Kinetics and Mechanism of *Candida antarctica* Lipase B Catalyzed Solution Polymerization of ϵ-Caprolactone

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ABSTRACT: Studies of the kinetics and mechanism of *Candida antarctica* Lipase B (CALB) catalyzed ϵ-caprolactone (ϵ-CL) polymerizations in toluene were performed. The kinetic plot of ln $([M]_0/[M]_t)$ vs time was carried out to 96% ϵ-CL conversion and M_n 11 970. The plot is linear ($r^2 = 0.998$), indicating that termination did not occur and the propagation rate is first order with respect to monomer concentration. Changes in the water (e.g., initiator) concentration did not change the polymerization rate but did change the number of chains [R−OH]. Thus, the polymerization is zero order with respect to [R−OH] and initiator concentration. A plot of ln k_{app} vs ln [enzyme] gave 0.7 as the reaction order of the enzyme concentration. The apparent activation energy for Novozyme-435 catalyzed ϵ-CL polymerization in toluene is 2.88 kcal mol^{-1}. This is well below 10.3 kcal mol^{-1}, the activation energy for aluminum alkoxide catalyzed ϵ-CL polymerization in toluene. Upward deviation from linearity for M_n vs fractional ϵ-CL conversion and decreases in the number of chains was accentuated by low enzyme water contents and high monomer conversion. These results are consistent with a competition between ring-opening chain-end propagation and chain growth by steplike polycondensations. CALB was irreversibly inhibited by modification with paraoxon at the lipase active site (Ser_{105}). The modified enzyme was no longer active for the polymerization. This supports that the polymerizations studied herein occurred by catalysis at the active serine residue (Ser_{105}) and not by other chemical or nonspecific protein-mediated processes.

Introduction

Certain lipases have been found to have an extraordinary ability to catalyze the synthesis of polyesters by in-vitro reactions with nonnatural substrates.[1−4] Our laboratory and others have begun to explore the kinetics and mechanism(s) of lipase-catalyzed lactone ring-opening polymerization. For porcine pancreatic lipase (PPL) catalyzed ϵ-caprolactone (ϵ-CL) polymerization in heptane, plots of log $\{[M]_0/[M]_t\}$ vs time and M_n vs conversion to about 85% monomer conversion were constructed. The linearity of these two plots indicated termination and chain transfer did not occur. Therefore, the system provided "controlled" polymerizations where the molecular weight was a function of the monomer-to-initiator stoichiometry.[2] The polymerization of ϵ-CL catalyzed by physically immobilized Lipase B from *Candida antarctica* (Novozyme-435) in bulk at 70 °C was investigated by Deng et al.[3] He reported that increased lipase concentration results in more rapid monomer conversion but decreased product molecular weight. A linear relationship between ln $\{([M]_0 - [M]_i)/([M]_t - [M]_i)\}$ vs reaction time and M_n vs monomer conversion (until 80%) was found. A kinetic analysis and study of products at low conversion for the bulk polymerization of ω-pentadeclactone (PDL) at 50 °C using an immobilized lipase from a *pseudomonas sp.* (I-PS-30) was also reported.[4] Kobayashi and co-workers analyzed ϵ-CL and 12-dodecanolide (DDL) polymerizations using Michaelis−Menten kinetics. Their studies were performed in isopropyl ether, at 60 °C, catalyzed by the lipase from *Pseudomonas fluorescens*. They reported that enhanced polymerizability of lactones with larger ring size is mainly due to the larger reaction rate (V_{max}) and not to differences in binding affinity. Furthermore, they reported that the transformation of the lipase−lactone complex to the acyl−enzyme intermediate is the key step for the lipase-catalyzed lactone polymerization.[5]

Work was performed to determine whether the known active site of a poly(β-hydroxybutyrate) depolymerase was also the site that catalyzed lactone ring-opening polymerizations in organic media. To this end, Doi and co-workers prepared three site-specific mutants and the wild-type poly(β-hydroxybutyrate) depolymerase from *A. Faecalis* T1.[6] The relative activities of the wild-type and mutant enzymes were compared for β-butyrolactone ring-opening polymerization in organic media. They found that none of the mutant enzymes showed polymerization activity whereas the wild-type enzyme actively catalyzed the polymerization. Their results proved that the active site, and not one or more remote sites, is responsible for the catalysis of β-butyrolactone polymerization.

The following defines the objectives of this study relative to previous reports:

1. Lipases from various origins including porcine pancreatic lipase (PPL), Lipase PF from *Pseudomonas fluorescens*, Lipase SPF from *Pseudomonas sp.*, and Lipase B from *Candida antarctica* (CALB) have been used as model enzymes. At this stage of our understanding, each lipase must be considered dissimilar in the mechanism of lactone polymerization until proven otherwise. Furthermore, CALB immobilized on Lewatit (Novozyme-435) is particularly attractive for more intense research due to its extraordinary activity for lactone polymerizations in addition to its ability to perform these polymerizations with high regioselectivity during initiation.[1,7]

2. The existence of transesterification and polycondensation between diacids and diols has been reported by others and our laboratory.[1,8] Although discussed elsewhere,[9] the effect of the concurrent processes of

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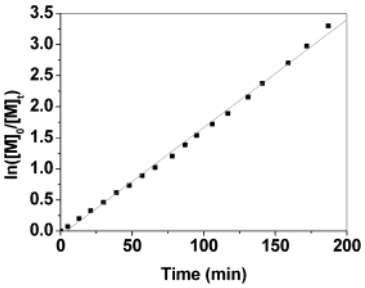


Figure 1. Semilogarithmic plot of Novozyme-435 catalyzed ϵ-caprolactone ring-opening polymerization at 60 °C, in toluene, with 1% (w/w) enzyme water content.

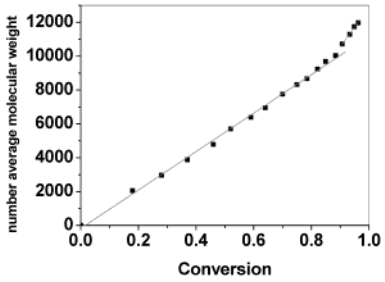


Figure 2. Dependence of molecular weight, M_n, upon monomer conversion of Novozyme-435 catalyzed caprolactone ring-opening polymerization at 60 °C, in toluene, with 1% enzyme water content.

lactone ring-opening and interchain condensations is in need of detailed studies.

3. Reaction temperature and water content are known to influence lactone conversion and product molecular weight.[10] However, the temperature dependence of the propagation kinetics has not yet been used to determine the activation energy. Furthermore, the influence of enzyme water content on the relative occurrence of chain growth by lactone ring-opening as opposed to interchain step condensation requires study.

4. Experiments thus far have not adequately addressed whether the lipase active site, and not one or more remote sites, is responsible for the catalysis of lactone ring-opening polymerizations.

Results and Discussion

1. Investigating the Factors Influencing Reaction Rate. Experiments were performed to determine the reaction order of monomer, initiator, and catalyst. Water was selected as the initiator since it is needed at low concentrations to stabilize the protein structure. Furthermore, even when water is replaced by other initiators, it is difficult to dispose of all the water molecules that can initiate ring-opening polymerizations.[10] Attempts to substitute water by other nucleophiles have in all cases resulted in products where some fraction, albeit low, of chains are initiated by water, giving a mixture of chain end groups.[2,11] The enzyme water content can strongly influence both the kinetics of the polymerization and product molecular weight. Since this can lead to difficulties in experimental analysis, we varied the water content of Novozyme-435 over the range from 0.6% to 2.0%, where it was shown that the enzyme activity remains unchanged.[10]

2. Derivation of an Experimental Rate of Propagation. The experiments were performed using ϵ-CL as the monomer and Novozyme-435 as the catalyst, in toluene-d_8, at 60 °C, with a monomer-to-catalyst ratio of 10:1 (10.6 mg of enzyme/mmol of CL; see Experimental Section). The monomer conversion, M_n, and the total number of polymer chains (N_p) at varying enzyme water content were monitored in situ by ^{1}H NMR.[10] To test for whether the polymerization occurs with or without termination reactions, an enzyme−water content of 1.0 wt % was selected, and plots of $\ln([M]_0/[M]_t)$ vs time and M_n vs fractional monomer conversion were constructed. The kinetic plot of $\ln([M]_0/[M]_t)$ vs time shown in Figure 1 was carried out to 180 min, corresponding to 96% ϵ-CL conversion and M_n 11 970. The linearity ($r^2 = 0.998$) of the plot in Figure 1 indicates that termination did not occur, and the propagation rate was first order with respect to monomer concentration. Also, the linearity infers that the polymerization rate is constant, which requires that the enzyme is not deactivated during the reaction. This indirect evidence of enzyme stability is supported by that, upon reuse, the enzyme activity appeared unchanged.[12] The plot in Figure 2 of M_n vs conversion was linear ($r^2 = 0.994$) up to ~88% conversion. However, above 88% conversion, the slope of the M_n vs conversion plots increased. The deviation from linearity at more than 88% monomer conversion is discussed below. Also, from Figure 2, one observes that the experimentally determined molecular weights were very close to the expected molecular weights based upon $([M]_0 - [M]_t)/[In]'$, where $[In]'$ is the additive amount of "free" and a fraction of "moderately bound" water.[10] Reference 10 gives a detailed description of the terminology "free" and "moderately bound" water. Briefly, the "free" and "moderately bound" water both can function as chain initiators. However, the "free" water is measured by the Karl Fisher titration method used whereas the "moderately bound" water is not detected by this method.

Previously we reported plots of $\log\{[M]_0/[M]_t\}$ vs time and M_n vs conversion (to 80%) for ϵ-CL polymerization catalyzed by PPL (166 mg of protein/mmol of CL) in heptane at 65 °C and catalyzed by Novozyme-435 (9.97 mg of protein/mmol of CL) in bulk at 70 °C.[2,3] Comparison between these studies shows that, by using the polymerization conditions herein, both a higher propagation rate and product molecular weight were attained. However, regardless of different propagation rates for these three systems that differ in the enzyme (Lipase B from *Candida antarctica* vs PPL) and reaction media (bulk vs toluene), plots of $\log\{[M]_0/[M]_t\}$ vs time and M_n vs conversion to 80% monomer conversion were all linear. Thus, even though the reactions were performed by using different lipases and reaction conditions, their mechanisms for lipase-catalyzed ring-opening polymerization still shares common features. Also, results discussed below show that, by not studying the relationship between M_n vs conversion beyond 80%, important information on the occurrence of interchain step condensation reactions may be missed.

The effect of the enzyme water content on the reaction kinetics was also studied. Polymerizations were performed at 0.6, 1.15, and 1.95% enzyme water contents and the corresponding plots of fractional ϵ-CL conversion vs reaction time and numbers of chains vs conversion are shown in parts a and b of Figure 3, respectively. Since water is the initiating species in the polymerizations,[10] Figure 3a proves that changes in the initiator concentration did not change the polymerization rate. However, Figure 3b shows that changes in the enzyme water content does change the number of chains [R−OH].

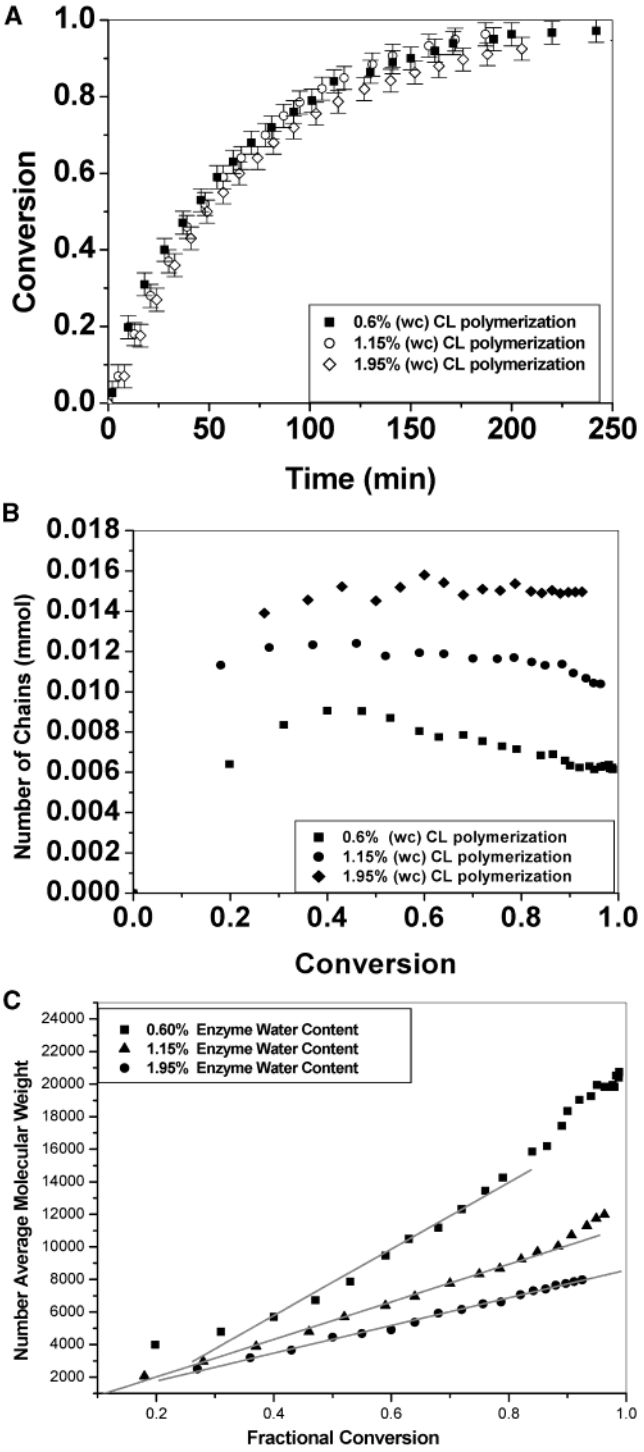


Figure 3. (A) Effect of the enzyme water content (given in wt %, see plot) on the time course of ϵ-caprolactone polymerization at 60 °C, in toluene, catalyzed by Novozyme-435. (B) Effect of the enzyme water content (given in wt %, see plot) on the total number of chains vs ϵ-caprolactone fractional conversion at 60 °C, in toluene, catalyzed by Novozyme-435. (C) Effect of the enzyme water content (given in wt %, see plot) on the plot of number-average molecular weight (M_n) vs ϵ-caprolactone fractional conversion at 60 °C, in toluene, catalyzed by Novozyme-435.

Therefore, the changes in the number of chains (N_p) did not affect the polymerization rate, and the polymerization is zero order with respect to [R−OH] and initiator concentration. These findings are consistent with the conclusions of our previous report where PPL was the catalyst for ϵ-CL polymerizations.

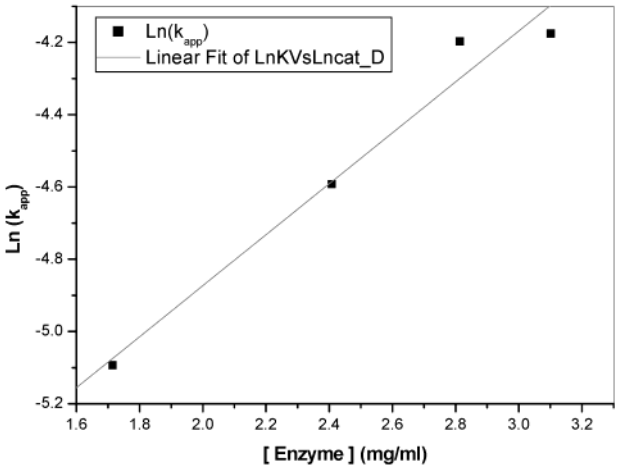


Figure 4. Plot of ln k_{app} vs ln(enzyme concentration) compiled from ϵ-caprolactone polymerizations at 60 °C, in toluene, catalyzed by Novozyme-435.

The effect of the enzyme concentration on the polymerization rate was studied. The enzyme concentration is defined as the *Candida antarctica* Lipase B weight divided by the total volume of ϵ-CL and toluene (mg/mL). To study the reaction order of the catalyst concentration, ln k_{app} vs ln [enzyme] was plotted (see Figure 4). Analysis of the data in Figure 4 by linear regression gave 0.70 for the slope. From these results, the experimental rate expression was found (see eq 1).

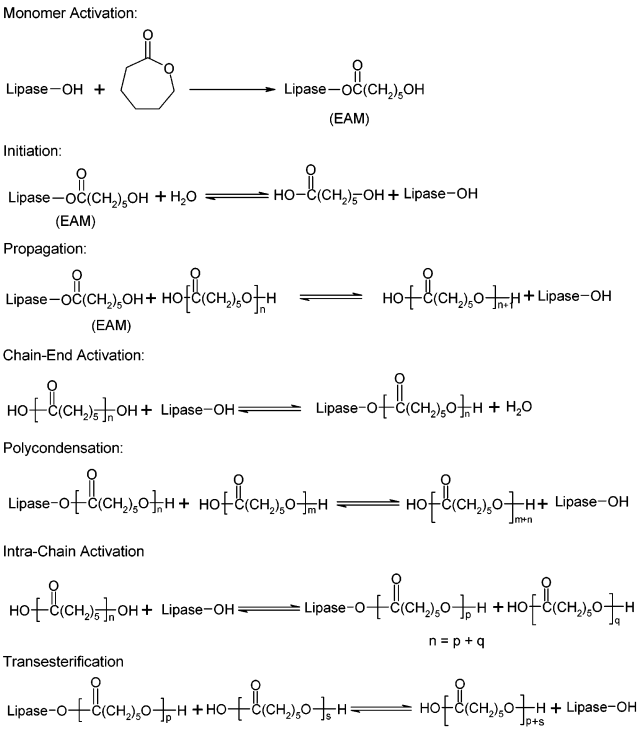
$$R_p = k_{app}[M] = k[\text{enzyme}]^{0.7}[M][R{-}OH]^0 = k[\text{enzyme}]^{0.7}[M][I']^0 \quad (1)$$

Our group previously reported the effect of Novozyme-435 concentration on the rate of bulk ϵ-CL polymerizations at 70 °C. Interestingly, when experimental data were imported and used to construct a plot of ln k_{app} vs ln [enzyme] (mg of Novozyme-435/mmol of ϵ-CL),[3] the apparent reaction order with respect to enzyme concentration was 1.4 (Supporting Information).[3] This prompted us to consider formulating an explanation for this observed difference in catalyst reaction order. Indeed, changes in reaction order as a function of the reaction medium have been discussed previously for lactone ring-opening polymerizations using chemical catalysts.[13] Although the possibility that diffusion limitations in the polymerization reaction caused the low value in the catalyst reaction order (0.7) was considered, the experimental results and discussion published elsewhere led us to reject this explanation.[10]

By in large, changes in the chemical catalyst reaction order for lactone polymerizations were interpreted on the basis of the extent of aggregation of the catalyst active species in the reaction medium. Although unlikely, it is possible that with changes in the reaction medium (e.g., bulk vs toluene solution) the physically immobilized lipase reorganizes at the resin−medium interface. Other explanations that appear more plausible to these authors are differences in matrix swelling and enzyme activity for the solvent-free and solution reactions. However, further work will be needed to determine the true explanation for the observed changes in the fractional reaction order of the enzymatic catalyst.

3. Ring-Opening Polymerization Mechanism and the Theoretical Rate of Propagation. In previous studies, a mechanism was proposed for lipase-catalyzed

Scheme 1



ϵ-CL ring-opening polymerization that involves monomer activation, chain initiation, propagation, and polycondensation.[9] As discussed below, the deviation from linearity of the number-average molecular weight vs fractional monomer conversion provided experimental support for events of polycondensation in addition to chainlike propagation reactions. Moreover, the occurrence of intrachain transesterification of poly(caprolactone) with hexanol/water catalyzed by Novozyme-435 was documented elsewhere by our group.[8] Therefore, in Scheme 1 is shown a revised mechanism that now takes into account processes of chain-end activation, intrachain activation, transesterification, and condensation. To explain that the propagation rate is independent of the number of chains or initiator concentration, Kobayashi and co-workers proposed that the monomer activation step is rate determining.[5] If it is assumed that monomer activation is rate determining, then the theoretical rate of propagation is as follows (eq 2).

$$-\frac{\mathrm{d[M]}}{\mathrm{d}t} = k[\text{enzyme}]^x[\mathrm{M}][\mathrm{I}']^0 = R_\mathrm{i} + R_\mathrm{p} \approx R_\mathrm{p} \quad (2)$$

Since eqs 1 and 2 are in agreement, this supports that monomer activation is indeed rate determining.

Previously, Henderson et al. and Matsumoto et al. proposed the following rate equations based on Michaelis−Menten kinetics:[2,14]

$$-\frac{\mathrm{d[M]}}{\mathrm{d}t} = k[\mathrm{M}][\mathrm{R{-}OH}] = k[\mathrm{M}][\mathrm{I}'] \quad (3)$$

This rate equation was not consistent with the experimental rate expression above since the reaction order with respect to both [R−OH] and [I′] is zero. However, if the explanation for zero reaction orders is that the concentration of propagation chain ends is above that where saturation of enzyme active sites occurs, then eq 3 may describe the propagation rate at lower reaction concentrations of [I′] and therefore [R−OH].

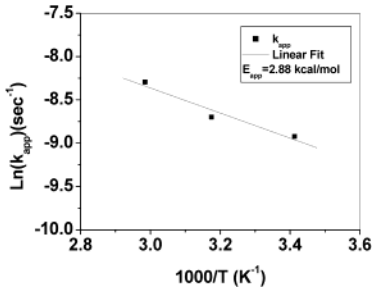


Figure 5. Arrhenius plot to determine the apparent activation energy for ϵ-caprolactone polymerizations in toluene, catalyzed by Novozyme-435.

4. Effect of Temperature and Enzyme Water Content. The temperature dependence of the polymerization rate was studied. The results were used to construct an Arrhenius plot (Figure 5). From the slope of the plot in Figure 5, the apparent activation energy for Novozyme-435 catalyzed ϵ-CL polymerization in toluene is 2.88 kcal mol^{-1}. Interestingly, this activation energy is well below that determined for aluminum alkoxide catalyzed ϵ-CL polymerization in toluene. For example, Dubois and co-workers found that $Et_2AlO(CH_2)_2Br$ catalyzed a "living" ϵ-CL polymerization in toluene with an apparent activation energy of 10.3 kcal mol^{-1} at 25 °C.[15] Since the lipase molecules are immobilized within a macroporous matrix, lipase molecules exist within microenvironments that may be chemically and physically nonequivalent. The extent that this variability in lipase environment causes differences in kinetics between lipase molecules that constitute the system studied is currently unknown. Thus, the activation energy measurements represent an average for lipase molecules that are in differing physical and chemical environments within the macroporous matrix.

The activity of lipases for the catalysis of step-condensation and transesterification reactions has been reported by us and others.[1,8] However, the few previous reports on the kinetics and mechanism of lipase-catalyzed lactone polymerization have not shown how these pathways affect the polymerization as viewed through characteristic plots such as M_n vs fractional conversion. Furthermore, the possibility exists that the relative occurrence of step-condensation reactions will be related to the enzyme water content.

Plots of the number of chains and M_n vs fractional ϵ-CL conversion for polymerizations conducted with different enzyme water contents are shown in parts B and C of Figure 3, respectively. Deviation from linearity of M_n vs fractional ϵ-CL conversion plots, for enzyme water contents of 0.60 and 1.15% and fractional conversions above about 80%, is viewed in Figure 3C. Values of M_n are above that expected from exclusively chain-type polymerizations. This is explained by steplike condensations that increase in prevalence as the concentration of ϵ-CL decreases. In accordance with this explanation, the number of chains decreased at similar water contents and fractional conversions as above. However, when the enzyme water content was 1.95%, there was no apparent upward deviation in the M_n vs fractional ϵ-CL conversion plot nor was there a decrease in the number of chains (Figure 3, C and B, respectively). Furthermore, comparison of the results at 0.60

and 1.15% enzyme water content shows that both the upward deviation in the M_n vs fractional ϵ-CL conversion and the decrease in the number of chains were more extreme at the lower enzyme water content (0.60%). These results are consistent with thermodynamic and kinetic arguments that favor condensation reactions at lower enzyme water content and a higher ratio of carboxyl/hydroxyl end groups relative to unreacted ϵ-CL. Conversely, at 1.95% enzyme water content, the tendency for condensation was so low that it was not manifested by inspection of the plots in Figure 3B,C.

Dong et al. used lipase PSL (from *Pseudomonas sp.*) to catalyze ϵ-CL polymerization in bulk at 45 °C with 3.8% (w/w) initial reaction water content.[9] The change of reaction water content and GPC profiles were used to study whether, in addition to ring-opening reactions, polycondensation occurs. They found that at about 80% monomer conversion propagation by polycondensation reactions dominated over lactone ring-opening chain-type polymerization. This finding is in excellent agreement with the results herein using a different lipase (CALB). Thus, at least for these two lipases of different origin, the polymerization mechanism for ϵ-CL polymerization shares common features.

Although the mechanism above includes transesterification, the experiments performed herein were not designed to determine the kinetics of transesterification or its effect on propagation and product structure (e.g., polydispersity). A detailed kinetic analysis of transesterification will be presented elsewhere.[16]

5. Mechanism of Enzyme-Catalyzed Ring-Opening Polymerization. Generally, control experiments to confirm that a reaction is catalyzed by a lipase at its alleged active site are performed by either (i) omission of the enzyme from the reaction mixture[17,18] or (ii) thermal deactivation of the lipase that is then included in the control experiment.[19,20] Both of these methods do not suitably address the above objective of the control. Omission of the lipase assumes that functional groups associated with the lipase at locations other than the lipase-active site do not participate in the reaction. Although inclusion of the thermally deactivated lipase in control experiments provides a more reasonable control experiment, it presumes that changes in protein conformation during deactivation do not change the reactivity of protein functional groups remote from the active site. To ensure that the polymerizations studied herein occurred by catalysis at the active serine residue (Ser_{105}) of the lipase box and not by other chemical or nonspecific protein mediated processes, Ser_{105} was modified by reaction with paraoxon (diethyl *p*-nitrophenyl phosphate).[21,22] In other words, paraoxon was used to modify and thereby irreversibly inhibit the activity of CALB. In-situ NMR was used to monitor the progress of this reaction. Briefly, to an NMR tube containing toluene-d_8 at 25 °C was added immobilized CALB (Novozyme 435) followed by an equimolar quantity of paraoxon (see Experimental Section for additional details). Figure 6 shows expansions of the 7.3−8.7 ppm regions of ^{1}H NMR spectra recorded at 0 and 30 min after the addition of paraoxon. The peaks corresponding to a and a′ at 7.5 ppm disappeared by 30 min. Concurrently, there appeared new signals at 7.8 ppm corresponding to protons b and b′ of *p*-nitrophenol. Further addition of a second equivalent of paraoxon to Novozyme-435 and incubation for 30 min at 25 °C did not result in any further reaction of paraoxon based on NMR studies. Thus, these results support that the reaction between paraoxon and CALB occurs selectively at Ser_{105}. Thus, if CALB catalyzed ϵ-CL polymerization occurs by catalysis at Ser_{105}, CALB that has been irreversibly inhibited by modification at Ser_{105} with paraoxon should no longer be active for this polymerization. Indeed, the attempted polymerization of ϵ-CL at 60 °C for 30 min in toluene using paraoxon inhibited CALB showed no monomer consumption.

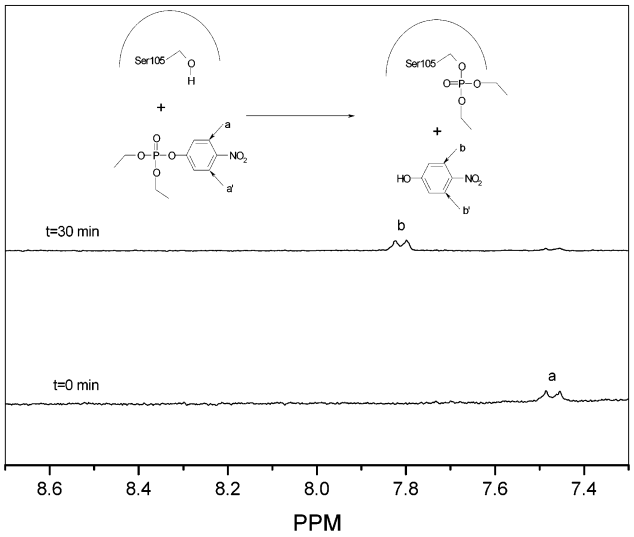


Figure 6. Proton (1H) NMR spectra recorded at time 0 (a) and 30 min (b) after beginning the reaction between paraoxon and immobilized Lipase B from *Candida antarctica* (Novozyme-435). The reaction was conducted at 25 °C in toluene.

Summary and Conclusions

A mechanism was proposed that expands on those previously by including formation of enzyme activated chain end complexes and intrachain scission reactions. In addition, the revised mechanism describes step-condensation reactions between activated chain ends and hydroxyl terminal groups. The inclusion of later steps in the mechanism was validated from inspection of M_n vs fractional ϵ-CL conversion plots at low enzyme water contents and high monomer conversion. An upward deviation from otherwise linear M_n vs fractional ϵ-CL conversion plots at the latter stage of the polymerization was seen. This was attributed to step-condensation reactions between enzyme-activated carboxyl chain ends and hydroxyl terminal groups.

Unlike previous studies, the kinetic plot of $\ln([M]_0/[M]_t)$ vs time was extended to 96% ϵ-CL conversion and M_n 11 970. By reaching this late stage in the polymerization, a sufficient effect was seen on M_n vs conversion plots to validate concurrent ring-opening and step-condensation reactions. Regardless of propagation rates that differ in this work from those previously where different enzymes (Lipase B from *Candida antarctica* vs PPL) and reaction media (bulk vs toluene) were studied, plots of $\log\{[M]_0/[M]_t\}$ vs time and M_n vs conversion to at least 80% monomer conversion were all linear. This infers that these different lipases and dissimilar reaction conditions proceed by mechanisms that share similar characteristics.

By study of the effect of enzyme water content on the time course of ϵ-CL polymerization, plots of $\log\{[M]_0/[M]_t\}$ vs time, and $\ln k_{app}$ vs ln [enzyme], an experimental rate equation was derived that is zero order with

respect to [R−OH] and initiator concentration, first order with respect to monomer concentration, and 0.7 order with respect to [enzyme], respectively. From an Arrhenius plot, an apparent activation energy of 2.88 kcal mol^{-1} was determined for Novozyme-435 catalyzed ϵ-CL polymerization in toluene. This value is well below 10.3 kcal mol^{-1} that is the activation energy for aluminum alkoxide catalyzed ϵ-CL polymerization in toluene.

Finally, by irreversibly inactivating *Candida antarctica* Lipase B site by reaction with paraoxon at Ser_{105} active, it was shown that the polymerizations studied occurred by catalysis at the active serine residue (Ser_{105}) of the lipase box and not by other chemical or nonspecific protein mediated processes.

Experimental Section

Polymerization grade ϵ-caprolactone, a gift from Union Carbide, was first dried over calcium hydride and then distilled under reduced pressure in a nitrogen atmosphere. Toluene-d_8 was purchased from Aldrich Chemical Co. Coulomat A and Coulomat C were purchased from EMscience. Paraoxon was bought from Sigma. Novozyme-435 (specified activity 7000 PLU/g) was a gift from Novozymes. Thermally deactivated Novozyme-435 was prepared following a procedure described elsewhere.[20] All liquid chemical transfers were performed by syringe through rubber septum caps under a nitrogen atmosphere. All other solvents and reagents were obtained commercially at the highest purity available and used without further purification.

General Procedure for Novozyme-435 Catalyzed Polymerization of ϵ-Caprolactone. Novozyme-435 (12 mg) was transferred under a nitrogen atmosphere into oven-dried 7 in. long premium (60−360 MHz) NMR tubes. The tubes were stoppered with rubber septa and sealed with Teflon tape, and ϵ-CL (0.12 mL, 1.13 mmol) and toluene-d_8 (0.6 mL) were added by syringe under nitrogen. The NMR tubes were placed in the NMR spectrometer at 60 °C. The temperature control was calibrated by using ethylene glycol as a standard sample. NMR data were recorded every 7−9 min, and the tube was taken out, shaken well, and put in the probe for the next recording.

Irreversible Inhibition by Reaction with Paraoxon. The immobilized *Candida antarctica* Lipase B marketed by Novozymes is Novozyme-435. Novozyme-435 consists of 20% (w/w) of *Candida antarctica* Lipase B. Thus, 12 mg of the catalyst (Novozyme-435) contains 2.4 mg of the lipase. Since the lipase molar mass is 33 kDa, 7.3×10^{-5} mmol of lipase was used.

Immobilized *Candida antarctica* Lipase B (12 mg of catalyst, 2.4 mg and 7.3×10^{-5} mmol of *Candida antarctica* Lipase B) was transferred into oven-dried 7 in. long premium (60−360 MHz) NMR tubes. The tubes were stoppered with rubber septa and sealed with Teflon tape, and toluene-d_8 (0.6 mL) was added by syringe under nitrogen. A solution of paraoxon (0.025 mL, 7.3×10^{-5} mmol**)** in toluene-d_8 was added to the NMR tube by syringe under nitrogen. The reaction between paraoxon and *Candida antarctica* Lipase B was maintained at 25 °C with agitation for 30 min.

Instrumentation Methods. Polymerization of ϵ-CL was monitored in situ by ^{1}H NMR to determine (i) monomer conversion, (ii) number-average molecular weight, and (iii) total number of polymer chains. ^{1}H NMR spectra were recorded on a Bruker NMR spectrometer (model DPX300) at 300 MHz. The chemical shifts in parts per million (ppm) for ^{1}H NMR spectra were referenced relative to tetramethylsilane (TMS, 0.00 ppm) as the internal reference. A ratio 5:1 (vol/vol) toluene-d_8 to ϵ-CL was selected for the NMR experiments. The NMR instrument was locked and was maintained at fine shim which avoided broadening of signals at high conversion due to increased viscosity. The signals at 3.99 (t, J 6.5 Hz, OCH_2), 2.48 (t, J 7.5 Hz, $C(O)CH_2$), and 1.71/1.52 (m, $(CH_2)_3$) were observed at zero time and were assigned to the protons of ϵ-CL monomer. Signals at 4.18 (t, 6.5 Hz, OCH_2), 3.62 (t, 6.5 Hz, $HOCH_2$), and 2.42 (t, J 7.5 Hz, $C(O)CH_2$) appeared after the onset of polymerization reactions and were assigned to PCL protons. The ratio of the signals at 4.18 to 3.99 and 4.18 to 3.62 were used to calculate the monomer conversion and M_n, respectively.

The different levels of enzyme water content were obtained by drying the enzyme (Lipase B from *Candida antarctica* physically immobilized on a macroporous poly(methyl methacrylate) resin) by one of the following methods: (1) the enzyme was used without drying and had 1.2% (w/w) enzyme water content; (2) the enzyme was dried over P_2O_5 using a pump with a drying pistol (0.1 mmHg; 48 h; room temperature) so that it had 0.6% (w/w) water; (3) the enzyme was suspended over distilled water in a desiccator at 5 °C to give enzyme water contents of 1.9% (w/w). Enzyme water contents (wt %) were measured by using an Aqua star C 3000 titrator with Coulomat A and Coulomat C from EMscience. Enzyme water content was determined by stirring 53 mg of Novozyme-435 in Coulomat A within the Aqua star closed septum container and titrating against Coulomat C. The water contents of toluene-d_8 and ϵ-CL prior to addition to the reactions were 0.040% and 0.050%, respectively. The water content of the reaction medium was determined as 0.033% after the polymerization. The water content of reaction medium was determined by stirring about 50 mg of reaction medium in Coulomat A within the Aqua star closed septum container and titrating against Coulomat C.

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Supporting Information Available: Plot of ln k_{app} vs ln [enzyme] (mg of Novozyme-435/mmol of ϵ-CL). This material is available free of charge via the Internet at http://pubs.acs.org.

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