ELSEVIER

Journal of Molecular Catalysis A: Chemical 134 (1998) 259–265

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

Transesterification of lactulose with ethyl butanoate catalysed by *Candida antarctica* lipase

A.M. van der Heijden, F.J. Zuijderduijn, F. van Rantwijk *

Department of Organic Chemistry and Catalysis, Delft University of Technology, Julianalaan 136, 2628 BL Delft, Netherlands

Received 18 September 1997; accepted 21 October 1997

Abstract

The enzyme-catalysed acylation of lactulose by ethyl butanoate was investigated. A slow conversion to a complex mixture of esters was observed at 40°C in *tert*-butyl alcohol in the presence of a number of lipases and a protease. With *Candida antarctica* B lipase, esterifications could also be performed at 82°C (reflux) to increase the rate. The reaction could be accelerated by a factor of 5 when 1,2-dimethoxyethane was used as solvent. In this way, a nearly quantitative conversion to a mixture of esters was accomplished within 24 h. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Transesterification; Ethyl butanoate; *Candida antarctica* lipase

1. Introduction

Fatty acid esters of disaccharides have several interesting applications. For example, α,α-trehalose-6,6′-dialkanoates show antitumor and antibiotic activity [1,2], and sucrose fatty acid esters are used as detergents and emulsifiers in food [3]. Moreover, disaccharide esters are biodegradable, essentially non-toxic, and can be synthesised from cheap renewable raw materials, namely carbohydrates and triglycerides. However, the selective acylation of carbohydrates is problematical due to their multifunctionality. The base-catalysed acylation of sucrose, for example, takes place with low selectivity and is accompanied by the formation of coloured byproducts. A solution for these problems was sought in the use of lipases and proteases, which are known to catalyse (trans)esterification selectively under mild conditions [4].

In an initial study, complex mixtures were obtained by esterification of sucrose with fatty acids mediated by various lipases in aqueous buffer [5]. In subsequent work, hydrolytic side-reactions were prevented by using polar, aprotic solvents such as pyridine and dimethylformamide as the reaction medium; activated acyl donors such as trichloroethyl butanoate were often applied to increase the reaction rate [6–8]. The projected use of disaccharide esters as food additives and pharmaceuticals is, however, incompatible with the use of these toxic solvents and activated esters.

It has been shown that lipase-catalysed esterification of carbohydrates proceeds efficiently in

* Corresponding author.

1381-1169/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII: S1381-1169(98)00044-2

medium–polarity reaction media, such as the biocompatible and non-toxic *tert*-butyl alcohol, in which the sugars are sparingly soluble [9]. Woudenberg et al. have shown that esterification of disaccharides, such as lactose, sucrose, trehalose, isomaltulose (Palatinose®) and leucrose, by non-activated acyl donors proceeds at a useful rate in *tert*-butyl alcohol [10]. We will now describe the esterification of lactulose (β-D-galactopyranosyl-(1 → 4)-D-fructose) using this procedure.

2. Experimental

2.1. Materials and analytical methods

Immobilised lipase from *Candida antarctica* (B type, SP435), a liquid lipase preparation from *Humicola* (SP398), a lyophilised lipase preparation from *Humicola* (SP523) and lyophilised subtilisin A were kindly donated by Novo Nordisk (Bagsvaerd, Denmark). Subtilisin Carlsberg from *Bacillus licheniformis* and *Pseudomonas alcaligenes* lipase were received from Gist-brocades (Delft, The Netherlands) as gifts. Cross-linked enzyme crystals of *P. cepacia* lipase, (ChiroCLEC™-PC), *C. rugosa* lipase (ChiroCLEC™-CR) and *Bacillus licheniformis* subtilisin (ChiroCLEC™-BL) were gifts from Altus Biologics (Cambridge, MA, USA). The lyophilised and liquid enzyme preparations were immobilised on EP100 according to a published procedure [11,12].

Ethyl butanoate was obtained from ACROS, lactulose from Solvay Duphar, and phenylboronic acid from Aldrich. Zeolite CaA was obtained from Uetikon and activated at 400°C for at least 24 h before use.

2.1.1. Analysis

Analysis of butanoyl esters of lactulose was performed by gas chromatography (GC) on a Hewlett-Packard (HP) 5890, Series II chromatograph, equipped with a 7673 auto injector and a Chrompack 50 m × 0.32 mm CP-Sil 5 CB, 0.12 μ column. The carrier gas was nitrogen at a flow of 1.5 ml/min. Temperature program: 60°C (5 min) to 300°C (10°C/min). Peaks were detected using flame ionisation detection (FID) and were integrated on a HP 3396A integrator. Tetradecane was used as internal standard. Samples were prepared by withdrawing 20-μl samples of the reaction mixture and treating them with 0.5 ml of a trimethylsilylating reagent. This reagent was a mixture of pyridine (104 ml), *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (26 ml), and trimethylsilyl chloride (13 ml).

Mass spectra of the derivatised samples were measured on a VG 70-SE mass spectrometer using EI + . Due to the lability of the trimethylsilylated products, their parent peaks could not be observed in the mass spectra. Hence, fragment peaks were used to characterise the butanoate esters of lactulose as follows: *Monoester*: m/z 813 ($M\text{-}CH_2OOCCH_2CH_2CH_3$), 415 (unacylated monosaccharide fragment); *Diester*: m/z 811 ($M\text{-}CH_2OOCCH_2CH_2CH_3$), 449 (monosaccharide fragment bearing a single butanoyl group); *Tetraester*: m/z 809 ($M\text{-}OSi(Me)_3$), 447 (monosaccharide fragment bearing two butanoyl groups).

^{11}B and ^{13}C NMR spectra were recorded in D_2O on a Varian Unity Inova 300 spectrometer. For ^{11}B NMR boric acid was used as external reference, for ^{13}C NMR *tert*-butyl alcohol was used as internal reference.

2.2. Transesterifications

A mixture of lactulose (40 mg, 0.12 mmol), a 12.5/87.5 (v/v) mixture of ethyl butanoate and a solvent (*tert*-butyl alcohol or dimethoxyethane) (4 ml, 32 equivalents of ethyl butanoate relative to lactulose), enzyme (10 mg) and zeolite CaA (0.4 g) was shaken on an orbit shaker at 400 rpm. If water was added to the mixture zeolite, CaA was omitted.

For reaction in refluxing solvents, the procedure was modified as follows: a mixture of lactulose (200 mg, 0.58 mmol), 12.5% (v/v) ethyl butanoate in a solvent (30 ml, 48.5 equiva-

lents of ethyl butanoate relative to lactulose), enzyme (100 mg), and zeolite CaA (2.0 g) were stirred. The reflux equipment was closed with a drying tube containing $CaCl_2$.

For transesterifications in the presence of phenylboronic acid mixtures of lactulose (50 mg, 0.15 mmol), a 12.5/87.5 (v/v) mixture of ethyl butanoate and *tert*-butyl alcohol (4 ml, 26 equivalents of ethyl butanoate relative to lactulose), *Candida antarctica* B lipase (SP435) (10 mg) and zeolite CaA (0.4 g) were shaken on an orbit shaker at 400 rpm at 40°C. Different amounts of phenylboronic acid were added (0, 2.7, 5.5, 9.3 and 19,2 mg, corresponding with 0, 0.15, 0.30, 0.52 and 1.07 molar equivalents relative to lactulose).

2.3. Phenylboronic acid esters of lactulose in water

A solution was prepared of lactulose (55 mg, 0.16 mmol) and phenylboronic acid (20 mg, 0.16 mmol) in D_2O. The pD was tuned at 7.0 with a 1 M sodium hydroxide solution. ^{11}B and ^{13}C NMR spectra were recorded: ^{11}B NMR (96.3 MHz, D_2O) δ −10.84 (phenylboronic acid ester of lactulose) 9.8 ($PhB(OH)_2$. ^{13}C NMR (75 MHz, D_2O) δ 62.70 (C6′) 64.90 (C6) 65.60 (C1) 70.24 (C4′) 72.29 (C2′) 74.18 (C3′) 76.85 (C5′) 82.67 (C4) 84.79 (C3) 88.60 (C5) 104.31 (C1′) 112.43 (C2) 128.14, 128.90, 133.0 (*Ph*B acid ester). Only three peaks are seen for the phenyl group due to overlap.

3. Results

In solution, lactulose exists as a mixture of three different configurations, the α- and the β-furanose and the β-pyranose configuration (see Fig. 1). Each configuration possesses two or three primary hydroxyl groups; hence, the formation of 17 different esters (mono- di- and tri-esters) is theoretically possible, when only the primary hydroxyl groups are acylated, so complex mixtures of esters are to be expected.

3.1. Screening of enzymes

Initially, we examined the activity and selectivity of different enzymes as catalyst for the transesterification of lactulose. Ethyl butanoate was chosen as acyl donor because the butanoate esters can be simply analysed by GC in contrast to long chain fatty acid esters. Small-scale acylations of lactulose were performed by shaking a suspension of lactulose and enzyme in a 87.5/12.5 (v/v) mixture of *tert*-butyl alcohol and ethyl butanoate. If the reactions were carried out under dry conditions, zeolite CaA was added. This removed any residual water and also adsorbed the liberated ethanol to shift the

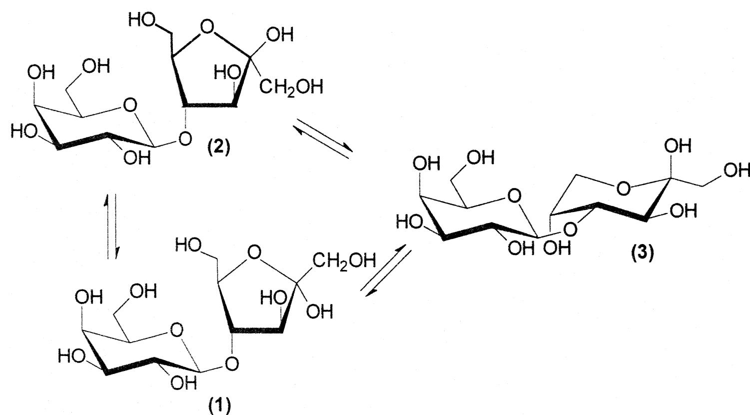


Fig. 1. Configurations of lactulose in solution.

Table 1
Enzyme catalysed acylation of lactulose by ethyl butanoate[a]

Enzyme source	Adjuvant[c]	Initial rate (pmol min^{-1} mg^{-1})	Conversion (%)
C. antarctica B (SP 435)	–	40	17[d]
	H_2O (50 μl)	29	3[d]
C. antarctica A (SP 526)[b]	H_2O (50 μl)	9	2
Subtilisin A[b]	–	180	9[e]
Humicola (SP 398)[b]	H_2O (50 μl)	540	15
Humicola (SP 523)[b]	H_2O (50 μl)	840	16
P. alcaligenes[b]	H_2O (50 μl)	110	6
	$Ca(OAc)_2$ [f]	13	4
P. cepacia, (ChiroCLEC™-PC)	–	82	4
	H_2O (50 μl)	–	0

[a]Reaction conditions: lactulose (0.12 mmol), ethyl butanoate (3.8 mmol), enzyme (10 mg), *t*-butyl alcohol 4 ml, 40°C, 210 h unless noted otherwise.
[b]Enzyme immobilised on Accurel EP100.
[c]If no activity without water is stated, the enzyme was inactive in anhydrous medium.
[d]Conversion after 170 h.
[e]Conversion after 240 h.
[f]20 μl saturated $Ca(Ac)_2$ in *tert*-butyl alcohol.

equilibrium to the right. The progress of the reaction was monitored by GC, which also showed that complex mixtures are formed. The conversions obtained with a number of enzymes are compiled in Table 1. Some enzymes, *C. rugosa* lipase (ChiroCLEC™-CR), *B. licheniformis* subtilisin (both immobilised on EP 100 and ChiroCLEC™-BL) showed no activity at all in the transesterification of lactulose.

From these results, it becomes clear that only four enzymes acylate lactulose at a useful rate. Within this group, the *Humicola* lipases require a small amount of water for activity, which is a disadvantage because the presence of water excludes the use of zeolite for adsorbing the liberated ethanol. Besides, water will cause the formation of butyric acid by hydrolysis of the ethyl butanoate, leading to deactivation of the enzyme and cleavage of the glycosidic bond of lactulose. Deactivation by butyric acid may be the reason why the relatively high initial rate of the *Humicola*, *P. alcaligenes* and *P. cepacia* lipases is not reflected in the conversion after 170–240 h. The two enzyme preparations that performed reasonably well, SP435 and subtilisin A, were selected for further study.

3.2. Effect of temperature

Since the conversion of lactulose is very low at 40°C, reactions have been carried out at reflux temperature in order to increase the solubility of lactulose. As explained above, SP435 and subtilisin A were used in these experiments (Table 2).

Table 2
Acylation of lactulose by ethyl butanoate at reflux temperature[a]

Enzyme source	Initial rate (pmol min^{-1} mg^{-1})	Conversion (%)
C. antarctica B (SP 435)	970	99
Subtilisin A	24	1[b]

[a]Reaction conditions: lactulose (0.58 mmol), ethyl butanoate (28 mmol), enzyme (100 mg), *t*-butyl alcohol 30 ml, mol sieve CaA (2 g), 144 h unless noted otherwise.
[b]Conversion after 240 h.

Table 3
Influence of solvent on the acylation of lactulose[a]

Solvent	Initial rate (pmol min^{-1} mg^{-1})	Conversion (%)
tert-Butyl alcohol	40	13
Dimethoxyethane	240	35
tert-Butyl alcohol with pinacol (5 wt.%)	510	16
Dimethoxyethane with pinacol (5 wt.%)	510	16

[a]Reaction conditions: lactulose (0.58 mmol), ethyl butanoate (28 mmol), enzyme (100 mg), *t*-butyl alcohol 30 ml, mol sieve CaA (2 g), 40°C, 144 h.

Reflux temperature improved the initial rate of SP435 by a factor of 25, and the reaction slowly proceeded towards complete conversion. Subtilisin apparently was deactivated at the reaction temperature, because the initial rate and the conversion were very low. These results reconfirm the extraordinary properties of *C. antarctica* lipase [11,13–16].

3.3. Influence of the solvent

Up to now, *tert*-butyl alcohol is the preferred solvent for esterification of carbohydrates [10], because it is slightly polar, inert and non-toxic. However, *tert*-butyl alcohol contains a hydroxyl group that can inhibit the active site of lipases [17,18]. We reasoned that a polar non-hydroxylic solvent, such as 1,2-dimethoxyethane, might give better results. At 40°C, the initial rate was enhanced by a factor 5 compared with *tert*-butyl alcohol and the conversion improved considerably (Table 3).

The inhibitory effect of alcohols was checked by performing reactions in the presence of a few % of pinacol. Although the initial rates went up (see also Fig. 2), the reactions soon slowed down and the conversion curves were similar to those obtained in *tert*-butyl alcohol. From these results, we conclude that tertiary alcohols inhibit *C. antarctica* lipase.

The beneficial effect of dimethoxyethane on the reaction rate enticed us to perform a reflux experiment. The results are presented in Fig. 3.

At 40°C, as well as reflux temperature, the initial reaction rate in dimethoxyethane was increased by a factor 5 compared with the reaction rate in *tert*-butyl alcohol. After 24 h in refluxing dimethoxyethane complete conversion of lactulose was obtained. During the reaction, a

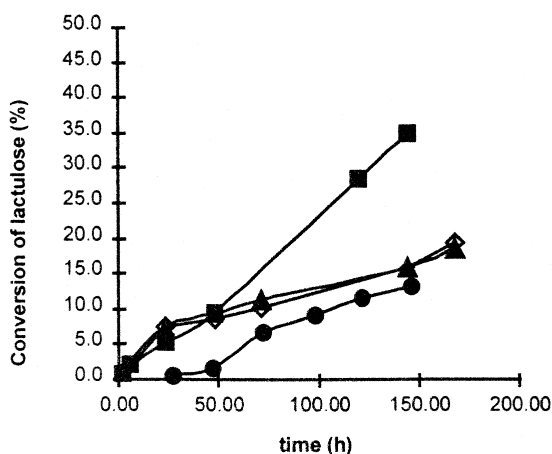


Fig. 2. Influence of solvent on the conversion of lactulose at 40°C, ●: *tert*-butyl alcohol; ■: 1,2-dimethoxyethane; ▲: *tert*-butyl alcohol with 5 wt.% pinacol; ◇: 1,2 dimethoxyethane with 5 wt.% pinacol.

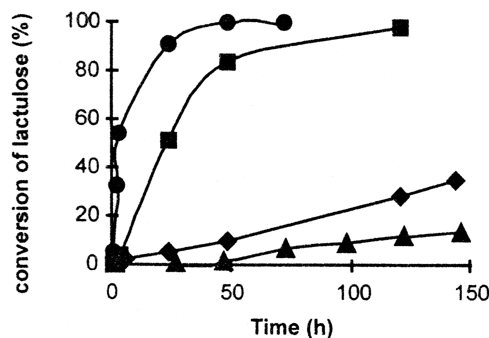
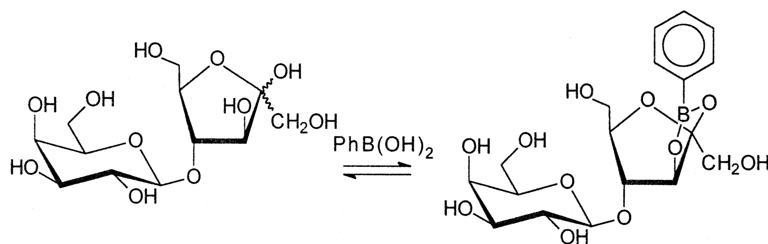


Fig. 3. Influence of solvent and temperature on the reaction rate, ▲: *tert*-butyl alcohol at 40°C; ◆: 1,2- dimethoxyethane at 40°C ■: *tert*-butyl alcohol at reflux temperature; ●: 1,2 dimethoxyethane at reflux temperature.



Scheme 1. Formation of the phenyl boronic acid ester of lactulose.

complex mixture was formed, in which tetra-esters predominated according to GC-MS, indicating that the lipase starts esterification of the secondary alcohol groups. It would seem likely that secondary alcohol groups in the galactose moiety are acylated in the tetraester, since esterification of secondary hydroxyl groups has also been observed in the acylation of methyl-β-D-galactopyranoside, but not of fructosides [18].

3.4. Influence of phenylboronic acid as adjuvant

The solubility of carbohydrates in organic media can also be increased by their conversion into the labile phenylboronic acid esters [19] (see Scheme 1). Although there are many organoboronic acids capable of forming reversible lipophilic complexes with sugars [21], phenylboronic acid was chosen because it is quite hydrophobic.

Van den Berg et al. [20] described the formation of boric acid esters of lactulose in water. The 2,3-di-O-β-furanose ester was exclusively formed at moderate pH; but a tridentate 2,3,6-ester of the β-furanose configuration was present at high pH. If this selectivity is in effect with phenylboronic acid esters, this adjuvant would be very useful to increase the selectivity. ^{11}B NMR proved only one phenylboronic ester type was in solution. From ^{13}C NMR, it becomes clear that only one ester was formed. From the chemical shifts in this spectrum (112 ppm for the anomeric carbon and 84 ppm for the C3, see also van den Berg et al. [20] for assignment of the chemical shifts) it can be concluded that the 2,3-di-O-β-furanose ester of phenylboronic acid predominated in water, but data on the formation of phenylboronic esters in *tert*-butyl alcohol could not be obtained, since the solubility of the lactulose and the phenylboronic acid esters of the lactulose was too low for NMR measurements.

To test the effect of phenylboronic acid on the reaction rate and conversion, experiments with different amounts were carried out. The initial rate and the conversion increased in almost linear fashion with the amount of phenylboronic acid (Table 4), but no effect on the selectivity towards the formation of mono- or diesters could be established, as judged by GC.

Table 4
Effect of phenyl boronic acid on the transesterification of lactulose with ethyl butanoate[b]

$PhB(OH)_2$ (molar equivalents)	Initial rate (pmol min^{-1} mg^{-1})	Conversion (%)
0	38	3
0.15	82	5
0.30	210	8
0.52	410	17
1.07	830	27

[b]Reaction conditions: lactulose (0.15 mmol), phenyl boronic acid as indicated, ethyl butanoate (3.9 mmol), lipase SP 435 (10 mg) *t*-butyl alcohol (4 ml), 40°C, 144 h.

4. Conclusion

We have shown that *C. antarctica* lipase is unique in catalysing the acylation of lactulose in refluxing *tert*-butyl alcohol. A mixture of monoesters and higher esters was obtained. A more rapid conversion could be obtained in refluxing dimethoxyethane. The acylation of lactulose could also be accelerated by its transformation into the labile lipophylic boronic acid esters, but the selectivity did not improve.

Acknowledgements

The authors wish to thank Novo Nordisk (Bagvaerd, Denmark) for generous donations of enzymes. A gift of enzymes by Gist-brocades (Delft, The Netherlands) is gratefully acknowledged. Samples of ChiroCLEC™ were kindly donated by Altus Biologics (Cambridge, MA, USA). Thanks are due to Mrs. A. Knol for recording the mass spectra. Financial support by the Netherlands Society for Technological Research SON-STW is gratefully acknowledged.

References

[1] H. Kohya, F. Ishii, S. Takano, T. Ebina, N. Ishida, Jpn. J. Cancer Res. 77 (1986) 602.
[2] E. Guillemard, M. Geniteau-Legendre, M. Mabboux, I. Poilane, R. Kergot, G. Lemaire, J.F. Petit, C. Labarre, A.M. Quero, Antiviral Res. 22 (1993) 201.
[3] W.F. Adams, G. Schuster, in: G. Schuster (Ed.), Emulgatoren für Lebensmittel, Springer, Berlin, 1985, pp. 151, 233, 406.
[4] M.C. de Zoete, F. van Rantwijk, R.A. Sheldon, Catal. Today 22 (1994) 563.
[5] H. Seino, T. Uchibori, T. Nishitani, S. Inamasu, J. Am. Oil Chem. Soc. 61 (1984) 1761.
[6] A.M. Klibanov, CHEMTECH 16 (1986) 354.
[7] S. Riva, J. Chopineau, A.P.G. Kieboom, A.M. Klibanov, J. Am. Chem. Soc. 110 (1988) 584.
[8] G. Carrea, S. Riva, F. Secundo, J. Chem. Soc. Perkin Trans. 1 (1989) 1057.
[9] N. Khaled, D. Montet, M. Farines, M. Pina, J. Graille, Oléagineux 47 (1992) 181.
[10] M. Woudenberg-van Oosterom, F. van Rantwijk, R.A. Sheldon, Biotechnol. Bioeng. 49 (1996) 328.
[11] S. Pedersen, P. Eigtved, Novo Nordisk, PCT Int. Appl. WO 90/15868.
[12] M.C. de Zoete, A.C. Kock-van Dalen, F. van Rantwijk, R.A. Sheldon, J. Mol. Catal. B Enzymatic 2 (1996) 19.
[13] F. Björkling, S.E. Godtfredsen, O. Kirk, J. Chem. Soc., Chem. Commun. (1990) 1301.
[14] F. Björkling, H. Frykman, S.E. Godtfredsen, O. Kirk, Tetrahedron 22 (1992) 4587.
[15] A.T.J.W. de Goede, W. Benckhuijsen, F. van Rantwijk, L. Maat, H. van Bekkum, Recl. Trav. Chim. Pays-Bas 112 (1993) 567.
[16] M.C. de Zoete, A.C. Kock-van Dalen, F. van Rantwijk, R.A. Sheldon, J. Chem. Soc., Chem. Commun. (1993) 1831.
[17] W. Chulalaksananukul, J.-S. Condoret, D. Combes, Enzyme Microb. Technol. (1992) 293.
[18] A.T.J.W. de Goede, M. van Oosterom, M.P.J. van Deurzen, R.A. Sheldon, H. van Bekkum, F. van Rantwijk, Biocatalysis 9 (1994) 145.
[19] I. Ikeda, A.M. Klibanov, Biotechnol. Bioeng. 42 (1993) 788.
[20] R. van den Berg, J.A. Peters, H. van Bekkum, Carbohydr. Res. 253 (1994) 1.
[21] R.J. Ferrier, Adv. Carbohydr. Chem. Biochem. 35 (1978) 31.