Polyamide synthesis by hydrolases
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Chapter 1
General introduction

1.1 ABSTRACT

Enzymes are the catalysts of nature and and they operate under ambient temperature and pressure mostly in aqueous environments. The high catalytic proficiency of enzymes under mild reaction conditions raised the interest in these green catalysts as alternatives for conventional processes. Stabilized by immobilization some enzymes are now commercially available for the in vitro use in organic media.

In recent years polymerizations with enzymes have been developed by different groups in the field. The enzymatic synthesis of polyamides however, has not been studied extensively. It is the aim of this thesis to expand the knowledge on the synthesis of polyamides by enzyme catalysis.

In the synthesis of polyamides and polypetides a lot can be gained by using enzymes in terms of waste reduction and the consumption of energy and chemicals. Typically, high temperatures and pressures and toxic chemicals are used in the synthesis of these compounds.

In this general introduction the choice of enzymes is illustrated and the basics of catalysis by enzymes and hydrolases in particular are explained. After this, it is shown what precautions are needed when enzymes need to function in organic media to be used in the synthesis of polymers. The chapter concludes with the aim and outline of the thesis in greater detail.
1.2 ENZYMATIC SYNTHESIS OF POLYAMIDES

In general enzymes can accelerate reactions by a factor $10^3$-$10^{17}$ under mild reaction conditions (ambient temperature and pressure in an aqueous environment). When applied to organic reactions the use of enzymes has ecological and economical benefits in reducing waste production, reaction steps and energy consumption.

In the synthesis of polyamides a lot can be gained by using enzymes as a catalyst. A distinction is made between polyamides (including all synthetic polymers that contain amide bonds) and polypeptides (polymers made from $\alpha$-amino acids).

Polyamides are produced in industry by amidation of dicarboxylic acid compounds with diamines, selfamidation of amino acids or ring-opening polymerization of lactams. The commercially most successful polyamide is nylon6,6 produced from hexamethylene diamine and adipic acid closely followed by nylon6 synthesized from $\varepsilon$-caprolactam. Together these nylon form 90% of the polyamides market. Other nylons are produced for special applications. Aromatic polyamides are more expensive to produce but offer a high heat and chemoresistance combined with ultra high strength. Kevlar and Twaron are tradenames of the polymer from terephthalic acid chloride and p-phenylenediamine. Nomex is produced from the meta substituted aromatic diacid and diamine. The synthesis of the above mentioned polyamides consumes a lot of energy in the form of heat as polymerizations are usually carried out at temperatures >200 °C.

Polypeptides are a class of polyamides with a wide variety of functions in living nature. They are part of the structure of living bodies (i.e. hairs, nails, tendons and muscles), regulate biological reactions (hormones, enzymes) and are used in construction (spider silk, mussel attachment fibers). The variety in structure and function combined with the biodegradability has made polypeptides interesting compounds for the use in cosmetics,$^{1,2}$ medicine$^3$ and fabrics.$^4$ The ring-opening polymerization of $\alpha$-amino acid N-carboxy anhydrides(NCA’s) is the preferred method for the in vitro polymerization of $\alpha$-amino acids. Although this method provides great control over chain length and composition a major drawback in the production of NCA’s is the use of toxic phosgene compounds.
1.3 ENZYMES

Almost all the reactions in living cells are catalyzed by enzymes. Enzymes are large proteins that consist of poly(α-amino acid) chains in secondary structures like the α-helix, β-sheet and random coil domains. The arrangement of these structures relative to each other is different in every protein and determines its shape and regulates its function. Sometimes two proteins combine to form an active complex.

Within each enzyme there is a small region were catalysis takes place, the so-called active site. The electrostatic properties and spatial arrangement of the amino acids surrounding the catalytically active residues determine the specificity of the enzyme.

The activity of the enzyme can be regulated by parts of the enzyme that are not in the active site. They control the accessibility of the active site by building an access channel and allosteric effects can turn the enzyme “on” and “off”5 In some enzymes the catalytic activity is assisted by co-factors. These co-factors can be iron, copper or zinc ions but also organic molecules or more complex prosthetic groups like the heme-group.6,7

1.3.1 Hydrolases are the enzymes of choice

Enzymes are categorized in six classes based on the type of reaction that is catalyzed as determined by the international union of biochemistry and molecular biology8

I. Oxido-reductases catalyze redox-reactions.

II. Transferases catalyze the transfer of a functional group.

III. Hydrolases catalyze the hydrolysis of various bonds.

IV. Lyases catalyze the cleavage of bonds but not by hydrolysis or oxidation.

V. Isomerases catalyze isomerizations of chiral substances.

VI. Ligases catalyze the bond formation coupled with ATP hydrolysis

Even though within each class enzymes evolved to catalyze very specific reactions some enzymes show a broad tolerance for substrates that can be converted using the same catalytic pathway. Also, enzymatic reactions are equilibria and by choosing the right conditions the reverse reaction can be catalyzed. For the synthesis of polyamides the obvious choice is to use enzymes that catalyze the amide bond formation in vivo.

The only polyamides synthesized in vivo are peptides. They are synthesized by expression of the genetic code with synthesis taking place on the ribosomes. Ribosomes are complex assemblies of rRNA’s and 50 kinds of proteins. For the synthesis of peptides in vitro this process is too complex.
Enzymes should be used that catalyze reactions by a pathway that can also accommodate the monomers for polyamides and peptides. From the six classes of enzymes introduced above the hydrolases are the enzymes of choice for the synthesis of polypeptides and polyamides. They are available in a large variety of specificities and they in general do not need co-factors for their function.9

1.3.2 Hydrolases used in this thesis

Hydrolases catalyze the hydrolysis of substrates and their names originate from the substrate that is hydrolyzed. Lipids are hydrolyzed by lipases and proteins are hydrolyzed by proteases.

Two lipases are used in chapter 2 and 3 (lipases A and B from Candida antarctica yeast) and the protease papain from Carica papaya fruit is used in chapters 4 and 5.

The lipase B and the papain were chosen mainly because they display a wide substrate tolerance (lipase B and papain) with respect to other enzymes belonging to the hydrolases. The lipase A was chosen for its reported high thermostability.

Candida antarctica lipase A

Only recently the structure of Candida antarctica lipase A was resolved.10 It is a 45 kDa enzyme with characteristic α/β-hydrolase structure. As many lipases it has a lid covering the active site entrance. Although reported by many as calcium dependent no calcium densities were detected in the x-ray study. The entrance to the active site is formed by a 30Å narrow tunnel with a 90° kink leading to an active site with a catalytic triad of Ser, His and Asp.

Candida antarctica lipase A is commercially available as a free enzyme and as crosslinked enzyme aggregates (CLEA), immobilized on celite with sucrose11, silicone particles12, polypropylene beads EP-10013 and magnetic particles.14 The most used methods of immobilization are the CLEA and the immobilization on Celite in the presence of sucrose. A cross-linked enzyme aggregate is obtained by cross-linking enzyme molecules.15 One CLEA particle contains approximately 8 million enzyme molecules and it has a size of 1μm. The particles do not behave like a solid, they can pass 0.2 μm pores in a filter.16 Candida antarctica lipase A was shown to have a ten-fold increased activity when it is used as a CLEA.17

The unique properties of the lipase A were reviewed by Kirk18 and de Maria et al.19 in the past. Cal-A is an enzyme with a high thermostability (>90 °C) with a selectivity for hydrolyzing the Sn-2 position of lipids. In synthesis reactions Cal-A has a preference for the N-acylation of β-amino acid esters and reactions with tertiary alcohols. Heldt-Hansen and co-workers reported a that esterifications with butyric acid and acetic acid gave no reaction whereas longer chain carboxylic acids are used with success.20
Reactions with activated esters (fluorinated butanoate and hexanoate) were performed by Kanerva et al.\textsuperscript{21}

\textit{Candida antarctica lipase B}

The yeast \textit{Candida antarctica} was isolated from sediments of lake Vanda in Antarctica and reported in 1969.\textsuperscript{22} From this organism two lipases (A and B) could be isolated\textsuperscript{20} and expression in \textit{Asperigillus oryzae}\textsuperscript{23} made the large scale production of the enzyme possible.

The crystal structure of the lipase B was elucidated by J.Uppenberg and coworkers.\textsuperscript{24} The lipase B is a 33 kDa enzyme with an active site that is accessible through a 12Å narrow channel. It lacks the interfacial activation found for other lipases\textsuperscript{25} and this is attributed to the absence of a “lid” that completely covers the active site entrance of most lipases. Although there are indications that the “lid” is actually an \(	extalpha\)-helix formed by residues 142-146\textsuperscript{24,26} it is however small and an open configuration of the active site is assumed. This greatly improves the accessibility of the active site compared to other lipases.

The active site has two distinct parts, the acyl-binding side and the alcohol binding side. The latter being stereospecific for secondary alcohols due to a stereospecific pocket that can accommodate a methyl side group.\textsuperscript{27}

\textit{Candida antarctica} lipase B is commercially available as the free enzyme or immobilized by absorption on macroporous beads known as Novozymes N435. The immobilization increases the thermostability of the lipase so that it can be used at temperatures up to 100 °C.\textsuperscript{28} Also the stability towards organic solvents is greatly improved by immobilization. Novozymes N435 is a versatile catalyst in terms of reaction conditions and the acceptance of various substrates e.g. it has been used to synthesize polyeesters from linear\textsuperscript{29} and cyclic\textsuperscript{30,28,31,32} starting materials. The best results for the polymerization of lactones were obtained in toluene.\textsuperscript{28}

The N435-beads consist of a crosslinked resin of poly(methyl methacrylate) and have a diameter ranging from 0.3 to 0.9 mm.\textsuperscript{31} The catalyst has an average loading of ~20% by weight\textsuperscript{32} with the lipase located in the outer 100 microns of the beads.\textsuperscript{34} The N435 catalyst has been employed in toluene, dioxane, hexane, iso-octane, cyclooctane, benzene, cyclohexane, ionic liquids\textsuperscript{35}, tert-butanol, iso-propylether and tetrahydrofuran.\textsuperscript{36} Care must be taken that the solvent itself cannot be hydrolyzed, amidated or transesterified by the N435.\textsuperscript{37}

\textit{Carica papaya papain}

Papain is a protease obtained from unripe papaya fruit. The structure of papain was published in 1968 by Drenth and co-workers\textsuperscript{38} at 2.8 Å resolution and refined to 1.65 Å by I.G. Kamphuis in 1983.\textsuperscript{39} It was shown that Papain is a 23 kDa protein of 212 amino
acid residues. Two domains can be distinguished a helical domain and a domain consisting of β-sheets. The active site is located at the surface of the enzyme in a groove running over the two domains.38,39

The optimum pH for hydrolysis by papain is 5.5 to 6.5. The optimum pH for the synthesis of peptides was determined to be 8-9.5.40 While the peptidase activity of the papain is negligible at pH >8 it retains its esterase activity.40

The catalytic action of papain to degrade peptides is used in the tenderization of meat.41,42

Specificity of papain
Before the structure of papain was resolved by X-ray crystallography Schechter and Berger identified recognition sites for amino acid residues on both sides of the active site cysteine by kinetic experiments.43,44 In Figure 1-1 three recognition sites on the amino side (S1-S3) of the peptide and 3 recognition sites on the carboxylic acid side (S1’-S2’) of the peptide are depicted.

Figure 1-1. Recognition sites around the papain active site according to Schlechter and Berger43,44

The only binding site in papain that has a distinct specificity is the S2 pocket build by two valine (Val133, Val157) residues and an aspartic acid (Asp158). The pocket was found by cocrystallizing the enzyme with a chloromethyl ketone substrate analog.45 Since these early findings published by Schechter and Berger more research pointed out that papain has a selectivity for binding aromatic hydrophobic residues in the S2 position.46,47,48,49,50 It prefers tyrosine over phenylalanine in peptides at P2.51 It was concluded by Kim et al.52 that the S2 and S3 form one big hydrophobic pocket.

The recognition sites S1, S1’ and S2’ are less well defined. In the S1’ site papain can bind hydrophilic and small hydrophobic side chains shown by Schuster et al.53 refined to leucine, alanine, serine and phenylalanine by Ménard and coworkers.54 While others determined a preference for tryptophan and leucine.55

The S2’ site binds small amino acids in the order Ala, Val, Asn, Leu and Phe. Shown with kinetic experiments of small peptides with chromophoric markers attached to
it.\textsuperscript{56} Schechter and Berger report also S3’ and S4’ but not much evidence for these subsites is found.

\section*{1.4 CATALYSIS BY ENZYMES}

In general enzymes catalyze reactions by introducing multiple reaction steps each with a lower activation energy than the uncatalyzed reaction ($\Delta G_{uc}^\circ$). In Figure 1-2 the energy diagram of the conversion of a substrate S into a product P is shown.

In the enzymatic reaction two intermediates are formed the enzyme-substrate (ES) and enzyme-product (EP). Each of these intermediates is formed through a transition state (TSi). Enzymes can specifically bind the transition states and reduce the energy cost to produce the ES and EP intermediates. The activation energy by the enzyme catalyzed reaction ($\Delta G_{c}^\circ$) is lower than the activation energy by the uncatalyzed reaction ($\Delta G_{uc}^\circ$).

![Energy profile of the conversion of S $\rightarrow$ P catalyzed by an enzyme](image)

Figure 1-2. Energy profile of the conversion of S $\rightarrow$ P catalyzed by an enzyme.

Enzymes can use hydrogen bonding and electrostatic interactions to bind the transition states. From equation 1-1\textsuperscript{7} that relates the acceleration by enzymes (V) to the reduction of the activation energy ($\Delta E_a$) it follows that a 10-fold acceleration requires \textasciitilde5.71 kJ mol\textsuperscript{-1}.

$$V \sim e^{\Delta E_a/RT}$$ \textsuperscript{1-1}

This is half the energy of a hydrogen bond\textsuperscript{57} easily provided by the amino acids in the active site of the enzyme. A million fold increase in reaction speed requires lowering
the activation energy by ~34 kJ mol⁻¹. Enzymes can use covalent bonds to stabilize intermediates and this lowers the activation energy with ~100 kJ mol⁻¹.

The combined stabilization of reaction intermediates by hydrogen bonding, covalent attachment and electrostatic interactions lowers the activation energy relative to the uncatalyzed reaction so that accelerations by a factor 10³ - 10¹⁷ are achieved. These rate enhancements are achieved in an aqueous environment at ambient temperatures and pressures.

1.4.1 Catalysis by hydrolases

A general hydrolase (e.g. proteases and lipases used in this thesis) catalyzed reaction proceeds as follows. First, the substrate is bound to the enzyme through a tetrahedral intermediate forming an acyl-enzyme intermediate (ES). In a second step the enzyme-substrate complex is attacked by water and a second tetrahedral intermediate is formed. In a last step the second intermediate falls apart and the product of hydrolysis is released and the enzyme is ready for a new cycle.

The sequence of catalytic steps is enabled by two distinct regions in the active site of hydrolases the catalytic triad and the oxyanion hole.

As an example of catalysis by hydrolases the function of both regions in the hydrolysis of esters by Candida antarctica lipase B is explained below.

**Catalytic triad**

All hydrolases share a common sequence of amino acids in the active center called the catalytic triad, first identified in α-chymotrypsin. The action of the catalytic triad of the Candida antarctica lipase B is depicted in Figure 1-3 and shows how the acyl-enzyme complex is formed.

The catalytic triad of Cal-B consists of a Ser105, His224 and Asp187 residue. Prior to binding the substrate the His224 accepts a proton from the Ser105 creating a nucleophile, the His224 tautomerizes and is stabilized by Asp187. At the same time Ser105 binds to the substrate. After the release of the products protons exchange to regain the situation depicted on the left in Figure 1-3.

![Figure 1-3. Catalytic triad without a substrate (left) and after binding the substrate (right).](image-url)
Although the principle of the catalytic triad is general it is not always performed by the same residues. In papain cystein performs the nucleophilic attack while aspartic acid has this function in pepsin and in other hydrolases threonine can act as the nucleophile.

**Oxyanion hole**

Close to the nucleophilic residue (serine or cysteine mostly) a so-called oxyanion hole exists that can bind the tetrahedral intermediates during hydrolysis. The first tetrahedral intermediate is formed when an ester is attacked by the activated Ser105. This intermediate is stabilized by binding in the oxyanion hole see Figure 1-4.

![Figure 1-4. Tetrahedral intermediate in the oxyanion hole of Candida antarctica lipase B.](image)

The oxyanion hole consists of the NH group of Gln106 and the OH and NH group of the main chain Thr40. Together they trap the negatively charged oxygen of the tetrahedral intermediate by hydrogen bonding. From this intermediate an alcohol is released and an acyl-enzyme complex is formed. The reaction of the acyl-enzyme complex with any nucleophile proceeds again through a tetrahedral intermediate stabilized by the oxyanion hole.

**Different fates of the acyl-enzyme complex**

The acyl-enzyme complex is attacked by water in hydrolysis (the natural action of hydrolases) but it can also be attacked by other nucleophiles. Reactions with water, amines, alcohols and hydrogen peroxide lead to hydrolysis, acyl transfer, aminolysis and peracid formation repectively as shown in Figure 1-5.

For the synthesis of polyamides the reaction with amines is the most important reaction. Amines are better nucleophiles than water but hydrolysis is always present as a possible side-reaction.
Figure 1-5. Hydrolysis, acyl transfer, aminolysis and peracid formation as the result of different nucleophiles attacking the acyl enzyme intermediate.

1.5 ENZYMES IN ORGANIC SOLVENTS

Two decades ago Alexander Klibanov and coworkers showed that porcine pancreatic lipase, subtilisin, chymotrypsin and horse radish peroxidase can perform their catalytic actions in dry organic solvents even at 100 °C. This turned out to be valid for more enzymes and stabilized by immobilization or cross-linking enzymes have become useful tools in organic chemistry. Especially in the field of enantiomeric resolution and enantioselective reactions enzymes are now widely employed.

Using enzymes in organic solvents rather than in water has several advantages:

1. Substrates and products usually dissolve better in organic solvents
2. Enzymes can be removed from the reaction medium since they do not dissolve
3. Undesired side-reactions with water are suppressed
4. Denaturation of enzymes is water dependent therefore the enzymes are expected to be more stable in a water-free environment
5. Thermodynamic equilibria can be shifted from hydrolysis towards synthesis

Enzymes can retain their catalytic function in organic solvents provided that a small amount of structural water is present in the enzyme. By non-covalent interactions these water molecules keep the enzyme together in its active conformation. The amount of essential water is not equal for each enzyme. Lipases and proteases only need traces of water while polyphenol oxidase needs 3.5x10⁷ molecules of water per enzyme.

The structural water is best retained in a hydrophobic solvent since hydrophilic solvents mix easily with water and are therefore able to strip all the water from the catalyst. A measure for the hydrophobicity of the solvent is the logarithm of the
partition coefficient (log P) of a solvent between 1-octanol and water. Solvents with a log P value higher than 2 are considered best for enzymatic synthesis.78

Enzymatic reactions are equilibrium reactions. In order to obtain yields that exceed the equilibrium reactions are performed under thermodynamic or kinetic control.

Thermodynamically controlled reactions are the reverse of the hydrolysis. The reaction conditions must be chosen to favour synthesis and usually this means choosing the right pH value. A drawback is the very slow reaction and low yield of the reaction. If the reaction products can accumulate by precipitation or by extraction, higher yields can be reached.

Kinetically controlled reactions fullfill two requirements. The formation of the acyl enzyme complex must be faster for the substrate than for the product. This can be met by using carboxylic esters in stead of acids. And secondly the rate of hydrolysis must be slower than the rate of aminolytic deacylation. If these two requirements are met an accumulation of the product polyamide occurs.

1.6 Enzymes in Polymer Synthesis

Enzymes are applied in polymer chemistry and a wide array of polymers has been synthesized by enzyme catalysis see Table 1-1.79 Enzymatic catalysis enabled scientists to synthesize polymers that are hard to synthesize by conventional catalysis like for instance polysaccharides.

Polyamides mentioned in Table 1-1 are published only recently by Gross80 in 2005 and Cheng81 in 2006. They synthesized polyamides (Mn ~3000 g/mol) in a condensation reaction of dialkylesters (among others: fumarate, malonate, adipate and oxalate) with diamines (e.g. diethylene triamine and diamine terminated polyethylene glycol).

Table 1-1. Polymers synthesized by enzymatic catalysis

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrolases, glycosidases, lipases, proteases</td>
<td>polysaccharides, polyesters, polycarbonates, poly(amoacid)s, polyamides</td>
</tr>
<tr>
<td>oxidoreductases</td>
<td>Polyphenols, polyanilines, vinyl polymers</td>
</tr>
<tr>
<td>horse radish and soybean peroxidase</td>
<td></td>
</tr>
<tr>
<td>laccase</td>
<td>polycarboxamides, cyclic oligosaccharides, polyesters</td>
</tr>
<tr>
<td>transferases</td>
<td></td>
</tr>
<tr>
<td>glycosyl-, acyltransferase</td>
<td></td>
</tr>
</tbody>
</table>

Two approaches were formulated at the start of the research. One is the polymerization analogous to the synthesis of polyesters by lipase (condensation of
diesters with diamines and the ring-opening polymerization of lactam rings) and the other one is starting from the reverse action of proteases \(^{82,83}\) and expand it to the formation of amide bonds between natural and non-natural substrates for proteases.

### 1.7 AIM AND OUTLINE OF THE THESIS

This thesis aims to develop and extend the knowledge of enzyme catalyzed ways to produce polyamides. The use of these highly selective catalysts that operate under mild conditions (aqueous media, ambient temperature and pressure) has developed over the last decades and offers new ways to synthesize polymers. These highly selective biocatalysts not just replace the conventional catalysts but also offer completely new routes to (bio)polymers that were not available before e.g. the synthesis of different polysaccharides.

By reducing energy consumption, waste production and the use of toxic chemicals enzymes can offer a green alternative for conventional processes to produce polyamides involving high temperatures (>200 °C) and toxic chemicals (like phosgene in peptide synthesis).

**Chapter 2** Describes the ring-opening polymerization of β-propiolactam by N435 to produce a nylon 3 characterized by MALDI-ToF MS and \(^{1}H\)-NMR spectroscopy. From the results we conclude that the polymerization does not proceed according to the reaction path accepted for enzyme catalyzed polymerization of lactones.

**Chapter 3** Enzyme catalyzed aminolysis is mainly used for the enantiomeric resolution of secondary amines but when applied to diesters and diamines the N435 can catalyze the synthesis of polyamides. The polycondensation of diesters with different diamines was studied with IR-spectroscopy.

**Chapter 4** Papain protease was used to synthesize a series of co-polyamino acids. The polymers are synthesized starting from a 1:1 molar mixture of two, three and four α-amino acids. MalDI-ToF mass spectrometry was used to determine the composition of these polymers.

**Chapter 5** The biodegradability of polymers can be enhanced by incorporating amino acids in the structure. By a reaction between aromatic diamines and protected amino acids diamines can be created that can serve as building blocks for biodegradable polymers. Papain was used to create a series of amido amines based on CBz-Gly, CBz-\(\text{-L-Leu}\) and CBz-\(\text{-L-Phe}\).
1.8 REFERENCES

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