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Ring-opening polymerization of ε-caprolactone catalyzed by a novel thermophilic esterase from the archaeon *Archaeoglobus fulgidus*

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ABSTRACT

The ring-opening polymerization of ε-caprolactone catalyzed by a novel thermophilic esterase from the archaeon *Archaeoglobus fulgidus* was successfully conducted in organic solvents. The effects of enzyme concentration, temperature, reaction time, reaction medium, and water activity on monomer conversion and product molecular weight were investigated. Poly(ε-caprolactone) was obtained in almost 100% of the monomer conversion, with a number-average molecular weight of 1400 in toluene at 80 °C for 72 h. Furthermore, the Michaelis–Menten kinetic analysis showed that the enzyme had the highest affinity for ε-caprolactone, with a K_m value of 0.093 mol/l compared with other reported lipases. The possible structural and energetic effects of the enzyme on the K_m value were investigated, using molecular docking studies.

1. Introduction

Poly(ε-caprolactone) (PCL) is an attractive aliphatic polyester due to its favorable biodegradability, biocompatibility, and permeability characteristics [1]. While these advantages make it a good candidate for many biomedical applications, it is especially well suited for tissue-engineering scaffolds [2]. Metallic catalysts are often used in ring-opening polymerization for PCL synthesis [3]. However, concern has been raised about the harmful effects of using PCL for medical applications because of its metallic residues. Enzymatic polymerization has been used as a substitute for the toxic, metal-based catalytic systems [4–7]. Moreover, it has the advantages of mild reaction conditions, recyclability of enzymes, and high enantio- and regioselectivity [7].

Various commercially available lipases have been employed in ring-opening polymerization of ε-caprolactone, such as porcine pancreatic lipase, *Candida rugosa* lipase (lipase CR), *Pseudomonas cepacia* lipase (lipase PC), *Candida antarctica* lipase B (lipase CA) and *Pseudomonas fluorescens* lipase (lipase PF) [8–12]. Among them, lipase CA showed high catalytic activity towards the polymerization of ε-caprolactone, and the number-average molecular weight (M_n) of PCL obtained was more than 4×10^4 [12]. Up to now, no research on esterase-catalyzed ring-opening polymerization of lactones has been reported except for that conducted on the cutinase from *Humicola insolens*, a fungal esterase with promising lactone ring-opening polymerization activity [13]. However, because of their lack of stability, these mesophilic enzymes are not well suited for the harsh reaction conditions (such as high temperature and exposure to organic solvents) required in industrial preparation of PCL. Recently, enzymes from extremophiles, especially thermophiles, have been recognized as having the potential for organic synthesis due to their high stability against organic solvents, high temperature, and chemical denaturants. Therefore, these enzymes permit the exploration of reactions over much wider ranges of pH, temperature, and solvents without significant loss in enzymatic activity [14]. The commercial thermophilic lipases ESL-001 were once employed in the polymerization of racemic β-butyrolactone and the production of optically active poly(3-hydroxybutyrate) [14].

To date, many cloned thermophilic esterases have been considered as potential catalysts in industrial processes [15]. Thermophilic esterase from the hyperthermophilic archaeon *Archaeoglobus fulgidus* (AFEST) has been cloned, well expressed in *Escherichia coli*, and characterized with respect to substrate specificity, crystal structure, and catalytic mechanism [16–19]. The enzyme was classified as a member of a hormone-sensitive lipase group of the esterase/lipase superfamily [18]. Its optimum temperature was 80 °C, and the $t_{1/2}$ value was 7.5 h at 70 °C [17]. It could catalyze the hydrolysis of a broad range of esters, including *p*-nitrophenyl esters, vinyl esters, and triacylglycerols of short chain, and its best substrate was *p*-nitrophenyl hexanoate among the *p*-nitrophenyl esters tested [17,19]. Its wide substrate specificity and unique stability make it a promising candidate for potential industrial applications.

In the present study, we first reported the ring-opening polymerization of ε-caprolactone catalyzed by thermophilic esterase

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AFEST. Reaction conditions were optimized to obtain high yield and high product molecular weight. Using Michaelis–Menten kinetic analysis, we gained deeper insight into the mechanism of enzymatic catalysis. In addition, we performed molecular docking studies to elucidate the possible structural and energetic basis of the high affinity of the enzyme for the monomer.

2. Materials and methods

2.1. Materials

ε-Caprolactone was purchased from Fluka and used without further purification. The organic solvents used in enzymatic polymerization were of analytical grade and dried over a 4 Å molecular sieve before use. Isopropyl β-D-thiogalactopyranoside (IPTG) and *p*-nitrophenyl caprylate were purchased from Sigma. All the other chemicals were of the highest reagent grade commercially available.

2.2. Purification of the recombinant enzyme AFEST

The recombinant *E. coli* BL21 harboring AF1716 from the archaeon *A. fulgidus* was a generous gift of Dr. Giuseppe Manco (Istituto di Biochimica delle Proteine, Italy). It was cultured in 2YT medium (1% yeast extract, 1.6% tryptone, and 0.5% NaCl) containing ampicillin (100 μg/ml) at 37 °C. After incubation with shaking at 37 °C until the OD_{600} (optical density of liquid medium at 600 nm) reached 1.8, the induction was carried out by adding IPTG at a final concentration of 1 mM and shaking for an additional 3 h at 37 °C. The cells were harvested by centrifugation (5000 rpm, 4 °C, 30 min), washed with 50 mM phosphate buffer (pH 8.0), and stored at −20 °C.

The frozen cells were melted and mixed with 50 mM phosphate buffer (pH 8.0). After ultrasonic cell disintegration, the suspension was thermoprecipitated by incubation at 80 °C for 30 min. The crude extract was centrifuged at 8000 rpm for 15 min, and the pellet was discarded. The enzyme solution was ultrafiltrated under a 20 kDa microporous filter membrane. The solution was then lyophilized, and the enzyme powder was used as the catalyst. SDS-PAGE and analysis of the gel with an ARTHUR 1442 multiwavelength fluoroimager indicated that the enzyme was about 92% pure, with a molecular mass of 35.5 kDa (data not shown).

2.3. Enzyme assay

The time course of the esterase-catalyzed hydrolysis of *p*-nitrophenyl caprylate was followed by monitoring the production of *p*-nitrophenyl at 405 nm in 1 cm path length cells with a double-beam Varian Cary 50 ultraviolet–visible spectrophotometer equipped with a temperature controller. The substrate *p*-nitrophenyl caprylate was dissolved in acetonitrile at a concentration of 10 mM. In the standard assay, 20 μl of 10 mM *p*-nitrophenyl caprylate solution was added to the reaction system to a final concentration of 0.2 mM in 50 mM phosphate buffer (pH 8.0) incubated at 80 °C; the reaction was initiated by adding 20 μl of enzyme solution. The background hydrolysis of the substrate was deducted using a reference sample of identical composition to the incubation mixture, except that the esterase was omitted. One unit of enzymatic activity was defined as the amount of protein releasing 1 μmol *p*-nitrophenyl from *p*-nitrophenyl caprylate per min.

2.4. Enzymatic polymerization of ε-caprolactone

The thermophilic esterase AFEST was dried in a desiccator overnight and transferred into a dried vessel containing 200 μl ε-caprolactone and 600 μl organic solvent. The vessel was sealed and then placed into a thermostatic reactor with stirring (180 rpm). Reaction was terminated by adding dichloromethane and filtrating to remove the enzyme. The enzyme was washed several times with dichloromethane, and the filtrates were collected and diluted to10 ml with dichloromethane. We used 1 ml of the organic phase to measure the monomer conversion by gas chromatography (GC). The other solution was evaporated under reduced pressure to remove dichloromethane, and the remaining viscous sample was precipitated in methanol at −20 °C. The cloudy solution was centrifuged (8000 rpm, 15 min, 4 °C), and the white precipitate was dried in a vacuum oven (0.1 mmHg, 25 °C, 24 h). The product obtained was then characterized by Fourier transform infrared spectra (FT-IR), ^{1}H and ^{13}C NMR, gel permeation chromatography (GPC), and differential scanning calorimeter (DSC).

2.5. Determination of monomer conversion

The monomer conversion was determined with a Shimadzu 2014 gas chromatograph equipped with an Rtx-1 capillary column (30 m × 0.25 mm × 0.25 μm) and hydrogen flame ionization detector. The temperatures of the injection pool and detector were set at 200 and 240 °C, respectively. The column oven temperature was held at 70 °C for 2 min and then programmed to rise at 10 °C/min to a final temperature of 140 °C, which was maintained for 2 min. The injection volume was 1.0 μl. Butyl acetate was used as the internal standard to determine the monomer conversion values, which were measured in duplicate.

2.6. Determination of molecular weight and polydispersity index

The M_n and polydispersity index (PDI) of the products were measured by GPC using a conventional calibration technique. The calibration curve was generated from polystyrene standards with molecular weights of 5.00×10^2, 2.10×10^3, 4.92×10^3, 9.92×10^3, 6.63×10^4, 9.60×10^4, and 9.56×10^5. Analyses were carried out using a Waters HPLC system equipped with a refractive index detector (model 410) and 10^3, 10^4, and 10^5 Å Ultrastyragel columns in series. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1.0 ml/min. The sample was filtrated under 0.22 μm micropore film. The sample concentration and injection volume were 0.3% (w/v) and 20 μl, respectively. The values were the average of duplicate measurements.

2.7. Structural and thermal analysis of PCL

The structure of the polymer was characterized by FT-IR, and ^{1}H and ^{13}C NMR. FT-IR spectrum was recorded in the range of 4000–400 cm^{-1} on a Nicolet 5700 Instrument (Thermo Electron Corporation, USA) using the standard KBr disk method. ^{1}H and ^{13}C NMR spectra were recorded in chloroform-d ($CDCl_3$) on an AVANCE DMX 500 spectrometer at 500 MHz. The chemical shifts for ^{1}H NMR spectrum were referenced relative to tetramethylsilane (TMS, 0.00 ppm). The ^{13}C NMR spectrum was recorded using the distortionless enhancement by polarization transfer including the detection of quaternary nuclei (DEPTQ) program at 25 °C.

Preliminary thermal analysis of PCL was carried out using a Perkin-Elmer differential scanning calorimeter. DSC scans were performed in the temperature range 0–100 °C, with a heating and cooling rate of 10 °C/min. The crystallization temperature T_c and melting temperature T_m were taken at the peak of the DSC exo- and endotherms, respectively.

2.8. Control of water activity

Enzyme preparations and reaction media were equilibrated with saturated salt solutions in 25 ml of separate containers,

according to Wehtje's method [20]. The water activities of the salts used were: LiCl (water activity, $\alpha_w = 0.11$); $MgCl_2$ ($\alpha_w = 0.33$); $Mg(NO_3)_2$ ($\alpha_w = 0.54$); NaCl ($\alpha_w = 0.75$); and Na_2SO_4 ($\alpha_w = 0.95$). Equilibration was performed at 25 °C for at least 16 h.

2.9. Michaelis–Menten kinetics

We followed the general procedure for the Michaelis–Menten kinetics described by Palmans and co-workers [21]. The Hanes–Woolf plot $[S]/V = (K_m/V_{max}) + ([S]/V_{max})$ was selected to calculate the maximal rate of reaction V_{max} and the Michaelis–Menten constant K_m, where [S] represents the concentration of ε-caprolactone. The initial rate of reaction V was calculated from the initial rate constants for each lactone concentration, which were derived from the slope of the ln(1 – conversion) versus time plot. In all cases, the amount of AFEST was kept constant at 100 mg, while the concentrations of ε-caprolactone were varied from 0.125 to 2.000 M in toluene. Ethylbenzene (100 μl) was added as an internal standard, and the total volume was 3 ml. Samples (50 μl) were taken at appropriate time intervals and analyzed by GC to determine the monomer conversion.

2.10. Molecular docking studies

The structural information from the theoretically modeled complex was performed with the InsightII package, version 2000 (Accelrys, San Diego, CA, USA). The 2.2 Å crystallographic structure of AFEST and the 2.1 Å crystallographic structure of lipase CA were used for the starting coordinates for calculations (Protein Data Bank, Brookhaven National Laboratory, code 1JJI and 1TCA [PDB]). The 3D structure of ε-caprolactone was built with the Builder program and optimized using AM1 method.

Docking experiments were performed with both Affinity and Autodock 4.0 programs [22,23] to increase the number of possible candidates of the binding complex. Affinity, which uses a combination of Monte Carlo-type and simulated annealing procedures to dock, is suitable for automatically docking a monomer to an enzyme [22]. The potential of complex was assigned by using the CVFF force field. To account for the solvent effect, the centered, enzyme–monomer complexes were solvated in a sphere of TIP3P water molecules with a radius of 10 Å. This provided 10 structures from SA docking, and the conformations generated were clustered according to RMS deviation. The global structure with the lowest energy was chosen for calculating the interaction energies of the monomer with enzymes. During the Autodock 4.0 process, a conformational search was performed using the Solis and Wets local search method and the Lamarkian genetic algorithm (LGA) was applied to identify the enzyme–monomer interactions [23]. Autodock calculated enzyme–monomer interaction energies over a grid, in which size was set to be 126 × 126 × 126 and the grid space was the default value of 0.375 Å. The Autodock method generated 10 enzyme–monomer configurations. Because the geometrical structures of these models were found to be similar, the global structure with the lowest energy was chosen to calculate the free energy of binding (ΔG). In the current version, the protein was treated as rigid, while the monomer was allowed torsional flexibility.

3. Results and discussion

3.1. Structural and thermal characterization of the product PCL

The recombinant thermophilic esterase AFEST, with a specific activity of approximately 168 U/mg for *p*-nitrophenyl caprylate, was used as a catalyst in the ring-opening polymerization of ε-caprolactone. The product was dried under reduced pressure and characterized by FT-IR, DSC, ^{1}H NMR, and DEPTQ ^{13}C NMR.

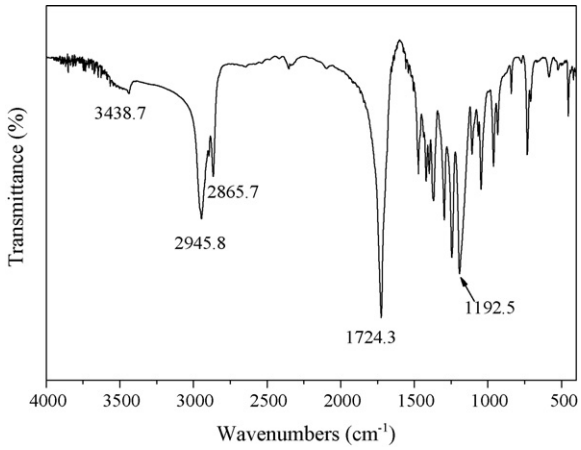

Fig. 1. FT-IR spectrum of the product synthesized by AFEST-catalyzed ring-opening polymerization of ε-caprolactone at 80 °C for 72 h in toluene.

The structure of PCL was confirmed by FT-IR, ^{1}H NMR, and DEPTQ ^{13}C NMR. The FT-IR spectrum of PCL was shown in Fig. 1. The bands near 1724 and 1192 cm^{-1} resulted from C=O and C—O stretching vibrations, respectively. The band at 3438 cm^{-1} indicated the presence of a hydroxyl group, which confirmed the formation of a linear polymer chain. PCL exhibited the bands at 2945 and 2865 cm^{-1}, assigned to C—H moiety. The ^{1}H NMR spectrum as shown in Fig. 2 confirmed the structure of PCL as follows: 1.38 (H_c, m, J = 8.54, 7.02 Hz); 1.65 (H_b and H_d, m, J = 7.32, 7.63, 4.88 Hz); 2.31 (H_a, t, J = 7.32 Hz); 3.65 (H_f, t, J = 6.71 Hz); and 4.06 (H_e, t, J = 6.71 Hz). The triplets at 4.16 ppm (trace amount) were the corresponding methylene protons in the dimmer [24]. Triplets at 2.31 and 2.37 ppm suggested the existence of a diverse mixture of linear and cyclic oligomers. By calculating the areas of peak e and f, the molecular weight of PCL obtained was 1270. The structure of PCL was characterized by DEPTQ ^{13}C NMR spectrum (Fig. 3) as follows: 173.8 (C=O, C-1), 34.5 (CH_2, C-2), 25.9 (CH_2, C-3), 24.9 (CH_2, C-4), 28.7 (CH_2, C-5), 64.5 (CH_2O, C-6), 62.8 (CH_2OH, end group). In the spectrum, the non-protonated carbons C=O (173.8 ppm) and $CDCl_3$ (76.9 ppm) were detected as negative resonances.

The preliminary thermal properties of PCL were characterized by DSC (Fig. 4). The curves indicated that a melting endotherm of PCL was at 58 °C, which was consistent with the reported values

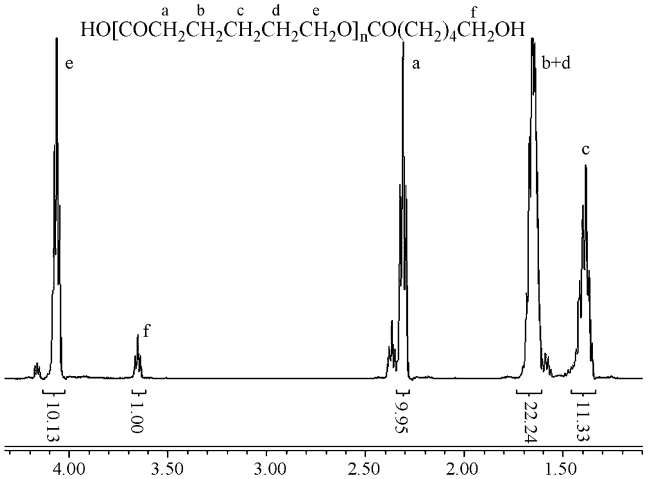

Fig. 2. ^{1}H NMR spectrum of the product synthesized by AFEST-catalyzed ring-opening polymerization of ε-caprolactone at 80 °C for 72 h in toluene. The numbers in the spectrum indicated the integral areas of the peaks.

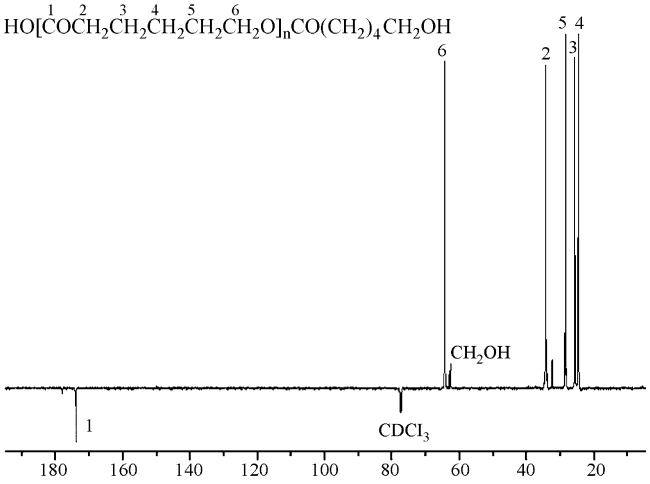

Fig. 3. DEPTQ ^{13}C NMR spectrum of the product synthesized by AFEST-catalyzed ring-opening polymerization of ε-caprolactone at 80 °C for 72 h in toluene.

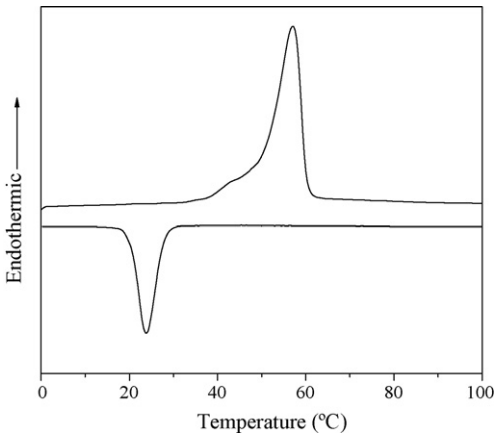

Fig. 4. DSC curve of PCL at the rate of 10 °C/min. The product was synthesized by AFEST-catalyzed ring-opening polymerization of ε-caprolactone at 80 °C for 72 h in toluene.

of PCL synthesized by lipase CA catalysis (54.4–60 °C) [1,21,25,26]. However, the crystallization temperature T_c = 25 °C was lower than that of PCL with M_n value of 9700 and PDI of 1.7 (35 °C) [25], probably due to the lower PDI of the product obtained in toluene (PDI = 1.2) (Table 1).

Table 1
Monomer conversion and product molecular weight M_n in various organic solvents at 80 °C for 72 h

Solvent	log *P*[a]	Monomer conversion[b] (%)	M_n[c]	PDI[c]
Dioxane	−1.10	84	890	1.33
Acetone	−0.23	96	820	1.18
THF	0.49	84	910	1.52
Dichloromethane	0.93	99	1000	1.24
Chloroform	2.00	98	910	1.25
Toluene	2.50	100	1400	1.21
Cyclohexane	3.09	97	1530	1.46
n-Hexane	3.50	100	1580	1.31
Solvent-free	–	99	1340	1.12

[a] log *P* values were cited from "Finding Physical and Chemical Properties" of science and engineering library of Vanderbilt University.
[b] Determined by GC.
[c] Determined by GPC.

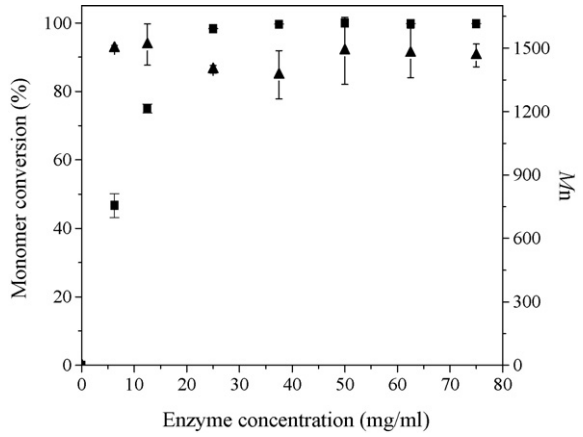

Fig. 5. Monomer conversion (■) and number-average molecular weight M_n (▲) as a function of enzyme concentration. The reactions were carried out using 200 μl ε-caprolactone and 600 μl toluene at 80 °C for 72 h.

3.2. Optimization of reaction conditions

3.2.1. Effect of enzyme concentration

In the enzymatic ring-opening polymerization of ε-caprolactone, the enzyme concentration had a profound effect on both the monomer conversion rate and product molecular weight [27]. Experiments were conducted with different concentrations of AFEST and the same amount of ε-caprolactone (200 μl) and toluene (600 μl). The reactions were performed at 80 °C for 72 h. Monomer conversion and M_n as a function of enzyme concentration were shown in Fig. 5. An increase in monomer conversion was observed with the increased concentration of the enzyme. When the enzyme concentration exceeded 25 mg/ml, the monomer conversion was more than 99%. However, the M_n value remained constant (ca. 1400) and independent of the enzyme concentration. Therefore, in subsequent research, 25 mg/ml of AFEST was employed in the ring-opening polymerization.

3.2.2. Effect of temperature

The effect of temperature on monomer conversion and product molecular weight was investigated at different temperatures in toluene for 72 h. As shown in Fig. 6, during the polymerization, the monomer conversion and product molecular weight were higher at 80 °C than at 45 °C and 60 °C. After 72 h, the monomer conversion increased from 84% at 45 °C to 100% at 80 °C. In addition, the molecular weight of product was also higher at 80 °C

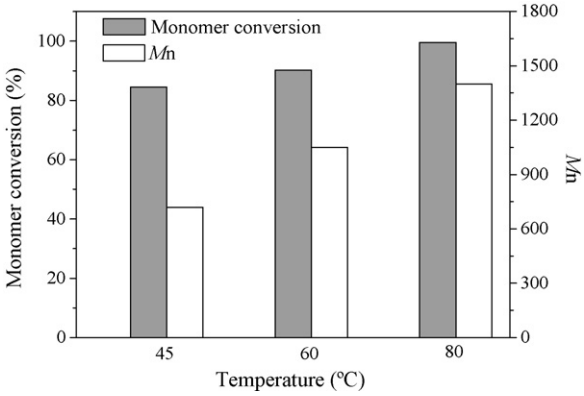

Fig. 6. Effect of temperature on monomer conversion and product molecular weight M_n. The reactions were carried out using 25 mg/ml AFEST, 200 μl ε-caprolactone and 600 μl toluene at 45 °C, 60 and 80 °C for 72 h.

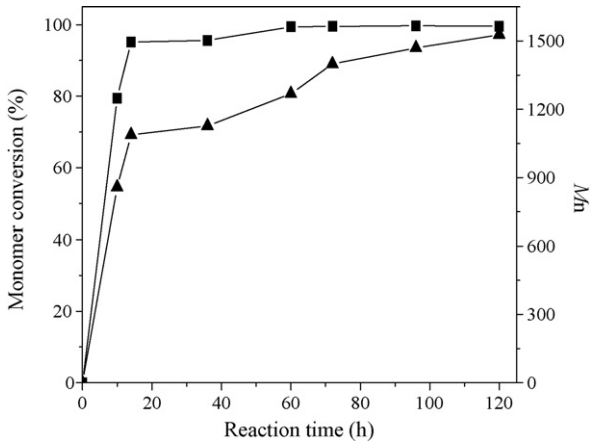

Fig. 7. Monomer conversion (■) and number-average molecular weight M_n (▲) as a function of reaction time. The reactions were carried out using 25 mg/ml AFEST, 200 μl ε-caprolactone and 600 μl toluene at 80 °C.

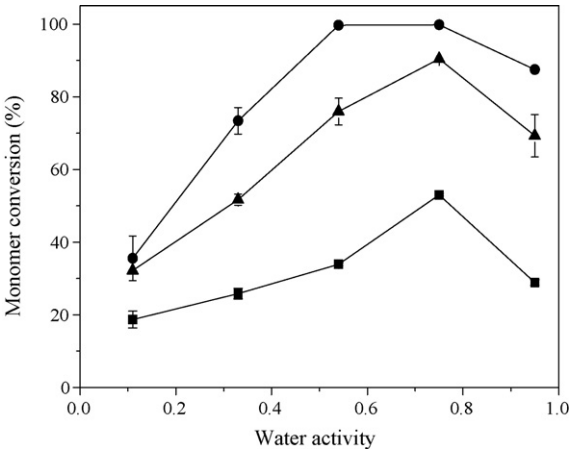

Fig. 8. Effect of water activity on the monomer conversion. The reactions were carried out using 25 mg/ml AFEST, 200 μl ε-caprolactone and 600 μl toluene at 80 °C for 1 h (■), 12 h (▲) and 24 h (●). The reaction systems were pre-equilibrated with saturated salt solutions of different water activity at 25 °C for at least 16 h.

(M_n = 1400, PDI = 1.21) than at 45 °C (M_n = 720, PDI = 1.24) and 60 °C (M_n = 1050, PDI = 1.30). The increased monomer conversions and molecular weights at higher temperatures were partly caused by the decreased diffusion constraints in the reaction mixture, resulting from high viscosity of the product. More importantly, in the ring-opening polymerization, water molecules may be increasingly accessible in the chain initiation reaction, which would accelerate the rate of conversion at higher temperatures [28]. As AFEST was thermal stable, a higher temperature was beneficial to the polymerization reaction because of the higher monomer conversion and molecular weight of the polymer.

3.2.3. Effect of reaction time

The polymerization reaction of ε-caprolactone for different time lengths was performed at 80 °C to investigate the effect of reaction time on monomer conversion and product molecular weight. Polymerizations were carried out using 25 mg/ml of AFEST, 200 μl ε-caprolactone and 600 μl toluene from 1 to 120 h. Monomer conversion and M_n as a function of reaction time were shown in Fig. 7. During the polymerization, the monomer conversion and M_n increased with the reaction time. The monomer conversion reached 95% rapidly within 14 h, in which almost all the monomer engaged in the initiation process of the polymerization. However, in the process of chain propagation, M_n increased slowly from 860 at the reaction time of 14 h to 1530 when the reaction was carried out for 120 h. A comparison of the rate of initiation with that of propagation suggested that the AFEST-catalyzed polymerization was characterized by rapid initiation and slow propagation. Compared with lipase CA-catalyzed synthesis of PCL at 70 °C for 4 h (yield 86%, M_n = 44,800, PDI = 1.7) [12], the products (yield 95%, M_n = 860, PDI = 1.2) catalyzed by AFEST at 80 °C for 14 h were of lower molecular weight during the reaction. Therefore, the thermophilic esterase AFEST was more suitable for preparing PCL of lower molecular weight, which was expected to be widely used as the drug carrier or soft segment of polyurethanes.

3.2.4. Effect of reaction medium

For enzymatic reaction in non-aqueous medium, the solvent played a crucial role in determining enzyme stability and regulating the partitioning of substrates and products between the solvent and the enzyme [28]. The effects of various organic solvents on monomer conversion and M_n at 80 °C for 72 h were summarized in Table 1. Relatively lower monomer conversion was observed in hydrophilic solvents such as dioxane and acetone. In solvents having log P values from 0.93 to 3.50, efficient production of the polymer was achieved, with nearly 100% monomer conversion. It was obvious that a higher molecular weight PCL was produced efficiently in hydrocarbon solvents (toluene, *n*-hexane, and cyclohexane). In toluene, PCL was obtained in almost 100% monomer conversion, with an M_n value of 1400. These results may be due to the deactivation of the enzyme by the hydrophilic solvent in a way that disrupted the functional structure or stripped off the essential water from the enzyme [28]. In addition, because dipole moment and solvent geometry might affect the enzymatic polymerization, log P values did not correlate completely with the monomer conversion and M_n.

Although higher monomer conversion and M_n were obtained in solvents with high log P values (*n*-hexane and cyclohexane), monomer and polymer were of lower solubility in these solvents. Therefore, toluene was the best solvent for producing PCL efficiently and the most appropriate for further kinetic studies.

3.2.5. Effect of water activity

A water molecule acting as a nucleophilic reagent played a key role in the chain initiation step [7,29]. Meanwhile, the limited water content was significant in controlling enzyme performance in organic medium [30]. Determining the optimum level of water in the enzymatic polymerization was desirable. In this study, we pre-equilibrated the reaction systems with saturated salt solutions

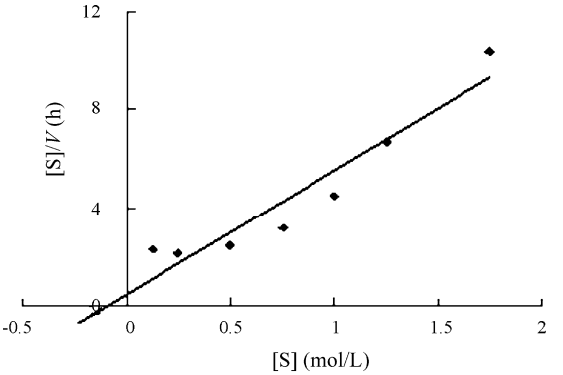

Fig. 9. Hanes–Woolf plot $[S]/V = (K_m/V_{max}) + ([S]/V_{max})$ in AFEST-catalyzed ring-opening polymerization of ε-caprolactone. The Michaelis–Menten constant K_m and the maximal rate of reaction V_{max} were calculated from the interaction with *y*-axis and the slope of the linear fit (R^2 = 0.91). The reactions were carried out at 80 °C with constant enzyme concentration and different lactone concentrations in toluene.

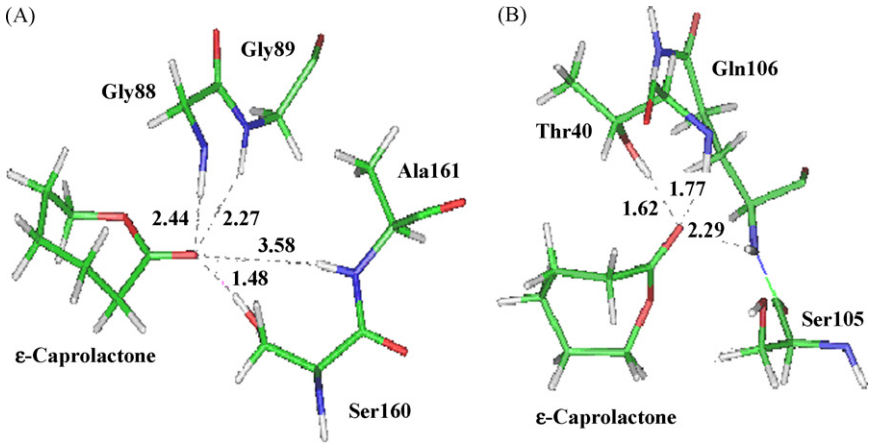

Fig. 10. The hydrogen bonding interactions of the theoretically modeled complexes, AFEST-ε-caprolactone (A) and lipase CA-ε-caprolactone (B). The conformations were developed by Affinity program, and the hydrogen bonds were represented in dashed lines (distances were shown in Å).

to keep the reaction medium with different values of water activity. The reactions were carried out for 24 h, and the effect of water activity on the monomer conversion at indicated time points was investigated. As shown in Fig. 8, the highest monomer conversions were observed at the water activity of 0.75, before the monomers were completely consumed (<24 h). When the reactions proceeded for 24 h, the monomer conversions were almost 100% at water activity of 0.54–0.75. These results showed that the thermophilic esterase AFEST exhibited the bell-shaped profile of water activity (similar to lipase CR), as opposed to many other enzymes that showed high activity in systems with higher water activity [20].

3.3. Michaelis–Menten kinetic evaluation

To get insight into the kinetics of AFEST-catalyzed ring-opening polymerization of ε-caprolactone, K_m and V_{max} values were determined. We used a Hanes–Woolf plot, shown in Fig. 9, to calculate V_{max} and K_m, which were 0.20 mol/(l h) and 0.093 mol/l, respectively. To calculate the turnover number k_{cat}, we determined an active protein content of 92% (w/w) of the enzyme preparation and a molecular weight of 35,500 g/mol of AFEST. The k_{cat} calculated from V_{max} was 0.064 s^{-1}. Compared with lipase CA-catalyzed polymerization of ε-caprolactone at 45 °C in toluene (K_m = 0.72 mol/l; V_{max} = 1.97 mol/(l h); and k_{cat} = 72.9 s^{-1}) [21] and lipase PF-catalyzed polymerization at 60 °C in isopropyl ether (K_m = 0.61 mol/l) [31], AFEST showed much higher affinity for ε-caprolactone. However, its catalytic efficiency (k_{cat}/K_m = 0.69 l/(mol s)) was much lower than that observed for lipase CA-catalyzed polymerization of ε-caprolactone (k_{cat}/K_m = 101.25 l/(mol s)).

It was generally accepted that, in enzymatic ring-opening polymerization, the lactone was activated via the formation of an acyl–enzyme intermediate by the nucleophilic attack of serine residue on the lactone carbonyl. The chain initiation and propagation occurred by the deacylation of the nucleophiles, such as water molecules and terminal hydroxyl group of the growing polymer chain [32]. According to this mechanism, our data suggested that, compared with lipase CA, AFEST could incorporate the monomer into its active site more rapidly, but the formation of acyl–enzyme intermediate, and the chain initiation and propagation were much slower.

3.4. Molecular docking studies

Studies of molecular docking of ε-caprolactone to AFEST and lipase CA were undertaken to investigate the structural and energetic basis of the high affinity of AFEST to the monomer ε-caprolactone. The model of the enzyme–monomer complex was developed using the Affinity module. Generally, hydrogen bonds play an important role for the stability of enzyme–substrate complex and were chosen to investigate the binding ability. As shown in Fig. 10A, hydrogen bonding interactions formed between the carbonyl group of the monomer and the active residues of AFEST. Three strong hydrogen bonds were formed between carbonyl O of ε-caprolactone and OH of active residue Ser160, and NH of residues of oxyanion hole (Gly88 and Gly89), respectively. In addition, NH of Ala161 of oxyanion hole formed a weak hydrogen bond with the carbonyl O of ε-caprolactone. Compared with AFEST, only three hydrogen bonds were formed between carbonyl O of ε-caprolactone and residues of oxyanion hole of lipase CA (Thr40 and Gln106) (Fig. 10B). Furthermore, the interaction energies, including the total, van der Waals and electrostatic, and the free energies of binding (ΔG) were calculated from Affinity and Autodock 4.0 programs, respectively (Table 2). The total energy E_{total} of AFEST to the monomer (−33.81 kcal mol^{-1}) was lower than that of lipase CA (−30.17 kcal mol^{-1}), and the free energy of binding of AFEST, calculated from Autodock 4.0, was −5.15 kcal mol^{-1}, which was also lower than that of lipase CA (−3.27 kcal mol^{-1}). These results suggested that AFEST had a stronger interaction with ε-caprolactone than lipase CA, which might lead to a higher affinity for the monomer, and they were consistent with the experimental results obtained by Michaelis–Menten kinetic analysis.

Table 2
The total energy E_{total}, van der Waal energy E_{vdw}, electrostatic energy E_{ele} and free energy of binding ΔG of the monomer ε-caprolactone tested for AFEST and lipase CA binding

Enzymes	E_{vdw}[a] (kcal mol^{-1})	E_{ele}[a] (kcal mol^{-1})	E_{total}[a] (kcal mol^{-1})	ΔG[b] (kcal mol^{-1})
AFEST	−19.31	−14.50	−33.81	−5.15
Lipase CA	−16.62	−13.55	−30.17	−3.27

[a] Calculated from Affinity program.
[b] Calculated from Autodock 4.0 program.

4. Conclusions

In this paper, PCL was successfully synthesized using a novel thermophilic esterase AFEST from the archaeon *A. fulgidus* as the catalyst. The synthesized PCL was of low molecular weight, and expected to be widely used as the soft segment of polyurethanes. Compared with the reported lipases, the enzyme AFEST had the

highest affinity for ε-caprolactone, and this finding was validated by the theoretical modeled complex. This work could provide a new route for polyester synthesis and help to broaden the application of thermophilic esterases.

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