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Papain-catalysed synthesis of Z-L-aminoacyl-antipyrine amides from Z-protected amino acid esters and 4-aminoantipyrine

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Abstract—The enzymatic synthesis of Z-L-aminoacyl-antipyrine amides from Z-protected amino acid esters and 4-aminoantipyrine (AAP) was accomplished by papain in aqueous-organic and biphasic media as well as in suspension. Product yields of 80% and 68% for Z-Gly-AAP and Z-Ala-AAP, respectively, could be obtained. The products were purified and characterised by polarimetry and NMR. The following results expand our knowledge of the catalytic potential of proteases, in particular the suitability of papain to accept as nucleophile the 1,2-amino ketone moiety of appropriate heterocyclic compounds.

The advantageous use of proteases as biocatalysts for specific formation of peptide bonds is currently well established. Protease-catalysed coupling reactions with protected amino acid or peptide derivatives may be carried out under mild conditions like room temperature and neutral pH value. This in combination with inherent properties of proteases such as stereospecificity and regiospecificity gives the chemist an instrument for faster and more economic syntheses compared to chemical conversions without catalyst.[1,2]

The esterase activity of cysteine (thiol-) proteases allows the kinetically-controlled approach starting with an ester substrate and the fast formation of an acylated enzyme as intermediate. This reacts in the second step with the nucleophilic component and often gives rise to temporary higher product concentrations than in thermodynamically-controlled synthesis. Secondary hydrolysis does not start until the donor ester is completely consumed because of the greater acceptance of the enzyme for ester than for peptide bonds.[3]

The thiol-endopeptidase papain is a natural product of *Carica papaya* and shows a primary specificity for aromatic or bulky aliphatic moieties in the P_2 position.[4] For amino acid derivatives this requirement is accomplished by using common protecting groups such as Z or Boc.[5] In the P_1 position small amino acids are favoured without any direct specificity.[6] Hydrophobic amino acid derivatives are the best nucleophiles to bind to the S'_1 position of papain.[4,7]

The following experiments demonstrate that the catalytic potential of papain with regard to *N*-components is broader than known so far. Thus, this protease is able to catalyse the reaction of *N*-protected amino acid esters with the heterocyclic 1,2-amino ketone 4-aminoantipyrine (AAP). There are so far no examples in literature for application of AAP in enzymatic synthesis.

AAP (CAS No. 83-07-8) is a metabolite of metamizole and has also analgesic, antipyretic and antiphlogistic effects with an activity lower than that of metamizole.[8] 4-Aminoantipyrine has already been chemically coupled to amino acid derivatives via the methods of classical peptide synthesis utilising *N*,*N*′-dicyclohexylcarbodiimide.[9]

On closer examination of the AAP structure we got the idea that some parts of it suggest a certain suitability for mimicking a substituted amino acid amide with a free α-amino group (Fig. 1). The side chain is part of a ring structure. Its aromaticity and that of the phenyl moiety make AAP almost planar. The free amino group and the protected carboxamide structure qualify 4-aminoantipyrine as a potential nucleophile in enzymatic synthesis.

Keywords: Papain; 4-Aminoantipyrine; Enzymatic synthesis.
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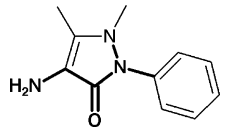


Figure 1. 4-Aminoantipyrine with a partial structure of an α-amino acid amide standing out in bold.

In this Letter we present the successful papain-catalysed synthesis of Z-L-aminoacyl-antipyrine amides utilising different strategies of medium engineering. Aqueous-organic and biphasic media as well as suspensious were applied as described elsewhere[6,7,10] to optimise product yield.

The reactions (Scheme 1) were performed in conus vessels of 5 mL volume placed in a magnetic stirring bath. Papain (Merck, 30000 USP-U/mg) was activated with 2–3 mg cysteine hydrochloride in 2 mL buffer for about 15 min. After addition of AAP while stirring with 640 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. We did not verify whether papain was totally deactivated, but the yields were slightly lower and absolutely reproducible compared to reactions without inactivating papain. After deactivation of the enzyme the solvent was evaporated under vacuum. The dried up reaction mixture was dissolved in 5 mL acetonitrile 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 papain we could not observe the formation of Z-L-aminoacyl-antipyrine amides.

In first experiments we used an aqueous-organic medium consisting of 20% methanol and 80% buffer with a pH of 5.0 near pH optimum of papain wherein all starting compounds were soluble. The organic solvent has the function of solubilising the ester substrate but the concentration is limited due to enzyme's denaturation. The reactions were conducted at 40 °C for 1 h with a ratio of acyl donor to nucleophile of 1:2. The results are presented in Table 1.

The reaction solution of the aqueous-organic medium became turbid immediately after addition of the dissolved **1a** and product **3a** precipitated. Products **3b** and **3c** remained dissolved in a clear or slightly turbid solution. In the case of the reaction in suspension none of the starting substances and products was completely dissolved. The suspension was made of the same solvent but with a 5-fold concentration of substrates and nucleophile. Working with a suspension led to higher product yields. Such reaction systems frequently approved to be advantageous because of reduced water activity resulting in lower hydrolysis of the acyl donor and secondary product cleavage.[10,11]

Table 1. Papain-catalysed synthesis of Z-L-aminoacyl-antipyrine amides in aqueous-organic medium and suspension[a]

Acyl donor	Product	Yield of Z-Xaa-AAP (%)	
		Aqueous-organic[b]	Suspension[c]
1a Z-Gly–OMe	**3a** Z-Gly-AAP	45	80
1b Z-Ala–OMe	**3b** Z-Ala-AAP	62	68
1c Z-Ser–OMe	**3c** Z-Ser-AAP	16	25

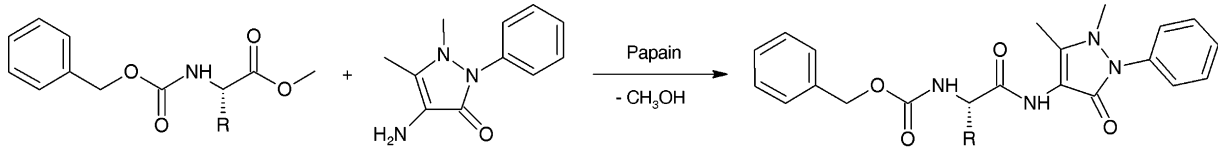
[a] 20 mg papain, 40 °C, reaction time: 1 h.
[b] 2 mL 0.1 M sodium citrate buffer (pH 5.0); 0.5 mL methanol; 0.1 M acyl donor; 0.2 M AAP.
[c] 2 mL 0.1 M sodium citrate buffer (pH 5.0); 0.5 mL methanol; 0.5 M acyl donor; 1 M AAP.

According to the substrate specificity, papain accepted **1a** and **1b** far better than **1c**. The side chain of **1c** is more bulky than that of the two other substrates.

In order to explore whether there is a kinetic maximum in the formation of **3b**, the time course of the reaction was followed and is shown in Figure 2.

As shown in Figure 2, **1b** reacts very fast and already after 5 min 35% yield of **3b** are formed. After approx. 4 h the kinetic maximum is reached and secondary hydrolysis starts.

To optimise the conditions from an economical point of view, we reduced the reaction time to 20 min and the enzyme amount to 10 mg papain. In addition, the following conversions were carried out under alkaline conditions (0.2 M KH_2PO_4; 0.2 M EDTA; pH 8.6) in order to obtain a higher concentration of AAP with unprotonated amino group. The results are presented in Table 2.



R = H	**1a**	*Z*-Gly-OMe	**3a**	*Z*-Gly-AAP
R= CH_3	**1b**	*Z*-Ala-OMe	**3b**	*Z*-Ala-AAP
R= CH_2OH	**1c**	*Z*-Ser-OMe	**3c**	*Z*-Ser-AAP

Scheme 1. Papain-catalysed synthesis of Z-L-aminoacyl-antipyrine amides (**3a**–**c**) from Z-L-amino acid esters (**1a**–**c**) and 4-aminoantipyrine (**2**).

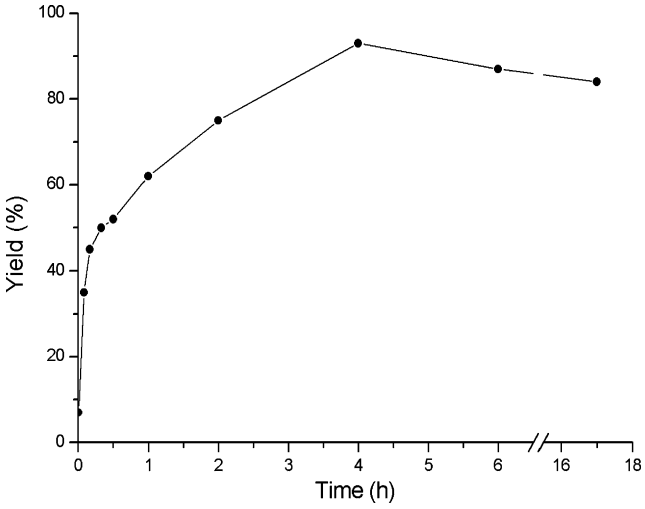


Figure 2. Time-dependent formation of Z-Ala-AAP from Z-Ala–OMe and AAP catalysed by papain (2 mL 0.1 M sodium citrate buffer (pH 5.0); 0.5 mL methanol; 20 mg papain; 40 °C; 0.1 M Z-Ala–OMe; 0.2 M AAP).

Table 2. Papain-catalysed synthesis of Z-L-aminoacyl-antipyrine amides in aqueous-organic and biphasic medium[a]

Acyl donor	Acyl donor–nucleophile ratio	Yield of Z-Xaa-AAP (%)	
		Aqueous-organic[b]	Biphasic[c]
1a Z-Gly–OMe	1:1	33	58
	1:2	66	67
1b Z-Ala–OMe	1:1	27	57
	1:2	36	50
1c Z-Ser–OMe	1:1	12	30
	1:2	13	34

[a] 10 mg papain, 40 °C, reaction time: 20 min.
[b] 2 mL buffer (0.2 M KH_2PO_4, 0.2 M EDTA, pH 8.6); 0.5 mL methanol; 0.1 M acyl donor.
[c] 0.25 mL buffer (0.2 M KH_2PO_4, 0.2 M EDTA, pH 8.6); 2.25 mL ethyl acetate; 0.1 M acyl donor.

In an aqueous-organic medium the higher pH value favoured remarkably the formation of **3a**, even at a considerably diminished amount of papain and at shorter reaction time. Scarcely influenced remained the synthesis of **3c**.

Using a biphasic reaction medium proved to be advantageous in comparison to an aqueous-organic reaction mixture. The yield of **3c** reached an acceptable value.

We consider a suspension of educts in buffer with slight addition of methanol as the most suited medium. The product yields obtained here are the highest so far for Z-Gly-AAP (80%) and Z-Ala-AAP (68%) (Table 1).

All the synthesised Z-L-aminoacyl-antipyrine amides were purified[12] and characterised by polarimetry, LC–MS, ^{1}H NMR and ^{13}C NMR.[13–15]

In conclusion, this work emphasises the far wider synthesis potential of proteases than that known so far. For the first time Z-L-aminoacyl-antipyrine amides could be synthesised by using papain as catalyst. Thus, one could be keen on the answer of the question: Is papain the only protease to accept 1,2-amino ketones in enzymatic synthesis? Further experiments in this line are currently under investigation in our group.

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12. Two batches of about 0.5 mmol product were evaporated under vacuum to dryness and then dissolved in 20 mL $CHCl_3$. The solution was washed twice with saturated sodium bicarbonate solution, and the two phases were separated. AAP was extracted with 1 M HCl from the organic phase as long as the reaction of the aqueous phase with Ehrlich reagent turned out positive. Then the organic layer was dried with sodium sulfate, filtered and evaporated. The obtained product was desiccated overnight at 40 °C.
13. Z-Ala-AAP: slightly yellow solid; $[\alpha]_D^{20}$ −31.7 (*c* 0.3, MeOH); ^{1}H NMR [500 MHz, $CDCl_3$] δ (in ppm) 1.35 (d, $^3J = 6.9$ Hz, 3H), 2.16 (s, 3H), 3.05 (s, 3H), 4.38–4.41 (m, 1H), 5.05, 5.15 ($^2J = 12.2$ Hz, 2H), 5.91 (d, $^3J = 7.5$ Hz, 1H), 7.24–7.40 (m, 10H), 8.79 (s, 1H); ^{13}C NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 12.23 (CH_3), 18.93 (CH_3), 35.85 (CH_3), 51.03 (CH), 66.72 (CH_2), 107.95 (C), 124.45, 127.10, 127.98, 128.00, 128.44, 129.25 (C_6H_5), 134.28 (C), 136.52 (C_6H_5), 150.06 (C_6H_5), 155.79 (CO), 161.61 (CO), 172.19 (CO).
14. Z-Gly-AAP: slightly yellow solid; ^{1}H NMR [500 MHz, DMSO-d_6] δ (in ppm) 2.10 (s, 3H), 3.04 (s, 3H), 3.79 (d, $^3J = 6.2$ Hz, 2H), 5.05 (s, 2H), 7.30–7.54 (m, 10H), 9.13 (s, 1H); ^{13}C NMR [125.75 MHz, DMSO-d_6] δ (in ppm) 11.20 (CH_3), 36.03 (CH_3), 43.40 (CH_2), 65.45 (CH_2), 107.24 (C), 123.46, 126.21, 127.71, 127.79, 128.35, 129.10 (C_6H_5), 135.03 (C), 137.07 (C_6H_5), 152.31 (C_6H_5), 156.52 (CO), 161.70 (CO), 168.65 (CO).

15. Z-Ser-AAP: yellow solid; $[\alpha]_D^{20}$ −23.4 (*c* 0.3, MeOH); ^{1}H NMR [500 MHz, $CDCl_3$] δ (in ppm) 2.15 (s, 3H), 3.07 (s, 3H), 3.71–3.78 (m, 2H), 3.95–4.02 (m, 1H), 4.42–4.44 (m, 1H), 5.05–5.22 (m, 2H), 6.54 (d, $^3J = 7.9$ Hz, 1H), 7.25–7.46 (m, 10H), 8.57 (s, 1H); ^{13}C NMR [125.75 MHz, $CDCl_3$] δ (in ppm) 11.69 (CH_3), 35.43 (CH_3), 56.89 (CH), 62.73 (CH_2), 66.89 (CH_2), 107.15 (C), 124.82, 127.58, 127.94, 128.08, 128.41, 129.32 (C_6H_5), 133.86 (C), 136.41 (C_6H_5), 150.65 (C_6H_5), 156.40 (CO), 161.69 (CO), 171.15 (CO).