# Probing Water-Temperature Relationships for Lipase-Catalyzed Lactone Ring-Opening Polymerizations

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ABSTRACT: Polymerizations of $\epsilon$-CL catalyzed by Novozyme-435 (immobilized Lipase B from *Candida antarctica*) were studied at temperatures between 20 and 108 °C. The monomer conversion to polymer was remarkably rapid at ambient temperature. At 20 °C by 7 h, $\epsilon$-CL conversion and product $M_n$ were >97% and 17 800, respectively. Contrary to previous reports, the number of chains formed, as well as the product molecular weight, was almost identical for polymerizations at constant enzyme water content between 60 and 108 °C. Thus, differences in reaction temperature over a 48 °C range did not "free" water from "bound" states so that it could function for chain initiation. At 60 °C, variation in the enzyme water content from 0.6 to 1.9% increased the number of chains formed but did not change the polymerization propagation kinetics. Therefore, the enzyme water content and not the reaction temperature regulated the product molecular weight. In contrast, at 108 °C, an increase in the reaction water content from 0.6 to 1.8% increased both the number of chains and the polymerization propagation kinetics. Explanations for these differences in behavior as a function of temperature and water contents are discussed.

## Introduction

Biotransformations represent an effective and sometimes preferable alternative to conventional chemical synthesis for the production of fine chemicals and optically active compounds.[1] Recently, efforts to exploit enzyme catalysis for polymer synthesis have received increased attention.[2] Lactone ring-opening polymerization offers the advantage that no leaving group is generated during the reaction that might limit monomer conversion or inhibit the enzymes activity.[3] Previous work by our laboratory established that lipase-catalyzed lactone polymerizations occur with some elements of "control". For porcine pancreatic lipase (PPL)-catalyzed $\epsilon$-CL polymerizations, the linearity of $\log\{[M]_0/[M]_t\}$ vs time plots indicated that the number of chains remained constant during polymerizations and chain termination did not occur. Furthermore, $M_n$ vs conversion plots were linear to high monomer conversion, showing that chain growth occurs predominantly from the chain end instead of by a step-growth mechanism.[4] Moreover, Hult and co-workers used MALDI−TOF to characterize the macrocyclic compounds formed during lipase-catalyzed lactone ring-opening polymerizations.[5]

To remain catalytically active in a nonaqueous environment, "dry" enzymes require a small amount of water, typically enough to provide at least a partial hydration layer around the enzyme molecule.[6] Furthermore, the amount of water adsorbed to an enzyme in organic solvents was found to be a more important determinant of enzyme activity than the water content of the solvent itself.[7] For Subtilisin Carlsberg, a certain population of this essential water is intricately associated with the enzyme and does not exchange with the water in the bulk organic solvent. Electron spin resonance (ESR) spectroscopy showed that further water bound to the enzyme causes an increase in active-site polarity, which correlates closely with a sharp increase in enzyme activity.[8] These bound or "essential" water molecules have been compared to lubricants, providing enzyme molecules with the flexibility necessary for enzyme catalysis. Mechanistically, it is believed that the "lubricant" properties of water result by its forming hydrogen bonds with enzyme functional groups. This hydrogen bonding serves to "unlock" the structure.[7] Moreover, water molecules can destabilize the ground state of a hydrophobic substrate but will stabilize the corresponding tetrahedral intermediates. This should result in a decrease in the activation barrier and hence increase the enzymatic reaction.[9]

Enzymes have been found with exceptional stability and activity under extreme conditions in aqueous environments. Examples are those from unusual environments such as hot springs.[10] Also, under exceptionally dry conditions (percent water in the medium and on the enzyme of 0.015% and 0.48% w/w, respectively), where the enzyme is very rigid, PPL powder has a half-life at 100 °C in toluene and tributyrin of 6 and 26 h, respectively. This very dry PPL was about 5 times more active at 100 °C than 20 °C. However, in contrast to 100 °C, at 20 °C the enzyme activity is increased dramatically at higher reaction water contents. This is due to the poor stability of PPL at 100 °C when the water contents in the medium and on the enzyme are about 0.7 and 3.6%, respectively.[11] Thus, it is important to study interactions between effects of temperature and water concentration for lipase-catalyzed transesterifications.

Thus far, little is known of how the concentration of water in reactions will alter the outcome of lipase-catalyzed ring-opening polymerizations. This in part is due to waters dual role in regulating lipase activity while also functioning as the polymerization initiator. Dong et al reported, for Lipase PSL (*Pseudomonas sp.*)-catalyzed $\epsilon$-caprolactone ($\epsilon$-CL) polymerization, that by using higher initial reaction water contents the polymerization rate and molecular weight increased and decreased, respectively.[12] They focused over a wide range of reaction water contents (0.1−16%) where medium polarity may have been altered.

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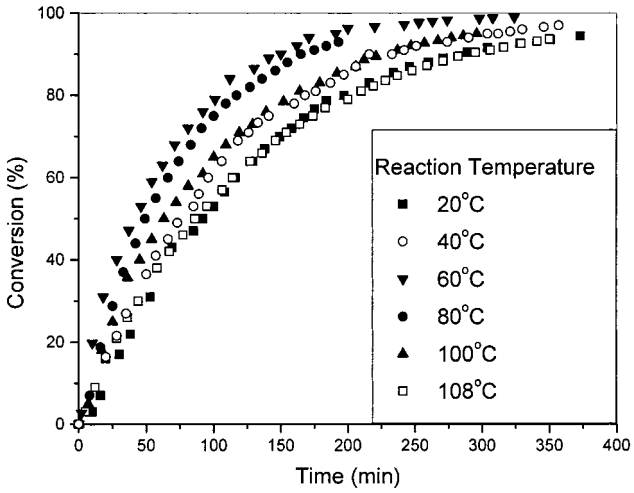


**Figure 1.** Monomer conversion as a function of time for the enzyme-catalyzed ϵ-caprolactone polymerizations at different reaction temperatures at constant water content (0.6 wt %).

This paper focuses on how lipase-catalyzed polymerizations are affected by small differences in enzyme water content and wide variations in reaction temperature. Studies were designed to explore interactions between the reaction parameters of temperature and enzyme water concentration. The enzyme water concentration and temperature were varied from 0.6 to 2.8% and 20 to 108 °C, respectively. Unexpectedly, small differences in the reaction water content caused large changes in product molecular weight. Furthermore, at 108 °C, using higher enzyme water content in the reactions increased the polymerization kinetics. Also, a surprising insensitivity of the product molecular weight to changes in the reaction temperature was found.

## Results and Discussion

**Effects of Temperature on Novozyme-435-Catalyzed ϵ-CL Polymerization**. A series of experiments were performed where the enzyme water content was fixed at 0.6% for polymerizations at 20, 40, 60, 80, 100, and 108 °C. These experiments were performed in NMR tubes placed within the NMR probe where the temperature was controlled (see Experimental Section). This permitted the in situ monitoring, by $^1$H NMR, of monomer conversion and $M_n$ as a function of reaction time.[13] Details of the NMR signals used for these determinations are given in the Experimental Section. Figure 1 shows that, for reaction temperatures from 20 to 108 °C, monomer conversions reached >80% by 4 h. The relative order of reaction rate as a function of reaction temperature is 60 ≅ 80 > 40 ≅ 100 > 20 ≅ 108.

The outcome for the ϵ-CL polymerization at 20 °C was unexpected and shows the potential use of this method at ambient temperatures. Furthermore, the ability to perform ring-opening polymerizations at or around 20 °C will be useful for polymerizations of monomers and polymers with low thermal stability. Previous studies assumed that such reactions at temperatures ≤60 °C were too slow, or the results obtained showed poor monomer conversion rates and low product molecular weights. For example, Kobayashi et al. studied ϵ-CL polymerization in carbon tetrachloride at 45 °C using the lipase from *Candida cylindracea*. They found that, after 5 days, monomer conversion and product $M_n$ were 41% and 710 g/mol, respectively.[14] In contrast, by variation of the catalyst and polymerization conditions as described herein, at 20 °C by 7 h ϵ-CL conversion and product $M_n$ are >97% and 17 800, respectively.

Previous studies of lipase-catalyzed polymerizations have avoided temperatures >90 °C. This is likely due to concerns over losses in catalyst activity. However, as was discussed in the Introduction, PPL stability and activity for transesterification reactions were found to be strongly related to the reaction water content and the solvent used.[11] Figure 1 shows that, at 0.6% by enzyme weight water, ϵ-CL polymerizations at 20 and 108 °C progress at similar rates. For the polymerization at 108 °C, by 5 h, ϵ-CL conversion and product $M_n$ were >93% and 18 000, respectively. CALB was irreversibly thermally deactivated to test the possibility that the polymerization at 108 °C was non-enzyme-mediated. When the active enzyme was replaced with thermally deactivated CALB, after 30 min at 108 °C no monomer consumption was found. The reason that ϵ-CL polymerization occurs more slowly at 108 °C than at 40, 60, and 100 °C may lie in a lower retention of CALB activity at 108 °C than the corresponding lower polymerization temperatures. However, further studies will be needed to define the precise values of CALB stability at elevated reaction temperatures.

**Effects of Enzyme Water Content on Novozyme-435-Catalyzed ϵ-CL Polymerization at 60 °C**. The water molecules in reactions can be separated into two categories: water associated with enzyme and water in the reaction medium. We found that the additive water content of ϵ-CL and toluene, prior to mixing them with the enzyme, was 0.0123 mmol. After CALB was mixed with ϵ-CL and toluene and the reaction was performed, analysis of the resulting reaction medium showed that it contained 0.0119 mmol. Within experimental error these water values are indistinguishable. Thus, the water associated with the enzyme prior to adding toluene/ϵ-CL and after mixing remained unchanged. This is largely attributed to the fact that toluene, ϵ-CL, and PCL create a medium of low polarity relative to the enzyme surface. Therefore, water content given in this paper is that measured for the catalyst alone over the total catalyst weight times 100 (percent by weight water). The enzyme water contents in reactions were varied from 0.6 to 1.9% while the reaction temperature was fixed at 60 °C. These reactions were performed in NMR tubes that facilitated direct monitoring by $^1$H NMR. The resulting plots of monomer conversion as a function of reaction time are shown in Figure 2. Inspection of Figure 2 shows that, within this range of enzyme water contents, the rate of monomer conversion did not significantly differ. The plots of the number of chains vs fraction monomer conversion at different enzyme water contents are displayed in Figure 3. These plots show that the number of chains increased significantly with increased enzyme water content, despite that the polymerization rates were invariable. The corresponding $M_n$ values at 80% monomer conversion increased from about 7000 to 15 000 with a decrease in the enzyme water content from 1.9 to 0.6%, respectively. We believe this is due to that the monomer activation step is rate-determining. This explanation will be further supported in a later publication.[15]

The number of chains was recorded at 80% monomer conversion for polymerizations at 60 °C that differed in the initial enzyme water content. These data were used

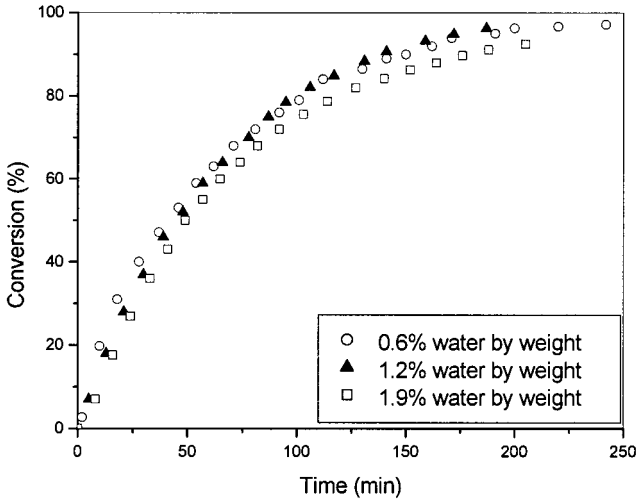


**Figure 2.** Monomer conversion as a function of time for the enzyme-catalyzed $\epsilon$-caprolactone polymerizations at 60 °C with different water contents.

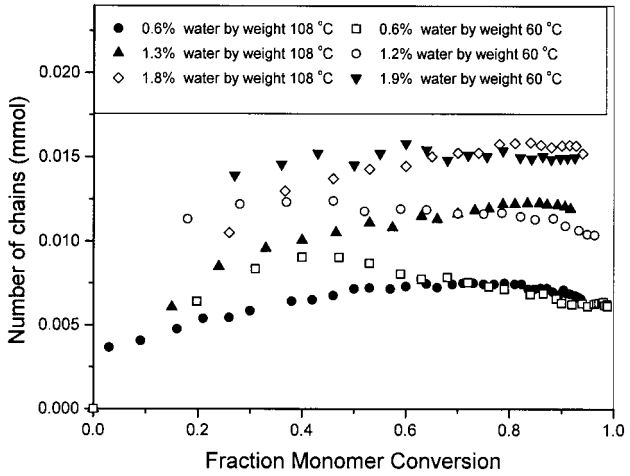


**Figure 3.** Number of chains as a function of water contents at two different reaction temperatures: 60 and 108 °C in the enzyme-catalyzed $\epsilon$-caprolactone (0.1236 g) polymerizations.

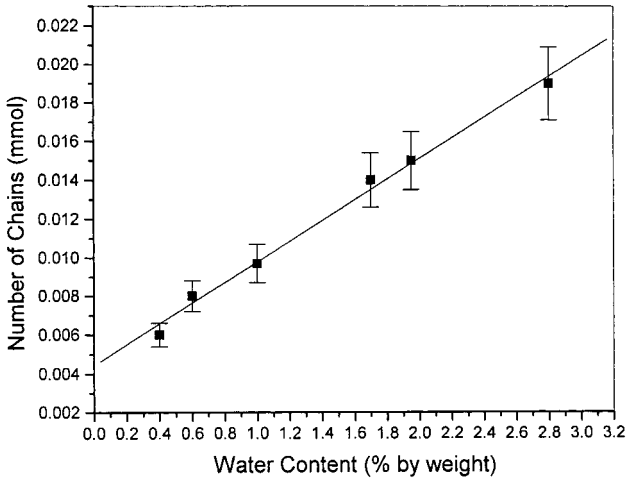


**Figure 4.** Number of chains as a function of water content in the enzyme-catalyzed $\epsilon$-caprolactone (0.1236 g) polymerization. The error bar is given from the triplicate runs.

to create the plot in Figure 4 of the total chain number (at 80% monomer conversion) vs the initial enzyme water content. Inspection of Figure 4 shows that the total number of chains increases regularly with increased initial enzyme water content. At 0.4 and 2.8% enzyme water content, the corresponding $M_n$ values are about 21 000 and 5000, respectively. When the data in Figure 4 are analyzed by linear regression, the extrapolated line to 0.0% enzyme water content did not pass through the origin. Thus, it appears that some of the water molecules not counted by the Karl Fisher titrator method (see Experimental Section) participated in chain initiation or transacylation reactions. In other words, some water that was unavailable to diffusible electrolytes during the Karl Fisher titration analysis was still reactive. Although the explanation for this is still unclear, it may be due to a simple electrostatic interaction that was previously described (see ref 16 for additional details).

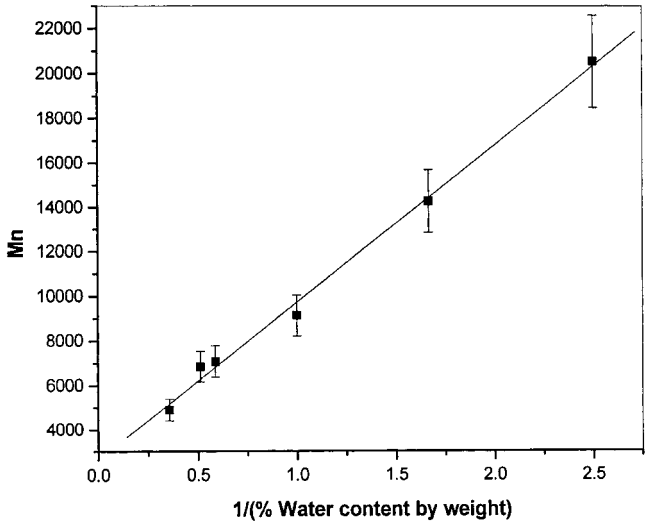


**Figure 5.** Number-average molecular weight as a function of reciprocal of percent water content in the enzyme-catalyzed $\epsilon$-caprolactone (0.1236 g) polymerizations. The error bar is given from the triplicate runs.

The following describes a model proposed by Clark et al based on a multinuclear NMR study that describes various states of water present during enzyme hydration in an organic medium.[17]

$$(H_2O)_{b1} \not\leftrightarrow (H_2O)_{b2} \leftrightarrow (H_2O)_f$$

The subscript "b1" denotes the tightly bound, nonexchanging hydration state, "b2" denotes the second hydration state, and "f" represents free water. They suggested that the role of "b1" state water molecules was to maintain the enzyme's catalytically active conformation. The roles of "b2" and free water molecules were to increase the enzyme flexibility and active site polarity. Based on this model, our current hypothesis is that water molecules active in chain initiation or transacylation reactions during CALB-catalyzed lactone polymerizations are due to some fraction of the "b2" state water molecules that could not be titrated and the "f" state water molecules that are easily titrated.

The plot of the product number-average molecular weight ($M_n$) vs the reciprocal enzyme water content is shown in Figure 5. This plot was constructed by analyzing the number-average molecular weight from the identical samples at 80% monomer conversion that were used to create Figure 4. Figure 5 show that, by regulation of the enzyme water content, predictable product molecular weights can be obtained without variation in the polymerization kinetics (see Figure 2). By changing

the enzyme water contents within the narrow range of 0.4−2.8 wt %, the product $M_n$ was varied between ~5000 and 21 000.

Previously, we defined the following equation to calculate the number-average molecular weight ($M_n$) values:[18]

$$M_n = [M]_0/[I']C_m m_{CL} \quad (1)$$

where $C_m$ is the fractional monomer conversion, $m_{CL}$ is the molar mass of $\epsilon$-CL, and [I′] is the total initiator concentration (reacted water). Then, [I′] is the sum of the fraction $x$ of moderately bound water molecules that could not be titrated and the "free" water ($x$b2 + "free") that initiates chain growth. By extrapolation of the line in Figure 4, the value of the $x$-intercept is −0.85. This value, 0.85% of the enzyme weight, is the amount of the water molecules that were not titrated but still were available to initiate chain growth. In other words, the enzyme water content that reacts for chain initiation is the value measured by titration plus 0.85%.

**Effects of Enzyme Water Content on Novozyme-435-Catalyzed $\epsilon$-CL Polymerization at 108 °C.** The interactions between water and catalytic proteins are believed to be principally due to hydrogen-bonding interactions.[7] Thus, changes in the reaction temperature should shift the relative populations of "free", "moderately bound", and "tightly bound" water. Then, by increasing the reaction temperature, some water molecules that are "moderately bound" may become more available or even "free", allowing their inclusion as available water molecules that can function as nucleophiles during lipase-catalyzed reactions. On the basis of this model, by increasing the reaction temperature at constant enzyme water concentration, increases in the total number of chains should result. Furthermore, the total chain number should be dependent on both the reaction temperature and the initial enzyme water content in the reactions. Water-initiated lactone polymerizations offer a convenient way to test this hypothesis by simply following the total number of water-initiated chains. Therefore, $\epsilon$-CL polymerizations were conducted at 60 °C with 0.6, 1.2, and 1.9% enzyme water content. Similarly, $\epsilon$-CL polymerizations were conducted at 108 °C with 0.6, 1.3, and 1.8% enzyme water content. The total numbers of chains for these reactions were plotted as a function of $\epsilon$-CL fractional conversion, and the results are displayed in Figure 3. By 60% conversion, reactions carried out at 60 or 108 °C with almost the same enzyme water content had total chain numbers that were identical. Thus, the input of thermal energy by raising the reaction temperature from 60 to 108 °C was insufficient to shift water molecules from "tightly" enzyme bound to "moderately bound" or "free" water. This is consistent with Vulfson et al., who reported that temperatures in excess of 200 °C are necessary to remove tightly bound water from proteins.[19] Thus, within the range of reaction conditions described above, and $\epsilon$-CL conversion values >60% where a plateau in the number of chains is observed, the number of chains and therefore the product molecular weight are determined by the enzyme water content and not the reaction temperature. Differences in the chain number as a function of reaction temperature at fractional monomer conversions between 0.2 and 0.6 are attributed to differences in the initiation kinetics (see Figure 3).

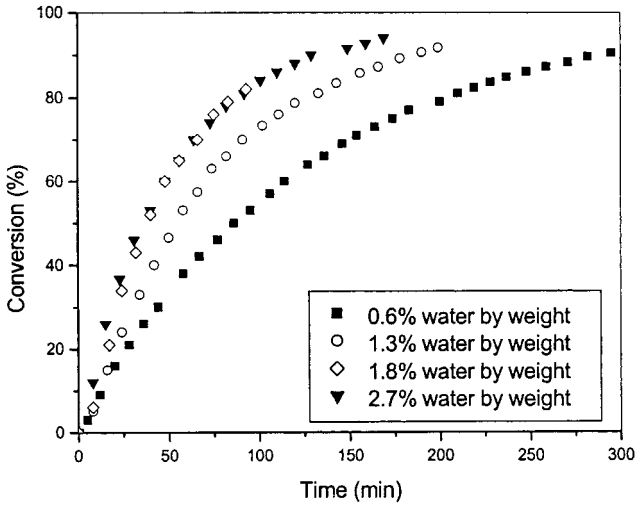


**Figure 6.** Monomer conversion as a function of time for the enzyme-catalyzed $\epsilon$-caprolactone polymerizations at 108 °C with different water contents at 108 °C.

Figure 2 showed that, although the enzyme water content was varied between 0.6 and 1.9%, the propagation kinetics for polymerizations conducted at 60 °C was essentially unchanged. In contrast, Figure 6 shows that, at 108 °C, increase in the enzyme water content from 0.6 to 1.8% resulted in faster polymerizations. Figure 4 showed that, by increasing the enzyme water content, an increase in the number of carboxyl chain ends results. These chain ends, more abundant at higher water concentration, may protect CALB from thermal deactivation by binding to the enzyme active site. This is consistent with a previous study that showed the importance of reactive intermediates in the stabilization of *Candida rugosa* against thermal deactivation.[20]

**Comments on Heterogeneous Lipase-Catalyzed Polymerizations in NMR Tubes.** The above studies were carried out in NMR tubes that allowed in-situ monitoring of reaction progress. The reactions are heterogeneous, and the observed rates will be a function of the agitation or mixing. To ensure that the reaction was not diffusion-limited under these conditions, a plot of $\ln([M_0]/[M_t])$ vs time was constructed for a reaction of $\epsilon$-CL in toluene-$d_8$ (1:5 v/v) at 25 °C (see Figure 1 in Supporting Information). The reaction was conducted to >90% monomer conversion for over 300 min. The polymer formed at the end of this reaction had a $M_n$ of 17 500. Despite the apparent increase in viscosity during the polymerization, the plot of $\ln([M_0]/[M_t])$ vs time was linear. This proves that the reaction was not diffusion-limited. By increasing the agitation in reactions, much faster kinetics can be achieved. For example, when the identical reaction was conducted in a round-bottom flask with rapid magnetic stirring, a linear plot of $\ln([M_0]/[M_t])$ vs time was obtained (see Figure 2 in Supporting Information) but with a much greater slope than for the corresponding reaction in the NMR tube.

## Summary of Results

The enzyme water content was fixed at 0.6% for polymerizations at 20, 40, 60, 80, 100, and 108 °C. The relative order of the reaction rate as a function of reaction temperature was 60 ≅ 80 > 40 ≅ 100 > 20 ≅ 108. Even at 20 °C, by 7 h, $\epsilon$-CL conversion and product $M_n$ were >97% and 17 800, respectively. This bodes well

for the potential use of this method at ambient temperatures. When the reactions were conducted at 60 °C, the number of chains increased significantly with increased enzyme water content, despite the fact that the polymerization rates were invariable. We believe this results from the fact that the monomer activation step is rate-determining. The product $M_n$ was varied between ~5000 and 21 000 by changing the enzyme water contents from 0.4 to 2.8 wt %. Thus, predictable product molecular weights will be obtained by determining the enzyme water content. Raising the reaction temperature from 60 to 108 °C did not shift water molecules from "tightly" enzyme bound to "moderately bound" or "free" water. Thus, it is the enzyme water content and not the reaction temperature that determines the product molecular weight. In contrast to the results at 60 °C, at 108 °C, increase in the reaction water content resulted in faster polymerizations. This is believed to result from increased stabilization of the enzyme by carboxyl chain ends that are more abundant at higher water concentration.

## Experimental Section

Polymerization grade $\epsilon$-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. Novozyme-435 (specified activity 7000 PLU/g) was a gift from Novozymes. Thermally deactivated Novozyme-435 was prepared following a procedure described elsewhere.[10] All liquid chemical transfers were performed by syringe through rubber septum caps under a nitrogen atmosphere.

**Novozyme-435-Catalyzed Polymerization of $\epsilon$-Caprolactone.** Novozyme-435 (12 mg) with different water content was transferred under a nitrogen atmosphere into an oven-dried 7 in. long premium (60−360 MHz) NMR tube. The tubes were stoppered with rubber septa and sealed with Teflon tape, and $\epsilon$-CL (0.12 mL) and toluene-$d_8$ (0.6 mL) were added by a syringe under nitrogen. The NMR tubes were placed in the NMR spectrometer at a constant temperature between 20 and 108 °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. The control experiments on Novozyme-435-catalyzed $\epsilon$−CL polymerization were performed in a 50 mL round-bottom flask instead of NMR tubes with stirring (220 rpm) with a batch size of 17.7 mL of CL. A small amount of reaction mixture was withdrawn by syringe at specified time periods, and the monomer conversion was analyzed by NMR.

**Instrumentation Methods.** $\epsilon$-CL polymerization 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 $\epsilon$−CL was selected in the NMR experiments. The NMR instrument was locked and was maintained at fine shim that avoided broadening of signals at high conversion due to increased viscosity. The signals at 3.99 (t, $J$ 6.5 Hz, O$CH_2$), 2.48 (t, $J$ 7.5 Hz, C(O)C$H_2$), and 1.71/1.52 (m, $(CH_2)_3$) were observed at zero time and were assigned to the protons of $\epsilon$−CL monomer. Signals at 4.18 (t, 6.5 Hz, O$CH_2$), 3.62 (t, 6.5 Hz, HOC$H_2$), and 2.42 (t, $J$ 7.5 Hz, C(O)C$H_2$) appeared after the onset of polymerization reactions and were assigned to PCL protons. The ratios 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 it 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 or room temperature to give enzyme water contents of 1.9 and 2.8% (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 it against Coulomat C. The water contents of toluene-$d_8$ and $\epsilon$-caprolactone prior to addition to the reactions were 0.0295% and 0.05%, 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 it against Coulomat C.

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**Supporting Information Available:** Semilogarithmic kinetic plot of Novozyme-435-catalyzed $\epsilon$-caprolactone ring-opening polymerization at 25 °C in toluene in a NMR tube and round-bottom flask. This material is available free of charge via the Internet at http://pubs.acs.org.

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