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Lipase-catalysed formation of macrocycles by ring-opening polymerisation of ϵ-caprolactone

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Studies were undertaken to gain mechanistic information on lactone ring-opening polymerisation reactions using *Candida antarctica* lipase B (Novozym 435) as the catalyst and ϵ-caprolactone as the monomer. Polymerisations were performed in organic solvents as well as without solvent at 60°C. *Candida antarctica* lipase B catalysed concurrently with the intermolecular ring-opening polymerisation, and also the formation of macrocycles by an intramolecular condensation reaction. *Candida antarctica* lipase B had the highest initial rate of consumption of ϵ-caprolactone (1.2 μmol mg^{-1} min^{-1}) in the bulk polymerisation, without solvent. Under these conditions, the highest average M_w, 4701 D, of poly(ϵ-caprolactone) was obtained. There were small amounts of cyclic oligomers present. When comparing the polymerisations performed in dioxane, acetonitrile and THF after 24 h reaction time with the bulk polymerisation, the average M_w of poly(ϵ-caprolactone) [2984, 1297, 1862 D, respectively] and the initial rates of monomer conversion of the enzyme (0.1, 0.05, 0.013 μmol mg^{-1} min^{-1}, respectively) were lower, however, the formation of cyclic oligomers was high. In dioxane, macrocycles of up to 2623 D corresponding to 23 monomer units were formed, and in acetonitrile there were mostly cyclic oligomers present.

(Keywords: enzymatic ring-opening polymerisation; ϵ-caprolactone; macrocycles)

INTRODUCTION

Lipase-catalysed ring-opening polymerisation of lactones has received increased attention over the past few years. It is a special type of transesterification, since no leaving group is released as a separate molecule. The reaction has been investigated by Uyama *et al.*[1,2], Maconald *et al.*[3] and Henderson *et al.*[4] who reported the polymerisations of ϵ-caprolactone (ϵ-CL) to the corresponding polyester with molecular weights of up to 7700 D. Knani *et al.*[5] studied the ring-opening polymerisation of ϵ-CL catalysed by porcine pancreatic lipase initiated by methanol. To achieve a complete conversion of the monomer, the reaction had to be carried out for 26 days at 40°C. The products were di-lactone, poly(ϵ-caprolactone) [PCL] and small amounts of larger cyclic oligomers. Uyama *et al.*[6] also polymerised macrolides to polyesters, and Bisht *et al.* investigated the bulk polymerisation of ω-pentadecalactone to give a M_w of 62 000 D[7]. Lipase-catalysed polymerisation of chiral lactones has also been reported[8,9]. Our group has previously synthesised methyl 6-O-poly(ϵ-caprolactone)-β-D-glucopyranoside by combining stereoselective acylation of methyl β-D-glucopyranoside with ring-opening polymerisation of ϵ-CL catalysed by *Candida antarctica* lipase B[10]. A bi-product, obtained when the synthesis was performed in acetonitrile, was di-lactone. There were also diminutive amounts of macrocycles formed (10-mer to 11-mer). In this paper, we have investigated the effects of ring-opening polymerisation of ϵ-CL in different organic solvents, using *C. antarctica* lipase B as the catalyst, for the formation of macrocycles and PCL. The products were characterised by matrix-assisted laser desorption and ionisation time of flight mass spectroscopy (MALDI-TOF MS), and n.m.r.. Especially soft ionisation mass spectroscopy techniques are suitable for characterising mixtures of linear chains and oligomeric cycles of polyesters, e.g. PCL[11,12]. Laser desorption mass spectroscopy enables structural and size characterisation of intact low molecular polymers with high accuracy[13].

EXPERIMENTAL

Materials

Candida antarctica lipase B, Novozym 435, (7000 U g^{-1}), an immobilised enzyme, was a gift from Novo Nordisk A/S. ϵ-CL was obtained from Aldrich Chemical. The ϵ-CL was dried by activated molecular sieves. Methyl-8 reagent was obtained from Pierce. All solvents used were of analytical grade, and prior to use, dried by shaking with molecular sieves.

Lipase-catalysed polymerisation

Polymerisations in organic solvents. A mixture (1 ml) of ϵ-CL (0.44 M) in THF, dioxane or acetonitrile was added to capped vials, each containing lipase (10 mg), which had previously been dried in a desiccator over P_2O_5. The vials were shaken at 120 rpm for different periods of time at 60°C. Samples were withdrawn from the reaction mixture and immediately analysed

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Table 1 Ring-opening polymerisation of ϵ-caprolactone in various organic solvents and without solvent at 60°C, catalysed by *C. antarctica* lipase B (Calb)

Solvent	Time (h)	Lipase	ϵ-CL[a] (%)	Products				
				Di-lactone[a] (%)	Cyclic[b] (%)	PCL[b] (%)	M_w, cyclic[c] (D)	M_w, PCL[c] (D)
Acetonitrile	48	Blank	0	0	0	0	0	0
Acetonitrile	24	Calb	76	17	53	30	808	1297
Acetonitrile	48	Calb	92	20	40	40	938	1907
THF	48	Blank	0	0	0	0	0	0
THF	24	Calb	39	36	28	36	948	1862
THF	48	Calb	84	31	47	22	940	1932
Dioxane	72	Blank	0	0	0	0	0	0
Dioxane	24	Calb	98	36	34	30	1255	2984
Dioxane	47	Calb	99	36	35	29	1080	2870
Isooctane	24	Calb	97	3		97[d]	1034	3957
Bulk	24	Calb	98	2		98[d]	1336	4701

[a]Consumed % ϵ-CL and % di-lactone of the total products were measured by GC.
[b]% Cyclic oligomers and % PCL of the total products were estimated using the ratios of Σa_i cyclic/Σa_i PCL, a_i = peak area, for the different MALDI-spectra and extrapolating to calibrated weight ratios.
[c]The average M_w of cyclic oligomers and PCL was determined by MALDI-TOF MS.
[d]Only the % polymers is shown, since in these cases the % cyclic oligomers could not be estimated.

by GC (Hewlett Packard 5890 Chromatograph, equipped with a 25 m × 0.32 mm CP Sil-8 CB column). The consumption of ϵ-CL and the production of di-lactone were analysed using hexadecane as the internal standard. Samples (40 μl) were withdrawn for MALDI-TOF MS analysis.

An experiment was also performed in isooctane. ϵ-CL (0.44 mmol) and isooctane (0.95 ml) were added into a dry vial containing lipase (10 mg), and the vial was incubated for 24 h (120 rpm, 60°C). Only a small amount of ϵ-CL, cyclic products and linear products were soluble in this system. Excess dry dichloromethane was therefore added to dissolve the reaction components, prior to GC and MALDI-TOF MS analyses.

Bulk polymerisation. Dried ϵ-CL (4.4 mmol) was added to dried vials, each containing *C. antarctica* lipase B (10 mg). The polymerisation was performed at 60°C (120 rpm). The contents of the incubated vials were, at different periods of time, immediately dissolved in dry dichloromethane and the lipase filtered off. The product mixture was directly analysed either by GC or MALDI-TOF MS.

PCL as the substrate. A fraction of PCL (4 mg), with an average M_w of 1362 D and a polydispersity of 1.2, which had been dried in a desiccator over P_2O_5, was dissolved in 1 ml of acetonitrile. The mixture was added to a dried vial containing lipase (3 mg). The vial was incubated at 60°C and 120 rpm. Samples (5 μl) were withdrawn after different periods of time and the M_w of the products determined by MALDI-TOF MS.

Characterisation of products

The product mixture from a dioxane polymerisation was fractionated on a silica gel column (ethyl acetate–methanol–water 100:10:1). Fractions were screened by MALDI-TOF MS.

Methylation analysis. To distinguish the cyclic products from the non-cyclic ones, methylation with Methyl-8 reagent (DMF-dimethylacetal) according to Thenot *et al.*[14] was performed and analysed by MALDI-TOF MS.

Table 2 The initial rates of ϵ-CL consumption and di-lactone production for *C. antarctica* lipase B in organic solvents and without solvent

Solvent	$V_{\epsilon\text{-CL}}$ (nmol mg^{-1} 1 min^{-1})	$V_{\text{di-lactone}}$ (nmol mg^{-1} min^{-1})
Dioxane	100	35
Acetonitrile	50	11
THF	13	4
no solvent	1200	not detected

The initial rates were calculated using the linear parts of curves for ϵ-CL consumtion and di-lactone production under different conditions (e. g. Fig. 1b) and dividing with the total weight of the Novozym 435 (10 mg) preparation.

n.m.r. analysis. All analyses were performed on a Brüker AM 400 spectrometer at ambient temperature using a $^1H/^{13}C$ dual probe head at a magnetic field strength of 9.4 T (400 MHz ^1H resonance frequency).

MALDI-TOF MS analysis. Samples (40 μl) from the different reactions were withdrawn and the solvent evaporated. The same volume of ethyl acetate was added and 5 μl was mixed with an equal volume of matrix [gentisic acid (DHB) dissolved in a 1:1 mixture of methanol and water]. An aliquot (0.5 μl) was applied to the sample probe, the solvent evaporated by vacuum, and the probe inserted into the spectrometer (Hewlett Packard G20205 A LD-TOF system). The average M_w of the cyclic oligomers and PCL as well as the polydispersity were determined. The ratio of cyclic products to linear products was estimated by using the ratios of Σa_i cyclic/Σa_i straight (a_i = peak area) for the different MALDI-TOF MS spectra and extrapolating to calibrated weight ratios.

RESULTS AND DISCUSSION

Lipase-catalysed ring-opening polymerisations

In *Table 1*, the results from the *C. antarctica* lipase B-catalysed ring-opening polymerisations of ϵ-CL performed in organic solvents as well as in bulk are presented. In the bulk polymerisations, the highest average M_w (4701 D) of PCL and the fastest reaction rate of ϵ-CL consumption (*Table 2*) were obtained. There were only small amounts of di-lactone and macrocycles present,

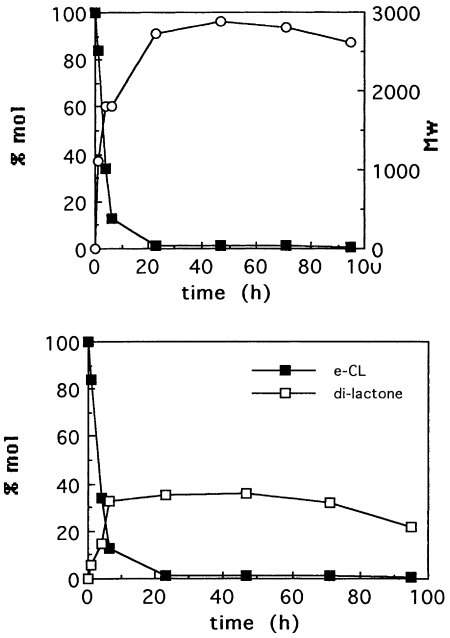


Figure 1 *Candida antarctica* lipase B (10 mg) catalysed polymerisation of ϵ-CL (0.44 M) at 60°C in dioxane. (a) Average M_w of PCL and ϵ-CL consumption as a function of time determined by MALDI-TOF MS and GC, respectively. (b) Time dependence of ϵ-CL consumption and ϵ-CL incorporation into di-lactone determined by GC

which is in correspondence to previously performed polymerisations with *C. antarctica* lipase B[10]. In the case of the polymerisation in isooctane, only a small amount of ϵ-CL was soluble. The lipase was present in the ϵ-CL phase. Consequently, the system was similar to the bulk polymerisation. The polymerisation reactions in dioxane, acetonitrile and THF resulted in lower average molecular weights of PCL, although substantial amounts of di-lactone and macrocycles compared to the bulk polymerisation were formed. The results of MALDI-TOF MS for the cyclic products larger than di-lactone in dioxane, acetonitrile and THF after 24 h were the following. In dioxane, the MALDI-TOF MS registered detectable peaks of cyclic products from 343 D (tri-mer) to 2623 D (23-mer), with a monomer repeat mass of 114 D and an average M_w of 1255 D. In acetonitrile, peaks were registered from 343 D (tri-mer) to 2167 D (19-mer) and with an average M_w of 830 D. In THF, peaks were registered from 343 D (tri-mer) to 2396 D (21-mer) with an average M_w of 948 D. The polydispersity was between 1.2 and 1.3 in all organic solvents. The higher percentage of cyclic products (e.g. di-lactone, *Table 1*) in the organic solvent polymerisation could be a result of the fact that the opportunity for intermolecular reaction is less favourable at higher dilution[15]. The order of magnitude of the initial rates (*Table 2*) of ϵ-CL consumption and di-lactone production for *C. antarctica* lipase B in the three solvents were: dioxane > acetonitrile > THF.

Figure 1a and *b* represent the consumption of ϵ-CL, the formation of di-lactone and the average M_w of PCL as a function of time in dioxane. Both the average M_w of PCL and the amount of di-lactone increased until almost all ϵ-CL was consumed. Then, as time proceeded, competing reactions, e.g. chain cleavage, and the use of di-lactone as a substrate became increasingly important. The result was a slight decrease in average M_w.

Structural characterisation

MALDI-TOF MS. Positive ion MALDI-TOF MS spectra were obtained for all products larger than tri-mer. *Figure 2* shows a spectrum of the products from a 24 h polymerisation in dioxane. It typifies the asymmetric and dispersed oligomer distributions of the polyester products obtained under the different reaction conditions. The

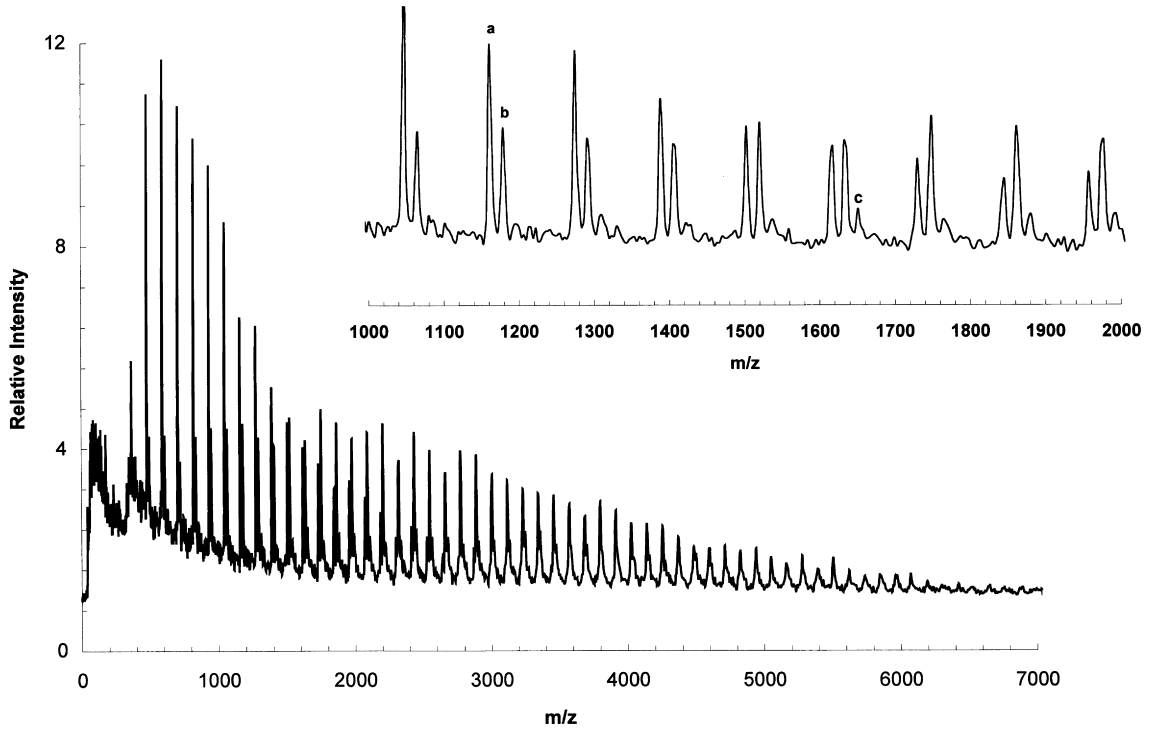


Figure 2 MALDI-TOF MS spectrum of the products from a 24 h polymerisation in dioxane with a DHB matrix. The inset shows an expanded view of the 1000–2000 D region of the spectrum. (a) Na^+ cationised cyclic oligomer, (b) Na^+ cationised straight chain oligomer, (c) K^+ straight chain oligomer

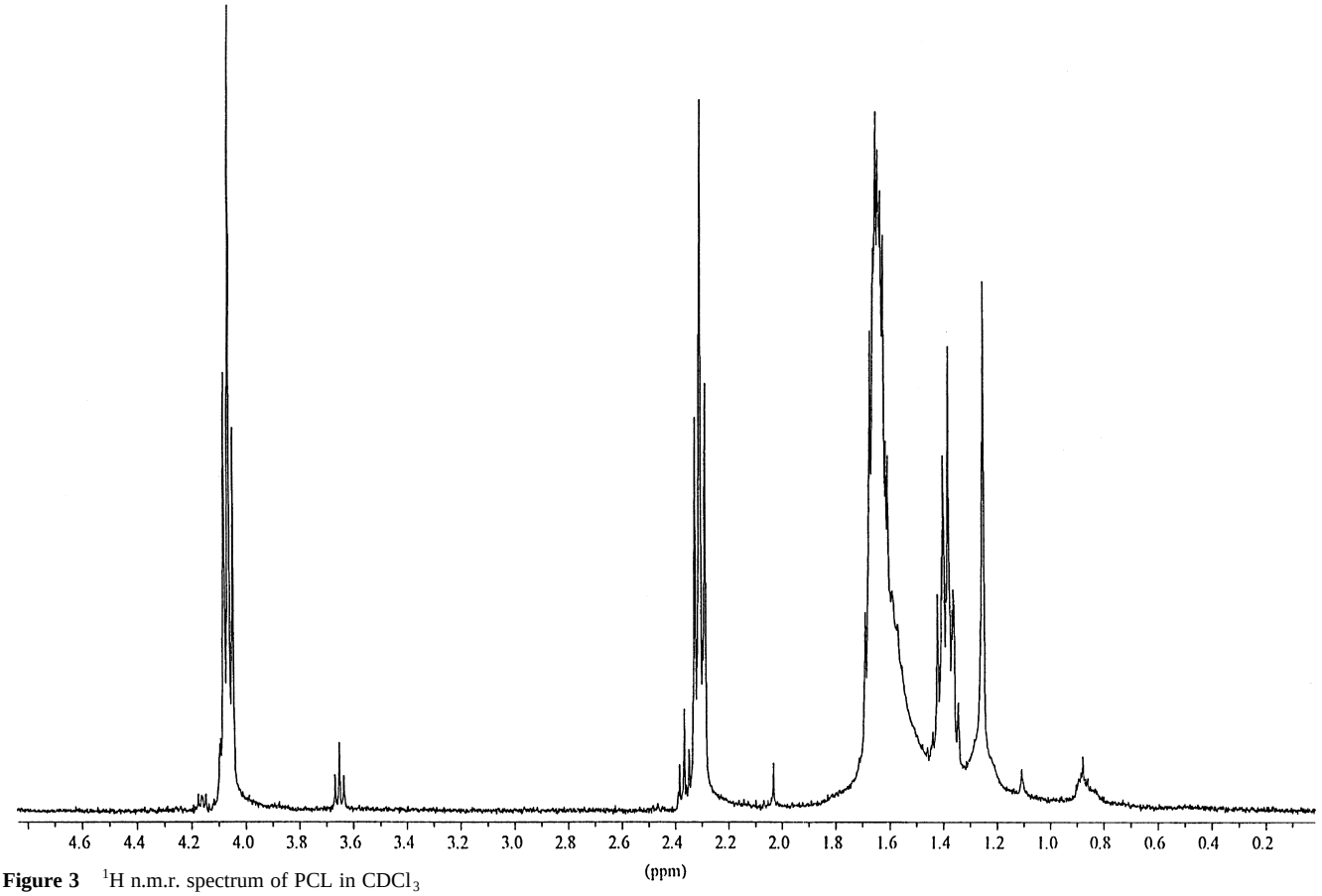


Figure 3 ^{1}H n.m.r. spectrum of PCL in $CDCl_3$

mass range below 300 D was dominated by peaks resulting from matrix, matrix-fragments, clusters and metal ions. Thus, a mass filter up to 300 D was put on the detector. In the spectrum, there are two different main product distributions, both with a repeat unit of 114 D. The first distribution with the highest intensity peaks results from Na^+-cationised cyclic oligomers (a) and extends over the mass range of 300–2700 D. The second distribution with peaks results from Na^+-cationised straight chain oligomers and extends over the mass range of 300–7000 D (b). K^+-cationised oligomer peaks show a lower intensity (c).

n.m.r.. ^{1}H n.m.r. analyses were performed on linear oligomers, cyclic oligomers and product mixtures. The solvent was $CDCl_3$. The ^{1}H n.m.r. spectrum of a pure fraction of PCL with an average M_n of 1512 D (determined by MALDI-TOF MS) is presented in *Figure 3*. ^{1}H n.m.r.: δ (ppm): 4.05 (t, CH_2O), 3.65 (t, CH_2OH, end group), 2.30 (t, CH_2CO), 1.6–1.7 (m, $2 \times CH_2$), 1.37 (m, CH_2). By comparing the intensities of the methylene protons (CH_2OH, end group) at 3.65 ppm with respect to the methylene protons (CH_2O) at 4.05 ppm, the degree of polymerisation (DP) could be calculated. DP corresponds to M_n, hence the n.m.r. data could be compared to MALDI-TOF MS values. The DP was 17 with n.m.r. and 13 by MALDI-TOF MS.

Figure 4 is the ^{1}H n.m.r. spectrum of a fraction of cyclic oligomers. A MALDI-TOF MS analysis of the same fraction detected peaks from 343 D (tri-mer) to 1253 D (11-mer) with an average M_n of 872 D. In the ^{1}H n.m.r. spectrum (*Figure 4*), the triplets at 4.14 (CH_2O) and 2.35 (CH_2CO) ppm correspond to the methylene groups of di-lactone[5]. The cluster of triplets at 4.05 (CH_2O) and 2.30 (CH_2CO) ppm correspond to the methylene groups of the cycles. The cluster is probably due to the fact that it is a disperse mixture of oligomeric cycles. There is no end group triplet at 3.65 ppm, confirming the absence of linear oligomers.

Methylation analysis. To confirm that cyclic products were obtained in the synthesis, crude mixtures of cyclic and linear oligomers, isolated cyclic oligomers and isolated linear oligomers were reacted with excess of Methyl-8 reagent (DMF-dimethylacetal) and characterised by MALDI-TOF MS. The MALDI-TOF MS spectrum of the methylated product mixture showed no change in the peaks corresponding to the cyclic oligomers, while the linear oligomer peaks disappeared and a new series of peaks appeared at masses that were 28 D higher. This shows that the linear oligomers had been methylated at both end groups, and was confirmed by ^{1}H n.m.r. ($CDCl_3$) analysis of the methylated linear oligomers [δ (ppm): 3.22 (3 H, s, $ROCH_3$), 3.65 (2 H, t, CH_2O), 3.67 (3 H, s, $COOCH_3$]. When a fraction of only cyclic oligomers, according to MALDI-TOF MS, was reacted, no difference in the masses of the peaks could be detected after the reaction. Finally, a fraction of linear oligomers was methylated and only new peaks at masses that were 28 D higher than the masses of the linear oligomers appeared in the spectrum.

PCL as substrate

Figure 5 shows the different MALDI-TOF MS spectra acquired at different times of an experiment, starting with linear oligomers of PCL as the starting material in

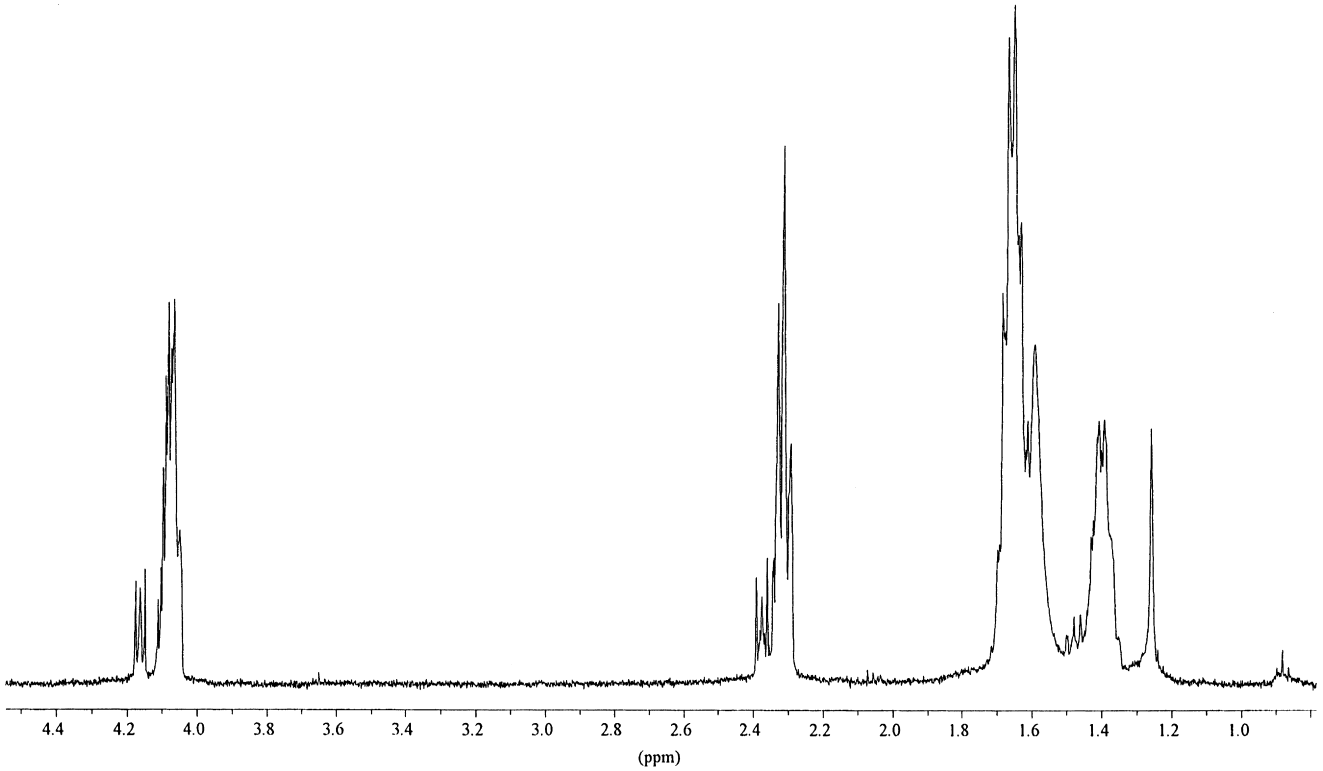


Figure 4 ^{1}H n.m.r. spectrum of a disperse mixture of macrocycles in $CDCl_3$

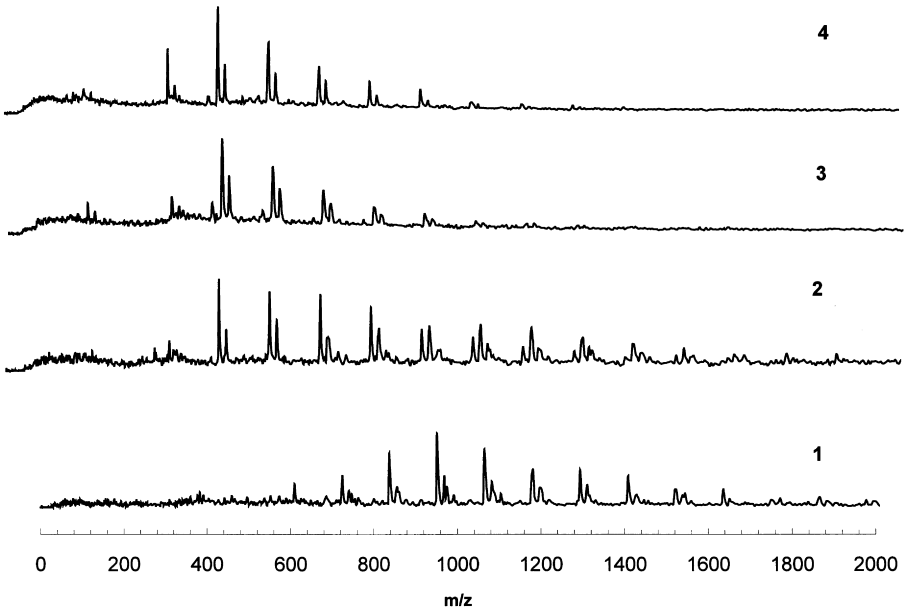
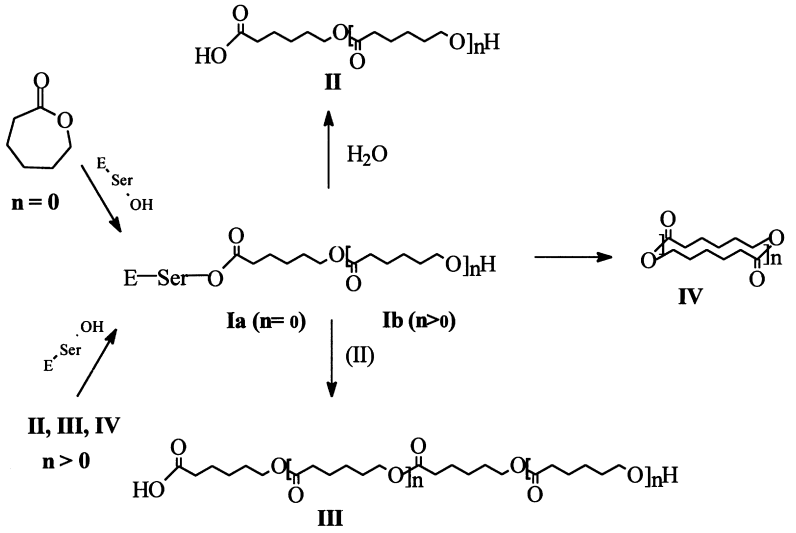


Figure 5 MALDI-TOF MS spectra of the products of a reaction in acetonitrile catalysed by *C. antarctica* lipase B, starting with linear PCL, acquired at different times. Spectrum 1 = 0 h, Spectrum 2 = 1 h, Spectrum 3 = 6 h and Spectrum 4 = 24 h

acetonitrile, with *C. antarctica* lipase B as the catalyst. Spectrum 1 was acquired at 0 h. There are only linear oligomers present with an average M_w of 1362 D. Spectrum 2 was acquired at 1 h, and there are new peaks of Na^+- and K^+-cationised cyclic oligomers appearing with the highest intensities between a mass range of 300 and 800 D. Spectrum 3 was acquired after 6 h reaction time. There are only cyclic oligomers present at this point of the reaction. Spectrum 4 was acquired at 24 h reaction time. There are only cyclic oligomers present with the same distribution as in spectrum 3. It should be mentioned that certain spectra, acquired after 24 h of the ring-opening polymerisations of ϵ-CL in acetonitrile, were identical to spectrum 4. The ^{1}H n.m.r. of these reaction mixtures showed no end-group triplet at 3.65 ppm. There were only oligomeric cycles present in these cases as well. As already stated by others, the water activity is important for the lipase-catalysed polymerisation[3]. It contributes to the DP of the oligomers as well as to the products that are formed. The water activity could be an explanation for the



Scheme 1 Structure **II**, with $n > 0$, corresponds to **III**, with a value of n one unit lower, e.g. **II** ($n = 1$) = **III** ($n = 0$)

observed variations of the ratios between cyclic oligomers and PCL in acetonitrile experiments.

Reaction mechanism

Scheme 1 shows the different types of reactions that compete during the polymerisation of ϵ-CL, catalysed by *C. antarctica* lipase B. In the initial step, the serine 105 of the lipase[16] will make a nucleophilic attack on ϵ-CL and the formation of an acyl complex **Ia** ($n = 0$) is achieved. The acyl complex **Ia** can then be deacylated by water to form **II** ($n = 0$)[10]. Compound **II** will then deacylate **Ia** to produce **III**. Compound **III** will be one monomer unit larger than **II**. Deacylation of **Ia** by **III** will lead to further propagation with one monomer unit added each time. The major chain propagation will follow this mechanism. The system is, however, more complex, since *C. antarctica* lipase B can form acyl complexes **Ib** ($n > 0$) with oligomer units. This is necessary for the formation of cyclic oligomers **IV**. At low concentrations of ϵ-CL in organic solvent polymerisation, the formation of **IV** was significant, since the chance of intramolecular deacylation increased.

CONCLUSION

Candida antarctica lipase B was efficient in catalysing the ring-opening polymerisation of ϵ-CL. The highest average M_w of PCL was obtained when the polymerisation was performed as a bulk polymerisation without solvent. When the polymerisation was performed in organic solvents, *C. antarctica* lipase B was able to catalyse the formation of cyclic oligomers of PCL in high yield. Macrocycles of PCL of up to 2623 D (23-mer) were synthesised in dioxane.

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