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Protease-catalysed coupling of N-protected amino acids and peptides with 4-aminoantipyrine

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Abstract The enzymatic synthesis of N-protected L-aminoacyl- and L-peptidyl-antipyrine amides was accomplished by proteases from different classes. Serine and cysteine proteases proved to be suitable tools for the production of amino acids and peptides conjugated to 4-aminoantipyrine, whereas metalloproteases do not seem to be very qualified for accepting this nucleophile. The product yields were optimised by applying ample opportunities of medium engineering, e.g. aqueous-organic, biphasic, suspension and solid-to-solid reaction systems. Thus, yields up to 100% could be obtained. The products were purified and characterised by polarimetry and NMR spectroscopy. These results broaden the common knowledge of the catalytic potential of proteases, in particular with regard to the suitability of a special heterocyclic 1,2-amino ketone as a nucleophile for the biocatalytic amidation of amino acids and peptides.

Keywords Amidation · Amino acids · 4-aminoantipyrine · Enzymatic synthesis · Peptides · Proteases

Abbreviations

AAP	4-aminoantipyrine
ACN	Acetonitrile
Boc	*Tert*-butoxycarbonyl
Bzl	Benzyl

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ESI/APCI-MS	Electrospray ionisation/atmospheric pressure chemical ionisation mass spectrometry
HPLC	High performance liquid chromatography
Me	Methyl
NMR	Nuclear magnetic resonance
TFA	Trifluoroacetic acid
Z	Benzyloxycarbonyl

Introduction

More than 100 years ago already van't Hoff (1898) pointed out the possibility of enzymatic peptide bond formation by reversal of the hydrolytic reaction of proteases. Since then the protease-catalysed synthesis has been widely investigated and is well established (Jakubke 1995; Bordusa 2002; Tai 2003). These catalysed coupling reactions with protected amino acid or peptide derivatives are carried out under mild conditions and avoiding the necessity of expensive and possibly toxic auxiliary agents. Stereospecificity and regiospecificity of proteases guarantee fast and economic syntheses compared to conventional chemical conversions.

Meanwhile it has become evident that the catalytic potential of proteases is not limited to the formation of peptide bonds. For example, coupling reactions between N-protected amino acids or esters and glycerine (Mitin et al. 1997), sorbitol (Mitin et al. 1999) or fatty alcohols (Clapés and Infante 2002) were catalysed by papain. There are also subtilisine-catalysed couplings using 2,2,2-trifluoroethyl butyrate with phenylalanine amide and other functionalised amines (van Rantwijk and Sheldon 2004).

Recently we found, that the cysteine protease papain catalyses the reaction of Z-protected amino acid esters with

the heterocyclic 1,2-amino ketone 4-aminoantipyrine (AAP) (Lang et al. 2007). A closer look at the AAP structure reveals that in part it is able to mimic a substituted amino acid amide with a free α-amino group (highlighted in Scheme 1). 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.

According to a method of classical peptide synthesis, 4-aminoantipyrine has already been bound to amino acid derivatives by applying N,N'-dicyclohexylcarbodiimide. In this way the amides of Z-Gly, Z-Ala, Z-DL-Ala, Z-DL-Val, Z-DL-Met, and Z-Leu (Kwapiszewski 1971) and of Z-Pro-Leu and Z-Tyr(Bzl)-Gly-Gly (Rips and Morier 1976) have been obtained. Because AAP shows analgesic, antipyretic and antiphlogistic effects, these derivatives were thought and tested as drugs, but with no significant improved action compared to AAP and its derivatives (Jeske and Misterek 1974). Today AAP is scarcely administered as analgetic but mostly found as an active metabolite of metamizole sodium (EMEA 1999, 2003).

As the free amino group and the modified carboxamide structure qualify 4-aminoantipyrine as an accepted nucleophile in enzymatic synthesis with papain, the question arises, whether papain is the only protease to catalyse coupling reactions with AAP.

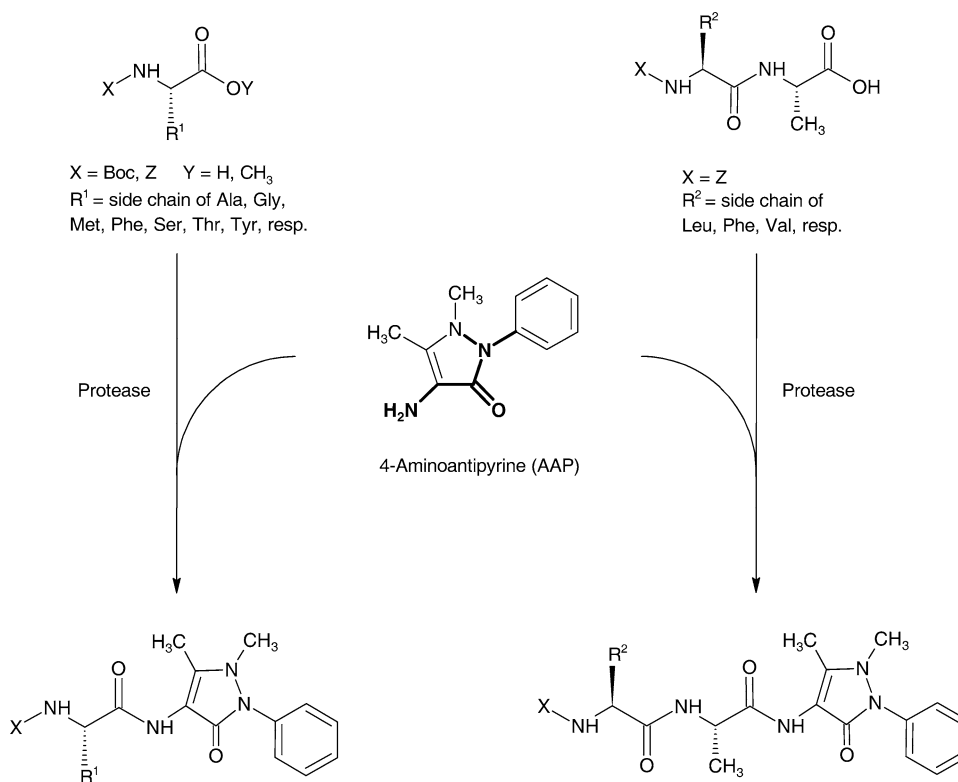Papain shows a primary specificity for aromatic or bulky aliphatic moieties in the P_2 position which are provided by common protecting groups like Z or Boc (Schechter and Berger 1967; Barbas and Wong 1987), whereas small amino acids are favoured without any distinctive specificity in the P_1 position (Kuhl and Jakubke 1990). Hydrophobic amino acid derivatives are the most accepted nucleophiles to bind to the S'_1 subsite of papain (Mitin et al. 1984). Because of the similar substrate specificity of bromelain and ficin (Tai 2003; Tai and Fu 2003) these thiol endopeptidases might also be able to catalyse the described type of coupling reaction.

To extend the number of amino acids which can be amidated by AAP, we chose the serine endopeptidase α-chymotrypsin. This enzyme favours aromatic amino acids and Leu in the P_1 position (Kuhl and Jakubke 1990; Kuhl et al. 1990). Therefore, it offers the right specificity for the synthesis of the antipyrine amides of Tyr, Trp, Phe, and Leu.

From the group of metalloproteases two enzymes were chosen, thermolysin and pronase. Thermolysin prefers hydrophobic amino acids in P_1 as well as in P'_1 position (Riechmann and Kasche 1986). The specificity of the downstream position may qualify AAP as nucleophile. The microbiologically produced pronase has a broad substrate specificity, but also favours hydrophobic amino acids in the same positions as thermolysin does (Fernandez et al. 1995; Lobell and Schneider 1998).

The esterase activity of cysteine and serine proteases enables both the thermodynamically- and the kinetically-

Scheme. 1 Overview of the protease-catalysed syntheses of N-protected aminoacyl- and peptidyl-antipyrine amides. Proteases = papain, bromelain, ficin, α-chymotrypsin, thermolysin and pronase, respectively

X = Boc, Z   Y = H, CH$_3$
R^1 = side chain of Ala, Gly, Met, Phe, Ser, Thr, Tyr, resp.

X = Z
R^2 = side chain of Leu, Phe, Val, resp.

4-Aminoantipyrine (AAP)

Protease

Protease

controlled approach. Utilising an ester substrate results in fast formation of an acylated enzyme, 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 (Jakubke 1995). Hence cysteine and serine proteases allow for two approaches to synthesising protected L-aminoacyl-antipyrine amides.

When using papain as biocatalyst we applied aqueous-organic, biphasic and suspensious reaction media. The latter turned out to be the best (Lang et al. 2007).

In this paper we present the successful protease-catalysed synthesis of new L-aminoacyl- and L-peptidyl-antipyrine amides using the thiol proteases papain, ficin and bromelain. In addition, α-chymotrypsin, thermolysin and pronase were probed for catalysing activity in that particular amidation reaction. For this, different strategies of medium engineering were applied, like the use of aqueous-organic and biphasic media. Prompted by the high yields with suspensious media we adopted the solid-to-solid technique (Erbeldinger et al. 2001). By adjusting this method to the synthesis of protected L-aminoacyl- and L-peptidyl-antipyrine amides we could enhance the yields perceptibly.

Materials and methods

Materials

Papain (EC 3.4.22.2) from *Carica papaya* (30,000 USP U/mg, casein assay) and α-chymotrypsin (EC 3.4.21.1) from bovine pancreas (400 U/mg protein, *N*-acetyl-L-tyrosine ethyl ester (ATEE) assay) were obtained from Merck. Bromelain (EC 3.4.22.32) from pineapple stem (2,480 U/g protein, gelatine assay) and thermolysin (EC 3.4.24.2) from *Bacillus thermoproteolyticus rokko* (55 U/mg protein, casein assay) were from Sigma. Ficin (EC 3.4.22.3) from *Ficus carica* (0.8 DMC U/mg, dimethyl casein assay) was obtained from Serva. Pronase (EC 3.4.24.31) from *Streptomyces griseus* (6.9 DMC U/mg, dimethyl casein assay) was from Fluka. All amino acid derivatives were purchased from Bachem except for Z-Ala-OMe and Z-Gly-OMe which were from Senn Chemicals. 4-Aminoantipyrine (puriss. p.a.) was obtained from Riedel-de Haën. TFA (p.a.) was purchased from Acros Organics. All other chemicals and solvents used were of analytical grade.

HPLC analysis

HPLC system: LC 1110 HPLC Pump, LC 1610 Advanced HPLC Autosampler, GAT-LCD 500 UV Variable LC Detector (254 nm), DP 800 Chromatography Data Processing System, Gamma Analysen Technik GmbH

Pre-column: Nucleosil C18, 5 μm, 14 × 4 mm, Bischoff Chromatograph

Column: Nucleosil C18, 5 μm, 250 × 4 mm, Bischoff Chromatograph

Solvent system: 71% water, 29% ACN, 0.1% TFA (v/v), flow rate: 1.0 mL/min

Enzymatic synthesis

Papain, bromelain, ficin

The reactions were performed in 25 mL round-bottom flasks attached to a rotary evaporator and placed in a water bath at 40°C. The protease (20 mg) was activated with 2–3 mg cysteine hydrochloride in 2 mL buffer (0.1 M sodium citrate buffer, pH 5.0) for 15 min. After addition of AAP and further rotating with 150 rpm for additional 5 min, the acyl donor dissolved in 0.5 mL methanol was added to start the reaction. In case of suspensions the concentrations of substrates and nucleophile were five-fold that of aqueous-organic mixtures. The flasks of solid-to-solid reactions were immediately evacuated after adding the acyl donor to 15 mbar and the solvent was removed within 5 min. Finally, the enzyme was inactivated by heating up to 70°C for 10 min. We did not verify whether the thiol proteases were totally deactivated, but the yields were slightly lower and absolutely reproducible compared to reactions without inactivating in the case of papain. After deactivation of the enzyme the solvent was evaporated under vacuum. The residue was dissolved in 5 mL acetonitrile and analysed by HPLC against authentic samples. Without protease we did not observe formation of L-aminoacyl-antipyrine amides.

Thermolysin, α-chymotrypsin

The reactions were performed in cylindrical glass vessels of 5 mL volume with a conic bottom placed in a magnetic stirring bath. Thermolysin (3 mg) was incubated in 0.25 mL buffer (0.05 M HEPES, 0.01 M CaCl$_2$, pH 7.0, adjusted with NaOH) and 1.75 mL ethyl acetate for 15 min at 40°C, whereas α-chymotrypsin (10 mg) was stirred at 25°C in a mixture of 0.2 mL buffer (0.2 M sodium carbonate, pH 9.0) and 1.3 mL ethyl acetate for 10 min. After addition of AAP the mixture was stirred with 920 rpm for additional 5 min. The acyl donor dissolved in 0.5 mL ethyl acetate was added to start the reaction. Finally, thermolysin was inactivated by heating up to 70°C for 10 min, whereas the reactions with α-chymotrypsin were stopped by adding 2 mL 1 M HCl. After deactivation of the enzyme the

solvent was evaporated under vacuum. The residue was dissolved in 5 mL acetonitrile and analysed by HPLC against authentic samples.

In analogy to the procedure with cysteine proteases we also performed the solid-to-solid reactions with thermolysin.

Pronase

The reactions were performed in 1.5 mL Eppendorf tubes in an Eppendorf thermomixer at 25°C. Pronase (5 mg) was incubated in 0.8 mL buffer (0.013 M sodium borate, pH 9.0) for 10 min at 1,400 rpm. After addition of AAP the mixture was stirred with 1,400 rpm for additional 5 min. The acyl donor was dissolved in 0.2 mL ACN and added to start the reaction. After deactivation of the enzyme the solvent was evaporated under vacuum. The residue was dissolved in 5 mL acetonitrile and analysed by HPLC against authentic samples. Thermodynamically controlled reactions were carried out in a biphasic mixture (2.7 mL 0.067 M potassium phosphate, pH 6.0; 0.3 mL ethyl acetate) in cylindrical glass vessels of 5 mL volume with a conic bottom.

All given yields in this work are obtained from triplicate determination. The average values have an error of ±3%.

Purification and characterisation of N-protected L-aminoacyl- and L-peptidyl-antipyrine amides

Several reaction batches were pooled to gain an amount of about 0.5 mmol product. The mixture was evaporated to dryness under vacuum. The crude product was dissolved in 20 mL $CHCl_3$. The solution was washed twice with saturated sodium bicarbonate solution, and the two phases were separated. The aqueous phase was discarded to waste, and from the organic layer AAP was extracted with 1 M HCl as long as the reaction of the aqueous phase with Ehrlich reagent turned out positive. Then, the organic phase was dried with sodium sulfate, filtered, and evaporated. The obtained product was desiccated overnight at 40°C.

^{1}H 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.

Z-Ala-AAP, Z-Gly-AAP and Z-Ser-AAP have already been described (Lang et al. 2007)

Boc-Ala-AAP: slightly yellow solid; $[\alpha]_D^{20}$ −45.3 (c 0.2, MeOH); ^{1}H-NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.38 (d, ^3J = 7.0 Hz, 3H, CH_3 (Ala)), 1.44 (s, 9H, CH_3 (Boc)), 2.22 (s, 3H, CH_3 (AAP)), 3.07 (s, 3H, CH_3 (AAP)), 4.34 (m, 1H, CH (Ala)), 5.27 (m, 1H, NH (Ala)), 7.25–7.46 (m, 5H, C_6H_5 (AAP)), 8.05 (s, 1H, NH (AAP)); ^{13}C-NMR

[125.75 MHz, $CDCl_3$] δ (in ppm) 12.27 (CH_3), 18.60 (CH_3), 28.33 (CH_3), 36.04 (CH_3), 50.48 (CH), 79.98 (C), 108.05 (C), 124.31, 126.99, 129.25 (C_6H_5), 134.50 (C), 149.93 (C_6H_5), 155.37 (CO), 161.58 (CO), 172.13 (CO); ESI-MS m/z 375.2 [M + H$^+$], m/z 397.2 [M + Na$^+$], $C_{19}H_{26}N_4O_4$ requires 374.2.

Boc-Gly-AAP: slightly yellow solid; ^{1}H-NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.44 (s, 9H, CH_3 (Boc)), 2.20 (s, 3H, CH_3 (AAP)), 3.08 (s, 3H, CH_3 (AAP)), 3.94 (d, ^3J = 5.4 Hz, 2H, CH_2 (Gly)), 5.45 (m, 1H, NH (Gly)), 7.25–7.46 (m, 5H, C_6H_5 (AAP)), 8.76 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 12.09 (CH_3), 28.35 (CH_3), 35.80 (CH_3), 44.06 (CH_2), 79.83 (C), 107.68 (C), 124.64, 127.23, 129.31 (C_6H_5), 134.30 (C), 150.21 (C_6H_5), 155.98 (CO), 161.84 (CO), 169.21 (CO); ESI-MS m/z 361.2 [M + H$^+$], m/z 383.2 [M + Na$^+$], $C_{18}H_{24}N_4O_4$ requires 360.2.

Boc-Met-AAP: yellow solid; $[\alpha]_D^{20}$ −29.1 (c 0.4, MeOH); ^{1}H-NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.44 (s, 9H, CH_3 (Boc)), 1.92–1.96 (m, 1H, CH_2 (Met)), 2.09 (s, 3H, CH_3 (Met)), 2.09–2.13 (m, 1H, CH_2 (Met)), 2.22 (s, 3H, CH_3 (AAP)), 2.56–2.59 (m, 2H, CH_2 (Met)), 3.08 (s, 3H, CH_3 (AAP)), 4.44–4.45 (m, 1H, CH (Met)), 5.47 (d, ^3J = 7.7 Hz, 1H, NH (Met)), 7.25–7.46 (m, 5H, C_6H_5 (AAP)), 8.04 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 12.21 (CH_3), 15.32 (CH_3), 28.32 (CH_3), 30.23 (CH_2), 31.96 (CH_2), 35.99 (CH_3), 53.78 (CH), 80.07 (C), 107.86 (C), 124.35, 127.05, 129.28 (C_6H_5), 134.46 (C), 150.08 (C_6H_5), 155.48 (CO), 161.54 (CO), 170.98 (CO); ESI-MS m/z 435.2 [M + H$^+$], m/z 457.1 [M + Na$^+$], $C_{21}H_{30}N_4O_4S$ requires 434.2.

Boc-Ser-AAP: yellow solid; $[\alpha]_D^{20}$ −27.9 (c 0.2, MeOH); ^{1}H-NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.45 (s, 9H, CH_3 (Boc)), 2.21 (s, 3H, CH_3 (AAP)), 3.11 (s, 3H, CH_3 (AAP)), 3.71–3.73 (m, 2H, CH_2 (Ser)), 4.05–4.07 (m, 1H, CH (Ser)), 4.35 (m, 1H, OH (Ser)), 6.01 (d, ^3J = 7.3 Hz, 1H, NH (Ser)), 7.25–7.47 (m, 5H, C_6H_5 (AAP)), 8.15 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 11.59 (CH_3), 28.35 (CH_3), 35.57 (CH_3), 56.14 (CH), 62.81 (CH_2), 80.05 (C), 107.27 (C), 124.75, 127.55, 129.37 (C_6H_5), 134.01 (C), 150.82 (C_6H_5), 155.83 (CO), 161.82 (CO), 171.73 (CO); ESI-MS m/z 391.2 [M + H$^+$], m/z 413.1 [M + Na$^+$], $C_{19}H_{26}N_4O_5$ requires 390.2.

Z-Met-AAP: yellow solid; $[\alpha]_D^{20}$ −26.7 (c 0.2, MeOH); ^{1}H-NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.91–1.96 (m, 1H, CH_2 (Met)), 2.04 (s, 3H, CH_3 (Met)), 2.08–2.18 (m, 1H, CH_2 (Met)), 2.18 (s, 3H, CH_3 (AAP)), 2.51 (m, 2H, CH_2 (Met)), 3.06 (s, 3H, CH_3 (AAP)), 4.50–4.51 (m, 1H, CH (Met)), 5.06, 5.15 (^2J = 12.3 Hz, 2H, CH_2 (Z)), 6.04 (d, ^3J = 8.2 Hz, 1H, NH (Met)), 7.25–7.42 (m, 10H, C_6H_5 (AAP), (Z)), 8.56 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 12.23 (CH_3), 15.25 (CH_3), 30.08 (CH_2), 32.11 (CH_2), 35.84 (CH_3), 54.32

(CH), 66.88 (CH$_2$), 107.77 (C), 124.51, 127.19, 128.02, 128.04, 128.47, 129.30 (C$_6$H$_5$), 134.24 (C), 136.38 (C$_6$H$_5$), 150.02 (C$_6$H$_5$), 156.02 (CO), 161.47 (CO), 170.87 (CO); ESI-MS m/z 469.2 [M + H$^+$], m/z 491.1 [M + Na$^+$], C$_{24}$H$_{28}$N$_4$O$_4$S requires 468.2.

Z-Thr-AAP: slightly orange solid; $[\alpha]_D^{20}$ −25.9 (*c* 0.2, MeOH); ^{1}H-NMR [500 MHz, DMSO-d$_6$] δ (in ppm) 1.14 (d, ^3J = 6.3 Hz, 3H, CH$_3$ (Thr)), 2.10 (s, 3H, CH$_3$ (AAP)), 3.04 (s, 3H, CH$_3$ (AAP)), 3.97–3.99 (m, 1H, CH (Thr)), 4.11–4.13 (m, 1H, CH (Thr)), 4.82 (s, 1H, OH (Thr)), 5.06 (s, 2H, CH$_2$ (Z)), 7.00 (d, ^3J = 8.7 Hz, 1H, NH (Thr)), 7.30–7.52 (m, 10H, C$_6$H$_5$ (AAP), (Z)), 9.11 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, DMSO-d$_6$] δ (in ppm) 11.21 (CH$_3$), 20.15 (CH$_3$), 36.04 (CH$_3$), 60.69 (CH), 65.52 (CH$_2$), 67.04 (CH), 107.34 (C), 123.41, 126.18, 127.65, 127.79, 128.34, 129.07 (C$_6$H$_5$), 135.04 (C), 137.00 (C$_6$H$_5$), 152.28 (C$_6$H$_5$), 156.11 (CO), 161.68 (CO), 169.83 (CO); ESI-MS m/z 439.2 [M + H$^+$], m/z 461.1 [M + Na$^+$], C$_{23}$H$_{26}$N$_4$O$_5$ requires 438.2.

Z-Tyr-AAP: slightly orange oil; $[\alpha]_D^{20}$ −6.9 (*c* 0.26, MeOH); ^{1}H-NMR [500 MHz, DMSO-d$_6$] δ (in ppm) 2.05 (s, 3H, CH$_3$ (AAP)), 2.69–2.74 (m, 1H, CH$_2$ (Tyr)), 2.93–2.96 (m, 1H, CH$_2$ (Tyr)), 3.04 (s, 3H, CH$_3$ (AAP)), 4.36–4.40 (m, 1H, CH (Tyr)), 4.96 (s, 1H, OH (Tyr)), 4.96, 4.98 (^2J = 8.2 Hz, 2H, CH$_2$ (Z)), 6.65, 6.67 (d, 2H, ^3J = 8.5 Hz, C$_6$H$_5$ (Tyr)), 7.13, 7.14 (d, 2H, ^3J = 8.4 Hz, C$_6$H$_5$ (Tyr)) 7.24–7.52 (m, 10H, C$_6$H$_5$ (AAP), (Z) and 1H, NH (Tyr)), 8.31 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, DMSO-d$_6$] δ (in ppm) 11.25 (CH$_3$), 36.08 (CH$_3$), 37.13 (CH$_2$), 56.54 (CH), 65.21 (CH$_2$), 107.34 (C), 114.90, 123.52, 127.48, 127.70, 128.33, 129.14, 130.28 (C$_6$H$_5$), 135.09 (C), 137.01, 137.13, 152.16, 155.87 (C$_6$H$_5$), 155.97 (CO), 161.71 (CO), 171.35 (CO); ESI-MS m/z 501.2 [M + H$^+$], m/z 523.1 [M + Na$^+$], C$_{28}$H$_{28}$N$_4$O$_5$ requires 500.6.

Z-Leu-Ala-AAP: yellow solid; $[\alpha]_D^{20}$ −54.5 (*c* 0.3, MeOH); ^{1}H-NMR [500 MHz, DMSO-d$_6$] δ (in ppm) 0.85–0.87 (m, 6H, CH$_3$ (Leu)), 1.30 (d, ^3J = 7.0 Hz, 3H, CH$_3$ (Ala)), 1.42–1.45 (m, 2H, CH$_2$ (Leu)), 1.62–1.65 (m, 1H, CH (Leu)), 2.07 (s, 3H, CH$_3$ (AAP)), 3.04 (s, 3H, CH$_3$ (AAP)), 4.05–4.08 (m, 1H, CH (Leu)), 4.41–4.44 (m, 1H, CH (Ala)), 5.02 (s, 2H, CH$_2$ (Z)), 7.28–7.52 (m, 10H, C$_6$H$_5$ (AAP), (Z) and 1H, NH (Leu)), 8.07 (d, ^3J = 7.2 Hz, 1H, NH (Ala)), 9.16 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, DMSO-d$_6$] δ (in ppm) 11.15 (CH$_3$), 18.54 (CH$_3$), 21.44 (CH$_3$), 23.18 (CH$_3$), 24.20 (CH), 36.09 (CH$_3$), 40.65 (CH$_2$), 48.16 (CH), 52.97 (CH), 65.38 (CH$_2$), 107.27 (C), 123.48, 126.25, 127.67, 127.80, 128.38, 129.14 (C$_6$H$_5$), 135.09 (C), 137.13 (C$_6$H$_5$), 152.32 (C$_6$H$_5$), 156.01 (CO), 161.70 (CO), 171.92 (CO), 172.13 (CO); ESI-MS m/z 522.3 [M + H$^+$], m/z 544.2 [M + Na$^+$], C$_{28}$H$_{35}$N$_5$O$_5$ requires 521.3.

Z-Phe-Ala-AAP: slightly yellow solid; $[\alpha]_D^{20}$ −33.2 (*c* 0.2, MeOH); ^{1}H-NMR [500 MHz, DMSO-d$_6$] δ (in ppm) 1.34 (d, ^3J = 7.1 Hz, 3H, CH$_3$ (Ala)), 2.09 (s, 3H, CH$_3$ (AAP)), 2.71–2.74 (m, 1H, CH$_2$ (Phe)), 3.03–3.06 (m, 1H, CH$_2$ (Phe)), 3.04 (s, 3H, CH$_3$ (AAP)), 4.28–4.32 (m, 1H, CH (Phe)), 4.44–4.47 (m, 1H, CH (Ala)), 4.93 (s, 2H, CH$_2$ (Z)), 7.17–7.53 (m, 15H, C$_6$H$_5$ (AAP), (Phe), (Z) and 1H, NH (Phe)), 8.29 (d, ^3J = 7.2 Hz, 1H, NH (Ala)), 9.14 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, DMSO-d$_6$] δ (in ppm) 11.19 (CH$_3$), 18.54 (CH$_3$), 36.09 (CH$_3$), 37.39 (CH$_2$), 48.36 (CH), 56.04 (CH), 65.21 (CH$_2$), 107.25 (C), 123.50, 126.26, 127.46, 127.71, 128.07, 128.34, 129.14, 129.27 (C$_6$H$_5$), 135.09 (C), 137.08 (C$_6$H$_5$), 138.26 (C$_6$H$_5$), 152.37 (C$_6$H$_5$), 155.93 (CO), 161.72 (CO), 171.46 (CO), 171.93 (CO); ESI-MS m/z 556.3 [M + H$^+$], m/z 578.2 [M + Na$^+$], C$_{31}$H$_{33}$N$_5$O$_5$ requires 555.3.

Z-Val-Ala-AAP: yellow solid; $[\alpha]_D^{20}$ −62.1 (*c* 0.2, MeOH); ^{1}H-NMR [500 MHz, DMSO-d$_6$] δ (in ppm) 0.84 (d, ^3J = 6.7 Hz, 3H, CH$_3$ (Val)), 0.88 (d, ^3J = 6.8 Hz, 3H, CH$_3$ (Val)), 1.30 (d, ^3J = 7.0 Hz, 3H, CH$_3$ (Ala)), 1.97–1.98 (m, 1H, CH (Val)), 2.07 (s, 3H, CH$_3$ (AAP)), 3.03 (s, 3H, CH$_3$ (AAP)), 3.89–3.92 (m, 1H, CH (Val)), 4.43–4.46 (m, 1H, CH (Ala)), 5.03 (s, 2H, CH$_2$ (Z)), 7.29–7.51 (m, 10H, C$_6$H$_5$ (AAP), (Z) and 1H, NH (Val)), 8.10 (d, ^3J = 7.1 Hz, 1H, NH (Ala)), 9.17 (s, 1H, NH (AAP)); ^{13}C-NMR [125.75 MHz, DMSO-d$_6$] δ (in ppm) 11.91 (CH$_3$), 18.11 (CH$_3$), 18.51 (CH$_3$), 19.26 (CH$_3$), 30.42 (CH), 36.08 (CH$_3$), 48.18 (CH), 59.91 (CH), 65.42 (CH$_2$), 107.27 (C), 123.48, 126.24, 127.69, 127.80, 128.39, 129.13 (C$_6$H$_5$), 135.08 (C), 137.14 (C$_6$H$_5$), 152.29 (C$_6$H$_5$), 156.19 (CO), 161.69 (CO), 170.90 (CO), 171.84 (CO); ESI-MS m/z 508.3 [M + H$^+$], m/z 530.2 [M + Na$^+$], C$_{27}$H$_{33}$N$_5$O$_5$ requires 507.3.

Results and discussion

Cysteine proteases

The experiments were accomplished according to Scheme 1 using the single nucleophile AAP but a choice of acyl donors. N-protected amino acid methylesters were applied in a kinetically-controlled approach, whereas in thermodynamically-controlled reactions N-protected amino acids or peptides with a free C-terminal carboxy group featured the acyl donor substrates. Two different types of protecting groups were investigated for their acceptance in the P$_2$ position, the planar Z-group and the bulky and spheric Boc-group.

At first we started with an aqueous-organic reaction medium consisting of 80% citrate buffer, 20% methanol, protease, acyl donor and AAP. The organic solvent has the function of solubilising the ester substrate but the concentration is limited due to enzyme's denaturation. We obtained comparable results for ficin and bromelain

referring to papain (Table 1). Merely in the case of Z-Ala-AAP papain afforded a 20% higher product yield.

In the following experiments we applied a suspension consisting also of 80% citrate buffer, 20% methanol, protease, but with a five-fold amount of acyl donor and AAP. This reaction system had given the best results in former experiments with papain (Lang et al. 2007). The results are comparable for these three enzymes (Table 2). The kinetically-controlled reactions were performed with a 1:2-ratio of acyl donor to nucleophile over one hour and led even in this short reaction time to reasonable yields up to 88%. Z-Gly-AAP could be synthesised in nearly the same yield with each of these three proteases. Z-Ser-AAP is the least formed product, which is according to the substrate specificity of papain for small amino acids in the P_1 position. Obviously bromelain and ficin share the same preference. The results are compiled in Table 2.

The findings from reactions in suspension and in aqueous-organic medium suggest that a consequent reduction of the water activity in those mixtures increases the product yields. Hence, we changed the procedure and went on with a solid-to-solid reaction model. In that case, the amino acid esters did not prove as first choice, because the yield was not enhanced remarkably. Therefore we abandoned the idea of short reaction times by kinetically-controlled synthesis and switched to equilibrium-controlled reactions. Amino acid and peptide derivatives with an unprotected C-terminus were dissolved in methanol, whereas the proteases and AAP got solved in citrate buffer (pH 5.0) separately. We chose the same composition as in aqueous-organic medium (20% methanol, 80% buffer), so that all substances were completely dissolved. After mixing the solutions, the solvent was immediately evaporated within 5 min. In this way a completely homogeneous mixture of all compounds can be guaranteed, which increases the contact area in the solid state. Finally a highly viscous, glue-like mass was formed with only remainders of buffer. After 7 h the reactions were stopped and analysed. The results are presented in Table 3.

Table 1 Cysteine protease-catalysed synthesis of Z-L-aminoacyl-antipyrine amides from Z-L-aminoacyl methylesters and AAP in aqueous-organic media

Product	Yield of Z-Xaa-AAP (%)		
	Papain[a]	Bromelain	Ficin
Z-Ala-AAP	62	38	44
Z-Gly-AAP	45	45	49
Z-Ser-AAP	16	13	13

20 mg protease; 40°C; reaction time: 1 h; 2 mL 0.1 M sodium citrate buffer (pH 5.0); 0.5 mL methanol; 0.1 M acyl donor; 0.2 M AAP

[a] Data published by Lang et al. (2007)

Table 2 Cysteine protease-catalysed synthesis of Z-L-aminoacyl-antipyrine amides from Z-L-aminoacyl methylesters and AAP in suspension

Product	Yield of Z-Xaa-AAP (%)		
	Papain[a]	Bromelain	Ficin
Z-Ala-AAP	68	88	53
Z-Gly-AAP	80	81	83
Z-Ser-AAP	25	41	20

20 mg protease; 40°C; reaction time: 1 h; 2 mL 0.1 M sodium citrate buffer (pH 5.0); 0.5 mL methanol; 0.5 M acyl donor; 1 M AAP

[a] Data published by Lang et al. (2007)

There is no significant difference in the yields of products derived from Boc- and Z-protected amino acid substrates. Obviously, no preference for the protecting group seems to exist, although the Boc- and Z-group are quite different in their space-filling properties. It is also remarkable, that under these conditions both papain and bromelain accept Z-Ser far better than in suspension. Likewise amino acid derivatives with bulky or branched side chains (Boc-Met, Z-Met, Z-Thr) are very well accepted by papain and bromelain.

Compared to papain and bromelain, the outcome with ficin is somewhat different. Just the Ala and Gly derivatives are very well accepted, lower yields are obtained for Z-Met-AAP and Z-Ser-AAP, but Z-Thr is not well suited (11% yield). This finding is in agreement with the P_1–$P_1^{'}$-matrix presented by Tai (2003). Herein most of the ficin-catalysed coupling reactions with amino acid-based

Table 3 Cysteine protease-catalysed synthesis of Boc-/Z-L-aminoacyl- and Z-L-dipeptidyl-antipyrine amides from Boc-/Z-L-aminoacids and Z-L-dipeptides, respectively, and AAP using the solid-to-solid approach

Product	Product yield (%)		
	Papain	Bromelain	Ficin
Boc-Ala-AAP	100	100	81
Boc-Gly-AAP	96	100	99
Boc-Met-AAP	88	87	54
Boc-Ser-AAP	100	100	52
Z-Ala-AAP	100	93	100
Z-Gly-AAP	82	81	82
Z-Met-AAP	100	97	73
Z-Ser-AAP	87	87	61
Z-Thr-AAP	83	92	11
Z-Leu-Ala-AAP	44	59	22
Z-Phe-Ala-AAP	52	59	59
Z-Val-Ala-AAP	36	27	38

20 mg protease; 40°C; reaction time: 7 h; 0.25 mmol acyl donor; 0.5 mmol AAP

nucleophiles were accomplished with Gly in P_1 position, followed by Ala and Cys. Obviously ficin is less prone to accept amino acids with bigger side chains, whereas AAP seems to be tolerated.

In another set of experiments we used Z-protected dipeptides as acyl donors. All peptide derivatives contained Ala in P_1 position and Leu, Phe or Val, respectively, in P_2 position (Table 3). The obtained product yields are lower (22–59%) compared to those for Z-Ala-AAP (93–100%). This might be due to less favoured interactions between substrates and the catalytic site. For papain and bromelain the trend is as expected with product yields for Phe > Leu > Val when in P_2 position, for ficin it differs: Phe > Val > Leu. The aromatic side chain of Phe is close to the Z-group, whereas Val is more bulky near the peptide backbone because of the missing methylene group. So it might be difficult to orientate Val at the active site.

We were not successful in coupling Boc-Pro, Z-β-Ala, Z-Phe, Z-Tyr, Z-Val and Ala with AAP.

In summary nine new antipyrine amides could be synthesised in cysteine protease-catalysed reactions with product yields up to 100%. The results also emphasise the close similarity between papain and bromelain and the somewhat greater distance to ficin.

Serine protease

It can be suggested that α-chymotrypsin offers a chance to synthesise the antipyrine amides of Tyr, Trp, Phe, and Leu. Preliminary studies revealed however, that only Z-Tyr-OMe was accepted and resulted in no more than 5% of Z-Tyr-AAP. Because of the esterase activity of α-chymotrypsin we used the kinetically-controlled approach to optimise the product yield. Two media, aqueous-organic and biphasic, were chosen and the ratio of acyl donor to nucleophile was varied from 1:1 to 1:10. The following conversions were carried out under alkaline conditions (pH 9.0) for strengthening the nucleophilicity of AAP. The gained results are shown in Table 4.

According to the law of mass action, the yields of Z-Tyr-AAP increased with a higher supply of AAP and could be raised from 14 to 41% in the case of an aqueous-organic medium and from 10 to 32% in a biphasic mixture. Obviously the aqueous-organic medium is slightly favoured. Compared to the product yields obtained with cysteine proteases, α-chymotrypsin seems to accept AAP with a far less affinity.

Metalloproteases

Thermolysin and pronase were chosen as representatives of these enzymes. Thermolysin has no esterase activity and therefore only accepts N-protected α-amino acids with a free α-carboxy group. Pronase is a mixture of several *Streptomyces griseus* proteolytic enzymes and can hydrolyse ester bonds (Lobell and Schneider 1998).

In the case of thermolysin we selected a single substrate, Z-Ala. To prove that Z-Ala is well accepted, we carried out a model reaction with Leu-NH_2 as nucleophile and achieved a yield of over 70% in an aqueous-organic medium. When replacing Leu-NH_2 by AAP we could not find any product. Therefore we reduced the water content of the reaction mixture by applying a biphasic medium and the solid-to-solid technique. Product formation could be observed, but only to a very small degree (Table 5).

The catalytic potential of pronase has been investigated in two different media, aqueous-organic and biphasic. This metalloprotease seems also to be a weak catalyst for a coupling between amino acid derivatives and AAP.

Table 4 α-Chymotrypsin-catalysed synthesis of Z-Tyr-AAP in aqueous-organic and biphasic reaction media

Molar ratio Z-Tyr-OMe : AAP	Yield of Z-Tyr-AAP (%)	
	Aqueous-organic [a]	Biphasic [b]
1:1	14	10
1:2	21	14
1:3	29	19
1:5	36	21
1:10	41	32

[a] 10 mg protease; 25°C; reaction time: 2.5 h; 1.8 mL 0.2 M sodium carbonate (pH 9.0); 0.2 mL ACN; 0.125 M Z-Tyr-OMe

[b] 10 mg protease; 25°C; reaction time: 2.5 h; 0.2 mL 0.2 M sodium carbonate (pH 9.0); 1.8 mL ethyl acetate; 0.125 M Z-Tyr-OMe

Table 5 Metalloprotease-catalysed synthesis of Z-L-aminoacyl-antipyrine amides in different reaction media

Acyl donor	Product	Yield of Z-Xaa-AAP (%)	
		Thermolysin	Pronase
Z-Ala-OH	Z-Ala-AAP	5[a]	
Z-Ala-OH	Z-Ala-AAP	<5[b]	
Z-Ala-OH	Z-Ala-AAP		8[c]
Z-Phe-OH	Z-Phe-AAP		10[c]
Z-Tyr-OMe	Z-Tyr-AAP		8[d]

[a] Solid-to-solid synthesis; 3 mg protease; 40°C; reaction time: 22 h; 0.125 mmol acyl donor; 0.25 mmol AAP

[b] Biphasic synthesis; 3 mg protease; 40°C; reaction time: 22 h; 0.25 mL 0.05 M HEPES, 0.01 M $CaCl_2$ (pH 7.0); 2.75 mL ethyl acetate; 0.05 M acyl donor; 0.1 M AAP

[c] Biphasic synthesis; 5 mg protease; 30°C; reaction time: 24 h; 2.7 mL 0.067 M potassium phosphate (pH 6.0); 0.3 mL ethyl acetate; 0.1 M acyl donor; 0.3 M AAP

[d] Aqueous-organic synthesis; 5 mg protease; 25°C; reaction time: 3.5 h; 0.8 mL 0.013 M sodium borate (pH 9.0); 0.2 mL ACN; 0.05 M acyl donor; 0.25 M AAP

Product yields did not exceed about 10% (Table 5). With Z-Met and Z-Thr no product was formed.

These results let us suggest that the active site of these two metalloproteases is not able to bind AAP correctly for a catalysed synthesis.

Conclusion

In the course of this work, ten 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. There is nothing reported so far about these compounds neither in enzymatic nor in chemical synthesis.

We found that, beside 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 this heterocycle was evidenced by a model reaction with Z-Tyr-OMe. Compared to cysteine proteases however, far lower yields were obtained. Metalloproteases do not seem suitable catalysts for transferring AAP, the product yields being as low as 5–10%.

Thus, cysteine proteases are so far the most suited proteases for coupling reactions between amino acid-based acyl donors and AAP. Among thiol proteases papain and bromelain are very similar as known from other investigations. Ficin is close by, but has a distinctive preference for the smallest amino acids, Gly and Ala, in P_1 position.

As to the medium engineering, experimental results confirmed that reducing the water activity of aqueous-organic mixtures via suspensions to solid-to-solid systems may increase the product yield steadily. Using thiol proteases, we could in this way enhance the product yields for several compounds up to 100%.

In summary, this work expands the knowledge of the synthesis potential of proteases. It gives evidence that beside papain also bromelain, ficin and α-chymotrypsin, according to their acyl specificity, are able to accept AAP as nucleophile in the amidation of amino acid and peptide derivatives.

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