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Protease-catalysed synthesis of *Z*-L-aminoacyl-L-caprolactam amides from *Z*-protected amino acid esters and DL-α-amino-ε-caprolactam

Alexander Lang *, Peter Kuhl

Lehrstuhl für Biochemie/Naturstoffchemie, Fachrichtung Chemie und Lebensmittelchemie, Technische Universität Dresden, Bergstraße 66, D-01069 Dresden, Germany

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ABSTRACT

The enzymatic synthesis of *Z*-L-aminoacyl-L-caprolactam amides from *Z*-protected amino acid esters and DL-α-amino-ε-caprolactam (ACL) was accomplished by the thiol proteases papain, bromelain and ficin in aqueous–organic media. Product yields of 96% and 87% for *Z*-Gly-L-ACL and *Z*-Ala-L-ACL, respectively, could be obtained. The products were purified and characterised by polarimetry, NMR and LC–MS. The suitability to accept a bulky 1,2-amino ketone as a nucleophile expands the general knowledge of thiol proteases and their catalytic potential.

Proteases as biocatalysts for the specific formation of peptide bonds are currently well investigated, established and applied in biotechnological processes.[1–3] The hypothesis of enzymatic peptide bond formation by reversal of the hydrolytic reaction of proteases dates back to 1898 and was supposed by van't Hoff.[4]

The advantage of protease-catalysed coupling reactions consists in environmentally friendly reaction conditions, avoiding extensive use of organic solvents and toxic auxiliary agents. Besides this, the conservation of stereospecificity and regiospecificity is an intrinsic attribute of proteases to favour enzymatic syntheses compared to conventional chemical conversions without catalysts.

As a matter of common knowledge the catalytic potential of proteases is not restricted to the formation of peptide bonds only. There is evidence of coupling reactions catalysed by papain between *N*-protected amino acids or esters and glycerine[5] or fatty alcohols.[6]

In addition, the thiol protease papain catalyses the reaction of *Z*-protected amino acid esters with the heterocyclic 1,2-amino ketone 4-aminoantipyrine (AAP).[7]

The free amino group and the modified carboxamide structure mimicking a substituted amino acid amide qualify 4-aminoantipyrine (Fig. 1) as an accepted nucleophile in enzymatic synthesis with papain. The aromaticity of the heterocyclic ring and the phenyl moiety makes AAP almost planar to approach the active site of papain more easily than a bulky molecule. The question arose, whether papain is the only protease to catalyse coupling reactions with AAP and whether there are further 1,2-amino ketones to be accepted by proteases.

In an extensive study the thiol proteases papain, bromelain and ficin, the serine endopeptidase α-chymotrypsin and the metalloproteases thermolysin and pronase were singled out to find answers to the above question. In the course of that work several new antipyrine amides with *N*-protected L-amino acid and L-peptide derivatives have been synthesised enzymatically. For the first time also *Boc*-protected amino acids and dipeptide derivatives were covalently attached to AAP resulting in the corresponding *Boc*-L-Xaa- and *Z*-L-Xaa-Ala-antipyrine amides.[8] It has been found that besides papain, also bromelain and ficin are able to couple amino acid or peptide derivatives to AAP. The ability of the serine protease α-chymotrypsin to aminoacylate the heterocycle resulted in far lower yields compared to cysteine proteases. Metalloproteases catalysed the described reaction with negligible yields lower than 10%.

The effort to understand why thiol proteases are so privileged to accept AAP brings substrate and nucleophile specificity into focus. Papain shows a primary specificity for aromatic and bulky aliphatic moieties in the P_2 position[9,10] which are provided by common

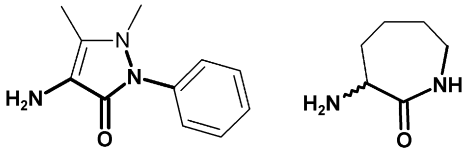

Figure 1. 4-Aminoantipyrine (left) and DL-α-amino-ε-caprolactam (right) with a partial structure of an α-amino acid amide highlighted in bold.

* Corresponding author. Tel.: +49 351 463 33805; fax: +49 351 463 35506.
E-mail address: alexander.lang@chemie.tu-dresden.de (A. Lang).

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protecting groups such as *Z* and *Boc*, whereas small amino acids are favoured without any distinctive specificity in the P_1 position.[11] Hydrophobic amino acid derivatives are the most accepted nucleophiles to bind to the S_1' subsite of papain.[12] Bromelain's and ficin's specificity are similar as stated in former investigations.[3,8,13]

Thus, cysteine proteases are so far the most suited proteases for coupling reactions between amino acid-based acyl donors and AAP and possibly could be a good choice for probing on other 1,2-amino ketones. One of these substances is α-amino-ε-caprolactam (ACL) which also has a carboxamide structure with a free amino group (Fig. 1). Compared to 4-aminoantipyrine this compound is not planar, but bulky, analogous to suberone, although there are no large substituents. Therefore, it is not apparent whether it fits to the active site of thiol proteases.

The experimental approach allows due to the esterase activity of thiol proteases both the thermodynamically and the kinetically controlled conversion. Utilising an ester substrate results in fast formation of an acylated enzyme intermediate, which subsequently acylates the amino component, thereby often yielding temporary higher product concentrations than in equilibrium-controlled synthesis. Only when the donor ester is completely consumed, secondary hydrolysis occurs because of the enzyme's greater acceptance for ester than for peptide bonds.[1]

In this Letter we present the first results of thiol protease-catalysed reactions of *Z*-protected amino acid esters and DL-α-amino-ε-caprolactam (ACL) which result in *Z*-L-aminoacyl-L-caprolactam amides.

The syntheses (Scheme 1) were performed in cylindrical glass vessels of 5 mL volume with a conical bottom placed in a magnetic stirring bath. Papain (Merck, 30,000 USP-U/mg), bromelain (Sigma, 2480 U/g) and ficin (Serva, 0.8 DMC-U/mg) were activated with 2-3 mg cysteine hydrochloride in 2 mL buffer for about 15 min. After addition of DL-ACL while stirring with 700 rpm for additional 5 min, the acyl donor ester dissolved in 0.5 mL methanol was added to start the reaction. Finally, the enzyme was inactivated by heating up to 70 °C for 10 min and the solvent was evaporated under vacuum. The reaction mixture was then dissolved in 5 mL acetonitrile, filtered and analysed by RP-HPLC (29% acetonitrile; 71% water; 0.1% TFA; v/v; flow 1 mL/min; Nucleosil 100, C18, 5 µm, 250 × 4 mm) against authentic samples. Without thiol proteases we could not observe the formation of Z-L-aminoacyl-L-caprolactam amides. All given yields in this work are obtained from triplicate determination. The average values have an error of ±3%.

To acquire characteristic data of the synthesised compounds, the Z-L-aminoacyl-L-caprolactam amides were purified[14] and characterised by polarimetry, LC–MS, ^{1}H NMR and ^{13}C NMR.[15,16]

By polarimetry an optical activity even for *Z*-Gly-ACL could be determined. Thus, the proteases seem to use only one enantiomer of the racemate DL-ACL. Usually, but not exclusively, proteases prefer L-configured isomers. To prove that assumption, the *Z*-L-aminoacyl-L-caprolactam amides of alanine and glycine were synthesised according to classical peptide chemistry by applying *Z*-Gly-OH, *Z*-Ala-OH, L-ACL·HCl, K_2CO_3, *N*,*N*′-dicyclohexyl-carbodiimide and 1-hydroxytriazole in tetrahydrofuran. The purified reaction products gave $[\alpha]_D^{20}$ 8.3 (*c* 0.5, MeOH) and $[\alpha]_D^{20}$ −14.2 (*c* 0.5, MeOH) for *Z*-Gly-L-ACL and *Z*-Ala-L-ACL, respectively. These data are in accordance with the values determined for enzymatically synthesised products.[15,16] This finding points out that indeed there is a preference for the L-isomer of α-amino-ε-caprolactam by thiol proteases.

In the beginning the enzymatic conversions were carried out under alkaline conditions (0.2 M KH_2PO_4; pH 8.6; 20% MeOH) to obtain a higher concentration of ACL with unprotonated amino group. The organic solvent has the role of solubilising the ester substrate but the concentration is limited due to enzyme's denaturation. The reactions were conducted at 40 °C for 2 h and 24 h with ratios of acyl donor to nucleophile of 1:2 and 1:5, respectively. Results obtained are presented in Tables 1 and 2.

Considering the results of chemical and enzymatic synthesis that just L-configurated isomers are utilised by the thiol proteases, the effective acyl donor–nucleophile ratio is reduced to 1:1 and 1:2.5, respectively. According to the law of mass action, the yields of *Z*-Xaa-L-ACL increased with a higher supply of DL-ACL and could be raised to nearly complete conversion in the case of *Z*-Gly-L-ACL with papain and ficin. Although papain and ficin generate the

Table 1
Protease-catalysed synthesis of *Z*-Gly-L-ACL (**3a**) in a basic aqueous–organic medium[a]

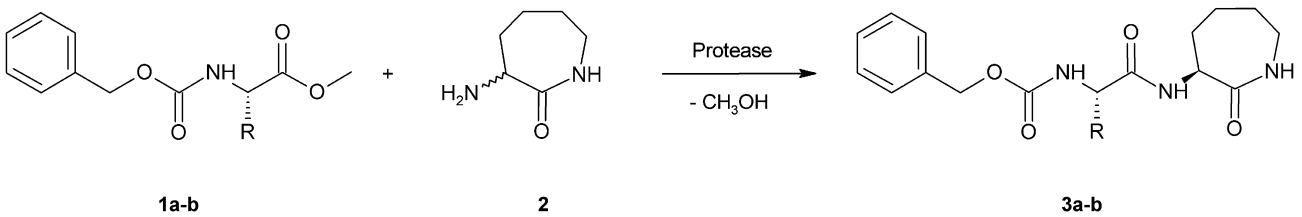
Acyl donor–nucleophile-ratio	Reaction time (h)	Yield of *Z*-Gly-L-ACL (%)		
		Papain	Bromelain	Ficin
1:2	2	72	49	91
	24	74	62	93
1:5	2	94	44	96
	24	92	89	96

[a] 20 mg protease, 40 °C, 2 mL buffer (0.2 M KH_2PO_4, pH 8.6); 0.5 mL methanol; 0.05 M *Z*-Gly-OMe (**1a**, acyl donor).

Table 2
Protease-catalysed synthesis of *Z*-Ala-L-ACL (**3b**) in a basic aqueous–organic medium[a]

Acyl donor–nucleophile-ratio	Reaction time (h)	Yield of *Z*-Ala-L-ACL (%)		
		Papain	Bromelain	Ficin
1:2	2	23	20	53
	24	9	16	45
1:5	2	70	56	87
	24	50	29	83

[a] 20 mg protease, 40 °C, 2 mL buffer (0.2 M KH_2PO_4, pH 8.6); 0.5 mL methanol; 0.05 M *Z*-Ala-OMe (**1b**, acyl donor).



R = H	**1a**	*Z*-Gly-OMe	**3a**	*Z*-Gly-L-ACL
R = CH_3	**1b**	*Z*-Ala-OMe	**3b**	*Z*-Ala-L-ACL

Scheme 1. Synthesis of *Z*-L-aminoacyl-L-caprolactam amides (**3a–b**) from *Z*-L-amino acid esters (**1a–b**) and DL-α-amino-ε-caprolactam (**2**).

Table 3
Protease-catalysed synthesis of *Z*-Ala-L-ACL (**3b**) in an acidic aqueous–organic medium[a]

Acyl donor–nucleophile-ratio	Reaction time (h)	Yield of *Z*-Ala-L-ACL (%)		
		Papain	Bromelain	Ficin
1:5	2	47	31	82
	24	43	23	69

[a] 20 mg protease, 40 °C, 2 mL buffer (0.1 M sodium citrate buffer, pH 5.0); 0.5 mL methanol; 0.05 M *Z*-Ala-OMe (**1b**, acyl donor).

highest yields, with *Z*-Ala-OMe the proteases show rather less conversion than with *Z*-Gly-OMe. For papain this is surprising because of its known far wider substrate acceptance than that of ficin. The formation of *Z*-Gly-L-ACL catalysed by bromelain seems to be the slowest process. After 2 h there is the lowest yield compared to the other proteases but it increases during the ongoing reaction until 24 h, whereas for papain and ficin the gain ratios remain nearly constant. The reaction time of 2 h is rather short and reasonable and gives for *Z*-Ala-L-ACL better conversion than in reactions over 24 h, without much doubt due to partial secondary hydrolysis in the latter case.

In additional experiments an aqueous–organic medium has been tested for the synthesis of *Z*-Ala-L-ACL consisting of 20% methanol and 80% buffer with a pH of 5.0 near the pH optimum of thiol proteases. The results are presented in Table 3.

Compared to the product yields obtained in basic buffer this medium can just compete in ficin-catalysed reactions. Papain- and bromelain-catalysed conversions differ from the results under basic conditions. In that acidic medium also secondary hydrolysis occurs. This is apparent from the yields after 24 h. All tested buffer-methanol compositions displayed this characteristic.

Summing up all reactions, ficin seems to be the best thiol protease for these types of substrates and this nucleophile. The difference with the three thiol proteases is significant in the case of *Z*-Ala-L-ACL, but more balanced in the formation of *Z*-Gly-L-ACL.

In summary, this work emphasises the far wider synthetic potential of proteases than that known so far. It gives proof of introducing another 1,2-amino ketone besides AAP as the nucleophile in the amidation of amino acid derivatives. For the first time *Z*-L-aminoacyl-L-caprolactam amides could be synthesised by using thiol proteases as catalysts, because there is nothing reported so far about these compounds neither in enzymatic nor in chemical synthesis. The findings accentuate the stereospecificity of proteases to prefer the L-configured isomer of a DL-ACL racemate. These results encourage the idea of finding new substances with possibly interesting properties, which can easily be prepared by protease-catalysed synthesis.

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14. Several reaction batches were pooled to gain an amount of about 0.5 mmol *Z*-Xaa-L-ACL. The mixture was evaporated to dryness under vacuum. The crude product was dissolved in 20 mL $CHCl_3$. The solution was washed thrice with 5 mL saturated sodium bicarbonate solution, and the two phases were separated. The aqueous phase was discarded to waste, and from the organic layer ACL was extracted twice with 5 mL 1 M HCl. Then, the organic phase was washed twice with 2 mL distilled water and dried afterwards with sodium sulfate, filtered and evaporated. The obtained product was desiccated overnight at 40 °C. ^1H and ^{13}C NMR spectra were recorded with DRX 500, Bruker. Polarimetry was performed with Model 341 LC, Perkin Elmer Instruments and ESI/APCI-MS data were acquired by LC/MS equipment HP 1100—Bruker Esquire Ion Trap.
15. *Z*-Gly-L-ACL: white solid; $[\alpha]_D^{20}$ 8.3 (*c* 0.4, MeOH); ^1H NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.37–1.47 (m, 1H, CH_2 (ACL)), 1.47–1.52 (m, 1H, CH_2 (ACL)), 1.79–1.82 (m, 1H, CH_2 (ACL)), 1.82–1.86 (m, 1H, CH_2 (ACL)), 1.98–2.01 (m, 1H, CH_2 (ACL)), 2.05–2.08 (m, 1H, CH_2 (ACL)), 3.23–3.28 (m, 2H, CH_2 (ACL)), 3.86–3.97 (dd, 2H, CH_2 (Gly)), 4.50–4.53 (q, 1H, CH (ACL)), 5.12 (s, 2H, CH_2 (*Z*)), 5.41 (t, 1H, NH (Gly)), 6.01 (t, 1H, NH (ACL)), 7.22 (d, 1H, NH (ACL-Ala)), 7.29–7.33 (m, 3H, C_6H_5 (*Z*)), 7.35 (d, ^3J = 4.4 Hz, 2H, C_6H_5 (*Z*)); ^{13}C NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 27.86 (CH_2, ACL), 28.82 (CH_2, ACL), 31.42 (CH_2, ACL), 42.16 (CH_2, ACL), 44.33 (CH_2, Gly), 52.25 (CH, ACL), 67.14 (CH_2, *Z*), 128.11, 128.17, 128.53, 136.18 (C_6H_5, *Z*), 156.39 (CO, *Z*), 167.77 (CO, Gly), 175.00 (CO, ACL); ESI-MS *m/z* 320.1 [M+H$^+$], *m/z* 342.1 [M+Na$^+$], $C_{16}H_{21}N_3O_4$ requires 319.4
16. *Z*-Ala-L-ACL: white solid; $[\alpha]_D^{20}$ −13.7 (*c* 0.4, MeOH); ^1H NMR [500 MHz, DMSO-d_6] δ (in ppm) 1.20 (d, ^3J = 12.6 Hz, 3H, CH_3 (Ala)), 1.13–1.24 (m, 2H, CH_2 (ACL)), 1.71–1.81 (m, 2H, CH_2 (ACL)), 1.85–1.88 (m, 2H, CH_2 (ACL)), 3.03–3.07 (m, 2H, CH_2 (ACL)), 4.02–4.06 (quin, 1H, CH (Ala)), 4.31–4.35 (q, 1H, CH (ACL)), 5.02 (s, 2H, CH_2 (*Z*)), 7.30–7.33 (m, 1H, C_6H_5 (*Z*)), 7.33–7.38 (m, 4H, C_6H_5 (*Z*)), 7.63 (d, ^3J = 7.6 Hz, 1H, NH (ACL-Ala)), 7.78 (d, ^3J = 6.5 Hz, 1H, NH (Ala)), 7.88 (t, 1H, NH (ACL)); ^{13}C NMR [125.75 MHz, DMSO-d_6] δ (in ppm) 18.06 (CH_3, Ala), 27.68 (CH_2, ACL), 28.87 (CH_2, ACL), 31.11 (CH_2, ACL), 40.65 (CH_2, ACL), 50.40 (CH, Ala), 51.38 (CH, ACL), 65.44 (CH_2, *Z*), 127.71, 127.83, 128.43, 137.07 (C_6H_5, *Z*), 155.74 (CO, *Z*), 171.49 (CO, ACL), 174.14 (CO, Ala); ESI-MS *m/z* 334.2 [M+H$^+$], *m/z* 356.2 [M+Na$^+$], $C_{17}H_{23}N_3O_4$ requires 333.4