Chapter 2
N435-catalyzed ring-opening polymerization of β-propiolactam

2.1 ABSTRACT

The successful polymerization of β-propiolactam to poly(β-alanine) catalyzed by the immobilized lipase B of Candida antarctica yeast (N435) is reported in this chapter. From a collection of lactam rings (4, 7, 9 and 13 membered rings) the 4-membered β-propiolactam is a good substrate for the enzyme. The synthesis of the monomers and the subsequent polymerization is reported and the influence of the reaction conditions is discussed. The polymer is characterized by 1H-NMR spectroscopy and MALDI-ToF mass spectrometry. Cyclic reaction products were removed and pure linear poly(β-alanine) is obtained.

Reaction conditions are of great influence on the activity of the N435 in this reaction and the resulting polymer. The best results were obtained with N435 (dried for 24 hours in vacuo at 46 °C) in toluene of 55 °C for a period of 96 hours. Control experiments showed that initiation by water or carrier material does not take place. Polymerization of the hydrolyzed lactam ring, the amino acid β-alanine was not feasible by the N435 catalyst. Showing that the β-propiolactam ring is the actual monomer for the reaction.

It was expected that the ring-opening polymerization of β-propiolactam proceeds according to the established mechanism for enzymatic ε-caprolactone polymerization. In this mechanism a hydroxy acid is the activated monomer, in our case this molecule is resembled by the amino acid β-alanine. From the observation that β-alanine cannot be polymerized it is concluded that the polymerization does not proceed according to the mechanism for enzymatic lactone polymerization. A new mechanism, developed by molecular modeling that explains the experimental results, is briefly discussed.
2.2 INTRODUCTION

The enzymatic ring-opening polymerization of lactones, lactides, cyclic carbonates and depsipeptides by various hydrolases has been studied extensively over the past decade as reviewed by different authors.\textsuperscript{1,2,3} To the best of our knowledge nothing was published on the enzymatic ring-opening polymerization of lactams. Enzymes catalyze reactions at ambient temperatures with a high selectivity and they can do so outside their natural environment in an organic solvent.\textsuperscript{4,5} When enzymes can be used in the synthesis of polyamides a reduction of the energy consumption and the use of less toxic compounds in the production process of polyamides will benefit the environment.

Poly(β-alanine) or nylon 3 is applied in in e.g. cosmetics, water purification and construction.\textsuperscript{6} The conventional synthesis of this polymer is by anionic polymerization of acrylamide in the presence of a strong base.\textsuperscript{9} Unfortunately, this method of polymerization leads to branched polymers.\textsuperscript{10} Direct polymerization of β-alanine or ring-opening polymerization of β-propiolactam by conventional methods is not possible.

This chapter introduces the enzymatic ring-opening polymerization of β-propiolactam as a new way to obtain linear poly-β-alanine.

2.2.1 Cal-B catalyzed ring-opening polymerization of lactones

Enzymatic ring-opening polymerization was first reported in 1993 and involved the ring-opening polymerization of ε-caprolactone and δ-valerolactone by lipases from Pseudomonas fluorescens, Candida cylindracea and porcine pancreatic lipase.\textsuperscript{3,11} Unsubstituted lactones with a ring size from 4 to 16 have since been polymerized by using Candida antarctica lipase B (Cal-B) among other lipases from different microorganisms see Figure 2-1. But also the methyl, ethyl and propyl substituted lactones were polymerized by lipases from different origins.

![Reaction scheme of the Cal-B catalyzed ring-opening polymerization of lactones.](image)

The reaction conditions for the ring-opening polymerization of ε-caprolactone have been studied towards the influence of water content, organic solvent and temperature.\textsuperscript{13,14} Decreasing the amount of water in the system produced the highest molecular weight polyester. Too much water present resulted in hydrolysis of the
desired polyester. However, not all the water should be removed since the enzymes need a small amount of water present as part of their structure referred to as bound or structural water.\textsuperscript{15}

Although the polymerization of lactones was the fastest at 90 °C, a higher degree of polymerization was found at 60 °C. This is attributed to the decreased amount of growing chains compared to the polymerization at 90 °C.\textsuperscript{13} The reaction speed however dropped significantly by lowering the temperature.

Mechanism of enzyme catalyzed lactone polymerization

The generally accepted mechanism for the ring-opening polymerization of ε-caprolactone by Cal-B proposed by different authors\textsuperscript{12,16} is depicted in Figure 2-2.

The first step is the formation of an acyl-enzyme intermediate (see chapter 1) by a nucleophilic attack of serine105 on the ε-caprolactone carbonyl group. Secondly a hydroxy acid is released from the serine moiety by hydrolysis of the acyl-enzyme intermediate. In the third step a new intermediate is attacked by the hydroxy acid and

**Figure 2-2. General accepted mechanism for the enzymatic ε-caprolactone polymerization**
the growing chain is released from the serine.

Polymers are formed when the serine forms an acyl-enzyme complex with a growing chain and is subsequently attacked by a hydroxy acid of one or more monomeric units. The acyl-enzyme complex can be formed with a carbonyl at the chain end and with a carbonyl group of the main chain.

It is a common feature of all polycondensations to form macrocycles\textsuperscript{17} and this was also found for enzymatic lactone polymerizations,\textsuperscript{18,19}

**β-Lactams are suitable substrates for Cal-B**

The enzymatic hydrolysis of β-lactams is a convenient method to obtain enantiomeric pure β-amino acids\textsuperscript{20,21,22} and shows that some enzymes are capable of binding β-lactams in their active site and able to perform reactions on them.

The number of lipases available for this reaction however, is limited due to the irreversible binding of β-Lactams and β-lactones to the serine in the active site of some lipases and proteases.\textsuperscript{23,24} For this reason β-lactams are used in antibiotics. They bind irreversibly to enzymes that are involved in cross-linking reactions in bacterial cell wall synthesis, the penicilne binding proteins,\textsuperscript{25,26} Limiting the growth of bacteria is closely related to the inhibition of these enzymes.\textsuperscript{25,27}

One of the lipases that is not inhibited by these compounds is Candidaantarctica lipase B. It is capable of performing the enantioselective ring opening of β-lactams without deactivation as was shown by Fülöp and coworkers.\textsuperscript{28} see **Figure 2-3**. Starting from a racemic mixture only one of the enantiomers was hydrolyzed. Substituents on the β-lactam can be cyclic and bicyclic with or without aromaticity.\textsuperscript{29,30,28,31}

**Figure 2-3. Hydrolysis of substituted β-lactams.**\textsuperscript{28}

The reaction was performed in the organic solvents toluene and isopropylether or a mixture of these solvents with different alcohols. Reasonable conversions (30 % or higher) were found with long chain alcohols and secondary or tertiary alcohols as a co-solvent,\textsuperscript{30} but hydrolysis was also performed in a solvent free system.\textsuperscript{28} In addition, the enzymatic formation of lactam rings (sizes 5-7) by ring closure of β-amino acid esters in organic solvents was reported by Arie Gutman and coworkers.\textsuperscript{22} The reaction was catalyzed by porcine pancreatic lipase and different proteases.
2.2.2 Lactams chosen are the analogues of polymerized lactones

For the enzymatic ring opening polymerization the 4, 7, 9 and 13 membered lactam rings were chosen as monomers. For each lactam monomer the lactone equivalent is listed in Table 2-1. All the mentioned lactones were already polymerized by others\(^2,33,34\) using Candida antarctica lipase B as the catalyst.

The \(M_n\) mentioned for the lactone polymers is reached by using N435 or free Cal-B except for the octanolide that was polymerized by porcine pancreatic lipase (PPL). Other lipases that polymerize lactones are Candida cylindracea lipase (\(\beta\)-propiolactone), porcine pancreatic lipase (\(\beta\)-butyrolactone) and Pseudomonas cepacia lipase (octanolide). Candida antarctica lipase B is not always the best catalyst but it is the most versatile of the lipases mentioned and accepts most of the lactones. The polymerizations of the lactones were performed in the bulk or in a hydrophobic solvent like toluene, isoctane or supercritical CO\(_2\).

**Table 2-1. Lactone monomers for Cal-B, the \(M_n\) reached and their lactam equivalents.**\(^2,33,34\)

<table>
<thead>
<tr>
<th>Ringsize</th>
<th>Lactam</th>
<th>Lactone</th>
<th>(M_n) (g mol(^{-1})) poly lactones</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>(\beta)-propiolactam</td>
<td>(\beta)-propiolactone</td>
<td>510</td>
</tr>
<tr>
<td></td>
<td>(\beta)-methyl-(\beta)-propiolactam(^1)</td>
<td>(\alpha)-methyl-(\beta)-propiolactone</td>
<td>(~10\ 000)</td>
</tr>
<tr>
<td>7</td>
<td>(\varepsilon)-caprolactam</td>
<td>(\varepsilon)-caprolactone</td>
<td>44800</td>
</tr>
<tr>
<td>9</td>
<td>(\eta)-caprylactam</td>
<td>octanolide(^2)</td>
<td>5200</td>
</tr>
<tr>
<td>13</td>
<td>(\omega)-laurolactam</td>
<td>dodecanolide(Cal-B)</td>
<td>6100</td>
</tr>
</tbody>
</table>

\(^1\) referred to as 4-methyl-azetidin-2-one in the following section, the \(\alpha\)-methyl-lactam was not available.

\(^2\) Polymerized by porcine pancreatic lipase (PPL)

From the mentioned lactams only the \(\omega\)-laurolactam and \(\varepsilon\)-caprolactam are commercially available. The \(\beta\)-propiolactam, 4-methylazetidin-2-one and the capryllactam were synthesized in our laboratory. Toluene and i-PrO\(_2\) are selected as possible solvents for the enzymatic polymerization of the mentioned lactams.

**Synthesis of \(\beta\)-propiolactam**

The lactam equivalent of \(\beta\)-propiolactone was produced by 2,2-cycloaddition of chlorosulfonylisocyanate to vinylacetate followed by two reduction steps (see Figure 2-4).\(^{35,36,37,38}\)
Synthesis of 4-methyl-azetidin-2-one

Two reactions were used to synthesize the 4-methyl-azetidin-2-one since the corresponding olefin was not available to use the cycloaddition method used for the synthesis of β-propiolactam. Two ring closure reactions starting with this monomer was synthesized by ring closure of D,L-3-amino butyric acid using triphenylphosphine / tetrachloromethane (method I, Figure 2-5) 39 or 2-chloro-1-methylpyridinium iodide (method II, Figure 2-6) 40 as a transfer agent.

Figure 2-5. Reaction scheme of the synthesis of 4-methyl-β-propiolactam by method I.

The 4-methyl-azetidin-2-one was obtained but the triphenylphosphine oxide produced in the reaction can hardly be removed from the reaction mixture. In method II 2-chloro-1-methylpyridinium iodide is used as the agent (Figure 2-6). 40

Figure 2-6. Reaction scheme of the synthesis of 4-methyl-β-propiolactam by method II.

In this reaction a N-methylpyridone is formed that can be removed from the product mixture by column chromatography to yield the desired product. For both methods it must be noted that a polymerization might start under basic reaction conditions as observed by Escalante and coworkers. 41
**Synthesis of capryllactam**

Capryllactam was synthesized according to the method of DSM\textsuperscript{42} and involves the Beckman rearrangement\textsuperscript{43} of cyclooctanoneoxime (Figure 2-7.). The reaction starts with the oximation of cyclooctanone to cyclooctanoneoxime by a reaction with hydroxylammonium sulphate under acidic (pH 4.5) conditions. The rearrangement was performed with sulfur trioxide. Capryllactam is extracted with toluene from the reaction mixture and obtained in 41 % yield.

![Reaction scheme for the synthesis of capryllactam via a Beckman rearrangement of the oxime.](image)

**Figure 2-7.** Reaction scheme for the synthesis of capryllactam via a Beckman rearrangement of the oxime.
2.3 EXPERIMENTAL

2.3.1 Materials and methods

Materials
Novozyme 435 (N435) kindly donated by Novozymes company was dried in vacuo for at least 24 hours over P₂O₅ (ACROS) before use. For the control reactions N435 was deactivated by heating it to 150 °C for 2 h. Toluene (Lab-Scan) was purified prior to use by distillation from sodium. e-Caprolactone (Union Carbide) and vinyl acetate (ACROS) were dried with CaH₂ (Merck) and distilled. β-Alanine (Fluka) and β-alanine ethylester (Sigma-Aldrich) were dried for 48 hours at room temperature over P₂O₅ in vacuo. e-Caprolactam, o-laurolactam were obtained from Sigma-Aldrich. β-Propiolactam was bought from Maybridge or synthesized in our laboratory. Capryllactam and 4-methyl-azetidin-2-one were synthesized in our laboratory. All lactam monomers were dried over P₂O₅ before use. Na₂SO₃·7H₂O was prepared from anhydrous Na₂SO₃ (Sigma-Aldrich). The water used is demineralized water from an in house tap.

Other chemicals were used as received from their suppliers: Trifluoroacetic acid, sulfuric acid (95-97%), Na₂SO₄, NaOH, ammonia, triphenylphosphine, NaCl, ethanol, and ortho-dichlorobenzene were obtained from Merck. Chlorosulfonyl isocyanate (98%), Acetonitril, NaHSO₃, ethyl acetate, di-amino butyric acid, triethylamine, ethylacetate and 1,4-dioxane were obtained from ACROS. Hydroxylamine sulfate, cyclooctanone, ammonium sulfate, NaHCO₃, α-cyano-4-hydroxycinnamic acid, diisopropylether and 2-chloro-1-methylpyridinium iodide were obtained from Sigma-Aldrich. Methanol and acetone were obtained from Lab-Scan and tetrachloromethane was obtained from Riedel de Haen.

Methods

¹H-NMR spectra were recorded using a 300 MHz and 400 MHz Varian VXR apparatus using deuterated chloroform or deuterium oxide as the solvent.

MALDI-ToF-MS measurements were performed on a Biosystems Voyager-DE PRO spectrometer in reflector mode with α-cyano-4-hydroxycinnamic acid as the matrix and acetonitril/H₂O (50/50) with 0,5 % TFA as the solvent. Calibration was performed with a mixture consisting of matrix dimer, bradykinin, angiotensin I and ACTH 18-39. The spots were created by mixing the matrix solution (10 mg mL⁻¹) with the sample solution (3 mg mL⁻¹) in a 1:1 ratio by volume. Sometimes a 10-fold dilution of the sample is needed to obtain a decent mass spectrum.
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 β-propiolactam.

Short-path distillation was carried out with a Büchi GKR-50 Kugelrohr apparatus. Elemental analysis was done using an EuroVector EA3000.

2.3.2 Synthesis of the monomers

Synthesis of β-propiolactam

Vinyl acetate (100 mL; 1.08 mol) was cooled using a acetone/liquid N: 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 minutes 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 sulphite 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 minutes until no more gas evolved. After filtration, the vinyl acetate phase was separated and dried over Na2SO4 and NaHCO3 and was filtrated again. The residual vinylacetate was removed by rotary evaporation at 40 °C. The water phase was extracted 5 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 KBH4 (1.5 moles 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 (1 M). After filtration the water was evaporated and the residue mixed with chloroform. Filtration and evaporation of the solvent yields β-propiolactam. The resulting crystals were purified by short path distillation, yielding a white crystalline product (20 %) m.p. 75 °C (lit 73-76 °C).

1H-NMR (CDCl3): δ= 5.72 (s, 1H; NH) 3.31(t, J=4.2 Hz 2H; CH2) ; 3.03(t, J=4.05 Hz, 2H; CH3)

Elemental analysis(C7H8NO2) : 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

- 25 -
*Synthesis of capryllactam*

The reactions are carried out in 500 mL 3-neck roundbottom flasks for efficient cooling. A solution of hydroxylammonium sulphate (30 g; 0.183 mole) in water (25 % m/v) was heated to 75 °C and while stirring vigorously small amounts of cyclooctanone (15.4 g; 0.121 mole) were added while maintaining a pH of 4.5 using a sodium hydroxide solution (25 % w/w). The mixture was stirred at 75 °C for 1 hour. The organic layer (cyclooctanone oxime) was separated and nitrogen was bubbled overnight through the reaction mixture at 55 °C to reduce the water content. A solution (0.6 %) of sulfur trioxide was created by dissolving oleum (21.6 mL, 0.16 mole) in cold (0 °C) sulfuric acid (65 mL). The solution was kept between 0 and 5 °C while adding the cyclooctanone oxime (14.24 g, 0.1 mole) in 3 hours time yielding a bright orange solution.

Sulfuric acid (30 mL) was cooled to 0 °C and a 65 % oleum solution (10 mL, 0.067 mole) was added dropwise to this to obtain a solution of 0.6 % free sulfur trioxide in sulfuric acid. To this the orange cyclooctanone oxime solution (65 mL, 0.1 mole in 0.6 % oleum) was added. The reaction was started by the addition of 10 mL of this solution and subsequent heating to 110 °C. The temperature was regulated by the dropwise addition of the remaining cyclooctanone oxime solution. After this the reaction mixture was stirred for an additional 30 minutes yielding a dark brown solution.

The brown capryllactam solution is added dropwise to a solution of ammonium sulphate (200 mL, 3.18 M) while maintaining a pH of 4.5 by adding ammonia with a second dropping funnel. During this reaction toluene is added (1:9 m/V) to solubilize the capryllactam. After separation the toluene is removed by rotary evaporation and the residue (7.3 g, 41 %) is stirred with a 4 mg equivalent sodium hydroxide solution as a 50 % (w/w) solution for 30 minutes. The water is removed and a white solid is obtained by distillation under reduced pressure.

$^1$H-NMR (CDCl$_3$): δ= 5.81 (s, 1H; NH) 3.73 (m, 2H; CH$_2$-NH) 2.42 (m, 2H; CH$_2$-C=O) 1.82 (m, 4H; CH$_2$) 1.58 (m, 6H; CH$_2$)

*Method I. Synthesis of 4-methyl-β-propiolactam*\(^9\)

To a suspension of DL-3-aminobutyric acid (212 mg; 2.0 mmol) and triphenylphosphine (624 mg, 2.4 mmol) in acetonitril (20 mL) were added tetrachloromethane (0.4 mL) and triethylamine (0.34 mL). The mixture is heated to 80 °C and stirred for 24 hours under a nitrogen atmosphere. When the reaction is finished the solvent is removed under reduced pressure and the residue is mixed with dichloromethane (40 mL). This solution is washed with brine (2x 10 mL). The organic layer is collected, dried with sodiumsulphate and the solvent is removed under reduced pressure. The residue has a large content of triphenylphosphineoxide that can be removed partially by dissolving everything in methanol and subsequently add
water until the phosphine compounds precipitate. After filtration the solvent is removed. During rotary evaporation more phosphine compounds precipitate and they are removed by filtration. The product mixture is yellowish oil. From this oil two drops of pure 4-methylazetidin-2-one could be obtained using short path distillation.

\[ ^1H\text{-NMR (D}_2\text{O): } \delta = 3.71 \text{ (m, 1H, CH)}; 2.99-2.93 \text{ (d, 2H, CH}_2); 2.94-2.93 \text{ (d, 1H, CH}_2); 2.45-2.40 \text{ (d, 2H, CH}_2); 1.21-1.19 \text{ (q, 3H, CH}_3) \]

**Method II. Synthesis of 4-methyl-\(\beta\)-propiolactam**

To a suspension of \(DL\)-3-aminobutyric acid (512 mg, 5 mmol) and 2-chloro-1-methylpyridinium iodide (1.405 g, 5.4 mmol) in acetonitril (90 mL) is added a triethylamine (1.54 mL) solution in acetonitril (10 mL). The mixture is heated to refluxing and stirred for 4 – 17 hours. After this the acetonitril is removed by distillation under reduced pressure. The residue is extracted with ethylacetate (3x 20 mL) filtered and the solution is concetrated by rotary evaporation under reduced pressure. The product is obtained by column chromatography using a silica 60 column material and ethylacetate as the eluent. Yield 24 %.

\[ ^1H\text{-NMR (D}_2\text{O): } \delta = 3.71 \text{ (m, 1H, CH)}; 2.99-2.93 \text{ (d, 2H, CH}_2); 2.94-2.93 \text{ (d, 1H, CH}_2); 2.45-2.40 \text{ (d, 2H, CH}_2); 1.21-1.19 \text{ (q, 3H, CH}_3) \]

**Polymerization of \(\beta\)-propiolactam**

The glassware was flame-dried before polymerization. A mixture of \(\beta\)-propiolactam (100 mg, 1.41 mmol), N435 (100 mg) and dry toluene (5 mL), was stirred for 96 h at 90 °C under a N\(_2\)-blanket. After cooling to room temperature the toluene was removed by rotary evaporation. The crude product can be purified by stirring with ethanol for 15 minutes and filtrate. By extracting the residu with water the pure poly(\(\beta\)-alanine) is obtained. (yield 30 %).

\[ ^1H\text{-NMR (D}_2\text{O): } \delta = 3.3 \text{ (m, 2H; CH}_2); 3.12 \text{ (t, 2H; CH}_2); 2.52 \text{ (t, 2H; CH}_2); 2.29 \text{ (m, 2H; CH}_2) \]

**Control reactions**

The following experiments were performed to validate that N435 is the catalyst and \(\beta\)-propiolactam the sole monomer in this reaction.

1. Polymerization without catalyst present
2. N435 was deactivated by heating the beads to 150 °C for two hours. After this treatment no residual activity was observed. To see if the carrier material influences the course of reaction the polymerization of \(\beta\)-propiolactam was repeated with the deactivated N435.
3. Polymerization with N435 and additional water
4. Polymerization with deactivated N435 and additional water
Polymerization of β-alanine and β-alanine ethylester was attempted
Polymerization of β-propiolactam in the presence of β-alanine and β-alanine ethylester

2.3.3 Activity assays

Hydrolytic assay
N435 catalyzes the transesterification of p-nitrophenyl acetate (pnpa) with methanol to yield p-nitrophenol (pnp). With UV spectrometry the concentration of pnp is determined and a rate of esterification is calculated.

A mixture of N435 (10 mg) and toluene (20 mL) stirred at 40 °C and a solution of p-nitrophenyl acetate (5 mL, 7.25 mmol L⁻¹) in toluene was added. Immediately following methanol (6 μL) is added to the mixture. After 15 minutes four samples were taken from the mixture and filtered over a plug of cotton to remove the catalyst particles. Of each sample 0.5 mL of the filtrate was dissolved in 9.5 mL of toluene and the resulting solution was used for the UV-absorption measurement.

The absorbance (304 nm) by pnp is related to its concentration by equation 2-1. With n = dilution factor (20), ε pnp (toluene, 304nm) = 9344.47 M⁻¹ cm⁻¹ and ε pnpa (toluene, 304nm) = 2469.08 M⁻¹ cm⁻¹ as determined from calibration curves.

The activity (a) was calculated see Equation 2-2 as nmol substrate converted by 1 mg N435 per minute during the first 15 minutes of the assay by using Equation 1-2. With

\[
c = \frac{n \cdot \text{Abs} - \varepsilon_{\text{pnpa}} \cdot \varepsilon_{\text{pnp}}}{\varepsilon_{\text{pnp}} - \varepsilon_{\text{pnpa}}} \tag{2-1}
\]

V, the reaction volume (25 mL) a factor x, (1*10⁹) to convert the value to nmol M, the mass of the N435 (10 mg) and t, the time of reaction (15 min.).

\[
a = \frac{c \cdot V \cdot x}{M_{\text{N435}} \cdot t} \tag{2-2}
\]

Synthetic assay
The enzymatic polymerization of ε-caprolactone was used as the synthetic activity assay. 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, 2 drops of the solution were withdrawn and the conversion of ε-caprolactone was determined with ¹H-NMR-spectroscopy. The signals of the CH₃ next to the carbonyl group (4.09 ppm) in the polymer backbone and in the monomer (4.22 ppm) are compared. The ratio of the integral of the backbone CH₃ over the total integral of the mentioned CH₃ groups is used to determine the conversion of ε-caprolactone.
2.4 RESULTS AND DISCUSSION

2.4.1 Polymerization of β-propiolactam

The polymerization of lactams was attempted with lactam rings of different ring sizes. Capryllactam, ε-caprolactam and valerolactam, were tried as a monomer but no polymer was obtained. The 4-methyl-azetidin-2-one polymerized by thermal treatment alone without an enzyme.

Only the β-propiolactam was a substrate for the enzyme and yielded polymer while no polymer was obtained in a blank reaction.

**Toluene is the best reaction medium for the polymerization of β-propiolactam.**

The first attempt to polymerize β-propiolactam via enzyme catalyzed polymerization was performed in diisopropyl ether at an oilbath temperature of 60 °C for 24 hours, to resemble the system used by Fülöp et al. A series of experiments was carried out to determine the appropriate reaction conditions summed in Table 2-2.

**Table 2-2. Solvent optimization for the polymerization of β-propiolactam.**

<table>
<thead>
<tr>
<th>entry</th>
<th>Solvent</th>
<th>t (h)</th>
<th>T (°C)²</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>i-Pr₂O</td>
<td>24</td>
<td>60</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>70</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>70</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>168</td>
<td>65</td>
<td>75 mg³</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>toluene</td>
<td>96</td>
<td>90</td>
<td>30 %⁴</td>
</tr>
<tr>
<td>6</td>
<td>1,4-dioxane</td>
<td>96</td>
<td>80</td>
<td>&lt; 5 %</td>
</tr>
<tr>
<td>7</td>
<td>o-dichlorobenzene</td>
<td>96</td>
<td>90</td>
<td>&lt; 5 %</td>
</tr>
</tbody>
</table>

1. ¹H-NMR spectra of entries 1-5 are shown in Figure 2-8
2. Temperatures are oilbath temperatures
3. Total amount of product still containing β-alanine
4. Purified polymer from ~70 mg crude product

The products from the experiments 1-5 in Table 2-2 were examined with ¹H-NMR spectroscopy in deuterium oxide, depicted in Figure 2-8. The first two experiments performed in i-Pr₂O yielded only little polymer and showed mainly monomer (δ=2.85 and 3.15 ppm).

The third experiment showed that elongation of the reaction to 72 hours in i-Pr₂O with the temperature raised to 70 °C leads to polymer formation. All the β-propiolactam is
now converted to β-alanine (δ=2.4 and 3.05 ppm) or polymer (δ=2.25 and 3.25 ppm). The fourth experiment shows that increasing the reaction time to 168 hours further increased the conversion of β-propiolactam. In order to increase the temperature of the reaction the solvent was changed to toluene. This solvent was chosen because of the high activity of N435 in this solvent at 90 °C reported in the enzymatic polymerization of ε-caprolactone.13

In the fifth experiment toluene was used at 90 °C, the reaction time could be reduced to 96 hours. From the 1H-NMR spectrum (Figure 2-8, nr 5) and the two assays it was concluded that further experiments should be done with toluene as the solvent and a reaction temperature of 90 °C.

![Figure 2-8. The 1H-NMR spectra of the reaction product after 24, 40, 72, 168 hours of reaction in i-Pr2O (1-4 respectively) and after 96 hours of reaction in toluene (5). The reaction conditions of these reactions are summarized in Table 2-2, entries 1-5.](image)

### 2.4.2 Cal-B remains active in toluene of 90 °C

Enzymes in general can denaturate when they are exposed to higher temperatures especially in aqueous environments. In organic solvents the activity is usually better retained under these conditions. In the former paragraph toluene is selected as the best reaction medium for the polymerization of β-propiolactam but deactivation over time can not be excluded. Two activity assays were used to evaluate the activity of the N435 after incubation at 90 °C in toluene for a maximum of 96 hours. From the results in Table 2-3 and Table 2-4 it can be concluded that the activity of N435 is only slightly affected by the thermal treatment.
Hydrolytic assay

N435 catalyzes a transesterification of p-nitrophenyl acetate with methanol to p-nitrophenol the rate of this transesterification can be used to determine the hydrolytic activity of the catalyst. The concentration of pnp was calculated from the absorption at 304 nm. The activity of the N435 is determined in the first 15 minutes because the conversion of pnpA is still linear with time in this region. The activity of the catalyst was determined after 0 and 96 hours of incubation at 90 °C and listed in Table 2-3. The activities are the mean value of 4 measurements.

Table 2-3. Hydrolytic activity of N435 after incubation in toluene. Calculated per mg of N435.

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Activity of N435 (nmol pnp min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>107.20 +/- 5.49</td>
</tr>
<tr>
<td>96</td>
<td>115.24 +/- 15.38</td>
</tr>
</tbody>
</table>

After 4 days in toluene of 90 °C the N435 has not lost any of its activity. It is concluded that N435 can be used for the enzymatic polymerization of β-propiolactam in this reaction medium and temperature.

Synthetic assay of poly-ε-caprolactone

Lactones are easily polymerized by the N435 and the conversion of ε-caprolactone over time is used to evaluate the ability of the catalyst to perform a ring-opening polymerization after incubation in toluene. In Table 2-4 the incubation times and % conversion of the ε-caprolactone are listed. The production of poly-ε-caprolactone is not affected by the treatment at 90 °C for 72 hours conversions remain 85 %.

Table 2-4. Synthetic activity of N435 after 0-72 hours incubation in toluene of 90 °C.

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>N435 (g)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0000</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0.0963</td>
<td>90</td>
</tr>
<tr>
<td>24</td>
<td>0.0974</td>
<td>74</td>
</tr>
<tr>
<td>48</td>
<td>0.0974</td>
<td>83</td>
</tr>
<tr>
<td>72</td>
<td>0.0986</td>
<td>85</td>
</tr>
</tbody>
</table>
From the results obtained from the two synthetic assays we conclude that the enzyme retains its activity after prolonged incubation in toluene at 90 °C. The enzyme is not denatured and can be used for polymerization reactions under these conditions.

2.4.3 Characterization of poly(β-alanine)

The poly(β-alanine) was characterized by both MALDI-ToF MS and 1H-NMR spectroscopy. It is shown below that pure poly-β-alanine can be obtained when cyclic products, monomer and β-alanine are removed from the crude polymer in a washing step with water and ethanol.

*Maldi-ToF Mass spectra of poly(β-alanine)*

In the MALDI-ToF mass spectrum of the crude polymer (Figure 2-9) cyclic structures appear as sodium adducts in the area of 300-600 m/z as expected for polycondensation reactions. Also in the lipase-catalyzed polymerization of β-propiolactone cyclic oligomers are formed as was reported by Namekawa et al. The insert explains the three typical peaks in this mixture [M-Na]⁺ ion (m/z = 609), [M-K]⁺ ion (m/z = 625) and the [M-Na]⁺ cyclic ions (m/z = 592).

![MALDI-ToF Mass spectrum of poly(β-alanine)](image)

*Figure 2-9. The MALDI-ToF mass spectrum of the crude poly(β-alanine). The insert explains the peaks for the [M-Na]⁺ ion (m/z = 609), [M-K]⁺ ion (m/z = 625) and the [M-Na]⁺ cyclic ions (m/z = 592).*

After purification of the poly(β-alanine) by introducing a washing step with ethanol a white solid is obtained and the MALDI-ToF mass spectrum (Figure 2-10) shows a mass increment of 71 m/z corresponding to one monomeric unit. In the ionization process adducts with sodium and potassium are formed. The sodium adducts form the primary distribution while the potassium adducts can be found as a second distribution of peaks between 300 and 700 m/z. The distribution has a maximum at 467 m/z (DP=6) and peaks up to 1319.7 m/z (DP=18) can be observed.
The MALDI-ToF mass spectrum of the purified poly(β-alanine). At a Δm/z of 71 the [M-Na]+ ions of the linear polymer are found. A small fraction of the [M-K]+ ions remains and the cyclic structures are removed.

**1H-NMR spectra of poly(β-alanine)**

The crude polymeric product was analyzed by 1H-NMR spectroscopy (Figure 2-11). The main chain protons (δ= 3.30, 2.29 ppm) and the protons next to the endgroups (amine δ= 3.12 ppm and carboxylic acid δ= 2.52 ppm) can be identified corresponding to literature. There is monomer (δ= 3.2, 2.85 ppm) left in the crude product and small traces of β-alanine (δ= 3.02, 2.4 ppm) formed by hydrolysis of β-propiolactam. Although polymerization is carried out successfully the conversion is not 100%. Low mass cyclic products and β-alanine are removed by washing with ethanol. After this purification the degree of polymerization is determined from the 1H-NMR spectrum (Figure 2-12) of the pure poly(β-alanine).

![Figure 2-10](image_url)

**Figure 2-10.** The MALDI-ToF mass spectrum of the purified poly(β-alanine). At a Δm/z of 71 the [M-Na]+ ions of the linear polymer are found. A small fraction of the [M-K]+ ions remains and the cyclic structures are removed.

![Figure 2-11](image_url)

**Figure 2-11.** The 1H-NMR spectrum of crude poly(β-alanine) showing: polymer (δ= 3.30, 2.29 ppm) end-groups (δ= 3.12, 2.52 ppm) monomer (δ= 3.2, 2.85 ppm) and β-alanine (δ= 3.02, 2.4 ppm).
The average degree of polymerization determined from the $^1$H-NMR-spectrum is $7$. This value is in good agreement with the maximum of the distribution found with MALDI-ToF MS (DP=6). The conversion of $\beta$-propiolactam is not complete as 30 wt% polymer is obtained after purification. Some of the monomer is hydrolyzed or left unharmed as was proven with $^1$H-NMR spectroscopy. MALDI-ToF mass spectrometry showed that some of the monomer is converted into cyclic products that are also washed away during purification.

Hydrolysis is caused by the presence of water in the catalyst. Although activity measurements showed that the N435 is active after long periods of time at a high temperature, some of the catalyst might be deactivated by these reaction conditions. Optimization of the reaction time and temperature as well as changing the drying regime for the catalyst is needed to improve both the yield and the degree of polymerization of the poly($\beta$-alanine).

### 2.4.4 Optimization of the reaction conditions

The time and temperature of the reaction and the drying conditions used to dry N435 were optimized with respect to the yield and degree of polymerization of the poly($\beta$-alanine).

Although the activity of N435 at 90 °C for 96 hours was shown in Paragraph 2.4.2 this is not necessarily the optimal combination of time and temperature. To prevent side reactions and thermal deactivation of the enzyme it is preferred to use a low reaction temperature.

The N435 catalyst can be deactivated by denaturing when too much water is present. Also hydrolysis will predominantly take place. Drying of the catalyst is therefore necessary. However not too much water should be removed since a small portion is needed for the structure of the enzyme and for catalysis.
Reaction time and temperature

The N435 was dried for 48 hours at 46 °C for all the optimizations regarding time and temperature. The enzymatic polymerization of β-propiolactam in toluene at 90 °C for 96 hours gave the poly(β-alanine) in 30 % yield with Dp 5. This represents the polymerization as described above. The temperature of the reaction medium was lowered to 55 °C. In the experiments the reaction time was either 96 hours or 144 hours. Table 2-5 sums the reaction conditions, the yield of polymer and the average degree of polymerization for these reactions.

The degree of polymerization did not change as a result of lowering the temperature or elongation of the reaction time to 144 hours. The yield of the polymerization did change from 30 % at 90 °C to 80 % at 55 °C.

Therefore, for the polymerization a temperature of 55 °C and a reaction time of 96 hours gives the best results in terms of DP and yield of the reaction.

Table 2-5. Influence of the time and temperature on the yield and DP of poly(β-alanine)

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>t (h)</th>
<th>Yield (%)</th>
<th>DP (H-NMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>96</td>
<td>81</td>
<td>5</td>
</tr>
<tr>
<td>55</td>
<td>144</td>
<td>83</td>
<td>5</td>
</tr>
<tr>
<td>90</td>
<td>96</td>
<td>30</td>
<td>5</td>
</tr>
</tbody>
</table>

Drying regime for the catalyst

Optimization towards the drying regime for the catalyst is done by evaluating the polymer (yield and DP) obtained after a reaction in toluene for 96 hours at 55 °C. Prior to the reaction the N435 was dried over P2O5 in vacuo using a temperature of 55 °C and 46 °C. After drying the catalyst for 2, 24 or 48 hours the polymerizations were started.

In Table 2-6 the results are summarized. Drying the enzyme for two hours already leads to a higher degree of polymerization, DP = 6.7 than drying for 48 hours as was presented in the former paragraph (DP = 5). After 24 hours of drying the polymers reached the highest degree of polymerization DP= 7. Changing to a slightly lower drying temperature (from 55 to 46 °C) did not influence the DP.

After drying for two hours hydrolysis limits the DP to 6.7. After 24 hours a DP of 7 was reached and this is the best result. After 48 hours too much water is removed leading to a denaturation of the enzyme.

After these two optimization steps it can be concluded that the polymerization is best performed at 55 °C for 96 hours using a catalyst that was dried over P2O5 in vacuo at 55 °C for 24 hours. Resulting in the highest yield of polymer with the longest chains.
Table 2-6. Influence of drying N435 on the DP of the poly-β-alanine.

<table>
<thead>
<tr>
<th>time (h)</th>
<th>T (°C)</th>
<th>DP (by $^1$H-NMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>55</td>
<td>6.7</td>
</tr>
<tr>
<td>24</td>
<td>55</td>
<td>7.3, 8.0</td>
</tr>
<tr>
<td>24</td>
<td>46</td>
<td>7.6</td>
</tr>
<tr>
<td>48</td>
<td>46</td>
<td>5.0</td>
</tr>
</tbody>
</table>

2.4.5 Control reactions and possible inhibition by intermediates

The polymerization of β-propiolactam by enzymatic catalysis was not reported before. Therefore it is of importance to show that the polymerization is only possible with the N435 catalyst present. This was indeed found because no polymer is formed without the N435 after 96 hours in toluene at 90 °C. Only the monomer was retrieved from the reaction mixture.

A reaction started with the deactivated catalyst (150 °C, 2h) shows no polymerization. The carrier material is therefore excluded as a possible catalytic agent.

Also it is known, that water alcohols and other nucleophiles can initiate the polymerization of lactones. Different experiments were performed that proof that catalysis by N435 is really the only way to obtain a polymer starting with β-propiolactam.

When the N435 catalyst is used, water is inevitably present in the catalyst and could act as the initiator for the polymerization. Two experiments were designed to see if this is the case. The polymerization was repeated without N435 but with added water and the polymerization was repeated with deactivated N435 and added water. In both cases no polymer was formed.

A second species that could act as the initiator of the polymerization is β-alanine. β-Alanine is formed when β-propiolactam is hydrolyzed. Since water is always present there will always be some β-alanine in the mixture. The polymerization was repeated without catalyst and added β-alanine, no polymer resulted.

Also it is possible that the enzyme polymerizes β-alanine that is first formed by hydrolysis of the β-propiolactam. This was ruled out by feeding the catalyst with either β-alanine or β-alanine ethylester. These experiments did not yield any polymer. When β-alanine was added to the polymerization of β-propiolactam the polymerization was not hindered another clue, that no intermediate is formed between β-alanine and the lipase.

Although poorly soluble in toluene (10⁻³ mol L⁻¹ determined with HPLC) the presence of β-alanine ethylester reduces the hydrolytic activity of N435 by 37% see Table 2-7.
The inhibitory effect is also found in the synthetic assay (polymerization of ε-caprolactone) and in the polymerization of β-propiolactam. In both cases no polymeric product was retrieved after the polymerization with β-alanine ethylester present.

From these results it can be concluded that neither the water, β-alanine or the carrier material starts the reaction. The N435 catalyzes the reaction. Because the β-alanine is not polymerized by the N435 it can be stated that the ring structure of the β-propiolactam is essential for the formation of polymer. It was found that β-alanine ethylester is an inhibitor for the polymerization of β-propiolactam and ε-caprolactone.

**Table 2-7. The activity of Cal-B decreases in the presence of β-alanine ethylester.**

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Activity of N435 + β-alanine ethylester (nmol pnp min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>67.95 +/- 2.48</td>
</tr>
<tr>
<td>96</td>
<td>45.18 +/- 8.97</td>
</tr>
</tbody>
</table>

2.4.6 Enzymatic lactam polymerization follows a different mechanism than ε-caprolactone polymerization

Lipase (N435) catalyzed amide bond formation is believed to proceed through the same intermediates as the formation of esters by N435. Prior to our experiments we expected that the lactam polymerization proceeds according to the mechanism of lactone polymerization. When the mechanism of the β-propiolactam polymerization is drawn according to the mechanism of lactone polymerization, Figure 2-13 is obtained.

In step 1 the first acyl-enzyme intermediate that is formed will undergo hydrolysis to form β-alanine \(n=1\). A newly formed intermediate can either be attacked by water or by a β-alanine molecule resulting in a growing chain \(n+1\). The chains grow by performing step 2 repeatedly.

**Figure 2-13. Hypothetical mechanism for the polymerization of β-propiolactam drawn according to the mechanism for enzymatic ε-caprolactone polymerization depicted in Figure 2-2.**
The mechanism in Figure 2-13 requires a β-alanine molecule, the analogue of the hydroxy acid in Figure 2-2, to be freed and attack a newly formed acyl-enzyme intermediate. From the control experiments we know that β-alanine is not polymerized by the Cal-B. Recent findings by Hollmann et al.⁴⁹ show that organic acids with a pKa < 4.8 protonate the His224 and thereby prevent the deprotonation of Ser105 needed to form the acyl-enzyme intermediate. Since β-alanine has a pKa of 3.6,⁵⁰ this could very well prevent the polymerization of this compound. In conclusion, β-alanine is not an intermediate in the lactam polymerization and therefore the lactam polymerization has to follow a different mechanism than the polymerization of ε-caprolactone.

2.4.7 A new mechanism developed by molecular modeling explains the β-propiolactam polymerization

Based on the experimental results outlined above Fels and Baum⁵¹, in close collaboration with us⁵², developed a mechanism for the enzymatic catalyzed ring-opening polymerization of β-propiolactam using molecular modeling. The authors explain the polymerization of β-propiolactam in eight steps, keeping in mind that the lactam ring is the sole monomer of the reaction and that β-alanine is not an intermediate nor a monomer in the reaction.

Figure 2-14 summarizes the sequence in four main steps. 1. Acyl-enzyme formation, 2. activation of the monomer, 3. elongation and 4. release of the polymer. Two features of the proposed mechanism in enable the polymerization of β-propiolactam without the release and reattachment of β-alanine or a growing chain.

Activation of the lactam ring by a molecule of water

The nucleophilicity of the β-propiolactam ring is not high enough for a direct attack on the acyl-enzyme intermediate. Activation by a water molecule increases the nucleophilicity of the monomer allowing a direct attack on the intermediate. Histidine224 accepts the proton and stabilizes the activated monomer by hydrogen bonding. In the lactone mechanism a hydroxy acid is released that attacks the next acyl-enzyme intermediate.

Insertion of the activated monomer between Ser105 and the growing chain

The accepted lactone polymerization suggests an attack of the released hydroxy acid or a growing chain on the acyl-enzyme intermediate with the release of elongated chain. In this new mechanism the activated monomer is inserted and the growing chain stays bonded to the serine.

A water molecule present near His224 in the active site (crystal structures 1TCA, 1TCB, 1TCC)⁵³ of Cal-B assists the transfer of protons between groups that are too far apart for direct hydrogen bonding and it enhances the nucleophilicity of the monomer. This was suggested for other Cal-B catalyzed reactions in molecular
Figure 2-14. Mechanism of enzyme catalyzed \( \beta \)-propiolactam polymerization developed by Baum et al.\textsuperscript{52} The use of water during polymerization is catalytic. Freeing the polymer from the enzyme however, is performed at the cost of one water molecule. In the optimization steps described earlier it was shown that drying the enzyme is necessary but not too much water should be removed. This can now be understood not only as a disturbance of the enzyme structure but also as removing water molecules that are necessary in performing the polymerization.

### 2.5 CONCLUSIONS

The successful enzymatic polymerization of the four membered \( \beta \)-propiolactam is reported. From a collection of lactam rings with 4, 6, 9 and 13 members only the 4-membered ring is a good substrate for the N435 catalyst.

The \( \beta \)-propiolactam ring can be polymerized in different solvents but the optimal reaction conditions are a polymerization in toluene at a temperature of 55 °C for 96 hours. The N435 should be dried before use for 24 hours over P\(_2\)O\(_5\) under reduced pressure.

Although a reasonable yield of polymer (30 % by weight) was obtained after a reaction at 90 °C at first, the yields almost tripled (83 %) when the reaction temperature was lowered to 55 °C. Also after longer reaction time (144 hours) the yields of polymer remained high. Changing the reaction time and temperature did
however not change the average degree of polymerization probably due to precipitation of the polymer.

Drying the N435 catalyst and the reagents before use is essential to prevent hydrolysis of the β-propiolactam. The drying conditions for the N435 were varied in both temperature (55 and 46 °C) and time (24, 48 h) over P2Os in vacuo. This has a clear effect on the degree of polymerization as it drops from 8 to 6 when the drying is continued from 24 to 48 hours. After 48 hours of drying the structural water is removed and the catalyst starts to lose its activity.

The polymer that was obtained could only be formed when the β-propiolactam was the monomer with N435 as the catalyst. The possible monomers β-alanine and β-alanine ethylester were not polymerized by the N435. Also possible initiation by water, β-alanine and carrier material was excluded.

Based on the experimental results that the possible intermediate β-alanine was not polymerized by the N435, it was concluded that the polymerization of β-propiolactam can not be explained by the mechanism accepted for the enzymatic ε-caprolactone polymerization. In close collaboration with us Baum et al., developed a mechanism by molecular modeling showing that the β-propiolactam can be the sole monomer in the polymerization when it is activated by a water molecule.

2.6 REFERENCES

8. Foley, K.M., Bell, R.H., McCombs, F.P., 1977, 620524
42. Loontjens, T. and DSM Geleen, Capryllactam synthesis, description of process-steps, personal communication, 1992
47. Applequist, J., Glickson, J.D., J. Am. Chem. Soc., 1971, 93, 3276-3281
51. Fels, G. and Baum, I. In Bioconversion in Polymer Chemistry, 349-368, Loos, K., Ed, Wiley-VCH, 2010