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Enzyme-catalysed deprotection of *N*-acetyl and *N*-formyl amino acids

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Abstract

To investigate the full potential of hydrolases for the removal of two amine-protecting groups, 15 different, commercially available lipases, acylases, proteases and esterases were studied for the hydrolyses of *N*-acetyl and *N*-formyl protecting groups. In addition to the well-known acylases from porcine kidney and *Aspergillus melleus*, this screening revealed that porcine liver esterase and the lipases from *Rhizomucor miehei* and *Pseudomonas stutzeri* are also catalysts for the hydrolysis of *N*-acetylalanine. The activity of lipases in this reaction was unexpected, since lipases are commonly believed not to hydrolyse amides. In addition, from these 15 enzymes, three were found to be active in the hydrolysis of *N*-formylalanine, i.e. porcine liver esterase and the two acylases. This is the first example where esterase is employed to deprotect *N*-formyl amides.

Keywords: Hydrolase; Acylase; Lipase; Deprotection; *N*-acetyl amino acids

1. Introduction

The enantioselective synthesis of amino acids has long been a topic that has attracted the attention of many chemists. Next to the large-scale industrial kinetic resolutions [1], countless synthetic methods have been developed ranging from transition metal catalysed reactions to the bis lactim ether approach [2–6]. Most of these very diverse methods have one thing in common: the enantiopure amino acid is not synthesised as the free compound, but it is masked by an attached chiral auxiliary or protecting group. Consequently, these very elegant enantioselective laboratory syntheses are often followed by rather harsh deprotection reactions.

However, this disadvantage of most amino acid syntheses can be turned into a virtue. When the protection group is removed with an enantioselective catalyst, it is possible to further amplify the enantiopurity of the target molecule [7,8]. Lipases and acylases fulfil all the criteria for such a catalyst, i.e. high enantioselectivity, low substrate specificity and high activity under very mild reaction conditions. So far, only acylases, particularly from *Aspergillus oryzae* and pig kidneys were shown to be able to readily remove *N*-acetyl protection groups [9–11]. The very selective hydrolysis achieved by these enzymes is already being utilised by Tanabe [12,13] and Degussa for the multi-tonnes scale resolution of several different amino acids [1,12,14–17]. For the catalytic deprotection of *N*-formyl protecting groups even fewer enzymes are available. Both acylases and deformylases are known for their activity in the hydrolysis of *N*-formyl acids [9]. But, to the best of our knowledge, the only example in organic synthesis of an enzymatic removal of a *N*-formyl group is by Sonke et al. utilizing a peptide deformylase [18].

Commonly it is assumed that lipases cannot cleave amides [19]. As they were optimised by nature for the cleavage of esters, they were supposed not to be able to attack the thermodynamically more stable amide bond. Recently, the very elegant *p*-acetoxybenzyloxycarbonyl (AcOZ) protecting group was introduced, which can be removed by lipases [20]. However, here the lipase does not directly hydrolyse the amide bond, but the amine group is liberated via a relay reaction.

To ensure that the full potential of the hydrolases is utilised for the gentle removal of the two above-mentioned amine-protecting groups, a wider screening of hydrolases was deemed necessary. We, therefore, studied 15 different, commercially available

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lipases, acylases, proteases and esterases for hydrolyses of *N*-acetyl and *N*-formyl protecting groups.

2. Experimental

2.1. General

Achrobacter sp. lipase (lipase AL, Meito Sangyo), *Alcaligenes* sp. lipase (lipase AL, Meito Sangyo), *Aspergillus melleus* acylase 1 (Fluka), *Candida antarctica* lipase B (Novozymes 435, Novozymes), *Candida rugosa* lipase (type VII, Sigma), α-chymotrypsin (from bovine pancreas, Sigma), *Humicola lanuginose* lipase (SP523, Novo Nordisk), penicillin G amidase (PGA-450, an immobilised *E coli* penicillin G acylase on a specially developed organic polymer containing 59% water, Roche Diagnostics), porcine kidney acylase 1 (Sigma), *Pseudomonas fluorescences* lipase (Fluka), *Pseudomonas stutzeri* lipase (lipase TL, Meito Sangyo), *Rhizomucor miehei* lipase (SP524, Novo Nordisk), Subtilisin (protease (subtilisin Carlsberg) from *Bacillus licheniformis*, Sigma), Trypsin II-S (Porcine Pancreas, Sigma), Trypsin IX-S (Porcine Pancreas, Sigma) were obtained from their respective suppliers. NMR spectra were recorded on a Varian Inova 300 MHz or a Varian VXR-400S spectrometer relative to *t*-BuOH.

2.2. Substrate synthesis

2.2.1. *N-acetyl*-D,L-*alanine*

D,L-Alanine (10 g, 112 mmol) and acetic anhydride (30 g, 0.3 mol) were stirred in methanol (50 mL) for 6 h under reflux, after which all volatiles were removed by evaporation. The crude product was triturated with ethyl acetate. The white solid was collected by filtration and dried under vacuum. Yield 98% (14 g, 74.8 mmol). ^{1}H NMR (300 MHz, D_2O): δ 4.29 (q, $J = 7.2$ Hz, 1H, $CHCH_3$), δ 1.98 (s, 3H, $COCH_3$), δ 1.38 (d, $J = 7.2$ Hz, 3H, CH_3CH).

2.2.2. *N-formyl*-D,L-*alanine*

N-formylalanine was prepared as described by Kolb et al. [21]. D,L-Alanine (3.8 g, 42.7 mmol), formic acid (8.6 g, 0.19 mol), and acetic anhydride (17 g, 0.17 mol) were stirred in acetic acid (100 mL) for 4 h at RT, after which all volatiles were removed by evaporation. The crude product was recrystallised from ethyl acetate. The resulting solid was further purified by ion-exchange. The DOWEX 50 (H^+) column was rinsed with water until neutral, followed by ammonia (1 M). The ammonia layer was lyophilised. Yield 40% (2 g, 17 mmol). ^{1}H NMR (D_2O): δ 8.04 (s, 0.9H, HCO, rotamer 1), δ 8.00 (s, 0.1H, HCO rotamer 2), δ 4.42 (q, $J = 7.2$ Hz, 0.9H, $CHCH_3$, rotamer 1), δ 4.38 (q, $J = 7.2$ Hz, 0.1H, $CHCH_3$, rotamer 2), δ 1.40 (d, $J = 7.2$ Hz, 2.7H, CH_3CH, rotamer 1), δ 1.43 (d, $J = 7.2$ Hz, 0.3H, CH_3CH, rotamer 2).

2.2.3. *N-acetyl*-D,L-*phenylalanine*

α-Acetamidocinnamic acid (5.0 g, 24.4 mmol) was hydrogenated with 10% Pd/C under 1 bar H_2 in ethanol (300 mL). After the reaction was finished (ca. 5 h) and filtered, the volatiles were removed by evaporation. The crystals were obtained by filtration and dried under vacuum. Yield 95% (4.8 g, 23.2 mmol). ^{1}H NMR (D_2O): δ 7.33–7.20 (m, 5H, C_6H_5), δ 4.63 (dd, $J = 5.0$ Hz, $J = 9.1$, 1H, $CHCH_2$), δ 3.18 (dd, $J = 5.1$ Hz, $J = 14.0$, 1H, $CHCH_2$), δ 2.92 (dd, $J = 9.1$ Hz, $J = 14.0$, 1H, $CHCH_2$), δ 1.88 (s, 3H, $COCH_3$).

2.3. Activity assessments

2.3.1. *Lipases and esterases*

The activities of the lipases and esterases were determined as described by Veum et al. [22]. Tributyrin (1.47 mL, 5.02 mmol) was added to 48.5 mL of a 10 mM potassium phosphate buffer, pH 7.0 (10 mM of potassium dihydrogen phosphate (100 mL) adjusted to pH 7.0 with 10 mM of dipotassium hydrogen phosphate (ca.100 mL)) in a thermostatted vessel at 25 °C, and the mixture was stirred mechanically. The pH was maintained at 7.0 with an automatic burette, and when the pH had stabilised, the enzyme was added (for example, 9 mg of CAL B). The consumption of 100 mM sodium hydroxide was monitored over 40 min and plotted against time. 1 μmol of NaOH consumed per min corresponds to 1 unit (1 U) of activity.

2.3.2. *Acylases, amidases and proteases*

The activities of the acylases were determined as described by Bakker et al. [23].

N-acetyl-L-methionine (15.7 mM) was dissolved in a Tris buffer (5 mL, 50 mM, pH 7.5), the pH was adjusted with NaOH (1 M) and enzyme was added. The reaction was performed at 25 °C and was quenched after 1 h by adding HCl (1 M, 5 mL) and the conversion was measured by reversed-phase HPLC using a custom-packed Symmetry C18 cartridge (Waters Radial-Pak, 8 mm × 100 mm, 7 μm) acetonitrile/phosphate buffer (50 mM, pH 2.2) of 7.5:92.5 (v:v) as eluent (flow 1.5 mL/min for the first 4 min followed by 3 mL/min), with detection at 210 nm. 1 unit (U) is the amount of enzyme hydrolyzing 1 μmol of *N*-acetyl-L-methionine per minute.

The activity of penicillin G acylase was determined as described by van Langen et al. [24].

To a 2% solution of penicillin G potassium salt in 0.1 M phosphate buffer at pH 8.0 and 34 °C enzyme was added. During the hydrolysis the pH was maintained at 8.0 with automated NaOH titration. 1 unit (U) of penicillin G acylase liberates 1 μmol of phenylacetic acid per min.

The activities of subtilisin and the various trypsins were determined as described by their supplier.

2.4. Hydrolysis of *N-acetyl*-D,L-*amino acids and N-formyl*-D,L-*alanine*

To 1.5 mmol substrate in 30 mL potassium phosphate buffer (0.1 M, pH 7.5) were added 333 units of enzyme. The mixture was shaken for the desired duration, after which samples were taken. The samples were adjusted to pH 5 with 1 M HCl, heated to 60 °C with Norit, filtered over Celite and lyophilised. Conversions were determined by ^{1}H NMR. Before determining the enantioselectivity, the samples were passed over a DOWEX-50

Table 1
Enzyme-catalysed hydrolysis of *N*-acetyl-D,L-alanine (**1a**)[a]

Enzyme[b,c]	Reaction time 3 h			Reaction time 24 h		
	Conversion (%)	Ee **2a** (%)	*E*	Conversion (%)	Ee **2a** (%)	*E*
RML	Traces	–	–	Traces	–	–
RML[d]	3	n.d.	–	11	65 (L)	5.1
PLE	25	77 (L)	9.9	50	79 (L)	20
HLL	3	n.d.	–	3	n.d.	–
TL	10	67 (L)	5.4	13	65 (L)	5.2
TL[d]	15	70 (L)	6.4	25	68 (L)	6.5
Trypsin IX	2	n.d.	–	4	n.d.	–
PenG amidase	12	80 (L)	10	50	88 (L)	45
Acylase 1 AM	48	99	>600	58	72 (L)	62
Acylase 1 PK	53	90 (L)	>110	53	90 (L)	>110

[a] 333 units, 1.5 mmol substrate, 30 mL potassium phosphate buffer (0.1 M, pH 7.5), RT.
[b] RML, *Rhizomucor miehei* lipase; PLE, porcine liver esterase; HLL, *Humicola lanuginose* lipase; TL, *Pseudomonas stutzeri* lipase; Trypsine IX, Trypsine type IX from porcine pancreas; PenG amidase, penicillin G amidase; Acylase 1 AM, *Aspergillus melleus* acylase 1; Acylase 1 PK, porcine kidney acylase 1.
[c] *Achrobacter* sp. lipase, *Candida antarctica* lipase B, *Candida rugosa* lipase, *Pseudomonas fluorescences* lipase, *Alcaligenes* sp. lipase, porcine pancreas Trypsin II, bovine pancreas α-chymotrypsin, *Bacillus licheniformis* subtilisin were also screened but no activity was observed.
[d] 10-Fold amount of enzymes added, 3333 units, 1.5 mmol substrate, 30 mL potassium phosphate buffer (0.1 M, pH 7.5), RT.

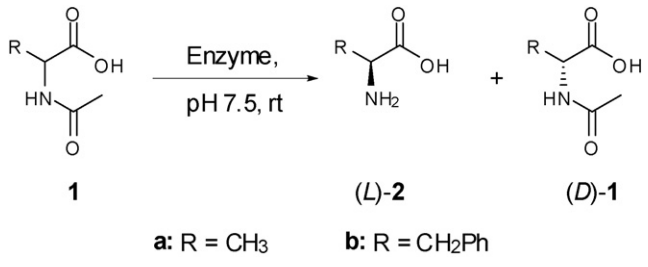
(H^+) column, which was rinsed with water until neutral, followed by 1 M ammonia. The ammonia layer was again lyophilised. Enantiomeric excesses of alanine were determined by chiral HPLC using a Crownpak CR (+) column (150 mm × 4 mm) with $HClO_4$ (pH 1) as eluens, a flow of 0.5 mL/min at 0 °C and UV detection at 215 nm. Retention times (min): D-alanine (4.6) and L-alanine (10.8). For phenylalanine the enantiomeric excesses were also determined by chiral HPLC using a Crownpak CR (+) column (150 mm × 4 mm) using a different eluent. $HClO_4$ (pH 2), a flow of 0.8 mL/min at 25 °C and UV detection at 215 nm. Retention times (min): D-phenylalanine (9.2) and L-phenylalanine (11.7).

3. Results and discussion

N-acetyl amino acids were chosen as model substrates to screen these 15 enzymes (Scheme 1), since the *N*-acetyl group is a standard functionality/protection moiety in the synthesis of amino acids (which is commonly removed under harsh deprotection conditions, including refluxing in concentrated acids). Although aminoacylases are ideally suited for the cleavage of the *N*-acetyl group, they do also have significant drawbacks. As with most enzymes, aminoacylases have limited substrate tolerance and only two of them are commercially available. Furthermore, these commercially available acylases are both L-specific, ruling out their application for the deprotection of D-amino acids.

To overcome this synthetic limitation, a broad range of enzymes was evaluated for their selective hydrolytic capabilities of the amide bond in *N*-acetyl amino acids. However, an accurate comparison between different families of enzymes, e.g. lipases and acylases is complicated, since the activity tests for those families are not related. To obtain the best possible assessment, the activities of the lipases and esterases were determined with the hydrolysis of tributyrin [22] and those of the acylases with the hydrolysis of *N*-acetyl-L-methionine [23]. The amidases and proteases gave no practical activity values when the *N*-acetyl-L-methionine test was used, thus the activity tests recommended by their suppliers were used.

The 15 commercially available enzymes were first screened for their hydrolytic activity towards the amide bond in *N*-acetyl-D,L-alanine (**1a**) (Table 1). The acylases from porcine kidney and *A. melleus*, are clearly the best enzymes, with respect to activity and selectivity. This is not surprising, since these enzymes were evolved for this purpose. The activity of some of the lipases and porcine liver esterase, however, is unexpected, since it is commonly thought that lipases are not able to catalyse the hydrolysis



Scheme 1. Screening for hydrolase activity was performed with two model substrates.

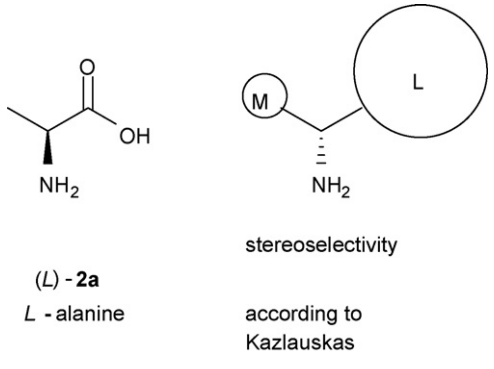


Fig. 1. According to the rule of Kazlauskas the opposite enantiomer of alanine should be a substrate for lipases.

Table 2
Enzyme-catalysed hydrolysis of *N*-acetyl-D,L-phenylalanine (**1b**)[a]

Enzyme[b,c]	Reaction time 3 h			Reaction time 24 h		
	Conversion (%)	Ee **2b** (%)	*E*	Conversion (%)	Ee **2b** (%)	*E*
RML[d]	3	n.d.	–	19	96 (L)	61
PLE	0	–	–	0	–	–
HLL[d]	0	–	–	0	–	–
TL[d]	0	–	–	0	–	–
Acylase 1 AM	49	99 (L)	>600	54	83 (L)	46
Acylase 1 PK	5	96 (L)	51	37	99 (L)	>300

[a] 333 units, 1.5 mmol substrate, 30 mL potassium phosphate buffer (0.1 M, pH 7.5), RT.
[b] RML, *Rhizomucor miehei* lipase; PLE, porcine liver esterase; HLL, *Humicola lanuginose* lipase; TL, *Pseudomonas stutzeri* lipase; Acylase 1 AM, *Aspergillus melleus* acylase 1; Acylase 1 PK, porcine kidney acylase 1.
[c] *Achrobacter* sp. lipase, *Candida antarctica* lipase B, *Candida rugosa* lipase, *Pseudomonas fluorescences* lipase, *Alcaligenes* sp. lipase, porcine pancreas Trypsin II, Trypsine type IX from porcine pancreas, bovine pancreas α-chymotrypsin, *Bacillus licheniformis* subtilisin were also screened but no activity was observed.
[d] 10-Fold amount of enzymes added, 3333 units, 1.5 mmol substrate, 30 mL potassium phosphate buffer (0.1 M, pH 7.5), RT.

of an amide bond [19]. One exception to this is the *C. antarctica* lipase B catalysed hydrolysis of *N*-acetyl-L-arylethylamines [25] which was recently re-confirmed [26]. It has also been found, in a related study carried out in our laboratory that *C. antarctica* lipase B hydrolyses alkyl- and alkylarylamides-derived from *N*-methylglycine and methoxyacetic acid [27]. However, *C. antarctica* lipase B is not among the active lipases for the hydrolysis of *N*-acetylalanine. *R. miehei* and *P. stutzeri* lipase are the ones that demonstrate a low activity for the amide hydrolysis, but only when a large amount of enzyme (3333 units) is added. Still, these two lipases and especially porcine liver esterase outperform most of the tested proteases, which nature engineered to hydrolyse amides. Trypsin XI gives very poor conversions, while the other Trypsin II, chymotrypsin and subtilisin show no activity at all. The only serine protease, which was able to catalyse the hydrolysis sufficiently, was penicillin G amidase.

All active enzymes show selectivity for the L-substrate; unfortunately no D-selectivity was observed. The acylases give, as expected, very high *E*-values (>110), the amidase is second best with an *E*-value of 45. Porcine liver esterase also displays a reasonable preference for the L-substrate (*E*-value = 20). The lipases demonstrate only modest selectivity for the L-amino acid. In the acylation of primary amines, lipases generally follow Kazlauskas' rule [28,29], where the enantiopreference is determined by the steric bulk of the substituents (Fig. 1). In the hydrolysis of **1a** the selectivity, opposite to the one predicted by Kazlaukas' rule, is observed, suggesting that electronic factors dominate the enantiodiscrimination of alanine.

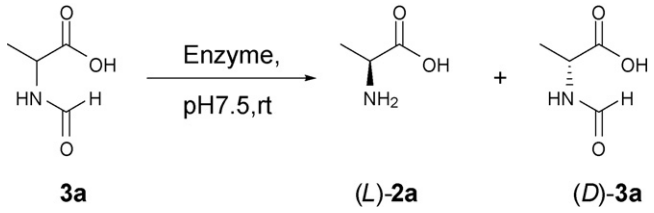
The enzymes were also tested with *N*-acetyl-D,L-phenylalanine (**1b**) as substrate (Table 2). Most of the enzymes, which showed activity for **1a**, e.g. porcine liver esterase and *P. stutzeri* lipase, are not able to hydrolyse **1b**. Both the acylases show a significant hydrolysis rate for **1b**, but porcine kidney acylase I is considerably slower for this substrate than for **1a**. This drop in activity for porcine kidney acylase I when changing from alanine to phenylalanine was also observed by others [9,30,31]. Both acylases show an excellent selectivity for the L-enantiomer of **1b**. Besides the acylases, *R. miehei* lipase is the only enzyme tested that is able to hydrolyse the amide bond in **1b**. Once more, this lipase demonstrates a better activity in the hydrolysis of amides as compared to the proteases. However, this activity is still well below that of the acylases. The *N*-acetyl group is removed by *R. miehei* lipase with good enantioselectivity (*E* = 61).

To investigate the potential of these 15, commercially available enzymes in the removal of the *N*-formyl protection groups, the *N*-formyl derivative of D,L-alanine (**3a**) was selected as model substrate (Scheme 2). Three of these enzymes were

Table 3
Enzyme-catalysed hydrolysis of *N*-formyl-D,L-alanine (**3a**)[a]

Enzyme[b,c]	Reaction time 3 h			Reaction time 24 h		
	Conversion (%)	Ee **2a** (%)	*E*	Conversion (%)	Ee **2a** (%)	*E*
RML[d]	0	–	–	0	–	–
PLE	3	n.d.	–	10	n.d.	–
HLL[d]	0	–	–	0	–	–
TL[d]	0	–	–	0	–	–
Acylase 1 AM	50	91 (L)	67	47	90 (L)	58
Acylase 1 PK	21	n.d.	–	50	95 (L)	145

[a] 333 units, 1.5 mmol substrate, 30 mL potassium phosphate buffer (0.1 M, pH 7.5), RT.
[b] RML, *Rhizomucor miehei* lipase; PLE, porcine liver esterase; HLL, *Humicola lanuginose* lipase; TL, *Pseudomonas stutzeri* lipase; Acylase 1 AM, *Aspergillus melleus* acylase 1; Acylase 1 PK, porcine kidney acylase 1.
[c] *Achrobacter* sp. lipase, *Candida antarctica* lipase B, *Candida rugosa* lipase, *Pseudomonas fluorescences* lipase, *Alcaligenes* sp. lipase, porcine pancreas Trypsin II, Trypsine type IX from porcine pancreas, bovine pancreas α-chymotrypsin, *Bacillus licheniformis* subtilisin were also screened but no activity was observed.
[d] 10-Fold amount of enzymes added, 3333 units, 1.5 mmol substrate, 30 mL potassium phosphate buffer (0.1 M, pH 7.5), RT.



Scheme 2. Screening for hydrolase activity for the *N*-formyl group.

found to be active in the hydrolysis of **3a** (Table 3). This is the first example where enzymes, other than deformylases [18], are employed to deprotect *N*-formyl amides.

The *N*-formyl is evidently more challenging to remove for most of the tested enzymes. The two lipases, which demonstrated reasonable activity in the *N*-acetyl deprotection, do not hydrolyse the *N*-formylamide of **3a**. This is unexpected since *N*-formyl is more readily hydrolysed under acidic conditions and, thus, less stable [32]. Two of the active enzymes, i.e. porcine liver esterase and porcine kidney acylase 1, also hydrolyse the *N*-formyl amide with a significant lower rate as compared to that of the *N*-acetyl group. Only *A. melleus* acylase 1 reaches the same conversion within 3 h for both of these substrates. Although the enantioselectivity of *A. melleus* acylase 1 is slightly decreased, this acylase is a good new catalyst for the hydrolysis of *N*-formylalanine (**3a**).

4. Conclusions

In conclusion, we have demonstrated for the first time that porcine liver esterase, *R. miehei* and *P. stutzeri* lipase are able to hydrolyse *N*-acetyl amides. The ability of these lipases to hydrolyse this very stable amide once again proves that the dogma "lipases cannot hydrolyse amides" is incorrect. As has been demonstrated before for other enzymes, the scope of an enzyme is not limited to its naturally occurring role [33]. Therefore, the catalytic promiscuity of enzymes in general should be investigated in more detail, extending their usefulness in organic synthesis significantly.

In this context, we demonstrated for the first time that an esterase is capable of deprotecting *N*-formyl amides. Next to porcine kidney acylase 1 and especially *A. melleus* acylase 1 AM, porcine liver esterase is quite efficient in the hydrolysis of the *N*-formyl bond. The fact that these three active enzymes are commercially available opens up new possibilities for their use in protection/deprotection chemistry. Hopefully this will further promote the use of enzymes in organic synthesis, since replacing chemical deprotection with biocatalytic deprotection is a major progress towards more efficient, 'greener' syntheses, and ultimately a more sustainable chemistry.

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