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Enzyme-Catalyzed Ring-Opening Polymerization of Unsubstituted β-Lactam

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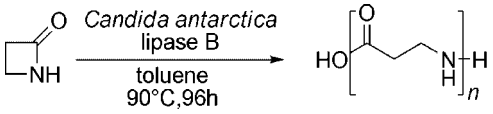
The synthesis of poly(β-alanine) by *Candida antarctica* lipase B immobilized as novozyme 435 catalyzed ring-opening of 2-azetidinone is reported. After removal of cyclic side products and low molecular weight species pure linear poly(β-alanine) is obtained. The formation of the polymer is confirmed with ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry. The average degree of polymerization of the obtained polymer is limited to $\overline{DP} = 8$ by its solubility in the reaction medium. Control experiments with β-alanine as a substrate confirmed that the ring structure of the 2-azetidinone is necessary to obtain the polymer.



Introduction

Enzymatic polymerizations proceed with high regio-, enantio-, and chemoselectivity under relatively mild conditions. Enzymes have been used so far to synthesize polyesters, polysaccharides, polycarbonates, polyphenols, polyanilines, vinyl polymers, and poly(amino acid)s.[1,2]

The lipase B of *Candida antarctica* immobilized on polyacrylic resin (Novozyme 435) has proven to be a very versatile catalyst in terms of reaction conditions and the acceptance of various substrates, e.g., this enzyme has been used successfully to synthesize polyesters from linear[3] and cyclic[4–7] starting materials. Little, however, has been reported on synthesizing polyamides catalyzed by enzymes.[8,9]

It is known that nylons can be produced from the corresponding amino acids or by anionic ring-opening polymerization of 5–13 membered unsubstituted lactams.

Poly(β-alanine) or nylon 3, however, cannot be obtained by either polymerization of β-alanine or the ring-opening of the unsubstituted 2-azetidinone (β-lactam). For application in, e.g., cosmetics,[10] water purification,[11] and construction,[12] poly(β-alanine) is synthesized by anionic polymerization of acrylamide in the presence of a strong base.[13] Unfortunately, this method of polymerization leads to branched polymers.[14]

In this communication, we present a new enzymatic route using *Candida antarctica* lipase B immobilized as novozyme 435 to produce unbranched poly(β-alanine) starting from unsubstituted 2-azetidinone. Ring-opening of substituted β-lactams with lipase was reported before[15] but not with the aim to polymerize.

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Experimental Part

Materials

Novozyme 435 (N435) was dried for 24 h at 55 °C over P_2O_5 under reduced pressure. For the control reactions N435 was deactivated by heating to 150 °C for 2 h. Chlorosulfonyl isocyanate 98% (Acros), potassium borohydride (Fluka), dichloromethane (Lab-Scan), ethanol (Merck), sulfuric acid 95–97% (Merck), chloroform (Analytical Sciences), 4-nitrophenylacetate (Sigma-Aldrich), deuterated chloroform (Aldrich), and deuterium oxide (Aldrich) were used as received. ε-Caprolactone (Union Carbide) and vinyl acetate (Acros) were distilled from CaH_2. Toluene was distilled from sodium. β-Alanine (Fluka) was dried for 24 h at 55 °C on P_2O_5 under

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reduced pressure. 2-Azetidinone was either bought (Maybridge) or synthesized according to literature procedures[16,17] and dried over P_2O_5 at room temperature under reduced pressure prior to polymerization. Sodium sulfite heptahydrate was prepared from anhydrous sodium sulfite (Fluka).

Methods

^{1}H NMR spectra were recorded using a 300 MHz Varian VXR-300 apparatus. MALDI-TOF-MS measurements were performed on a Voyager-DE PRO spectrometer in reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix. UV-vis measurements were carried out on a PYE UNICAM SP8-200 UV-Vis spectrophotometer. A TA instruments DSC Q1000 was used to determine the melting point of 2-azetidinone. All reactions were carried out in flame-dried glassware under a nitrogen atmosphere.

Synthesis of 2-Azetidinone

Vinyl acetate (100 mL, 1.08 mol) was cooled using an acetone/liquid N_2 mixture and to this chlorosulfonyl isocyanate (17.4 mL, 0.2 mol) was added while keeping the temperature between 20 and 25 °C. After addition of the isocyanate, the mixture was stirred for 20 min and subsequently cooled rapidly to −20 °C.

The obtained red-brownish chlorosulfonyl-β-lactam (I) solution was added dropwise to a mixture of water (20 mL), ice (90 g), sodium bicarbonate (47 g, 0.56 mol), and sodium sulfite heptahydrate (33 g, 0.13 mol) and stirred vigorously. The color of the reaction mixture changes to yellow. The reaction mixture was stirred for 15 min until no more gas evolved. After filtration, the vinyl acetate phase was separated, dried over Na_2SO_4 and $NaHCO_3$, and filtrated again. The residual vinylacetate was removed by rotary evaporation at 40 °C. The water phase was extracted five times with cold (−15 °C) dichloromethane. The dichloromethane solution was added to the residue of the organic phase. The solvent was removed by rotary evaporation, yielding the dark yellow oily 4-acetoxy-2-azetidinone (II) yield (40% determined by weight).

The crude 4-acetoxy-2-azetidinone was dissolved in 20 mL of water. This solution was added to KBH_4 (1.5 mol excess compared to II) in water (20 mL) whilst keeping the temperature of the reaction mixture at 30 °C. When no more gas evolved, the mixture was neutralized with a sulfuric acid solution. After filtration, the water was evaporated and the residue mixed with chloroform. Filtration and evaporation of the solvent yields 2-azetidinone. The resulting crystals were purified by short path distillation, yielding a white crystalline product (20%) m.p. 75 °C (lit 73–76 °C).

^{1}H NMR ($CDCl_3$): $\delta = 5.72$ (s, 1H, NH) 3.31(t, $J = 4.2$ Hz, 2H, CH_2); 3.03(t, $J = 4.05$ Hz, 2H, CH_2) ($C_3H_5NO_9$) Calcd. C 50.69, H 7.00, N 19.71, O 22.51; Found C 50.57, H 7.13, N 19.54, O 22.76.

Polymerization of 2-Azetidinone

A mixture of 2-azetidinone (100 mg, 1.41 mmol), N435 (100 mg), and dry toluene (5 mL) was stirred for 96 h at 90 °C. The suspension was allowed to cool and the toluene was removed by rotary evaporation. Ethanol (10 mL) was added to the white residue and the mixture was stirred for 15 min. After removal of the ethanol by rotary evaporation, the residue was washed with water yielding 73 mg of a yellow product (yield 73%).^{1}H NMR (D_2O): $\delta = 3.3$ (m, 2H, CH_2) 3.12 (t, 2H, CH_2) 2.52 (t, 2H, CH_2) 2.29 (m, 2H; CH_2)

Control Reactions

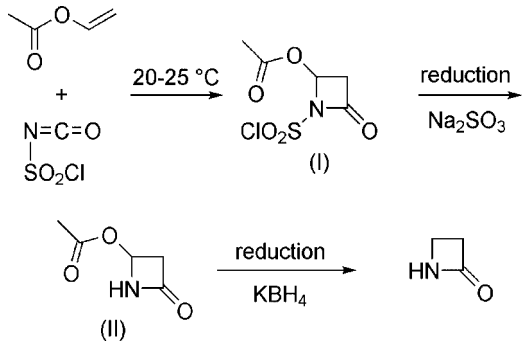
The polymerization was repeated with deactivated enzyme plus additional (10 times the amount present in the enzyme) water (0.025 mL, 1.4 mmol) resulting in less then 5% yield probably due to rest activity of the biocatalyst. The polymerization was repeated with β-alanine as a monomer which resulted in no yield. The polymerization was also repeated with β-alanine (100 mg) or water (0.025 mL, 1.4 mmol) as an initiator which also resulted in no yield.

Hydrolytic Activity Assay

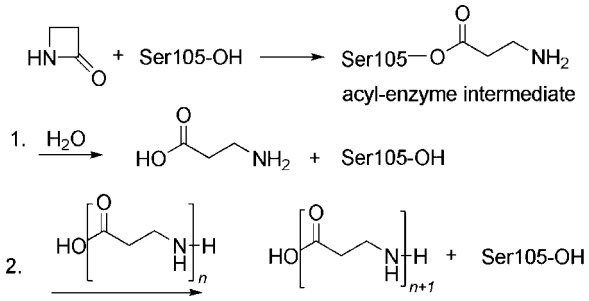
N435 (10 mg) and toluene (20 mL) were stirred at 90 °C for a definite period of time (0, 24, 48, and 72 h). The mixture was cooled to 40 °C and a solution of *p*-nitrophenyl acetate (5 mL, 7.25 mmol · L^{-1}) was added in toluene. Aliquots of 1 mL are withdrawn at 0, 15, 30, 45, 60, 90, and 120 min. Each sample was filtered and 0.5 mL of the filtrate was dissolved in 9.5 mL of toluene. The *p*-nitrophenol concentration was determined by UV photospectrometry. UV-vis (pNP): $\lambda_{max}(\varepsilon) = 304$ nm (10 349.543 $M^{-1} \cdot cm^{-1}$), (pNPA): $\lambda_{max}(\varepsilon) =$ 275 nm (2167.011 $M^{-1} \cdot cm^{-1}$). The activity was calculated as nmol substrate converted by 1 mg N435 per minute.

Synthetic Activity Assay

For each period (0, 24, 48, and 72 h), a mixture of N435 (100 mg) and toluene (5 mL) was stirred at 90 °C. ε-Caprolactone (1 mL, 9 mmol) was added and stirred for 5 h. After 5 h, two drops of the solution were withdrawn and the conversion of ε-caprolactone was determined with ^{1}H NMR.[18] The activity was determined by comparing the conversion after each time span with the conversion by untreated N435.



Scheme 1. Synthesis of 2-azetidinone.



Scheme 2. The proposed pathway of the ring-opening polymerization. The acyl-enzyme intermediate is formed and attacked. (1) By water to release β-alanine. (2) By a growing oligomer ($n = 1, 2, \ldots$) to yield the poly(β-alanine).

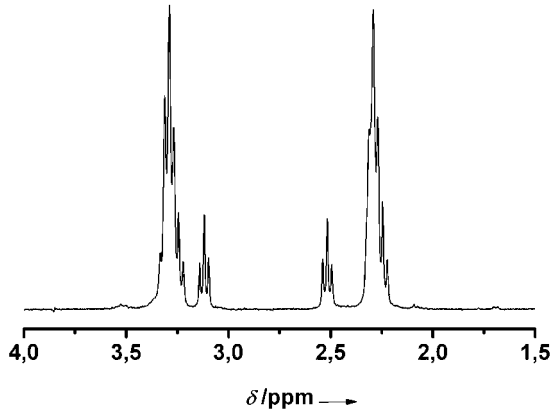


Figure 2. ^{1}H NMR spectrum of poly(β-alanine).

Results and Discussion

2-Azetidinone was synthesized by the addition of chlorosulfonylisocyanate to vinyl acetate followed by a reduction[16] with Na_2SO_3 to produce the 4-acyloxyazetidinone. This species is reduced[17] with KBH_4 to yield the 2-azetidinone, see Scheme 1. The ring-opening polymerization of 2-azetidinone (Scheme 2) proceeds readily with N435 at 90 °C for 96 h. The reaction is essentially carried out under anhydrous conditions; however, water can never be removed completely, as it will always be present as the structural water of the enzyme.

Great care was taken to rule out that the ring-opening polymerization proceeds via any other mechanism than via enzyme catalysis. Attempts to initiate the ring-opening polymerization of 2-azetidinone with water or β-alanine without enzyme resulted in no product. A control reaction with deactivated N435 resulted in less then 5% product probably due to rest activity in the beads. To check whether the enzyme is still active at the end of the polymerization we incubated N435 for 72 h in toluene and found that the hydrolytic activity decreased by just 9% and the synthetic activity was not changed when compared to the non-incubated enzyme. With these experiments we have proven that the ring-opening polymerization of 2-azetidinone indeed proceeds via enzyme catalysis even at longer reaction times.

The MALDI-TOF mass spectrum of the poly(β-alanine) shows a distribution with peaks up to 1 319.7 m/z corresponding to 18 monomeric units, see Figure 1. The mass increment of 71 m/z corresponds to one monomeric unit. In the ionization process adducts with sodium and potassium of the linear chains are formed. These adducts can be found as a second distribution of peaks between 300 and 700 m/z. The poly(β-alanine) was analyzed with ^{1}H NMR spectroscopy as well, see Figure 2. The main chain protons ($\delta = 3.3$, 2.29 ppm) and the protons next to the endgroups (amine $\delta = 3.12$ ppm) (carboxylic acid $\delta = 2.52$ ppm) can be identified. The average molecular mass of the polymer was determined to be 586 g · mol^{-1} ($\overline{DP} = 8$). This value is in good agreement with the maximum of the distribution found with MALDI-TOF-MS. At this stage the polymer precipitates from the reaction mixture.

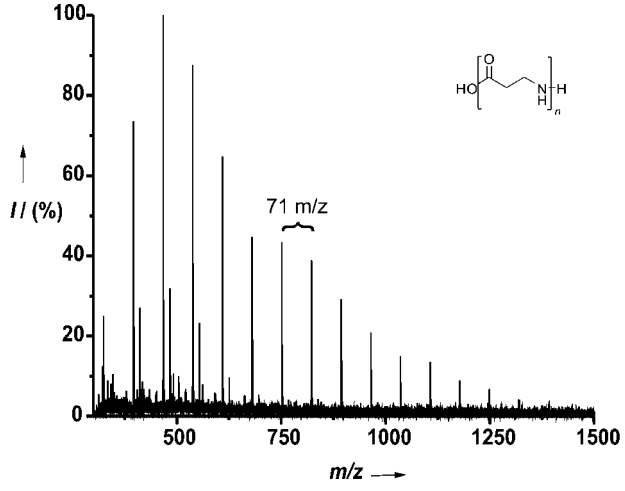


Figure 1. MALDI-TOF mass spectrum of poly(β-alanine).

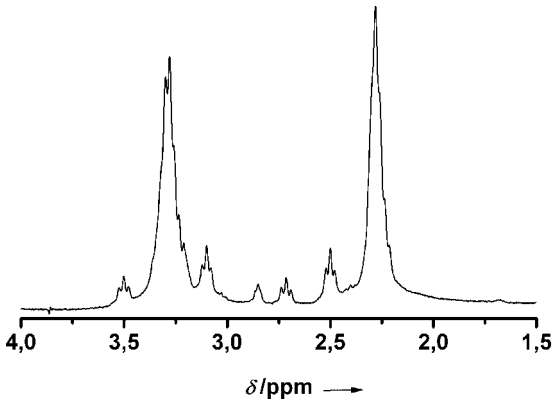


Figure 3. ^{1}H NMR spectrum of the crude polymer.

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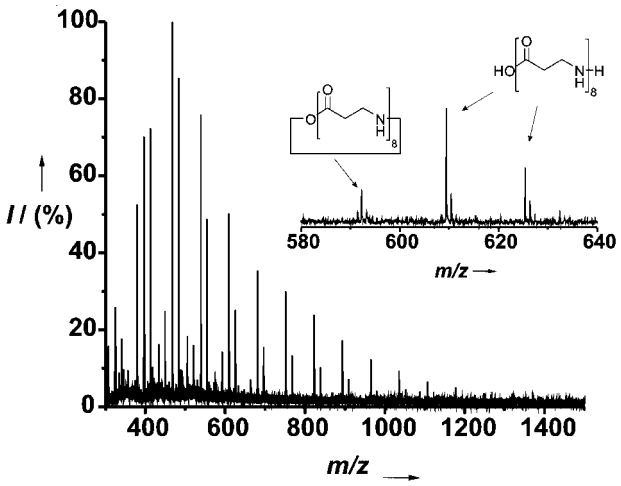

Figure 4. MALDI-TOF mass spectrum of the crude polymer.

The crude polymeric precipitate was also analyzed by ^{1}H NMR (Figure 3). According to the ^{1}H NMR spectrum there are small traces of β-alanine ($\delta = 3.02$, 2.4 ppm) and monomer ($\delta = 3.2$, 2.85 ppm) left in the crude product. In the MALDI-TOF mass spectrum (Figure 4) of the crude precipitate, the cyclic structures appear as sodium adducts in the area of 300–600 *m/z*.

We suggest that the polymerization proceeds analogous to the mechanism proposed for lipase catalyzed ε-caprolactone polymerization,[7] see Scheme 2. An acyl-enzyme intermediate is formed between the 2-azetidinone and the enzyme. In the first stage (route 1 in Scheme 2) of the reaction, this intermediate is attacked by water present in the enzyme, liberating β-alanine. The β-alanine is essentially a growing chain with $n = 1$. Growing chains of any length can attack a newly formed acyl-enzyme intermediate (route 2 in Scheme 2). An attempt to polymerize β-alanine with Novozyme 435 did not yield any polymer. This indicates that the ring-structure is essential in forming the acyl-enzyme intermediate. Currently we are further investigating the proposed mechanism.

Conclusion

In conclusion, we have shown that Novozyme 435 is able to catalyze the polymerization of 2-azetidinone to form a linear polyamide. The maximum chain length obtained is 18 monomeric units. Also we have proven that the enzyme stays active under the mentioned reaction conditions. β-Alanine is formed during the reaction but it cannot be polymerized by the enzyme. Water is present in the enzyme and necessary for the polymerization but is not able to initialize the polymerization. The results also show that our purification method is successful because neither β-alanine nor cyclic oligoamides can be observed in the product.

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