Polyamide synthesis by hydrolases
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Chapter 5
Papain catalyzed synthesis of protected amino acid amides

5.1 ABSTRACT

This chapter describes the synthesis of amido amines catalyzed by papain from aromatic diamines (aniline, o,m,p-phenylene diamine) and N-carbobenzyloxy (Z) protected amino acids (Gly, l-Leu, l-Phe) in phosphate buffer (pH 7, 1.0 M). The amides precipitate (yield 19 - 47 % depending on the amino acid used) from the reaction mixture after one amide bond is formed thus preventing the formation of diamides in all cases.

Papain retains its activity in buffers with a higher pH (9 and 12) shown by the amide bond formation between 1,3-phenylene diamine and Z-Gly and Z-Phe.

Aliphatic diamines(1,4-butanediamine and 1,6-hexanediamine) were also used but amide formation could not be observed in buffers of pH 7, 9 or 12 due to the selectivity of papain. The obtained amido amines are potential building blocks for biodegradable polymers.
5.2 INTRODUCTION

By incorporating amino acids into artificial polymers the biodegradability can be enhanced without the loss of properties due to the variety of functional side groups that can be found. Direct polymerization of amino acids leads to peptides or polyamino acids as shown in chapter 4. Oligopeptides could serve as monomers but they have two different functional end-groups.

The monomers can be applied more easily in polymerizations when the end-groups are of the same functionality e.g. diamines. Amino acids can be converted into diamines by protecting the amine functionality (to prevent polymerization) and a reaction of a diamine with the acid functionality. In this chapter we show that papain can catalyze the formation of amido amines starting from (CBz) protected amino acids and aromatic diamines.

Amides based on N-protected ε-amino acids and amines have been under investigation for the use as surfactants1-3 and pharmaceutics4,5,6,7. Enzymatic catalysis to obtain these compounds offers control over the stereochemistry and functional side groups are usually left unchanged.

Examples from literature of Z-protected amino acid based amides synthesized by papain catalysis are given in paragraph 5.2.1, Figure 5-1 (a,b,c and d). Followed by an overview of the papain catalyzed synthesis of NCBz protected amino acid amides performed in our laboratory in paragraph 5.2.2.

5.2.1 Examples of amides based on Z-protected amino acids

Surfactants are used on a large scale in the world today and end up in the aquatic environment with detrimental effects. Surfactants based on α-amino acids mimick natural lipoamino acids and thereby offer biodegradability and biocompatability that reduces the impact of these compounds on the environment. A surfactant based on NCBz-L-Arg synthesized by papain is depicted in Figure 5-1 a) The charged amino acid NCBz-L-Arg can be converted into surfactants by a reaction with amines of different lengths shown by Clapès et al.2 Figure 5-1 b) Gemini surfactants based on NCBz-L-Arg were synthesized by Piera et al.1 using diamines in the same reaction.

Pharmaceutics based on N-carbobenzyloxyglycine and aniline have been under investigation as anti-epileptics reported by Geurts et al.3 Also other amides based on protected amino acids were under investigation for the same application shown by Conley.7 In Figure 5-1 c) amides of different NCbz-amino acid esters (Gly, L-Ala, L-Ser) with the pharmacologically active 4-aminoantipyrine4 reported by Lang and coworkers are shown. Figure 5-1 d) Proteolytically stable peptides that can be used as building blocks for protease inhibitors were synthesized by a reaction between NCBz-Gly and a series of ketoamines by Schuster.8

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5.2.2 Protected amino acids and aromatic (di)amines

In this chapter papain is used to synthesize amides based on the N-protected amino acids (Gly, L-Leu and L-Phe) and amines, summarized in Figure 5-2. Amines used in this reaction are aromatic (aniline, o, p, m-phenylenediamine) or aliphatic (tetra- and hexamethylene diamine). Amides were formed by the aromatic amines only. The product amides all precipitate from the reaction mixture as the monoamide. In Figure 5-2 the reaction schemes are depicted.

The CBz-protecting group was used because it can bind in the S2 binding position in the active site of papain (see Figure 1-1). It is known that bulky hydrophobic residues like phenylalanine bind preferentially at this position. The protecting group therefore binds at the S2. Since for the S1 position the papain is less selective all amino acids can be used connected to this protecting group.

The selectivity on the other side of the cystein is less well understood. To adress the presumed preference for aromatic hydrophobic substances aromatic amines were used. However it could be possible that aliphatic amines can also be accepted in this position.

Figure 5-1. Examples of Z-amino acid based amides synthesized using papain as a catalyst.
The reaction medium used is phosphate buffer (pH 7, 1.0 M) known to be a good medium for papain\(^{10,11}\). Later the buffer system was changed to pH 9 and 12 to ensure the presence of NH\(_2\) groups on the aliphatic diamines with pKa of 11.15 and 9.71 for 1,4-butanediamine and 11.85 and 10.76 for hexamethylene diamine. Papain is known to be active at alkaline pH without modification.\(^{12}\)

**Figure 5-2. Reaction scheme of the papain catalyzed formation of Z-protected amino acid amides**

\(\text{R}=\text{H, CH}_2\text{C}_8\text{H}_5, \text{CH}_2\text{CH(CH}_3\text{)}_2\)

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5.3 EXPERIMENTAL

5.3.1 Materials and methods

Materials
Papain lyophilized powder, N-carbobenzyloxy- (NCBz)-Gly, Z-I-Leu and Z-Phe were used as obtained from ACROS. Ortho-, meta- and para-phenylenediamine (ACROS), 1,4-Butanediamine and 1,6-hexanediamine (Fluka) were purified by sublimation. Dimethylsulfoxide(DMSO)-d6 (Sigma-Aldrich) and aniline (Merck) were used as received. The phosphate buffers (1.0 M, pH 7) (0.1 M, pH 12) and TRIS (0.1 M; pH 9) were prepared in the laboratory.

Methods
$^1$H-NMR spectra were recorded on a Varian 400 MHz spectrometer using DMSO-d6 as the solvent.

Mass spectra were recorded on a Thermoscientific LTQ XL/Orbitrap with positive ion detection.

Synthesis of Z-protected-amino acid amides
In a 50 mL flask, equipped with a stirring egg, a mixture of the N-protected amino acid (10 mmol), (di)amine (5 mmol), 20 mL of buffer and papain (150 mg) were placed. Z-Leu needed 30 mL of buffer solution in order to dissolve the reactant. The mixture was kept at 40 °C and stirred for 24 hours. The solids were separated by centrifugation, after decanting the solvent the solids were washed with a hydrochloric acid solution (0.1 M) and subsequently centrifuged. The washing procedure was repeated twice with water. The resulting powdery solid was dried in vacuo. The following structures were prepared:

1. Z-glycidyl-anilide

\[
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{O} \\
\text{H} \\
\text{H} \\
\text{O}
\end{array}
\]

$^1$H-NMR (DMSO-d6 $\delta=2.49$): $\delta=9.94$ (s, 1H); 7.55 (t, J=9.17, 3H); 7.38-7.32 (m, 5H); 7.29(t, J=7.71, 2H); 7.03 (tJ=7.18, 1H); 5.03 (s, 2H); 3.80 (d, J=6.04, 2H)

m/z: [M-H]$^-$ : 285.12
### 2. 1-amino-4-[Z-glycidylamido]-phenylene

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>NMR (DMSO-d6) δ = 2.49</th>
<th>Mass Spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>δ = 9.51 (s, 1H); 7.47 (t, 1H); 7.40 - 7.30 (m, 5H); 7.19 (d, J = 8.6, 2H); 6.48 (d, J = 8.6, 2H); 5.03 (s, 2H); 3.71 (d, J = 6.1, 2H)</td>
<td>m/z [M-H]^+: 300.13</td>
</tr>
</tbody>
</table>

### 3a. 1-amino-3-[Z-glycidylamido]-phenylene

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>NMR (DMSO-d6) δ = 2.49</th>
<th>Mass Spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>δ = 9.61 (s, 1H); 7.48 (s, 1H); 7.40 - 7.30 (m, 5H); 6.89 (m, 2H); 6.67 (d, J = 7.5, 2H); 6.24 (d, J = 7.6, 2H); 5.03 (s, 2H); 3.75 (d, J = 6.1, 2H)</td>
<td>m/z: [M-H]^+: 300.13</td>
</tr>
</tbody>
</table>

### 4a. 1-amino-2-[Z-L-glycidylamido]-phenylene

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>NMR (DMSO-d6) δ = 2.49</th>
<th>Mass Spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>δ = 9.13 (s, 1H); 7.52 (s, 1H); 7.40 - 7.30 (m, 5H); 7.12 (d, J = 7.6, 1H); 6.89 (t, J = 7.56, 1H); 6.7 (d, J = 7.98, 1H); 6.52 (t, J = 7.46, 1H); 5.04 (s, 2H); 4.86 (s, 2H); 3.82 (d, J = 5.83, 2H)</td>
<td>m/z: [M-H]^+: 300.13</td>
</tr>
</tbody>
</table>

### 3b 1-amino-3-[Z-L-leucidylamido]-phenylene

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>NMR (DMSO-d6) δ = 2.49</th>
<th>Mass Spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>δ = 10.35 (s, 1H); 7.92 (s, 1H); 7.50 (1H); 7.40 (t, J = 8.01, 2H); 7.35 - 7.25 (m, 5H); 7.04 (d, J = 7.68, 1H); 5.04 (s, 2H); 4.19 (m, 1H); 1.65 (m, 1H); 1.56 (m, 2H); 0.91 (s, 6H)</td>
<td>m/z: [M-Na]^+: 378.18</td>
</tr>
</tbody>
</table>

### 4b. 1-amino-2-[Z-L-leucidylamido]-phenylene

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>NMR (DMSO-d6) δ = 2.49</th>
<th>Mass Spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>δ = 9.84 (s, 1H); 7.62 (1H); 7.40 - 7.20 (m, 5H); 7.13 (m, 3H); 7.02 (m, 1H); 5.02 (s, 2H); 4.24 (s, 1H); 1.68 (s, 1H); 1.57 (s, 2H); 0.91 (m, 6H)</td>
<td>m/z: [M-Na]^+: 378.18</td>
</tr>
</tbody>
</table>
3c. 1-amino-3-[Z-L-phenylalanidylamido]-phenylene

\[
\begin{align*}
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{H} & \quad \text{N} \\
\text{H} & \quad \text{N} \\
\text{NH}_2
\end{align*}
\]

\[ ^1H\text{-NMR (DMSO-d6 } \delta=2.49): \delta= 9.80 (s, 1H); 7.62 (d, J=8.43, 1H); 7.38-7.22 (m, 10H); 7.20 (d, J=6.91, 1H); 6.93 (s, 1H); 6.70(d, J=7.75, 1H); 6.26(d, J=7.80, 1H); 4.95 (s, 2H); 4.39 (m, 1H); 3.12-2.99 (m, 1H); 2.82 (t, J=11.85, 1H) \\
m/z: [M-H]^+ 390.18
\]

4c. 1-amino-2-[Z-L-phenylalanidylamido]-phenylene

\[
\begin{align*}
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{H} & \quad \text{N} \\
\text{H} & \quad \text{N} \\
\text{NH}_2
\end{align*}
\]

\[ ^1H\text{-NMR (DMSO-d6 } \delta=2.49): \delta= 9.30 (s, 1H); 7.67(d, J=7.56, 1H); 7.38-7.08 (m, 10H); 7.02 (d, J=7.51, 1H); 6.89 (t, J=7.61, 1H); 6.70 (d, J=8.18, 1H); 6.52 (t, J=7.60, 1H); 4.97 (d, J=7.20, 2H); 4.43 (m, 1H); 3.10-3.0(2d, J=16, 2H D-Phe) 2.92-2.76 (m, 2H) \\
m/z: [M-H]^+ 390.18
\]

Table 5-1. Yields and mass spectrometry data of the obtained amides

<table>
<thead>
<tr>
<th>Structure</th>
<th>N-CBZ-</th>
<th>Nucleophile</th>
<th>Yield (%)</th>
<th>Mass m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>glycine</td>
<td>aniline</td>
<td>47</td>
<td>285.12</td>
</tr>
<tr>
<td>2</td>
<td>glycine</td>
<td>1,4-phenylenediamine</td>
<td>19</td>
<td>300.13</td>
</tr>
<tr>
<td>3a</td>
<td>glycine</td>
<td>1,3-phenylenediamine</td>
<td>8,11,13(^1)</td>
<td>300.13</td>
</tr>
<tr>
<td>4a</td>
<td>glycine</td>
<td>1,2-phenylenediamine</td>
<td>23</td>
<td>300.13</td>
</tr>
<tr>
<td>3b</td>
<td>leucine</td>
<td>1,3-phenylenediamine</td>
<td>41</td>
<td>378.18</td>
</tr>
<tr>
<td>4b</td>
<td>leucine</td>
<td>1,2-phenylenediamine</td>
<td>28</td>
<td>378.18</td>
</tr>
<tr>
<td>3c</td>
<td>phenylalanine</td>
<td>1,3-phenylenediamine</td>
<td>31,62,70(^1)</td>
<td>390.18</td>
</tr>
<tr>
<td>4c</td>
<td>phenylalanine</td>
<td>1,2-phenylenediamine</td>
<td>27</td>
<td>390.18</td>
</tr>
<tr>
<td>5a</td>
<td>Gly, Leu, Phe</td>
<td>1,4-butanediamine</td>
<td>--(^2)</td>
<td>--</td>
</tr>
<tr>
<td>5b</td>
<td>Gly, Leu, Phe</td>
<td>1,6-hexanediame</td>
<td>--(^2)</td>
<td>--</td>
</tr>
</tbody>
</table>

\(^1\) yields at pH 7.9 and 12 respectively

\(^2\) only the reactants were retrieved
5.4 RESULTS AND DISCUSSION

5.4.1 Product amides precipitate after monamidation

After a reaction time of 24 hours a white solid has precipitated from all the reaction mixtures. The products are then collected by centrifugation. Analysis of the products by $^1$H-NMR spectroscopy shows that the aromatic diamines formed an amide bond using one out of the two available amine groups.

In a first reaction the N-carbobenzoxyglycine (Z-Gly) was converted to Z-glycidylanilide 1 by a reaction with aniline. The amide precipitated in $47\%$ yield. Both $^1$H-NMR spectroscopy ($\delta=10.0$ (s, 1H, ArNCO aniline NH)) and mass spectrometry confirmed the structure of the amide. This reaction was repeated with para-, meta-, and ortho-phenylene diamine leading to the amides 3 and 4 see Figure 5-2.

![Figure 5-2](image)

**Figure 5-2.** $^1$H-NMR spectrum of 1-amino-4-[Z-glycidylamido]-phenylene

In the $^1$H-NMR spectra of these compounds the signals of the aromatic protons indicate monoamidation of the diamines as confirmed by mass spectrometry.

This is best illustrated with the $^1$H-NMR spectrum of 1-amino-4-[Z-glycidylamido]-phenylene Figure 5-3. The aromatic protons are split up in signals d and e while a diamide would show only one type of protons in the aromatic ring of the amide. The 1:1 ratio of the integrals d and e together with the mass spectrometry data confirms
the formation of one amide bond. For all the structures the \([M-H]^+\) or \([M-Na]^+\) masses are those of monoamides see Table 5-1.

Mono amidation is most probably the result of the poor solubility of the products. For future research it is recommended that the experiments are repeated with amino acids, diamines and reaction media that increase the solubility of the products.

Also a protecting group should be used that can be removed more easily than the CBz-group usually removed by catalytic hydrogenation. For the use in other media it might be necessary to increase the stability of papain. This can be achieved by immobilization\(^{13,14,15}\), modification with polyethylene glycol\(^{16,17,18}\) or site directed mutagenesis.\(^{19}\)

5.4.2 Papain prefers aromatic amines over aliphatic amines

Two aliphatic diamines were used in this reaction, 1,4-butanediamine and 1,6-hexanediame see entry 5 in Figure 5-2. No amides were obtained with these reactants. The reactions with these amines were repeated at pH 9 and 12 to ensure the presence of NH\(_2\) groups since the pKa values of the aliphatic diamines are 11.15 and 9.71 for 1,4-butanediamine and 11.85 and 10.76 for hexamethylene diamine.

It was reported that papain retains its esterase activity at alkaline pH with\(^{19}\) or without\(^{12}\) modification. To confirm the activity of the papain the synthesis of the amides 3a and 3c was repeated at pH 9 and 12 and yielded the amides in comparable yields as at pH 7 see Table 5-1. Therefore papain is not deactivated by the high pH and the amines should be able to react with the protected amino acids.

Selectivity of papain at the S1’ position (the other side of the amide bond to be formed) can be such that aliphatic diamines are not accepted as substrates. However very little is known about the selectivity of papain at this position. From the results presented here it is concluded that papain prefers aromatic amines over aliphatic amines at the S1’ position.
5.5 CONCLUSIONS

Papain is able to catalyze the formation of an amide bond between Z-gly, Z-Phe and Z-Leu and aniline, ortho-, meta- and para-phenylene diamine. In a phosphate buffer of pH 7 the structures $\text{1,2,3a,}\text{b,}\text{c}$ and $\text{4a,}\text{b,}\text{c}$ were obtained. The amides precipitate after the formation of one amide bond shown by $\text{^1H-NMR}$ spectroscopy and mass spectrometry measurements.

Aliphatic diamines were not accepted as a substrate in this reaction not even at higher pH (9 and 12) used to ensure nucleophilic NH$_2$ groups on the aliphatic amines. Activity of papain in these media is confirmed by repeating the synthesis of the amides from 1,3-phenylene diamine in combination with Z-Gly and Z-Phe with yields comparable with the reaction at pH 7.

The selectivity of the papain is known for the S2 position where it prefers aromatic hydrophobic amino acids like phenylalanine. Selectivity on the S1 is less pronounced (charged amino acids can also be used). The selectivity of papain on the S1’ site is unclear from literature but from our results it is concluded that papain prefers aromatic amines over aliphatic amines in this position.

The obtained amido-amines can be used fo polymerization once the solubility problems are solved. After removal of the N-carbobenzoxy protecting group a large diamine is obtained that can be used as a monomer in polymerization reactions.

Larger amido amines are obtained when the diamides can be synthesized by papain catalysis as well. Future research should be directed to change monomers and reaction media that increase the solubility of the products and therefore allow the formation diamides. This includes modification of papain and the search for other enzymes that show better reactivity in organic solvents.
5.6 REFERENCES
