Chapter 4
Papain catalyzed (co)polymerization of \(\alpha\)-amino acids

4.1 ABSTRACT

Four hydrophobic amino acids (Leu, Tyr, Phe, Trp) were polymerized by the protease papain in homopolymerization, binary copolymerization and ternary copolymerization. After 24 hours solid polydisperse reaction products of the homopolymerization are obtained in yields ranging from 30 – 80 % by weight. A \(\text{DP}_{\text{avg}}\) was calculated based on MALDI-ToF MS results using the ion counts for the chains in the product. Based on the \(\text{DP}_{\text{avg}}\) and the yield of the homopolymerization it is determined that the amino acids can be ranked according to reactivity in the order: Tyr > Leu > Phe > Trp. Thermal degradation of the homopolymers shows two degradation steps, at 178-239 °C and at 300-330 °C. All the products left a significant amount of char ranging from 18 - 57 % by weight at 800 °C.

Binary copolymers are obtained as a polydisperse precipitate with a compositional distribution of the chains. Both the compositional and chain length distribution are calculated from MALDI-ToF mass spectra. By comparing the amount of each amino acid present in the chains it was determined that the amino acids are incorporated with a preference: Leu > Tyr > Phe > Trp.

Ternary copolymers were also obtained as a precipitate and analyzed by MALDI-ToF MS. The compositional distribution and the chain length distribution are calculated from the MALDI-ToF data. The amount of every amino acid in the chains was determined. Also the influence on the \(\text{DP}_{\text{avg}}\) when the polymers are compared with the corresponding binary copolymers. From the combined results it is concluded that in the copolymerization of three amino acids the preference is Leu > Tyr > Phe > Trp. Thermal degradation of all the copolymers showed a weight loss of 2 wt% before the main polymer degradation step at 300 - 325 °C.
4.2 INTRODUCTION

The enzymatic polymerization of \( \alpha \)-amino acid esters was studied by many authors for a wide array of proteases and amino acid esters over the years. The first reports by Brenner et al.\(^1\) in 1950 showed the polymerization of threonine and methionine esters by \( \alpha \)-chymotrypsin. Others used bovine lung proteinase,\(^4\) ficin and Cathepsin-C\(^5\) a protease from Streptomyces sp. was used for the oligomerization of diethyl \( \alpha \)-aspartate by Matsumura et al.\(^6\) and Soeda et al.\(^7\)

One of the most versatile proteases is papain, it was used in the past to catalyze the polymerization of leucine\(^8,9\) methionine\(^8,10\) glycine,\(^9\) tyrosine\(^8,11\) and glutamic acid diethylester.\(^11\) The polymerizations were carried out in citrate buffer pH 5-6 \(^8,10\) or phosphate buffer pH 7\(^11\) leading to polyaminoacids with a degree of polymerization (DP) of 5-9 amino acids.

Copolymers of glutamic acid diethylester with ethyl- or methylesters of alanine, leucine, phenylalanine, tyrosine and aspartic acid ethylester were reported by Uyama et.al.\(^12\) The copolymers were exclusively \( \alpha \)-linked and the yield of polymeric product depended on the comonomer used. Copolymers with leucine were obtained in the highest 70 % yield with DP 8 while a copolymer with alanine gave chains with DP 15 but in a lower 31 % yield.

\[
\text{H}_2\text{N}\bigg\}^\text{O}\bigg\}^\text{O}\bigg\}^\text{O}\bigg\}^\text{CH}_3 + \text{H}_2\text{N}\bigg\}^\text{O}\bigg\}^\text{O}\bigg\}^\text{O}\bigg\}^\text{CH}_3 \xrightarrow{\text{Papain pH 7}} \text{H} \bigg\}^\text{N}\bigg\}^\text{O}\bigg\}^\text{CO}\bigg\}^\text{N}\bigg\}^\text{O}\bigg\}^\text{O}\bigg\}^\text{CH}_3
\]

**Figure 4-1.** Reaction scheme of enzyme catalyzed amino acid (co)polymerization \( R_1=R_2 \) and \( R_1 \neq R_2 \).

Papain has a known preference for hydrolysis of the peptide bond one amino acid away from hydrophobic aromatic residues in peptides. Therefore it is expected that the hydrophobic amino acids are also polymerized by papain. In this chapter we add the papain catalyzed homo- and copolymerization of Leu, Tyr, Trp and Phe (see Figure 4-1) to this field.

Polyamino acids containing 1 to 4 of the listed amino acids were prepared in phosphate and tris(hydroxymethyl)aminomethane buffers and a suitable reaction medium is chosen from this.

Since a selectivity of papain is reported for hydrolysis and binding sites are identified in the active site, it is expected that the amino acids will not be polymerized equally well. MALDI-ToF mass spectrometry is used to determine the composition of the polyamino acids. From the composition of the polymer chains conclusions are drawn regarding the reactivity order in which the amino acids are polymerized. The thermal stability of polyamino acids was determined by thermogravimetric analysis.
4.3 EXPERIMENTAL

4.3.1 Materials and methods

Materials
Papain was purchased from ACROS Organics as a lyophilized powder. The methyl ester hydrochlorides of L-leucine, L-phenylalanine, L-tryptophan and L-tyrosine, α-cyano-4-hydroxycinnamic acid, DMSO-d6, tris(hydroxymethyl)aminomethane and 2,2,2-Trifluoroethanol (TFE) were obtained from Sigma-Aldrich. N-Carbobenzoxyglycine (Z-Gly), trifluoroacetic acid (TFA), Na2CO3 and acetonitrile are used as received from Acros Organics. Hydrochloric acid (37 %), NaH2PO4·H2O, Na2HPO4·7H2O, citric acid monohydrate and sodium citrate dihydrate were obtained from Merck. Water is purified by reversed osmosis from an in-house tap. Methanol was bought from Lab-Scan. The MALDI-ToF calibration mixture (bradykinin, angiotensin I and ACTH 18-39) was obtained from Sigma-Aldrich. Citrate buffer (pH 5.6; 2.0 M), phosphate buffer (pH 7; 1.0 M) and TRIS buffer (pH 7; 1.0 M) were prepared in the laboratory.

Methods
1H-NMR spectra were recorded on a Varian 400 or 300 MHz spectrometer. Either DMSO-d6 or a 9:1 mixture of DMSO-d6 with trifluoroacetic acid was used as a 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.

Thermogravimetric analysis measurements were carried out using a Perkin-Elmer TGA7 with a heat rate of 10 °C min⁻¹ and N2 flow from 18-800 °C. The samples were dried over P₂O₅ prior to the measurement.

Synthesis of Z-Gly-(Leu)₅-OMe
Papain (38 mg) was dissolved in 10 mL buffer solution (citrate buffer pH 5.6) and to this Z-Gly-OH was added (140 mg; 6.69·10⁻⁴ mol) followed by Leu-OMe·HCl (1.229 g; 6.77·10⁻³ mol). The clear solution is stirred for 66 hours at room temperature. After this a white precipitate is collected by filtration and washed twice with 2 mL of a Na₂CO₃ solution (5 %) and twice with a 1 M solution of HCl in water and finally with water. The product is dried overnight under reduced pressure at 46 °C and gave a crude yield of 49 %. The crude product is purified by dissolving it in hot methanol. Water is added dropwise to the hot solution until a precipitate forms. After cooling in an ice bath for 45 minutes the product is collected by filtration and dried in vacuum at 46 °C overnight (yield 36 %).
\(^1\)H-NMR (1 drop CF₃COOH in CDCl₃): \(\delta = 7.8\) (s, 1H, CONHCH (Gly)); 7.5 (s, 1H, CONHCH (Leu)); 7.3 (m, 5H, Ar); 5.1 (s, 2H, Ar-CH₂O); 4.5 (s, 1H, NCHRCO); 4.0 (s, 2H, NCH₂CO); 3.8 (s, 3H OCH₃); 1.5 (m, 3H, CHCH₂CH(CH₃)₂); 0.8 (m, 6H, CH(CH₃)₂)

Polymerization and copolymerization of \(\alpha\)-amino acids

All the homopolymerizations and copolymerizations where conducted according to the following experimental.

In a 50 mL flask, equipped with a stirring egg, a mixture of the amino acid ester hydrochloride (5 mmol), 25 mL phosphate buffer (pH 7.0; 1.0 M) and papain (300 mg) was placed. The mixture was kept at 40 °C and stirred for 24 hours in the air. Precipitates were collected by centrifugation (7500 rpm) and washed once with dilute HCl solution (0.1 M) and twice with water. The resulting white powder was dried in vacuo for three hours, recrystallized from 2,2,2-trifluoroethanol and subsequently dried to give 30 to 50 % yield, depending on the nature of the amino acid ester. In the case of copolymerization the amino acid esters were added in equimolar amounts.

MALDI-ToF MS sample preparation

For MALDI-ToF analysis the samples were dissolved (3 mg mL\(^{-1}\)) in TFE, acetonitrile/H₂O/TFA (30/70/0.1) mixtures or pure TFA depending on the copolymer. Sometimes a 10-fold dilution of the sample is needed to obtain a decent mass spectrum. The spots were created by 2 \(\mu\)L of a mixture containing matrix solution (10 mg mL\(^{-1}\)) and sample solution in a 1:1 ratio by volume. Calibration was performed with a mixture of matrix dimer, bradykinin, angiotensin I and ACTH 18-39 dissolved in a mixture of acetonitrile/H₂O/TFA (30/70/0.1). Each spectrum is obtained by a minimum of 600 shots.
MALDI-TOF MS data processing and related calculations

The polymers produced in this chapter are collected as a precipitate from the reaction medium. All the (co)poly amino acids are polydisperse in chain length and chain composition. It is assumed in the discussion that the distribution represents the way polymers grow. Although no time-resolved data is available the results are interpreted as such. Short chains are polymers that just started growing and represent the first stage of the polymerization, longer chains represent the last stages of the reaction.

The average degree of polymerization \( \langle DP \rangle \) weighted by the amount \( I \) of the molecular ions of a length \( i \) is calculated using Equation 4-1. All the ions of the same DP are counted irrespective of the endgroup or the type of molecular ion.

\[
DP_{avg} = \frac{\sum_{i=1}^{n} I_i DP_i}{\sum_{i=1}^{n} I_i}
\]

Within each DP a distribution is found in the ratio of the amino acids that are incorporated in the product. For each DP the average fraction of the amino acids \( F_{aa} \) is calculated weighted by the amount of chains \( I_i \) with a fraction amino acid \( faa_i \) using Equation 4-2. The ion counts for all the chains with the same amino acid composition are added irrespective of the endgroup or the type of molecular ion.

\[
F_{aa} = \frac{\sum_{i=1}^{n} I_i faa_i}{\sum_{i=1}^{n} I_i}
\]

The average fraction \( F_{aa} \) times the according DP gives the average number of every amino acid in a distribution of chains. This value is then plotted against the corresponding DP. A linear fit through the data points reveals how much on average is contributed by each containing amino acid. In total one amino acid is added for the elongation DP+1. When more than half of it is contributed by a certain amino acid is said to be preferred in the polymerization.

In the MALDI-TOF mass spectra the molecular ions [M-H]\(^+\) and [M-NA]\(^+\) were found, but potassium adducts, although reported by others,\(^{13}\) were not identified. Adducts with more than one metal atom\(^{13,14}\) were found only in the case of the leucine homopolymer, as well as the leucine copolymer with tyrosine.

\[ \text{- 65 -} \]
4.4 RESULTS AND DISCUSSION

4.4.1 Homopolymerization of α-amino acids

Homopolymers of leucine, tyrosine and tryptophan were synthesized with papain from the corresponding methylester hydrochlorides in phosphate buffer (pH 7; 1 M). After 24 hours of reaction the precipitate was collected by centrifugation while in the absence of papain no precipitation was formed. Yields depend on the amino acids used varying from 30 to 80% by weight see Table 4-1.

Table 4-1. Yield and DP_{avg} of the Leu, Phe, Tyr and Trp homopolymers.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Polymer</th>
<th>Yield(wt%)</th>
<th>DP by 1H-NMR</th>
<th>DP_{avg} by MALDI-ToF MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Z-Gly(Leu)ₙ</td>
<td>31</td>
<td>5</td>
<td>6³</td>
</tr>
<tr>
<td>2</td>
<td>(Leu)ₙ</td>
<td>55</td>
<td>12</td>
<td>6.86</td>
</tr>
<tr>
<td>3</td>
<td>(Phe)ₙ</td>
<td>60</td>
<td>6</td>
<td>5.35</td>
</tr>
<tr>
<td>4</td>
<td>(Tyr)ₙ</td>
<td>80</td>
<td>9</td>
<td>6.98</td>
</tr>
<tr>
<td>5</td>
<td>(Trp)ₙ</td>
<td>30</td>
<td>5</td>
<td>4.85</td>
</tr>
</tbody>
</table>

³ Determined as DP at the peak molar mass (Mp) of the distribution. Other DP_{avg} were calculated with equation 4-1.

The average chain lengths determined from 1H-NMR spectroscopy and MALDI-ToF MS are shown in Table 4-1. Signals from the methylester end-groups are used to determine the DP from 1H-NMR spectra. The overestimation of the degree of polymerization of polyleucine (DP 12) and polytyrosine (DP 9) by 1H-NMR spectroscopy can be explained by hydrolysis reactions creating acid end-groups. In MALDI-ToF spectra the resulting acid end groups are found in significant amounts as well.

To prove this, leucine was polymerized starting from a N-carbobenzoxy-glucose (Z-Gly) molecule (see Table 4-1 entry 1). The chain length (DP 5) can now be determined using the aromatic protons of the protecting group and the leucine main chain. Including the starting Z-Gly moiety, 6 amino acids are connected by papain in agreement with the DP_{avg} calculated from the MALDI-ToF mass spectra.

From the MALDI-ToF MS data DP_{avg} was calculated using Equation 4-1. Often the smallest chains detected are 4 or 5 amino acids length. Chains with a lower DP are not detected because of overlapping peaks with matrix fragments and polymer fragments.

From the results presented in Table 4-1 the following order of reactivity is determined Tyr > Leu > Phe > Trp. Polytyrosine is obtained in highest yield (80 %) combined with the highest DP_{avg} (6.98). Polyleucine was obtained with a DP_{avg} 6.86 but in comparable yield (55 %) as polyphenylalanine (60 %) with DP_{avg} 5.35. Polytryptophan is the
homopolymer that was obtained in the lowest yield of the series (30%) and formed the shortest chains with $\text{DP}_{\text{avg}}$ 4.85.

### 4.4.2 Copolymerization of $\alpha$-amino acid esters

The copolymers where synthesized by papain starting from a 1:1 molar ratio of the amino acids. After 24 hours of reaction in the buffer the precipitate is collected by centrifugation in yields 28 - 60% in phosphate buffer and 13 - 35% in TRIS buffer. Since the yields are lower in TRIS buffer than in the phosphate buffer see Table 4-2 TRIS is discarded as a suitable reaction medium and all further experiments are conducted in phosphate buffer (pH 7; 1.0 M).

TRIS buffer is used for some protease mediated reactions\textsuperscript{15,16,17,18} including papain\textsuperscript{19,20} so it is not likely that the enzyme looses its activity in this buffer. However, in the polymerization of amino acids\textsuperscript{8,9,11,12,10} TRIS was not reported, only citrate and phosphate buffers are mentioned.

### Table 4-2. Copolymer yields in phosphate and TRIS buffer.

<table>
<thead>
<tr>
<th>entry</th>
<th>Copolymer</th>
<th>Yield in phosphate buffer (wt%)</th>
<th>Yield in TRIS buffer (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tyr-Phe</td>
<td>44</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Tyr-Leu</td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Tyr-Trp</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Leu-Phe</td>
<td>52</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>Trp-Phe</td>
<td>35</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>Trp-Leu</td>
<td>60</td>
<td>41</td>
</tr>
<tr>
<td>7</td>
<td>Tyr-Phe-Trp</td>
<td>41</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Phe-Tyr-Leu</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>Trp-Tyr-Leu</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>Phe-Leu-Trp</td>
<td>39</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>Phe-Leu-Trp-Tyr</td>
<td>38</td>
<td>18</td>
</tr>
</tbody>
</table>

### 4.4.3 Composition of binary copolyamino acids

MALDI-ToF mass spectrometry provides detailed information about the composition and endgroups of the polymer chains. Hydrolysis of the ester endgroups leads to acid endgroups and both are found in the MALDI-ToF mass spectra. The most abundant molecular ion is the sodium adduct [M-Na]$^+$ but also some [M-H]$^+$ ions are detected. At low m/z values (< 700 m/z) the peaks overlap with matrix ions and
polymer fragments. Molecular ions with a lower m/z value can therefore not be identified.

**Table 4-3. Composition of binary copolymers composed of amino acids AA1 and AA2 at DP_{avg} and mass at DP_{avg}.**

<table>
<thead>
<tr>
<th>entry</th>
<th>Copolymer</th>
<th>DP_{avg}</th>
<th>AA1</th>
<th>AA2</th>
<th>M at DP_{avg}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tyr-Phe</td>
<td>6.9</td>
<td>4.14</td>
<td>2.74</td>
<td>1110.5</td>
</tr>
<tr>
<td>2</td>
<td>Tyr-Leu</td>
<td>7.7</td>
<td>3.94</td>
<td>3.78</td>
<td>1103.9</td>
</tr>
<tr>
<td>3</td>
<td>Tyr-Trp</td>
<td>5.9</td>
<td>2.50</td>
<td>3.35</td>
<td>1063.1</td>
</tr>
<tr>
<td>4</td>
<td>Leu-Phe</td>
<td>6.7</td>
<td>3.57</td>
<td>3.12</td>
<td>895.8</td>
</tr>
<tr>
<td>5</td>
<td>Trp-Phe</td>
<td>4.3</td>
<td>2.13</td>
<td>2.18</td>
<td>749.6</td>
</tr>
<tr>
<td>6</td>
<td>Trp-Leu</td>
<td>7.2</td>
<td>3.74</td>
<td>3.42</td>
<td>1114.8</td>
</tr>
</tbody>
</table>

In **Table 4-3** we can see the average degree of polymerization and the composition at DP_{avg} for all the binary copolymers. From the number of each containing amino acid and the assumption that all the chains have an ester endgroup the mass of the average chains is calculated. All the binary copolymers show an average composition of a random copolymer (a 1:1 ratio of the amino acids AA1 and AA2) in most of the chains at DP_{avg}.

From the homopolymerization it is expected that the amino acids do not have an equal reactivity in the copolymerization. By analyzing the composition of copolymers this can be verified.

Below, first the MALDI-ToF MS analysis of the copolymer poly(tyrosine-co-phenylalanine) is illustrated in detail. Second, a brief description of the other binary copolymers is given accompanied by the resulting graphs illustrating the composition of the binary copolymers. Finally the effect of combining amino acids with another is evaluated to rank the amino acids in order of reactivity.

1. **Poly(tyrosine-co-phenylalanine)**

The copolymer of tyrosine and phenylalanine was obtained in 44 wt% yield. The mass difference between Tyr and Phe causes the mass peaks to be grouped per DP ranging from 5 to 10 see **Figure 4-2**. Each peak can be explained as one of the four possible molecular ions, [M-H]^+ or [M-Na]^+ with either an ester or an acid endgroup.
For each DP the ions are identified and counted. This is illustrated for the 7-mer, Figure 4-3 zooms in on this fraction and its peaks are explained in Table 4-4. Peaks of all the possible chains with seven amino acid residues were found except the chains with a 1:6 and a 0:7 ratio of tyrosine to phenylalanine. Either they are not present or to small to distinguish them from the noise.

The chains with an average composition of 4 plus 3 amino acids are the most abundant, they make up around 55 % of the ion counts for this DP. In Table 4-4 the peaks (m/z value) are listed from 1102 to 1180 and the composition Tyr_Phe, ion-type, end-group, absolute I (counts) and relative (I %; ion counts divided by total counts per DP) are listed. Most of the ions are [M-Na]+ type with ester or acid endgroup.
Table 4-4. Identification of the ions found in the poly(Tyr-co-Phe) 7-mer.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Tyr_Phe</th>
<th>Ion</th>
<th>Endgroup</th>
<th>I (counts)</th>
<th>I(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1102</td>
<td>2_5</td>
<td>[M-Na]^+</td>
<td>Acid</td>
<td>597.77</td>
<td>2.14</td>
</tr>
<tr>
<td>1112</td>
<td>4_3</td>
<td>[M-H]^+</td>
<td>Acid</td>
<td>347.79</td>
<td>1.25</td>
</tr>
<tr>
<td>1116</td>
<td>2_5</td>
<td>[M-Na]^+</td>
<td>Ester</td>
<td>690.90</td>
<td>2.47</td>
</tr>
<tr>
<td>1118</td>
<td>3_4</td>
<td>[M-Na]^+</td>
<td>Acid</td>
<td>2533.93</td>
<td>9.07</td>
</tr>
<tr>
<td>1124</td>
<td>4_3</td>
<td>[M-H]^+</td>
<td>Ester</td>
<td>300.12</td>
<td>1.07</td>
</tr>
<tr>
<td>1128</td>
<td>5_2</td>
<td>[M-H]^+</td>
<td>Acid</td>
<td>200.40</td>
<td>0.72</td>
</tr>
<tr>
<td>1132</td>
<td>3_4</td>
<td>[M-Na]^+</td>
<td>Ester</td>
<td>2320.56</td>
<td>8.31</td>
</tr>
<tr>
<td>1134</td>
<td>4_3</td>
<td>[M-Na]^+</td>
<td>Acid</td>
<td>5170.25</td>
<td>18.51</td>
</tr>
<tr>
<td>1140</td>
<td>5_2</td>
<td>[M-H]^+</td>
<td>Ester</td>
<td>1213.88</td>
<td>4.35</td>
</tr>
<tr>
<td>1148</td>
<td>4_3</td>
<td>[M-Na]^+</td>
<td>Ester</td>
<td>3710.17</td>
<td>13.28</td>
</tr>
<tr>
<td>1150</td>
<td>5_2</td>
<td>[M-Na]^+</td>
<td>Acid</td>
<td>3915.33</td>
<td>14.02</td>
</tr>
<tr>
<td>1156</td>
<td>6_1</td>
<td>[M-H]^+</td>
<td>Ester</td>
<td>1965.10</td>
<td>7.03</td>
</tr>
<tr>
<td>1164</td>
<td>5_2</td>
<td>[M-Na]^+</td>
<td>Ester</td>
<td>2046.85</td>
<td>7.33</td>
</tr>
<tr>
<td>1166</td>
<td>6_1</td>
<td>[M-Na]^+</td>
<td>Acid</td>
<td>1205.64</td>
<td>4.32</td>
</tr>
<tr>
<td>1172</td>
<td>7_0</td>
<td>[M-H]^+</td>
<td>Ester</td>
<td>1233.70</td>
<td>4.42</td>
</tr>
<tr>
<td>1180</td>
<td>6_1</td>
<td>[M-Na]^+</td>
<td>Ester</td>
<td>481.27</td>
<td>1.72</td>
</tr>
</tbody>
</table>

For every observed DP the ion counts are added and the resulting chain length distribution is plotted in Figure 4-4. The DP$_{avg}$ is calculated weighted by the number of ions using Equation 4-1 to be 6.9.
The average amount of the two amino acids was calculated from the average fraction (from Equation 4-2) and plotted in Figure 4-5. The chains grow by adding on average 0.92 units of Tyr and 0.08 units of Phe (slope of the lines). Showing the low reactivity of phenylalanine when copolymerized with a tyrosine. Tyrosine is preferred over phenylalanine as it was observed for the reactivity in the case of the homopolymers.

Figure 4-5. Average number amino acid residues in poly(Tyr-co-Phe) for every observed DP.
2. Poly(tyrosine-co-leucine)

The copolymer of tyrosine and leucine was obtained in 40 % yield. The MALDI-ToF MS spectrum shown in Figure 4-6 A shows peaks with m/z values 769 – 1656. The peaks belong to chains with a DP 6 -12. The DP_avg was calculated as 7.8 corresponding to the maximum of the distribution shown in Figure 4-6 B at DP 8.

![MALDI-ToF mass spectrum and chain length distribution of poly(Tyr-co-Leu)](image-url)
In Figure 4-7 the average number of tyrosine and leucine residues for every DP are plotted. The chains grow by adding preferentially leucine. The slopes of the two amino acids (determined from a linear fit) show an increase of 0.37 per DP for Tyr and 0.72 for Leu. Leucine is therefore preferred over tyrosine in this reaction. This result is not in accordance with the reactivity found for homopolymerization of leucine and tyrosine.

**Figure 4-7.** Average number of amino acids in poly(Tyr-co-Leu) for every observed DP.
3. Poly(tyrosine-co-tryptophan)

The copolymer of tyrosine and tryptophan was produced in 36 % yield. The MALDI-ToF MS spectrum of this copolymer is shown in Figure 4-8 A. The peaks with values 799 – 1717 m/z were analyzed and belong to chains with a DP 4-10. The most abundant ion is the 939 m/z, 2_3 [M-Na]^+ ester terminated molecular ion.

![MALDI-ToF mass spectrum](image)

**Figure 4-8. A) MALDI-ToF mass spectrum and B) Chain length distribution of the poly(Tyr-co-Trp).**

In Figure 4-8 B the chain length distribution of the poly(Tyr-co-Trp) is plotted. The DP_{avg} of the polymer is calculated at 5.5. This is a slight overestimation (DP 1,2 and 3 are missing) caused by the lack of MALDI-ToF peaks below 700 m/z that could not be distinguished from polymer fragments and noise.

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In **Figure 4-9** the average amount of tyrosine and tryptophan are plotted. The amount of tyrosine residues in the chains rises by 0.75 per DP and the amount of tryptophan in the chains with 0.25 per DP. Tyrosine is more reactive than tryptophan, which was also the amino acid with the smallest yield and shortest chains in the homopolymerization of the amino acids.

![Graph showing the average number of amino acids in poly(Tyr-co-Trp) for every observed DP.](image)

**Figure 4-9.** Average number of amino acids in poly(Tyr-co-Trp) for every observed DP.
4. Poly(leucine-co-phenylalanine)

Leucine and phenylalanine were copolymerized in 52% yield. The MALDI ToF MS spectrum of this copolymer is shown in Figure 4-10 A. The peaks with m/z values 753 – 1027 were analyzed and could be assigned to chains with a DP 5-9. The most abundant ion is the 948 m/z, 4_3, [M-Na]+ ester terminated molecular ion.

Figure 4-10. A) MALDI-ToF mass spectrum and B) Chain length distribution of the poly(Leu-co-Phe).
An ion of a chain with a DP 9 was found at 1276 m/z belonging to the \(_{3,6} [M-\text{Na}]^+\) ester terminated molecular ion. However, not enough data points of the 9-mer could be found to include these in the discussion.

The chain length distribution is plotted in Figure 4-10 B and shows a maximum between at DP 7. A \(\text{DP}_{\text{avg}}\) of 6.4 was calculated from the ion counts as described before.

In Figure 4-11 the average amount of each amino acid per DP is plotted. The chains grow by adding more leucine (slope 0.76) than phenylalanine (slope 0.24). Leucine is therefore more reactive than phenylalanine.

**Figure 4-11.** Average amount of amino acids in poly(Leu-co-Phe) for every observed DP.
5. Poly(tryptophan-co-phenylalanine)

Poly(Trp-co-Phe) was obtained in 35 % yield. The MALDI-ToF MS spectrum of this copolymer is shown in **Figure 4-12 A**. The peaks with m/z values 499-1201 were assigned to chains with DP 4-7. The only chains that were found with DP 7 have a composition 5_2 and 4_3. In **Figure 4-12 B** the distribution of the chain lengths in the sample is plotted with a maximum DP 4 with a calculated DP_{avg} 4.4.

![MALDI-ToF mass spectrum and chain length distribution of poly(Trp-co-Phe).](image)

**Figure 4-12.** A) MALDI-ToF mass spectrum and B) chain length distribution of poly(Trp-co-Phe).
The DP rises by adding on average 0.25 tryptophan and 0.90 phenylalanine. The slopes add up to more than 1 amino acid residue per DP. This is not possible since chains are elongated with only one amino acid at a time. It is probably the result of a linear fit through all the data points while the values at DP 3 are too low for both amino acids. Phenylalanine is more reactive than tryptophan in this combination.
Poly(tryptophan-co-leucine)

Poly(Trp-co-Leu) was obtained in 60 % yield. The MALDI-ToF MS spectrum of this copolymer is shown in Figure 4-14 A. The peaks with m/z values of 704-1745 were assigned to chains with a DP of 4-13. The most abundant ion is the 1025 m/z, 2_4, [M-Na]⁺ ester terminated molecular ion.

![MALDI-ToF mass spectrum](image1.png)

A

![Chain length distribution](image2.png)

B

Figure 4-14. A) MALDI-ToF mass spectrum and B) chain length distribution of the poly(Trp-co-Leu).

- 80 -
In the high-end tail of the distribution the 1744 m/z, $^{10}\text{[M-Na]}^+$ ester terminated molecular ion can be found but it is only one of the possible 13-mer ions and therefore not included in the discussion. The chain length distribution (Figure 4-14 B) shows a maximum at DP 7 in good agreement with the calculated the DP$_{avg}$ of 7.2.

The average amount of each amino acid was calculated and plotted against DP in Figure 4-15. The amount of leucine rises with DP and as the chains grow after DP 8 the amount of Trp in the chains drops. One explanation could be that the amount of Trp decreases after some time when the chains redistribute by transamidation or hydrolysis reactions. Leucine has a higher reactivity than tryptophan.

Figure 4-15. Average number of amino acids in poly(Trp-co-Leu) for every observed DP.
4.4.4 Reactivities of the amino acids in binary copolymerization

In the four graphs below the number of Tyr, Phe, Leu or Trp in combination with the other amino acids per degree of polymerization are presented. A linear fit through the data points shows how much of each amino acid is added when the chains grow by one amino acid residue. From the slopes a reactivity order is derived that concludes this paragraph.

Reactivity of leucine in copolymerization with Tyr, Phe or Trp

Figure 4-16 shows the average amount of leucine in chains obtained when leucine is copolymerized with tyrosine, phenylalanine or tryptophan. It can be concluded that leucine is the amino acid that is the most reactive of the four amino acids. This is shown in two ways. First, in all cases the slope of the amount of leucine with increasing DP is higher than 0.5. The slopes are 0.72 in combination with tyrosine and 0.76 in combination with phenylalanine. The slope is 1.16 in combination with tryptophan which can be explained by redistribution reactions and hydrolysis. Second, copolymers with leucine always show a higher DP_{avg} than the homopolymers.

![Graph showing reactivity of leucine](image)

**Figure 4-16.** Average number of leucine residues in copolymers with Tyr, Phe or Trp.

Reactivity of tyrosine in copolymerization with Phe, Leu or Trp

Figure 4-17 shows the average amount of tyrosine in chains obtained when it is copolymerized with Phe, Leu or Tryp. The chains grow by the addition of 0.92 tyrosine residues in combination with Phe, 0.75 with Trp and 0.37 with Leu. From the slopes it can be concluded that Tyr is preferred over Phe and trp while it is less reactive than Leu.
Figure 4-17. Average number of tyrosine residues in copolymers with Phe, Leu or Trp.

Compared to the homopolymerizations of Leu, Phe and Trp the binary copolymers with tyrosine always have a higher $\text{DP}_{\text{avg}}$. However this effect of Leu is stronger. Tyrosine is therefore the second most reactive amino acid.

_Reactivity of phenylalanine in copolymerization with Tyr, Leu or Trp_

Figure 4-18. Average number of phenylalanine residues in copolymers with Tyr, Leu or Trp.

Figure 4-18 shows the average amount of phenylalanine in the chains obtained when phenylalanine is copolymerized with Tyr, Leu and Trp. The slope of the lines show
that the copolymers of phenylalanine grow by adding 0.90 units per DP when combined with Trp, 0.24 with Leu and 0.08 with Tyr. Phenylalanine is more reactive than Trp but less than Tyr and Leu. Compared to the homopolymers phenylalanine does not really influence the DP$_{avg}$. In conclusion phenylalanine is the third most reactive amino acid in the synthesis of binary copolymers.

Reactivity of tryptophan in copolymerization with Tyr, Phe or Leu

Figure 4-19 shows the amount of tryptophan found in the chains after copolymerization with Tyr, Phe and Leu. In the homopolymerization tryptophan was polymerized in the lowest yield and with the lowest DP$_{avg}$. This is also found in combination with other amino acids. The chains grow by adding on average 0.25 Trp units per DP in combination with Tyr and Phe. In combination with leucine the number of tryptophan residues is decreasing for chains with DP 9-12. The decreasing amount of tryptophan can be explained with chain elongation by transamidation or by partial hydrolysis of the chains. From the slopes it is concluded that in all copolymerizations the other amino acid is more reactive.

![Figure 4-19](image.png)

Figure 4-19. Average number of tryptophan residues in copolymers with Tyr, Phe or Leu.

The reactivity of the amino acids in the copolymerization of two amino acids can be summarized as Leu > Tyr > Phe > Trp. This result is almost in accordance with the reactivity series found for the homopolymerizations. Leucine and tyrosine are very near in reactivity because they reach a comparable DP$_{avg}$ in the synthesis of homopolymers. The copolymers with leucine are probably better soluble and therefore reach higher DP$_{avg}$. 

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4.4.5 Composition of ternary copolyamino acids

In Table 4-5 the DP_{avg} of the ternary copolyamino acids and the composition at DP_{avg} are listed. Unlike the copolymerization with two amino acids the chains do not represent the composition of a random copolymer. The reactivity differences are more pronounced in these copolymers.

Table 4-5. Average composition of copolymers containing three amino acids.

<table>
<thead>
<tr>
<th>entry</th>
<th>% homo</th>
<th>Copolymer</th>
<th>DP_{avg}</th>
<th>AA1</th>
<th>AA2</th>
<th>AA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>9.5</td>
<td>Phe-Tyr-Trp</td>
<td>6.0</td>
<td>2.13</td>
<td>1.88</td>
<td>1.99</td>
</tr>
<tr>
<td>8</td>
<td>3.5</td>
<td>Phe-Tyr-Leu</td>
<td>8.3</td>
<td>3.08</td>
<td>1.84</td>
<td>3.37</td>
</tr>
<tr>
<td>9</td>
<td>1.3</td>
<td>Trp-Tyr-Leu</td>
<td>6.0</td>
<td>2.21</td>
<td>1.92</td>
<td>1.90</td>
</tr>
<tr>
<td>10</td>
<td>5.8</td>
<td>Phe-Leu-Trp</td>
<td>5.2</td>
<td>1.48</td>
<td>1.26</td>
<td>2.44</td>
</tr>
</tbody>
</table>

Below the composition of the different copolymers and the reactivity differences are discussed in more detail.

7. Poly(phenylalanine-co-tyrosine-co-tryptophan)

In phosphate buffer this copolymer was obtained in 41 % yield. The MALDI-ToF MS spectrum of this copolymer is shown in Figure 4-21. Peaks with m/z values of 656-1445 were assigned to the chains with a DP 4-8 see Figure 4-21. From the chain length distribution DP_{avg} is calculated to be 6.0.

Figure 4-20. MALDI-ToF mass spectrum of poly(Phe-co-Tyr-co-Trp).
Figure 4-21. A) and B) chain length distribution of poly(Phe-co-Tyr-co-Trp).

In Figure 4-22 the average amount of the amino acids in this copolymer is plotted for each DP. The chains contain progressively more Phe and Tyr. As more redistribution reactions take place the amount of tryptophan drops and again after DP 6. The same reactions cause an increase in the amount of Tyr and Phe. Trp is the least reactive amino acid in the formation of binary copolymers and that trend is reflected here.

Figure 4-22. Average number of amino acids in poly(Phe-co-Tyr-co-Trp) for every observed DP.
8. Poly(phenylalanine-co-tyrosine-co-leucine)

The copolymer of phenylalanine, tyrosine and leucine was synthesized in 28% yield. The MALDI-ToF MS spectrum of this copolymer is shown in Error! Reference source not found. A. The peaks with an m/z value 753-1523 were assigned to chains with a DP 6-11. The chain length distribution of the polymer was determined and plotted in Error! Reference source not found. B it shows a maximum ion counts at DP 8. The DP$_{avg}$ of the copolymer was calculated as 8.3.

![MALDI-ToF mass spectrum](image1)

![Chain length distribution](image2)

**Figure 4-23.** A) MALDI-ToF mass spectrum and B) chain length distribution of Poly(Phe-co-Tyr-co-Leu).
In Figure 4-24 the average number of each amino acid in the copolymer is plotted for every DP. As was found also for the copolymers derived from two amino acids Leu is the most reactive amino acid in this product. Because, after DP 9 the amount of Phe and Tyr drop and these amino acids are replaced by leucine showing an increase in the polymers with DP 9-12.

![Figure 4-24. Average number of amino acids in Poly(Phe-co-Tyr-co-Leu) for every observed DP.](image)

9. Poly(tryptophan-co-tyrosine-co-leucine)

The copolymer of tyrosine, leucine and tryptophan was obtained in 36 % yield. The MALDI-ToF MS spectrum of this copolymer is shown in Figure 4-25A. The peaks with m/z values 703-1329 were assigned to chains with DP 4-9 with a distribution as plotted in Figure 4-25B with a D_{avg} 6.0.

For every DP the average number of every containing amino acid was calculated and depicted in Figure 4-26. The average amount of leucine is rising throughout the distribution with 0.95 residues per DP. Tyrosine shows a maximum number of residues at DP 7 and a decrease for DP 8 and 9. Tryptophan seems to arrive at around 2.3 residues on average per chain. Decreasing amounts of any amino acid can be the result of redistribution reactions like hydrolysis or transamidation.
Figure 4-25. A) MALDI-ToF mass spectrum and B) chain length distribution of poly(Trp-co-Tyr-co-Leu).
10. Poly(phenylalanine-co-leucine-co-tryptophan)

Phenylalanine, leucine and tryptophan were co-polymerized in 39 % yield. The MALDI-ToF MS spectrum of this copolymer is shown in Figure 4-28A. Peaks with an m/z value 673-1123 were assigned to chains with DP 4-7 with its distribution depicted in Figure 4-28. The DP$_{avg}$ was calculated as 5.2.

Figure 4-26. Average number of amino acids in poly(Trp-co-Tyr-co-Leu).

Figure 4-27. MALDI-ToF mass spectrum of poly(Phe-co-Leu-co-Trp).
Figure 4-28. Chain length distribution of poly(Phe-co-Leu-co-Trp).

For every DP the average amount of the amino acids is plotted in Figure 4-29. The amount of Leu is rising in the polymer with every elongation. The amount of Trp and Phe in the chain arrives at a constant value.

Figure 4-29. Average amount of amino acids in poly(Phe-co-Leu-co-Trp).
4.4.6 Reactivities of the amino acids in ternary copolymerization

In the four graphs below the number of Leu, Tyr, Phe or Trp in combination with the other amino acids per degree of polymerization is presented as already shown for the binary copolymers. The plots show how much of each amino acid is added when the chains grow by one amino acid residue (Dp+1). From the average amount of every amino acid and the effect of adding an amino acid to the binary copolymer on the DP_{avg} an order of reactivity was determined.

Leucine containing ternary copolymers

All the copolymers containing leucine have more leucine residues incorporated as the chains grow longer see Figure 4-30. Elongation with leucine is often combined with a reducing amount of one of the other components like tyrosine when combined with Leu-Trp and Phe in combination with Leu-Trp and Leu-Tyr. This was also observed for Trp in combination with Leu in the serie of binary copolymers.

In comparison with the binary copolymers the influence of leucine on the DP_{avg} is very clear. Adding leucine produces ternary copolymers that have a higher DP_{avg} than the corresponding binary copolymers. In accordance with the results from the binary copolymers leucine has the highest reactivity in the ternary copolymerizations.

![Graph showing reactivities of amino acids in ternary copolymerization](image)

Figure 4-30. Average amount of leucine residues in ternary copolymers.
Figure 4-31. Average amount of tyrosine residues in ternary copolyamino acids.

Tyrosine containing ternary copolymers

Tyrosine is present in the chains varying from 1.0 to 3.0 residues (see Figure 4-31). However most chains contain 1.7 - 2.2 tyrosine residues and this seems to be rather constant for all the copolymers throughout the distribution.

Only combined with Trp-Leu (triangles in Figure 4-31) the amount of tyrosine rises with DP and drops again after DP 7. Again this is explained by redistribution reactions taking place.

When the DP_{avg} of the copolymers is compared to the DP_{avg} of the binary copolymers the addition of tyrosine leads to a lower DP_{avg} in combination with Trp-Leu and an increasing DP_{avg} for the combinations with Phe-Leu and Phe-Trp. From this it is concluded that Tyr is the second most reactive amino acid in the ternary copolymerizations.

Phenylalanine containing ternary copolymers

The amount of phenylalanine rises with DP and starts to go down again after DP 8 for the combination with Leu-Tyr (see Figure 4-32). Again the explanation for this is a hydrolysis or transamidation reaction leading to a redistribution of the amino acids. In combination with Trp-Tyr and Leu-Trp the amount of phenylalanine seems to reach a maximum but not enough data points are available (due to precipitation) to conclude if it is there.

Comparing the DP_{avg} of the ternary copolymers with the binary copolymers shows that phenylalanine had no influence on the DP_{avg} when combined with Tyr-Trp, it
increased the DP_{avg} when combined with Tyr-Leu and decreased DP_{avg} in combination with Leu-Trp. In conclusion phenylalanine is the third most reactive amino acid, as was also seen for the binary copolymers.

![Figure 4-32. Average amount of phenylalanine in ternary copolyamino acids.](image)

**Tryptophan containing ternary copolymers**

The number of tryptophan residues in the ternary copolymers lies between 1.8 and 2.4 (see Figure 4-33). In the binary copolymer of Trp-Leu a redistribution was seen. The redistribution reactions are not obvious in the ternary copolymers. When the DP_{avg} of the ternary copolymers is compared to the DP_{avg} of the corresponding binary copolymers Trp lowers the DP_{avg} for all chains. Tryptophan is again found to be the least reactive amino acid.

Based on the above discussed reactivities of the amino acids in the ternary copolymerizations it is concluded that the reactivity order of the amino acids is Leu > Tyr > Phe > Trp for these polymerizations.
4.4.7 Thermogravimetric analysis of peptides

The polyamino acids polyleucine, polytyrosine, polyphenylalanine and polytryptophan were pyrolyzed by heating the samples from 20 to 800 °C. In Table 4-6 the TGA data are presented. The polyamino acids have a main degradation step between 300-320 °C. The polyleucine and the polytryptophan and polyphenylalanine have an extra degradation step between 160 °C and 239 °C.

Table 4-6. Polymer degradation temperatures of polyamino acids

<table>
<thead>
<tr>
<th>Polymer</th>
<th>T1</th>
<th>T2</th>
<th>wt% char</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>178</td>
<td>306</td>
<td>57</td>
</tr>
<tr>
<td>Tyr</td>
<td>158</td>
<td>317</td>
<td>40</td>
</tr>
<tr>
<td>Phe</td>
<td>239</td>
<td>331</td>
<td>24</td>
</tr>
<tr>
<td>Trp</td>
<td>302</td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

In Figure 4-34 the TGA-curves of the homopolymers are presented. All the polymers except polyTrp show weight loss step before the main degradation step (300-340 °C). After the thermal treatment a significant amount of char is left. Especially poly(leucine) degrades only half since 57 % by weight is left as a char.

The weight loss before the main degradation is caused by the loss of water while cyclic peptides are formed\(^{13}\) the so-called diketopiperazides. During the main degradation step these cyclic structures fall apart and a char is left. The main degradation step at around 300-350 °C can be explained by two processes. The
pyrolysis of polytyrosine\textsuperscript{21} and polyleucine\textsuperscript{13} proceeds through the degradation of cyclic structures under the release of ammonia and water. Polyphenylalanine\textsuperscript{21,13} degrades by falling apart in nitrile and olefin fragments that further degrade by the loss of CN, HCN and CH\textsubscript{2}R (R = amino acid side group).\textsuperscript{21}

Char left after the thermal treatment can be stabilized by phenolic residues in the case of polytyrosine.\textsuperscript{22,21} The other polyamino acids also leave a char behind.

\textit{Pyrolysis of copolyamino acids}

The copolyamino acids were also subjected to thermal degradation. The results of this degradation are summarized in Table 4-7. All the copolymers show the main degradation between 300 and 325 °C. After heating the samples to 800 °C still at least 10 \% char was left for all the polymers. A combination of phenylalanine and tryptophan left 29 wt\% char and a copolymer of tyrosine, phenylalanine and tryptophan left 26 wt\% char. The other copolymers left <20 wt\% char. Degradation already starts at temperatures between 150-200 °C.

![Figure 4-34. TGA curves of polyLeu, polyTyr, polyTrp and polyPhe](image)

The degradation of peptides proceeds through the formation of cyclic peptides and the formation of diketopiperazines. The formation of the diketopiperazines is accompanied by the release of water, in the TGA diagrams this first step was visible as a small decrease of ~2 wt\% before the start of the main degradation step.
Table 4-7. Degradation temperatures of the copolymers

<table>
<thead>
<tr>
<th>Entry</th>
<th>Copolymer</th>
<th>PDT(^1) (°C)</th>
<th>loss (wt%)</th>
<th>Char (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tyr-Phe</td>
<td>320</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Tyr-Leu</td>
<td>312</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Tyr-Trp</td>
<td>308</td>
<td>81</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>Phe-Leu</td>
<td>301</td>
<td>88</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Phe-Trp</td>
<td>314</td>
<td>69</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>Leu-Trp</td>
<td>313</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Tyr-Phe-Trp</td>
<td>322</td>
<td>63</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>Tyr-Phe-Leu</td>
<td>316</td>
<td>86</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>Tyr-Leu-Trp</td>
<td>311</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>Phe-Trp-Leu</td>
<td>307</td>
<td>85</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>Phe-Leu-Trp-Tyr</td>
<td>302</td>
<td>83</td>
<td>13</td>
</tr>
</tbody>
</table>

1. PDT\(^1\) = Polymer Degradation Temperature

4.4.8 Parameters that influence the reactivity of the amino acids

From the polymerizations described above a reactivity order was deduced. This is an apparent reactivity since it is not known if characteristics of the enzyme, the intrinsic reactivity of the amino acids or the solubility of monomers and products determines the course of the reaction. These effects described below and future recommendations are given when appropriate.

Selectivity by papain

The observed reactivity differences of the monomers can be the result from the inherent selectivity of papain (described in chapter 1). It is known that papain has a preference for aromatic hydrophobic residues in the S2 position (see Figure 1-1),\(^{23,24}\) with a preference for tyrosine over phenylalanine.\(^{25}\) Cysteine proteases and papain in particular, are reported to be tolerant with respect to binding amino acids in other subsites.\(^{26}\) In the homopolymerizations this preference for tyrosine is found. In the copolymerizations leucine is found in a higher ratio than the other amino acids with tyrosine as the second most reactive amino acid.

Future research on this topic could reveal if the selectivity for the synthesis of polyamino acids is different from the selectivity found for hydrolysis. The exact order
of the amino acids and the consumption of monomers over time can provide information for this. The order of amino acids can be determined by post source decay MALDI-ToF MS as used for proteins. Difficulties arise because a monodisperse sample is needed for this. Time resolved analysis of reaction products and monitoring the monomer consumption during the course of reaction are recommended.

**Reactivity of the amino acids**

The nucleophilicity of the amino acids does not vary that much judged by there pK values listed in Table 4-8. Based on this data it is not expected that there is a big difference in reactivity of the amino acids. In addition, the order of Pk values does not represent the order of reactivity found for the amino acids in any of the polymerizations described.

<table>
<thead>
<tr>
<th></th>
<th>pK1 (α-COOH)</th>
<th>pK2 (α-NH₃⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>2.33</td>
<td>9.74</td>
</tr>
<tr>
<td>Trp</td>
<td>2.46</td>
<td>9.41</td>
</tr>
<tr>
<td>Phe</td>
<td>2.20</td>
<td>9.31</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.20</td>
<td>9.21</td>
</tr>
</tbody>
</table>

**Precipitation of polyamino acids**

The polymerizations described in the former paragraphs all yield polymers with an insoluble product after 24 hours of reaction. Since all homopolymers are obtained as polydisperse solid precipitates, it is assumed that the precipitation starts and the chains present at that time are dragged out of the solution. This seems a reasonable assumption for the copolymers as well. According to literature the precipitates of polypeptides contain a distribution of chains as shown by Uyama and Li for the enzymatic (co)polymerization of glutamic acid derivatives²¹,²² and by Fukuoka for the enzymatic polymerization of tyrosine derivatives.²³

Chains of amino acids precipitate as a solid powder or in crystal structures. The crystal structures peptides and polyamino acids usually form are the α-helix and the β-sheet. An α-helix is only stabilized when at least two turns can be formed each containing 3.6 amino acids. Chains with a DP 5-10 usually form β-sheets. The polypeptides in this research all have a DP₉₅% of 8 or lower and therefore precipitate predominantly in β-sheets.

Not all the amino acids used like to form β-sheets. The propensities of amino acids to form β-sheets was discussed in a review article by Neslonsey and Kelly.²⁴ From a series of amino acids the four amino acids used in this chapter can be ranked shown in Table 4-9 according to four different studies. Each group used different computations...
to find energy minima for the β-sheet formation. The mentioned studies agree that polyleucine has the least propensity to form β-sheets. For the other amino acids it is not clear which is the right order when looking at the β-sheet propensities.

**Table 4-9. β-sheet propensities of Tyr, Trp, Phe and Leu ranked from high to low propensity.**

<table>
<thead>
<tr>
<th></th>
<th>Chou and Fasman30,31</th>
<th>Kim and Berg32</th>
<th>Smith and Regan33</th>
<th>Minor and Kim34,35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr</td>
<td>Phe</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Tyr</td>
</tr>
<tr>
<td>Trp</td>
<td>Tyr</td>
<td>Phe</td>
<td>Phe</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>Trp</td>
<td>Trp</td>
<td>Trp</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Leu</td>
<td>Leu</td>
<td>Leu</td>
<td></td>
</tr>
</tbody>
</table>

For α-helix formation similar studies were reported in literature summarized in **Table 4-10.** It can be seen that leucine has the highest propensity to form α-helical structures. The order of the other three amino acids is not that clear. As well in α-helix as in β-sheet propensities leucine stands out and the others have a similar preference for β-sheets. Chains with a DP > 10 are found for a few (co)polymers, these chains either precipitate as an α-helix or, they are included in the β-sheets.

**Table 4-10. α-helix propensities of Tyr, Trp, Phe and Leu**

<table>
<thead>
<tr>
<th></th>
<th>Pace and Scholtz36</th>
<th>Rohl et al.37</th>
<th>Munoz and Serrano38</th>
<th>Williams et al.39</th>
<th>Chou and Fasman30,31</th>
<th>Luque et al.45</th>
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A polydispersity is also found in the precipitate obtained in the homopolymerization of Leu, Phe, Tyr and Trp. Once β-sheet formation is started polymers of different DP are included in the precipitate.

Copolymers will precipitate in β-sheets for two reasons. First, amino acids show similar propensities towards β-sheet formation. Second, the DP_{avg} found for the copolymers is 8.3 or less and this implies β-sheet formation for all the copolymers.

In order to obtain longer polymers in buffer systems monomers can be included that are more hydrophilic. Another approach is changing to non-aqueous media. This also needs enhancement of papain for the use in these media. From literature it is known that this can be achieved by immobilization41,42,43, modification with polyethylene glycol44,45,46 or site directed mutagenesis47.
4.5 CONCLUSIONS

Papain catalyzed the polymerization of the amino acids leucine, tyrosine, phenylalanine and tryptophan. Also the binary and ternary copolymerization of the mentioned amino acids was catalyzed by the enzyme. The observed reactivity of the amino acids is the result from an interplay between, the selectivity of the enzyme, reactivity of the amino acids and the solubility of the polymers. Further research needs to be done to clarify the contribution of each of these factors.

The homopolymerization of the amino acids revealed that papain catalyzes the polymerization with a reactivity order of Tyr > Leu > Phe > Trp. Although the DP$_{avg}$ of polyTyr (6.98) and polyLeu (6.85) is comparable the yields of both polymerizations (55 % and 80 % respectively) determined the order presented here.

Binary copolymerization lead to polymers obtained in yields of 35 – 60 % and DP$_{avg}$ varying between 4.3 and 7.7. The variations in chain length and in composition of the chains were determined and evaluated.

The reactivity of the amino acids was determined based on the changing amount of each amino acid with DP. The reactivity of the amino acids was Leu > Tyr > Phe > Trp. Also in relation to the corresponding homopolymers this order of reactivity was found.

Ternary copolymers were synthesized by papain, the composition of the chains was calculated. Leucine turned out to be most present at high DP. The amount of leucine shows an increase per DP for all the polymers. Tryptophan showed to be present in constant, but low amounts. Phenylalanine and tyrosine were less pronounced.

However when the effect of the amino acids on the DP of the parent 2-amino acid copolymer was evaluated it showed that the reactivity order was the same as for the 2-amino acid copolymers Leu > Tyr > Phe > Trp.

The degradation of the homopolymers proceeded in one or two steps. The main degradation step is similar for the homopolymers and the copolymers, ranging from 300-325 °C. All the polymers left char behind after heating to 800 °C. Homopolymers left 18-57 % char while the copolymers left only 10-29 % char behind. Char formation is explained by cross-linking reactions by the phenolic residues of tyrosine. 

Copolymerization enabled more complete degradation of the products.

In the future, research should be directed to improve the solubility of reaction products in aqueous media by incorporating hydrophilic amino acids or by changing to non-aqueous media with challenges for the activity of papain. Experiments towards the consumption of monomers during the reaction can reveal a selectivity by the papain in this reaction.
4.6 REFERENCES

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