100*, 4465–4493.
18. Klohr, E. A.; Koch, W.; Klemm, D.; Dicke, R. *European Patent Office*, EP 1 035 135, 2000.
19. Akkara, J. A.; Kaplan D. L.; Ferdinando, F.; Jonathan, D. S. U.S. Patent 6,063,916, 2000.
20. Plou, F.; Cruces, M. A.; Pastor, E.; Ferrer, M.; Bernabé, M.; Ballesteros, A. *Biotechnol. Lett.* **1999**, *21*, 635–639.
21. Xiao, Y.; Wu, Q.; Wang, N.; Lin, X. *Carbohydr. Res.* **2004**, *339*, 1279–1283.
22. Siuzdak, G. *Mass Spectrometry for Biotechnology*; Academic: San Diego, 1996; pp 105–111.
23. Dubes, A.; Bouchu, D.; Lamartine, R.; Parrot-Lopez, H. *Tetrahedron Lett.* **2001**, *42*, 9147–9151.
24. Dicke, R. *Cellulose* **2000**, *11*, 255–263.
25. Degn, P.; Zimmermann, W. *Biotechnol. Bioeng.* **2001**, *74*, 483–491.
26. Plou, F.; Cruces, M. A.; Ferrer, M.; Fuentes, G.; Pastor, E.; Bernabé, M.; Christensen, M.; Comelles, F.; Parra, J. L.; Ballesteros, A. *J. Biotechnol.* **2002**, *96*, 55–66.
27. Pleiss, J.; Fischer, M.; Schmid, R. D. *Chem. Phys. Lipids* **1998**, *93*, 67–80.
28. Arroyo, M.; Sinisterra, J. V. *J. Org. Chem.* **1994**, *59*, 4410–4417.
29. Pattekhan, H. H.; Divakar, S. *Ind. J. Chem.* **2002**, *418*, 1025–1027.
30. Jindrich, J.; Pitha, J.; Lindberg, B. *Carbohydr. Res.* **1995**, *275*, 1–7.
31. Kussmann, M.; Nordbo, E.; Rahbek-Nielsen, H.; Haebek, S.; Rossel-Larsen, M.; Jacobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kroll-Kristensen, A.; Palm, L.; Poepstorff, P. *J. Mass Spectrom.* **1997**, *32*, 593–601.