N-Acyl glycinates as acyl donors in serine protease-catalyzed kinetic resolution of amines. Improvement of selectivity and reaction rate†

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Enzymatic kinetic resolution of aliphatic and benzylic amines leading to (*S*)-amides was achieved by using alkaline protease as the catalyst and *N*-octanoyl glycine trifluoroethyl ester as the acyl donor; enantioselectivity ranged between 4 to 244, while reaction times were dramatically shortened and ranged between 15 min to 6 h.

Introduction

Serine proteases constitute the most important class of commercially available proteases.[1] In organic solvent, these proteases accept a broad range of substrates. Besides their activity as catalysts for peptide synthesis, subtilisins can be used for the resolution of both alcohols and amines.[2,3] There are rather few examples of protease-mediated resolution of amines in the literature.[4] In the kinetically controlled formation of an amide link, an activated ester reacts with the serine residue in the active site of the enzyme to form an acyl–enzyme complex which then reacts with the amine to form the desired amide. The spatial arrangement of the catalytic triad in the protease active site is the mirror image of that in lipases. As a consequence, their enantiopreference is opposite to that of lipases.[5] However, substilisins are generally less reactive, less selective and less stable in an organic medium than lipases.[5b]

The search for fast and selective enzymatic kinetic resolution (KR), compatible with thiyl radical mediated-racemization is ongoing in our research group.[6] We have recently performed the (*R*)-selective dynamic kinetic resolution (DKR) of amines by associating CAL-B catalyzed-KR with *in situ* radical racemization.[7,8] In order to broaden the scope of the reaction, we concentrated our efforts on developing a process leading to the opposite selectivity, *i.e.*, (*S*)-selective amide synthesis. However, before investigating any DKR protocol, it was necessary to optimize an enzymatic KR procedure obeying first and foremost the following specifications: the highest possible (*S*)-selectivity, and a reaction time inferior or equal to 4 h in order to prevent side reactions like oxidative degradation to occur when the KR is coupled to the fast thiyl radical-mediated racemization process.

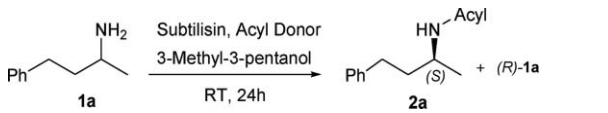
We report in this communication our preliminary results on protease-catalyzed resolution of chiral amines in which the stereogenic center is directly adjacent to the reactive amine moiety.

Results and discussion

Optimizing the KR protocol implied the screening of a series of acyl donors and different proteases. Phenylbutyl amine (**1a**) was used as a model compound throughout the optimization studies. The best experimental conditions were then applied to a series of related amines.

The results obtained for the resolution of **1a** by subtilisin A, treated and co-lyophilized with two surfactants according to Bäckvall's protocol,[9,10] are given in Table 1. The reactions were performed in 3-methyl-3-pentanol.[4a,b] Within 44 and 24 h, respectively, low conversion and low enantioselectivity was observed when using either trifluoroethylbutyrate or ethyl methoxyacetate as the acyl donor (entries 1, 2).

Table 1 Influence of the acyl donor on subtilisin A-catalyzed kinetic resolution of phenylbutylamine (**1a**)[a]



1a **2a**

Entry	Acyl donor	**1a** ee (%)[b]	**2a** ee (%)[c]	*C* (%)[d]	*E*[e]
1	$C_3H_7CO_2CH_2CF_3$[f]	11	74	13.0	7
2	$CH_3OCH_2CO_2Et$	27	86	23.9	17
3	$AcNHCH_2CO_2Et$	40	nd	35.0[g]	10
4	$BzNHCH_2CO_2Et$	23	nd	32.0[g]	3
5	$C_3H_7CONHCH_2CO_2Et$	61.5	84	42.2	22
6	$C_7H_{15}CONHCH_2CO_2Et$	40.5	92.6	30.4	39
7	$C_{11}H_{23}CONHCH_2CO_2Et$	46	92.5	33.2	40

[a] Standard procedure: reactions were performed on a 0.5 M solution of **1a** (0.25 mmol) in 3-Me-3-pentanol at room temperature for 24 h, with 12 mg of coated subtilisin A (co-lyophilized in phosphate buffer 0.1 M pH 7.2 with Brij®56 and *n*-octyl β-D-glycopyranoside 4 : 1 : 1 w/w/w) and 1.5 equiv of acyl donor. [b] Determined by GC after derivatization. [c] Determined by HPLC. [d] Calculated according to $C = ee_{amine}/(ee_{amine} + ee_{amide})$. [e] Enantioselectivity factors were calculated according to $E = \ln[(1 - C)(1 - ee_{amine})]/\ln[(1 - C)(1 + ee_{amine})]$ unless otherwise stated. [f] Reaction was performed at 35 °C for 44 h. [g] Determined by GC using an internal reference.

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Table 2 Screening other serine proteases for the kinetic resolution of **1a** using *N*-octanoyl glycine ethyl ester as an acyl donor[a]

Entry	Surfactant treated enzyme	**1a** ee (%)[e]	**2a** ee (%)[f]	*C* (%)	*E*
1	Alkaline protease (4 : 1 : 1)	74.1	93	44.3	61
2	Savinase (4 : 1 : 1)	46	95.5	32.5	60
3	Alkaline protease (pH 9)[b]	61	93	39.6	52
4	Alkaline protease[c]	72.1	92	43.0	50
5	Alkaline protease[d]	60	90	40.0	32

[a] Standard procedure given in Table 1, except that *n*-octyl β-D-glycopyranoside was replaced by *n*-octyl α,β-D-glycopyranoside (reaction time: 24 h).[18] [b] Co-lyophilized in phosphate buffer at pH 9. [c] Reaction was performed at 35 °C. [d] Reaction was performed at 45 °C. [e] Determined by GC after derivatization. [f] Determined by HPLC.

Table 3 Influence of the nature of the leaving group, concentration and temperature on the KR of **1a** with $C_7H_{15}CONHCH_2CO_2R$ donors

Entry	R	[**1a**]/M	Time/h	**1a** ee (%)[e]	**2a** ee (%)[f]	*C* (%)	*E*
1	H[a]	0.5	24	0	nd	0	—
2	Et[a]	0.5	24	74.1	93	44.3	61
3	CH_2CONH_2[a]	0.16	24	49	94	34.2	55
4	CH_2CF_3[b]	0.1	3	96	90	51.6	75
5	CH_2CF_3[b]	0.5	0.5	92.3	89	50.7	57
6	CH_2CF_3[b]	1	0.3	>99.5	80	55.5	52
7	CH_2CN[b]	1	0.3	>99.5	79.5	55.3	50
8	CH_2CF_3[c]	1	5	97.5	87.3	52.7	65
9	CH_2CF_3[d]	1	1	>99.5	83	54.5	65

[a] Standard procedure given in Table 2. [b] 1 equiv of acyl donor. [c] Reaction was performed at 2 °C. [d] Reaction was performed at 10 °C. [e] Determined by GC after derivatization. [f] Determined by HPLC.

Since the reaction rate is determined mainly by the specificity of the enzyme toward the acyl donor, the search for acyl donors better recognized at the S_1 site of the enzyme channel[11,12] led us to test a series of glycine derivatives.[13] Although the selection of glycine esters might not be considered as the best choice according to the enzyme order of preference,[14] the latter were selected because they did not contain any stereogenic center.[15] The best conversion and the best enantioselectivity factor ($E = 39$–40) were registered with *N*-acyl glycine ethyl esters derived from long chain linear carboxylic acids (entries 6, 7). This might be related to subtilisin specificity toward substrates having hydrophobic residue at the P_4 site.[16]

We then screened the efficiency of *N*-octanoyl ethyl glycinate as an acyl donor with different proteases. The best results are summarized in Table 2.[17]

The best results, regarding both the selectivity and the rate of conversion, were obtained with alkaline protease, co-lyophilized with Brij®56 and *n*-octyl α,β-D-glycopyranoside in phosphate buffer at pH 7.2. When the enzyme was lyophilized at pH 9, both the conversion, and the enantioselectivity were lowered (entry 3). Increasing the temperature up to 45 °C (entries 4–5/1) led to even worse results. It can be noted that additives like KCl,[19] and water[13,20] did not improve the KR. Even though the enantioselectivity factor was acceptable, the time needed to reach 50% conversion was far superior to the upper limit fixed in our specifications.

Alkaline protease was selected to further optimize the acyl donor. The data are summarized in Table 3 (for the sake of clarity, the results obtained with glycine ethyl ester are repeated in that table in entry 2).

Attempts to acylate amine (**1a**) directly with *N*-protected glycine failed (entry 1). The *E* factor for the carbamoylmethyl ester[13] was slightly lower than that of the ethyl ester and little acceleration was registered (entry 3/2). Owing to its low solubility, this donor could not be used at higher concentration. Strong acceleration was expected with trifluoroethyl ester.[4a,21] Indeed, with this acyl donor the reaction time dropped down and ranged between 20 min to 3 h depending on the concentration (entries 4–6). Although the reaction was slower at 0.1 M concentration, under these conditions the enantioselectivity factor reached the highest value ($E = 75$, entry 4). Thereby, one can alter the concentration depending on whether *E* or the reaction time needs to be optimized. It can be noted that lowering the temperature could also be used to improve *E* without slowing down the reaction too much (entries 8–9/6).

Since the results obtained with the more toxic cyanomethyl ester were very close to those obtained for the trifluoroethyl ester under similar conditions (entry 7/6),[22] the latter was selected to extend this KR protocol to a series of non-benzylic and benzylic amines, including some biologically active compounds (**1b–m**, Fig. 1). The results obtained at 2 mmol scale are reported in Table 4.

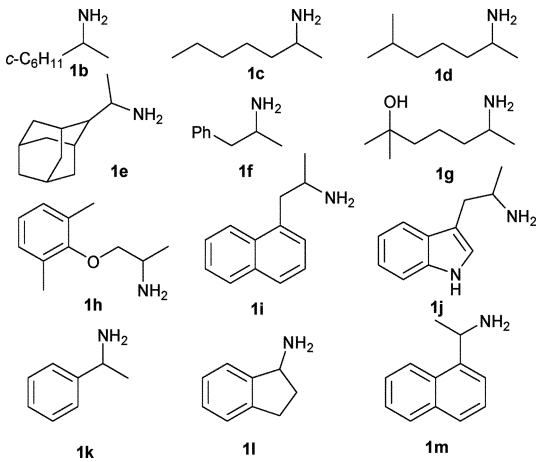

Fig. 1 Amine structures.

As previously mentioned, enantioselectivity increased at the expense of reaction time upon decreasing the concentration from 1 M to 0.1 M (entries 1, 3, 4, 7, 11). Amines structurally close to **1a**, *i.e.*, **1c**, **1d**, **1g**, led to enantioselectivity factors ranging from 32 to 53 when carrying the KR on 1 M solutions; they ranged from 57–92 when using 0.1 M solutions (entries 1, 3, 4, 7). The highest enantioselectivity factors ranging between 185 and 244 were observed when the difference in the steric bulk of the two substituents at the stereogenic center was at a maximum, *i.e.*, for amines **1e**, **1h**, **1i**, and **1m** (entries 5, 8, 9, 13). The lowest discrimination between the two enantiomers was registered for amines **1k** and **1f** (entries 11 and 6). *E* fell to the range of 19–26 for amines **1b**, **1j**, and **1l**. In most cases, the selectivity was similar or far superior to that reported in the literature for the same amines and the rate of conversion was strongly accelerated.[4]

Table 4 Alkaline protease catalyzed kinetic resolution of amines **1a–m**

			(*R*)-Amine		(*S*)-Amide			
Entry	Amine	Time/min	ee (%)[c]	Yield (%)[d]	ee (%)[e]	Yield (%)	*C* (%)	*E*
1	**1a**	40	>99.5	43	80.5	51	55.5	53
	1a[b]	180	96	48	90	47	51.6	75
2	**1b**	180	90	44	73	55	55.2	19
3	**1c**	20	>99.5	40	78	54	56.1	40
	1c[b]	330	96	44	92	50	51.1	92
4	**1d**	15	99	38	74	55	58.0	32
	1d[b]	240	>99.5	44	88	52	53.2	88
5	**1e**	360	90	52	99	47	48.0	244
6	**1f**	240	91.5	37	60	52	60.4	12
7	**1g**	60	>99.5	41	78	52	56.7	40
	1g[b]	240	96	42	90	51	51.6	75
8	**1h**	180	96	48	96.2	49	49.9	214
9	**1i**	300	98[f]	43	96	52	50.5	229
10	**1j**	360	90[f]	44	79	51	53.2	26
11	**1k**	60	95	nd	18	nd	84.0	4
	1k[b]	180	68	45	66	50	50.7	10
12	**1l**	15	>99.5	41	68	48	59.0	25
13	**1m**	90	95	45	96	43	49.7	185

[a] All reactions were performed on a 1 M solution (unless otherwise stated) of amine (2 mmol) in 3-Me-3-pentanol at room temperature with 50 mg of surfactant treated alkaline protease (co-lyophilized in phosphate buffer 0.1 M pH 7.2 with Brij® 56 and α,β-D-glycopyranoside 4 : 1 : 1 w/w/w) and 1 equiv of acyl donor. [b] 0.1 M concentration. [c] Determined by GC after derivatization. [d] Isolated as BOC-derivatives. [e] Determined by HPLC. [f] Determined by HPLC after derivatization.

Conclusion

Alkaline protease-catalyzed KR of chiral amines, in which the stereogenic center is directly adjacent to the amine moiety, with *N*-octanoyl glycine trifluoroethyl ester as the acyl donor led to a good compromise regarding selectivity and reactivity. The enantioselectivity could reach extremely high values (>200) whereas the rate of the reaction could be as short as 20 min, and never exceeded 6 h. These results open perspectives regarding the possibility to achieve (*S*)-selective DKR by associating *in situ* thiyl radical-mediated racemization to this KR protocol. The screening of other enzymes and DKR experiments are in progress; they will be reported in due course.

Experimental

General experimental methods

See Electronic Supplementary Information (ESI).†

Immobilization of alkaline protease

Alkaline protease (120 mg) from Valley Research was dissolved in a solution of octyl α,β-D-glycopyranoside (15 mg) and Brij 56 (polyethylene glycol hexadecyl ether, 15 mg) in a phosphate buffer (pH 7.2 (unless otherwise stated), 6 mL) and the mixture was rapidly frozen in liquid N_2 and lyophilized for 12 hours.

General procedure for the kinetic resolution of amines

To a solution of 570 mg (2 mmol) *N*-octanoylglycine trifluoroethyl ester in 2 mL of 3-methyl-3-pentanol was added 50 mg of coated alkaline protease[23] and 298 mg (2 mmol) of phenylbutyl amine (**1a**). The resulting mixture was stirred at room temperature for 40 minutes. The amine ee was determined by GC after derivatization of an aliquot of the crude mixture in trifluoroacetamide with 1.5 equiv of *N*-methyl-bis-trifluoroacetamide. The enzyme was filtered off from the solution and washed with 10 mL of dichloromethane. Then 240 mg (1.1 mmol) of Boc_2O was added to the filtrate, and the mixture was stirred until complete consumption of amine monitored by TLC. The solvent was then evaporated and the crude mixture was purified on gel-silica (pentane–diethyl ether gradient 0 to 10% to afford the Boc-amine, then DCM–MeOH 98 : 2 to afford the amide).

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