Papain Catalyzed Synthesis of Protected Amino Acid Amides

Leendert W. Schwab, Wouter M. J. Kloosterman, Jakob Konieczny, and Katja Loos*

Department of Polymer Chemistry & Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747AG Groningen, The Netherlands

Received July 13, 2012; Accepted October 19, 2012

ABSTRACT: The papain catalyzed enzymatic synthesis of amido amines catalyzed from aromatic diamines and N-carbobenzyloxy (Z) protected amino acids (Gly, L-Leu, L-Phe) is described. 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) observable by the amide bond formation between 1,3-phenylene diamine and Z-L-Gly and Z-L-Phe. Aliphatic diamines (1,4-butanediamine and 1,6-hexanediamine) were used as well but amide formation could not be observed in buffers of pH 7, 9 or 12 due to the selectivity of papain.

KEYWORDS: Papain, biocatalysis, amino acid amide, selectivity

1 INTRODUCTION

Amides based on N-protected α-amino acids and amines have been investigated for the use as surfactants [1–3] and pharmaceutics [4–7]. Enzymatic catalysis to obtain these compounds offers control over the stereochemistry and functional side groups are usually left unchanged [8].

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 which reduces the impact of these compounds on the environment. A surfactant based on NCBz-L-Arg was synthesized by Clapès et al. [2] via papain catalysis. The charged amino acid NCBz-L-Arg was converted into surfactants by a reaction with amines of different lengths. 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 [5] and other amides based on protected amino acids [7] have been under investigation as anti-epilectics. Amides of different NCbz-amino acid esters (Gly, L-Ala, L-Ser) with the pharmacologically active 4-aminoantipyrine were reported by Lang and coworkers [4]. 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 [9].

Furthermore, papain was used to catalyze the polymerization of oligopeptide sequences of leucine, methionine, glycine, tyrosine and various amino acid ethylester [10–15]. Various copolymers of amino acids and amino acid ester could be synthesized as well [16–18]. The enzymatic polymerization [19–21] of polymers containing amide bonds [22–26] could be an important future process for a more sustainable production of commodity plastics.

Before the structure of papain was resolved by X-ray crystallography Schechter and Berger had already identified recognition sites for amino acid residues on both sides of the active site cysteine by kinetic experiments - 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 [27,28]. 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 [29]. Since these early findings further research pointed out that papain has a selectivity for binding aromatic hydrophobic residues in the S2 position[30–34]. It prefers tyrosine over phenylalanine in peptides at P2 [35]. It was concluded by Kim et al. [36] 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

*Corresponding author: k.u.loos@rug.nl

DOI:10.7569/JRM.2012.634102

small hydrophobic side chains as shown by Schuster et al [37]. This was refined to leucine, alanine, serine and phenylalanine by Ménard and coworkers [38], while others determined a preference for tryptophan and leucine [39]. The S2′ site binds small amino acids in the order Ala, Val, Asn, Leu and Phe, as shown with kinetic experiments of small peptides with chromophoric markers attached to it [40]. The selectivity on the other side of the cystein is less well understood. To address this issue we used papain to synthesize amides based on the N-protected amino acids (Gly, L-Leu and L-Phe) and amines, summarized in Figure 1. The CBz-protecting group was used as it can bind to the S2 binding position in the active site of papain. Furthermore, it is known that bulky hydrophobic residues like phenylalanine bind preferentially at this position [27,28]. The protecting group therefore binds at S2. Since papain is less selective at the S1 position all amino acids can be used connected to this protecting group.

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.

2 EXPERIMENTAL SECTION

2.1 Materials

Papain lyophilized powder, N-carbobenzyloxy-Z-L-Gly, Z-L-Leu and Z-L-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.

2.2 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.

2.3 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-L-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:

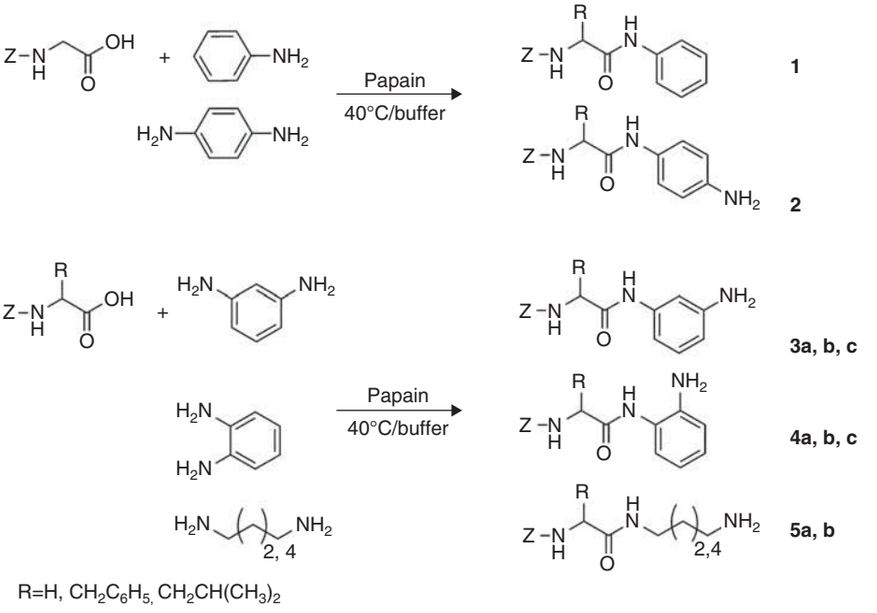
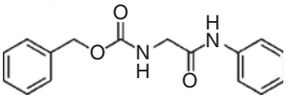


Figure 1 Reaction scheme of the papain catalyzed formation of Z-protected amino acid amides.

1. Z-glycidyl-anilide

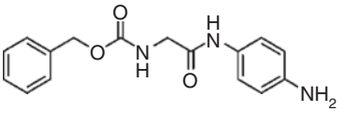


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 9.94 (s, 1H); 7.55 (t, J = 9.17, 3H); 7.38–7.32 (m, 5H); 7.29(t, J = 7.71, 2H); 7.03 (t,J = 7.18, 1H); 5.03 (s, 2H); 3.80 (d, J = 6.04, 2H)

m/z: [M-H]+ : 285.12

Yield: 47%

2. 1-amino-4-[Z-glycidylamido]-phenylene

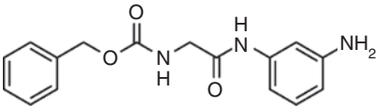


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 9.51(s,1H); 7.47 (t, 1H); 7.40 - 7.30(m, 5H); 7.19(d, J = 8.6, 2H); 6.48(d, J = 8.6, 2H); 5.03 (s, 2H); 3.71 (d, J = 6.1, 2H)

m/z [M-H]+: 300.13

Yield: 19%

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

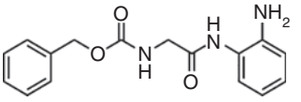


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 9.61(s, 1H); 7.48(s, 1H); 7.40–7.30 (m, 5H); 6.89(m, 2H); 6.67 (d,J = 7.5, 2H); 6.24(d, J = 7.6,2H); 5.03 (s, 2H); 3.75 (d, J = 6.1, 2H)

m/z: [M-H]+ 300.13

Yield: 8% (pH = 7); 11% (pH = 9); 13% (pH = 12)

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

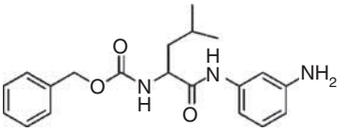


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 9.13 (s, 1H); 7.52(s, 1H); 7.40–7.30(m, 5H); 7.12 (d, J = 7.68, 1H); 6.89 (t, J = 7.56, 1H); 6.7(d, J = 7.98, 1H); 6.52 (t, J = 7.46, 1H); 5.04 (s, 2H); 4.86 (s,2H); 3.82(d, J = 5.83, 2H)

m/z: [M-H]+ 300.13

Yield: 23%

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

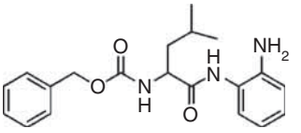


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 10.35 (s, 1H); 7.92 (s, 1H); 7.50 (1H); 7.40(t, J = 8.01, 2H); 7.35–7.25 (m, 5H); 7.04 (d, J = 7.68, 1H) 5.04 (s, 2H); 4.19 (m, 1H); 1.65 (m, 1H);1.56 (m, 2H); 0.91(s, 6H)

m/z: [M-Na]+ 378.18

Yield: 41%

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

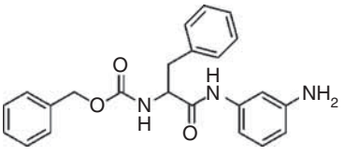


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 9.84 (s, 1H); 7.62 (1H); 7.40–7.20(m, 5H); 7.13 (m, 3H); 7.02(m, 1H); 5.02(s, 2H); 4.24(s, 1H); 1.68 (s, 1H);1.57 (s, 2H); 0.91(m, 6H)

m/z: [M-Na]+ 378.18

Yield: 28%

3c. 1-amino-3-[Z-L -phenylalanidylamido]-phenylene

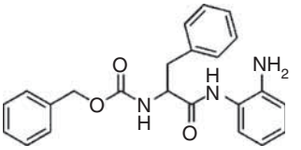


^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 9.80 (s, 1H); 7.62 (d, J = 8.43, 1H); 7.38–7.22 (m, 10H); 7.20 (d, J = 6.91, 1H); 6.93 (s, 1H); 6.70(d, J = 7.75, 1H); 6.26(d, J = 7.80, 1H); 4.95 (s, 2H); 4.39 (m, 1H); 3.12–2.99 (m, 1H); 2.82 (t, J = 11.85, 1H)

m/z: [M-H]+ 390.18

Yield: 31% (pH = 7); 62% (pH = 9); 70% (pH = 12)

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



^{1}H-NMR (DMSO-d6 δ = 2.49): δ = 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

Yield: 27%

3 RESULTS AND DISCUSSIONS

The mono-amidation products of N-protected amino acids (Gly, L-Leu and L-Phe) and aromatic amines (aniline, ortho-, para-, meta-phenylenediamine) were successfully synthesized via papain catalyzed amidation reactions.

The reaction medium used was phosphate buffer (pH 7, 1.0 M) known to be a good medium for papain [41–42]. After a reaction time of 24 hours a white solid precipitated from all reaction mixtures. Subsequently, the products were 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-L-Gly) was converted to Z-glycidylanilide **1** by a reaction with aniline. The amide precipitated in 47 % yield. Both ^{1}H-NMR spectroscopy (δ= 10.0 (s, 1H, ArNHCO aniline NH) and mass spectrometry confirmed the structure of the amide (see analysis details in the experimental part). This reaction was repeated with para-, meta-, and ortho-phenylene diamine leading to the amides 2, 3 and 4 see Figure 1 and the detailed analysis results in the experimental part.

The ^{1}H-NMR spectra of these compounds clearly 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, see Figure 2. The aromatic protons are split up in the signals d and e while a diamide would show only one type of proton of 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 detailed analysis results in the experimental part.

Mono amidation is most probably the result of the poor solubility of the products. Therefore, 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. However, for the use in other media it might be necessary to increase the stability of papain. This can be for instance achieved by immobilization [43–45], modification with polyethylene glycol [46–48] or site directed mutagenesis [49].

Two aliphatic diamines were tested in this reaction as well - 1,4-butanediamine and 1,6-hexanediamine see entry 5 in Figure 1. However, no amide bond formation was observable. Therefore, the reactions with these amines were repeated at pH 9 and 12 to ensure the presence of NH_2 groups - 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 diamines respectively. Papain is known to be active at alkaline pH [50] and to retain its esterase activity at alkaline pH

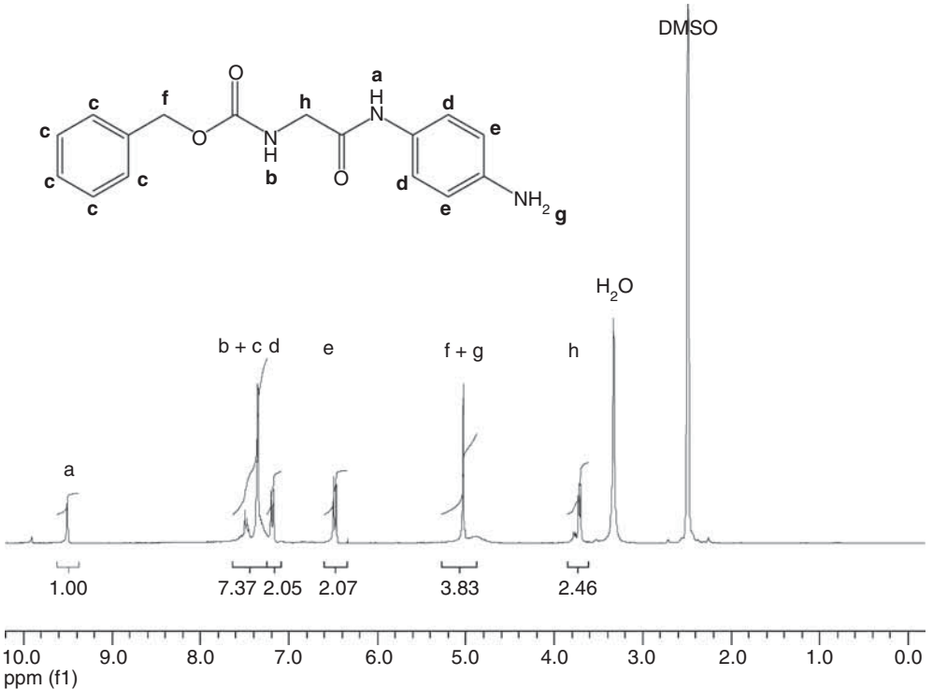


Figure 2 1H-NMR spectrum of 1-amino-4-[Z-glycidylamido]-phenylene.

with [51] or without [50] modification. To confirm this, the synthesis of the amides **3a** and **3c** was repeated at pH 9 and 12 and yielded the respective amides in comparable yields as at pH 7 see detailed analysis results in the experimental part.

Therefore, papain is still active at this high pH and the amines should be able to react with the protected aliphatic amino acids. With our obtained results it can be concluded that the selectivity of papain at the S1′ position (the other side of the amide bond to be formed) is such that aliphatic diamines are not accepted as substrates and that that papain prefers aromatic amines over aliphatic amines at the S1′ position.

4 CONCLUSIONS

Papain is able to catalyze the formation of an amide bond between Z-L-Gly, Z-L-Phe and Z-L-Leu and aniline, ortho-, meta- and para-phenylene diamine. The amides precipitate after the formation of one amide bond as shown by ^{1}H-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-L-Gly and Z-L-Phe with yields comparable with the reaction at pH 7.

The selectivity of the papain is known for the S2 position at which 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 can be concluded that papain prefers aromatic amines over aliphatic amines in this position.

The obtained amido-amines could be - after removal of the N-carbobenzoxy protecting group - used as monomers for polycondensation reactions. By incorporating amino acid structures like the ones synthesized here into artificial polymers their biodegradability can be enhanced without the loss of properties due to the variety of functional side groups that can be found.

REFERENCES

1. E. Piera, M. R. Infante, and P. Clapés, Chemo-enzymatic synthesis of Arg-based gemini surfactants. *Biotechnol. Bioeng.* **70**, 323–331 (2009).
2. P. Clapés, C. Morán, and M. R. Infante, Enzymatic synthesis of arginine- based cationic surfactants. *Biotechnol. Bioeng.* **63**, 333–343 (2009).
3. C. Morán, A. Pinazo, L. Pérez, P. Clapés, M. Angelet, T. García, P. Vinardell, and R. Infante, "Green" amino acid-based surfactants. *Green Chem* **6**, 233–240 (2004).
4. A. Lang, C. Hatscher, and P. Kuhl, Papain-catalyzed synthesis of Z-L-aminoacyl-antipyrine amides from Z-protected amino acid esters and 4-aminoantipyrine. *Tetrahedron Lett.* **48**, 3371–3374 (2007).
5. M. Geurts, J. H. Poupaert, G. K. E. Scriba, and D. M. Lambert, N-(benzyloxycarbonyl)glycine esters and amides as new anticonvulsants. *J. Med. Chem.* **41**, 24–30 (1998).
6. R. Paruszewski, M. Strupinska, G. Rostafinska-Suchar, and J. P. Stables. Anticonvulsant activity of benzylamides of some amino acids and heterocyclic acids. *Protein Peptide Let*, **10**, 475–482 (2003).
7. J. D. Conle, and D. H. Kohn, Functionalized D,L-amino acid derivatives. Potent new agents for the treatment of epilepsy. *J. Med. Chem.* **30**, 567–574 (1987).
8. K. Faber, *Biotransformations in Organic Chemistry: A Textbook*, Springer, Berlin Heidelberg (2004).
9. M. Schuster, B. Munoz, and W. Yuan, Papain catalyzed synthesis of peptide isosteres. *Tetrahedron Lett.* **34**, 1247–50 (1993).
10. G. Anderso, and P. L. Luisi, Papain-induced oligomerization of α-amino acid esters. *Helv. Chim. Acta.* **62**, 488–93 (1979).
11. L. A. Sluyterma, and J. Wijdenes, Sigmoidal progress curves in polymerization of leucine methyl-ester catalyzed by papain. *Biochim. Biophys. Acta.* **289**, 194 (1972).
12. J. Rolf, B. Edgardo, C. M. Julio, and L. L. Pier, Papain Catalyzed Oligomerization of α-Amino Acids. Synthesis and characterization of water-insoluble oligomers of L-methionine. *Helv. Chim. Acta.* **63**, 375–384 (1980).
13. G. Li, A. Vaidya, K. Viswanathan, J. Cui, W. Xie, W. Gao, and R. A. Gross, Rapid regioselective oligomerization of l-glutamic acid diethyl ester catalyzed by papain. *Macromolecules* **39**, 7915–7921 (2006).
14. K. Viswanathan, R. Omorebokhae, G. Li, and R. A. Gross, Protease-catalyzed oligomerization of hydrophobic amino acid ethyl esters in homogeneous reaction media using l-phenylalanine as a model system. *Biomacromolecules* **11**, 2152–2160 (2010).
15. X. Qin, W. C. Xie, Q. Su, W. Z. Du, and R. A. Gross, Protease-catalyzed oligomerization of l-lysine ethyl ester in aqueous solution. *ACS Cata.* **1**, 1022–1034 (2011).
16. H. Uyama, T. Fukuoka, I. Komatsu, T. Watanabe, and S. Kobayashi. Protease-catalyzed regioselective polymerization and copolymerization of glutamic acid diethyl ester. *Biomacromolecules* **3**, 318–323 (2001).
17. G. Li, V. K. Raman, W. C. Xie, and R. A. Gross, Protease-Catalyzed Co-Oligomerizations of l-Leucine Ethyl Ester with l-Glutamic Acid Diethyl Ester: sequence and chain length distributions. *Macromolecules* **41**, 7003–7012 (2008).
18. L. W. Schwab, W. M. J. Kloosterman, J. Konieczny, and K. Loos, Papain Catalyzed (co)Oligomerization of α-Amino acids. *Polymers* **4**, 710 (2012).
19. K. Loos, *Biocatalysis in Polymer Chemistry*, Wiley-VCH, Weinheim (2010).

20. A. R. A. Palmans and A. Heise, *Enzymatic Polymerization*, vol. 237, Springer-Verlag, Berlin (2010).
21. H. N. Chen, and R. A. Gross, (Eds.), *Green Polymer Chemistry: Biocatalysis and Biomaterials. ACS Symposium Series*, vol. 1043, American Chemical Society, Washington, DC (2010).
22. I. Baum, B. Elsasser, L. Schwab, K. Loos, and G. Fels, Atomistic Model for the Polyamide Formation from β-Lactam Catalyzed by *Candida antarctica* Lipase B. *Acs Catalysis* **1**, 323–336 (2011).
23. L. W. Schwab, I. Baum, G. Fels, and K. Loos, In Green Polymer Chemistry: Biocatalysis and Biomaterials Chapter 1, in *ACS Symposium Series*, H. N. Chen, and R. A. Gross, (Eds.), pp. 265–278, Vol. 1043, American Chemical Society, Washington, DC (2010).
24. H. N. Cheng, Enzyme-Catalyzed Synthesis of Polyamides and Polypeptides, in *Biocatalysis in Polymer Chemistry* K. Loos, (Ed.), Wiley-VCH, Weinheim, 131–141(2010).
25. H. N. Cheng, W. W. Maslanka and Q. M. Gu, Hercules Inc., invs. US Patent 6677427, (2004).
26. L. W. Schwab, R. Kroon, A. J. Schouten, and K. Loos. Enzyme-catalyzed ring-opening polymerization of unsubstituted β-Lactam. *Macromol. Rapid Com*. **29**, 794 (2008).
27. I. Schechte, and A. Berger, On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun*. **27**, 157–162 (1967).
28. I. Schechte, and A. Berger, On the active site of proteases. 3. Mapping the active site of papain; specific peptide inhibitors of papain. *Biochem. Biophys. Res. Commun*. **32**, 898–902 (1968).
29. J. Drenth, K. H. Kalk, and H. M. Swen, Binding of chloromethyl ketone substrate analogues to crystalline papain. *Biochemistry* **15**, 3731–3738 (1976).
30. H. E. Khouri, T. Vernet, R. Menard, F. Parlati, P. Laflamme, D. C. Tessier, B. Gour-Salin, D. Y. Thomas, and A. C. Storer. Engineering of papain: selective alteration of substrate specificity by site directed mutagenesis. *Biochemistry* **30**, 8929–8936 (1991).
31. J. R. Tchoupé, T. Moreau, F. Gauthier, and J. G. Bieth, Photometric or fluorometric assay of cathepsin B, L and H and papain using substrates with an aminotrifluoromethylcoumarin leaving group. *Bio chimica Biophys Acta*. **1076**, 149–151 (1991).
32. C. Garcia-Echeverri, and D. H. Rich. Photometric or fluorometric assay of Cathepsin-B, Cathepsin-L and Cathepsin-H and Papain using substrates with an aminotrifluoromethylcoumarin leaving group. *FEBS Let*, **297**, 100–102 (1992).
33. C. Serveau, L. Juliano, P. Bernard, T. Moreau, R. Mayer, and F. Gauthier, New substrates of papain, based on the conserved sequence of natural inhibitors of the cystatin family. *Biochimie*, **76**, 153–158 (1994).
34. R. Menard, H. E. Khouri, C. Plouffe, R. Dupras, D. Ripoll, T. Vernet, D. C. Tessier, F. Laliberte, D. Y. Thomas, and A. C. Storer, A protein engineering study of the role of aspartate 158 in the catalytic mechanism of papain. *Biochemistry* **29**, 6706–6713 (1990).
35. F. Lecaille, C. Serveau, F. Gauthier, and G. Lalmanach, Revisiting the S2 specificity of papain by structural analogs of Phe. *FEBS Lett*. **445**, 311–314 (1999).
36. M. J. Kim, D. Yamamoto, K. Matsumoto, M. Inoue, T. Ishida, H. Mizuno, S. Sumiya, and K. Kitamura, Crystal structure of papain-E64-c complex. Binding diversity of E64-c to papain S2 and S3 subsites. *Biochem*. **287**, 797–803 (1992).
37. M. Schuster, V. Kasche, and H. D. Jakubke, Contributions to the S'-subsite specificity of papain. *Biochim. Biophys. Acta*. **1121**, 207–212 (1992).
38. R. Menard, E. Carmona, C. Plouffe, D. Bromme, Y. Konishi, J. Lefebvre, and A. C. Storer, The specificity of the S1' subsite of cysteine proteinases. *FEBS Lett*. **328**, 107–110 (1993).
39. M. R. Alecio, M. L. Dann, and G. Lowe, The specificity of the S1' subsite of papain. *Biochem*. **141**, 495–501 (1974).
40. C. García-Echeverrí, and D. H. Rich. Effect of P2' substituents on kinetic constants for hydrolysis by cysteine proteinases. *Biochem. Biophys. Res. Commun*. **187**, 615–619 (1992).
41. G. Li, A. Vaidya, K. Viswanathan, J. Cui, W. Xie, W, Gao, and R. A. Gross. Rapid regioselective oligomerization of l-Glutamic acid diethyl ester catalyzed by papain. *Macromolecules* **39**, 7915–7921 (2006).
42. H. Uyama, T. Fukuoka, I. Komatsu, T. Watanabe, and S. Kobayashi. Protease-catalyzed regioselective polymerization and copolymerization of glutamic acid diethyl ester. *Biomacromolecules* **3**, 318–323 (2001).
43. H. Toshio, H. Chuichi, and I. Makoto. Papain immobilization onto porous poly(λ-methyl L-glutamate) beads. *J. Appl. Polym. Sci*. **44**, 143–150 (1992).
44. J. F. Dia, and K. J. Balkus, Enzyme immobilization in MCM-41 molecular sieve. *J. Mol. Catal. B: Enzyme* **2**, 115–126 (1996).
45. D. E. Stevenso, and C. S. Andrew. Papain in organic solvents: determination of conditions suitable for biocatalysis and the effect on substrate specificity and inhibition. *Biotechnol. Bioeng*. **37**, 519–527 (1991).
46. H. F. Gaertner, A. Ferjancic, and A. J. Puigserver. Papain-catalyzed peptide synthesis and oligomerization of amino acid amides in organic solvents. *Biocatal. Biotransfor*, **3**, 197–205 (1990).
47. K. Sakurai, K. Kashimoto, Y. Kodera, and Y, Inada, Solid phase synthesis of peptides with polyethylene glycol-modified protease in organic solvents. *Biotechnol. Lett*. **12**, 685–688 (1990).
48. H. Lee, K. Takahashi, Y. Kodera, K. Ohwada, T. Tsuzuki, A. Matsushima, and Y. Inada. Polyethylene glycol-modified papain catalyzes peptide bond formation in benzene. *Biotechnol. Lett*. **10**, 403–407 (1988).
49. H. E. Khouri, T. Vernet, R. Menard, F. Parlati, P. Laflamme, D. C. Tessier, B. Gour-Salin, D. Y. Thomas, and A. C. Storer, Engineering of papain: selective alteration of substrate specificity by site-directed mutagenesis. *Biochemistry* **30**, 8929–8936 (1991).
50. Y. V. Mitin, N. P. Zapevalova, and E. Y. Gorbunova. Peptide synthesis catalyzed by papain at alkaline pH values. *Int. J. Pept. Protein. Re*s. **23**, 528–534 (1983).
51. K. Sangeeth, and T. E. Abraham, Chemical modification of papain for use in alkaline medium. *J. Mol. Catal. B: Enzyme* **38**, 171–177 (2006).