Formation and hydrolysis of amide bonds by lipase A from *Candida antarctica*; exceptional features†

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Various commercial lyophilized and immobilized preparations of lipase A from *Candida antarctica* (CAL-A) were studied for their ability to catalyze the hydrolysis of amide bonds in *N*-acylated α-amino acids, 3-butanamidobutanoic acid (β-amino acid) and its ethyl ester. The activity toward amide bonds is highly untypical of lipases, despite the close mechanistic analogy to amidases which normally catalyze the corresponding reactions. Most CAL-A preparations cleaved amide bonds of various substrates with high enantioselectivity, although high variations in substrate selectivity and catalytic rates were detected. The possible role of contaminant protein species on the hydrolytic activity toward these bonds was studied by fractionation and analysis of the commercial lyophilized preparation of CAL-A (Cat#ICR-112, Codexis). In addition to minor impurities, two equally abundant proteins were detected, migrating on SDS-PAGE a few kDa apart around the calculated size of CAL-A. Based on peptide fragment analysis and sequence comparison both bands shared substantial sequence coverage with CAL-A. However, peptides at the C-terminal end constituting a motile domain described as an active-site flap were not identified in the smaller fragment. Separated gel filtration fractions of the two forms of CAL-A both catalyzed the amide bond hydrolysis of ethyl 3-butanamidobutanoate as well as the *N*-acylation of methyl pipecolinate. Hydrolytic activity towards *N*-acetylmethionine was, however, solely confined to the fractions containing the truncated form of CAL-A. These fractions were also found to contain a trace enzyme impurity identified in sequence analysis as a serine carboxypeptidase. The possible role of catalytic impurities *versus* the function of CAL-A in amide bond hydrolysis is further discussed in the paper.

Introduction

The physiological role of secreted lipases, such as lipase A from the yeast *Candida antarctica* (CAL-A), is to decompose triacylglycerols into free fatty acids and glycerol. Typically the lipase-catalyzed hydrolytic reactions of the carboxyester bond are highly regioselective and enantioselective with a substrate preference for long-chain acylglycerols (>10 carbons). As a group, lipases are known to be diverse catalysts which can hydrolyze a broad range of different fatty acid esters and, in reverse, catalyze esterification between carboxylic acids and alcohols. These features, combined with characteristic stability in demanding conditions, such as in organic solvents, render lipases useful for numerous applications in organic synthesis and industry.

The active site topology and hydrolytic mechanism of lipases is common to the members of the serine hydrolase family. The hydrolytic reactions of both ester and amide bonds proceed *via* a well-characterized double displacement mechanism involving a tetrahedral intermediate between the substrate and a serine hydroxyl at the enzyme active site. This is coupled to the formation of a covalent acyl-enzyme complex (acylation phase), which results in the release of the first product, the nucleophilic part of the acyl donor. In the subsequent step, the ester intermediate is hydrolyzed (deacylation phase) generating the second product, free carboxylic acid. Interestingly, although many amidases are known to readily hydrolyze both esters and amides, lipases generally do not cleave amide bonds. This may be due to the higher activation energy required for the formation of the enzyme-bound tetrahedral transition state of amides in comparison to the more polar ester substrates. Thus, contrary to in amides, in esters the vicinity of the relatively electronegative oxygen restricts electron delocalization over the adjacent bonds, resulting in the reduction of resonance stabilization at the ground state. A structural explanation for the ester *versus* amide specificity may be the efficiency of the protonation of the leaving group, which has been demonstrated to influence the enzymatic cleavage of unnatural amide substrates.[1] In addition, it has been shown that the presence of the transition-state stabilizing hydrogen-bonded water network reaching to the amide hydrogen is decisive for the amide hydrolyzing activity of esterase from *Bacillus subtilis*.[2]

Despite the relative stability of amides, some lipases and esterases have been reported to be capable of hydrolyzing amide bonds. Electrophilic substituents facilitating polarization of electrons (*e.g.* halogens) and structural features which prevent resonance stabilization can activate amide substrates towards

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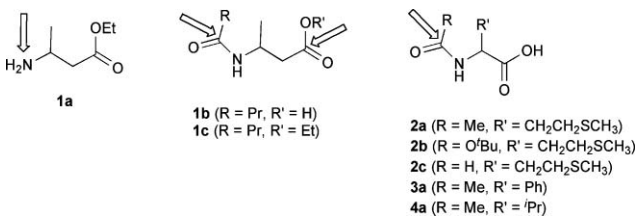
hydrolytic cleavage by lipases. Thus, many lipases cleave the strained four-membered β-lactam ring by water or alcohol[3-6] and lipase from *Candida rugosa* acts on *N*-trifluoroethyl 2-chloropropanamide.[7] Lipases from *Rhizomucor miehei* and *Pseudomonas stutzeri* have recently been reported to hydrolyze *N*-acetyl-DL-alanine, a natural amino acid amide, with low enantioselectivity.[8] In addition, a lipase from *Pseudomonas aeruginosa* has been shown to hydrolyze the amide bond of oleoyl 2-naphthylamide, an activity which has further been enhanced by directed evolution.[9] Perhaps the most extensively studied member of the esterase family with reported amide activity is the lipase B from *Candida antarctica* (CAL-B) which has been shown to hydrolyze substrates like *trans*-8-methyl-*N*-vanillyl-6-nonenamide, *N*-(1-(4-chlorophenyl)ethyl)acetamide and *N*-methylacetamide.[10-12] It is worth pointing out that many of the experiments have been carried out using semi-pure cell extracts or bulk-purified commercial enzyme preparations which may be expected to contain enzyme impurities. For instance, the amide bond hydrolytic activity observed for the lipase extract from porcine pancreas appeared to result from a contaminant enzyme in the preparation.[13] Thus, the possible contribution of trace protein activities should be considered and analyzed whenever unexpected activities are encountered.

As the starting point for this work, the lipase CAL-A was expected to be a potential candidate for hydrolyzing amides, as it has been previously shown to catalyze *N*-acylation of various amine precursors.[14-16] CAL-A is a unique member of the superfamily of ester hydrolases, and it has been extensively characterized for catalytic and physical features in parallel to recent studies on crystal structure.[17,18] CAL-A is a secreted enzyme composed of 431 amino acids in the mature form. In the primary translation product the sequence is preceded by an N-terminal signal peptide of 21 amino acids and a propeptide of 10 amino acids.[19] In primary sequence comparison CAL-A shares substantial amino acid similarity with a wide array of secreted lipases and esterases. However, related lipase homolog crystal structures have not been resolved, and the most closely related folds found in a DALI search are annotated as peptidases. The enzymes with the highest sequence identity in a BLAST search are lipase from *Kurtzmanomyces sp. I-11* (77%), protein from *Ustilago maydis* 521 with a conserved esterase/lipase domain (68%) and secreted lipase 1 precursor from *Malassezia furfur* (35%). There is no apparent amino acid sequence homology between the *Candida antarctica* lipases CAL-A and CAL-B (sequence identity 7.8% in EBI EMBOSS alignment). Thus, they are not directly related despite their similar nomenclature, function and the common origin.

As expected by the comparisons, the structure of CAL-A encloses the central structural features characteristic for the mechanistically related superfamilies of hydrolases, involving the α/β hydrolase fold and a catalytic triad of Ser, Asp and His arranged in the order typical to lipases.[18] The topology of the active site pocket of CAL-A appears to be well suited to accommodate long chain fatty acids and, in agreement with the putative function, the enzyme has been reported to have catalytic preference for long-chained carboxylic acids over smaller acyl groups.[20] Importantly, the structure also reveals a distinct lid domain characteristic of lipases, but without apparent sequence homology to previously known lid structures.[18] Together with the reported catalytic activation at the lipid–water interface (interfacial activation),[21] a related classical property of lipases associated with the hydrolysis of fatty acid glycerol esters, the 3D fold assigns CAL-A as the first representative of a novel subclass of lipases.

The interest towards CAL-A has largely evolved from its exceptional tolerance to heat, pH and organic solvents, which together with exceptionally broad substrate specificity and stereoselectivity make it a versatile biocatalyst for organic synthesis and biotechnological applications.[15,22] CAL-A has been shown to exhibit many unique catalytic properties, such as substrate preference for *trans*-fatty acids and the ability to catalyze highly enantioselective acylation reactions of bulky sterically hindered secondary and tertiary alcohols which are not usually accepted as substrates by lipases.[23-29] Of specific interest to this study, CAL-A preparations have also been shown to catalyze secondary *N*-acylation of α-amino esters,[30-32] and a range of other α- and β-amino esters in organic solvents.[14-16] All these features make CAL-A a potential candidate for protein engineering.[33] CAL-A has been heterologously expressed in *Aspergillus oryzae*,[34] *Pichia pastoris*[35] and *Escherichia coli*[36] and at present is commercially available from various companies as lyophilized preparations, cross-linked enzyme aggregates (CLEA preparation) as well as in immobilized form on different solid supports.

We have herein studied the behavior of CAL-A with amide substrates in detail (Scheme 1). New marketed CAL-A preparations were first screened for *N*-acylation of **1a**, followed by the analysis of selected candidates for the studies on amide bond hydrolysis of various racemic α- and β-amino acid derivatives **1b–c**, **2a–c**, **3a** and **4a**. A parallel objective was to investigate the possible effect of contaminating enzymes, because some commercial CAL-A preparations are known to contain enzyme impurities.[37] Based on detected activities of interest, the commercial CAL-A preparation Cat#ICR-112 was selected for fractionation, followed by comparison of the resolved catalytic activities and the different protein species found.

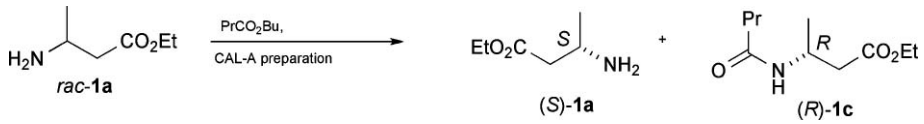


Scheme 1 The model compounds for the CAL-A reactions.

Results and discussion

β-Amino acid derivatives as substrates for CAL-A

As the first experiment, commercial CAL-A preparations were compared for their ability to catalyze the *N*-acylation of ethyl 3-aminobutanoate *rac*-**1a** in neat butyl butanoate, a reaction earlier reported for CAL-A (Scheme 2, Table 1).[38] All the enzyme preparations were in immobilized form as provided by the manufacturers or, in the case of the lyophilized Chirazyme L5 and Cat#ICR-112, immobilized on Celite as described earlier (entries 1 and 2).[39] The preparations clearly catalyzed the reaction, enantioselectivities (*E* around 40) being similar to those previously observed for



Scheme 2 CAL-A-catalyzed *N*-acylation of ethyl 3-aminobutanoate *rac*-**1a**.

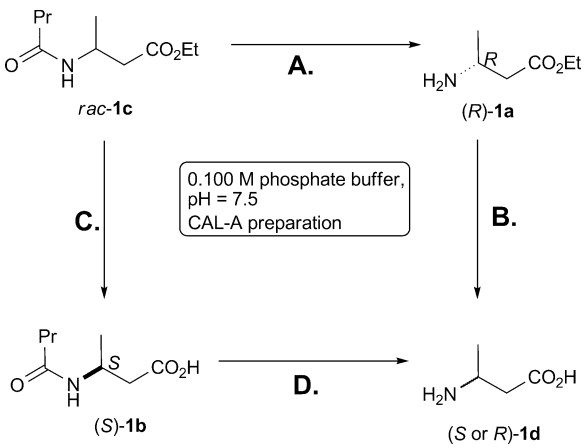
Table 1 *N*-Acylation of *rac*-**1a** (0.100 M) in butyl butanoate (1 cm^3) with CAL-A preparations (40 mg cm^{-3}) at 3 h. The CAL-A preparations tested later for hydrolysis of α- and β- amino acid derivatives are shown bold (entries 1–5)

Entry	CAL-A preparation	Conv. [%]	ee_s [%]	ee_p [%]	*E*
1	**Chirazyme L5 on Celite**[a,b]	51	88	83	32 ±1
2	**Cat#ICR-112 on Celite**[b]	48	82	88	39 ±1
3	**CAL-A-CLEA**	33	45	92	34 ±2
4	**Cat#NZL-101-IMB**	55	98	80	42 ±3
5	**Immozyme CAL-A-T2-150**	46	76	88	39 ±1
6	VZ1030-2	9	9	95	48 ±2
7	VZ1030-3	20	24	95	51 ±1
8	VZ1030-4	48	82	91	50 ±1
9	VZ1030-10	25	31	94	41 ±1
10	VZ1030-11	47	78	89	41 ±1
11	VZ1030-12	52	94	85	41 ±1
12	VZ1031-2	37	54	93	46 ±1
13	VZ1031-3	53	95	85	46 ±1
14	VZ1031-5	37	55	93	46 ±1

[a] Ref. 38, $t = 24$ h. [b] Commercial lyophilized CAL-A (10%) on Celite (78%) and sucrose (12%).

CAL-A on Celite (*cf.* Table 1; entry 1).[38] The immobilized CAL-As of ViaKit gave the highest *E* values of around 50 (entries 6–8 and 12–14).

Five of the CAL-A preparations analyzed above (Chirazyme L5, Cat#ICR-112, CAL-A-CLEA, Cat#NZL-101-IMB and CAL-A-T2-150) were selected for the trial to test the amide bond hydrolysis of *rac*-**1c**, the resolution product of Scheme 2 (Scheme 3). In contrast to the previous reaction series, the lyophilized preparations Chirazyme L5 and Cat#ICR-112 were analyzed without immobilization on Celite, as the enzymes may be detached from the solid support in aqueous reaction conditions. All the preparations yielded three different conversion products (*S*)-**1b**, (*R*)-**1a** and



Scheme 3 CAL-A-catalyzed sequential resolution of *rac*-**1c**.

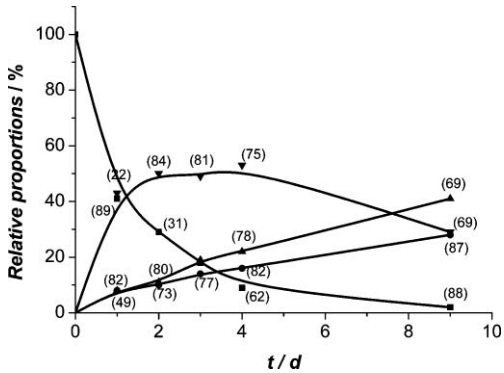


Fig. 1 Cat#NZL-101-IMB-catalyzed (100 mg cm^{-3}) hydrolysis of *rac*-**1c** (■, 0.050 M) and formation of the products (*S*)-**1b** (●), (*R*)-**1a** (▼) and (*R*)-**1d** (▲). Ee-values have been marked in parentheses.

(*S* or *R*)-**1d** (Scheme 3; Table 2; Fig. 1). Although the reactions proceeded slowly, this confirmed that both the amide and the ester groups of the substrate were hydrolyzed. The observed amide hydrolysis was highly enantioselective with the preparations Cat#ICR-112, Chirazyme L5 and CAL-A-CLEA, ee values being >95 for (*R*)-**1a** (Table 2; entries 1–3). With Cat#NZL-101-IMB and Immozyme CAL-A-T2-150 the corresponding reaction took place with only moderate enantioselectivity (entries 4 and 5). Configuration of the formed product **1d** was either *R* (Cat#NZL-101-IMB and CAL-A-CLEA) or *S* (CAL-A-T2-150, Cat#ICR-112 and Chirazyme L5) depending on the relative proportions and hydrolytic rates of (*S*)-**1b** and (*R*)-**1a** in each case.

α-Amino acid derivatives as substrates for CAL-A

In the subsequent reaction series the same five CAL-A preparations shown in Table 2 were analyzed for their ability to hydrolyze the amide bonds of N-protected methionine *rac*-**2a–c**, *N*-acetylphenylglycine *rac*-**3a** and *N*-acetylvaline *rac*-**4a** (Scheme 4, Table 3). Both lyophilized CAL-A preparations (Chirazyme L5 and Cat#ICR-112) were found to catalyze the hydrolysis of the amide substrates **2a**, **2c**, **3a** and **4a** in phosphate buffer (0.100 M, pH 7.5) (Table 3; entries 1–2). The reactions were highly enantioselective with *E* values of over 200, in contrast to the corresponding *N*-acylation reactions of α-amino esters earlier reported to proceed with low or moderate enantioselectivity.[31] The highest conversions were observed with *N*-acetylmethionine **2a**, whereas the hydrolysis of **3a** with a sterically more constrained α-phenyl substituent was less efficient. The only compound that was not accepted as a substrate by the lyophilized CAL-A preparations was *N*-Boc-methionine **2b** with the bulky *tert*-butoxycarbonyl substituent.

Immozyme CALA-T2-150 was also found to have activity toward some of the substrates tested; it slowly catalyzed the amide bond hydrolysis of **2a**, **2c** and **4a** with high enantioselectivity

Table 2 CAL-A-catalyzed hydrolysis of β-amino acid derivative *rac*-**1c** (0.050 M) in phosphate buffer (0.100 M, pH 7.5) at room temperature after 24 h

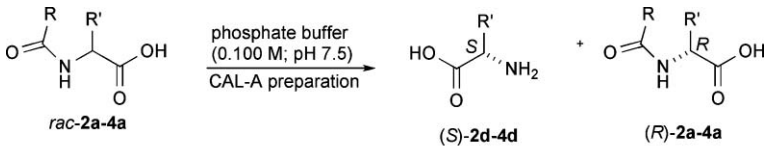
Entry	CAL-A preparation	ee_{1c} (%)/Yield [%]	$ee_{(R)\text{-}1a}$ (%)/Yield [%]	$ee_{(S)\text{-}1b}$ (%)/Yield [%]	ee_{1d} (%)/Yield [%]/Abs. config.
1	Chirazyme L5, lyo.[a]	53/35	98/44	71/18	-/3/*S*
2	Cat#ICR-112, lyo.[a]	77/33	>95/17	64/47	-/3/*S*
3	CAL-A-CLEA[c]	10/4	>95/22	29/73	-/1/*R*
4	Cat#NZL-101-IMB[b]	22/41	89/43	49/8	82/8/*R*
5	Immozyme CAL-A-T2-150[c]	4/39	50/19	67/37	8/5/*S*

[a] 5 mg cm^{-3}. [b] 100 mg cm^{-3}. [c] 75 mg cm^{-3}.

Table 3 Enzyme-catalyzed (5 mg cm^{-3}) hydrolysis of α-amino acid derivatives **2a–c**, **3a** and **4a** (0.050 M) in phosphate buffer (pH 7.5; 0.100 M) at room temperature (t = 24 h, except for **2c** 2 h)

Entry	CAL-A preparation	**2a** Conv. [%]/*E*	**2b** Conv. [%]/*E*	**2c** Conv. [%]/*E*	**3a** Conv. [%]/*E*	**4a** Conv. [%]/*E*
1	Chirazyme L5, lyo.	49/>200	0/-	17/>200	6/>200	37/>200
2	Cat#ICR-112, lyo.	50/>200	0/-	31/>200	2/>200	42/>200
3	CAL-A-CLEA[a]	0/-	0/-	0/-	0/-	0/-
4	Cat#NZL-101-IMB[b]	0/-	0/-	0/-	<1/>200[c]	0/-
5	Immozyme CAL-A-T2-150	14/>200	0/-	1/>200[c]	0/-	1/>200[c]

[a] 20 mg cm^{-3}. [b] 75 mg cm^{-3}. [c] determined by taking samples after several days at later stage of the reaction.



2a and **d**: R' = $CH_2CH_2SCH_3$, R = CH_3
2b and **d**: R' = $CH_2CH_2SCH_3$, R = O^{t}Bu
2c and **d**: R' = $CH_2CH_2SCH_3$, R = H
3a and **d**: R' = Ph, R = CH_3
4a and **d**: R' = ^{i}Pr, R = CH_3

Scheme 4 CAL-A-catalyzed hydrolysis of *N*-acyl-α-amino acids.

(E > 200), just as observed for the two lyophilized preparations (Table 3; entry 5).

The remaining immobilized preparation (Cat#NZL-101-IMB) and the cross-linked derivative (CAL-A-CLEA) were either inactive or had only very low activity towards the substrates tested (Table 3; entries 3–4). Similar results were obtained in an additional reaction series, in which the 18 immobilized CAL-As of ViaKit (not included in Table 3) gave only traces of the hydrolyzed product (*S*)-**2d** (c < 3% in 24 h) from *rac*-**2a**.

Fractionation and analysis of the commercial CAL-A preparation (Cat#ICR-112, Codexis)

Overview. Several CAL-A preparations in this study were found to have unexpected amidase activity. Of the enzymes which generally catalyze the hydrolysis of non-peptide carbon–nitrogen bonds of linear amides (E.C. 3.5.1), hydrolysis of **2a–4a** corresponds to that of acylase I-type enzymes (*N*-acyl-L-amino-acid amidohydrolases, E.C. 3.5.1.14) which by definition are specific for different *N*-acyl-L-amino acids.[40] Members of this functional class of amidohydrolases have been widely used as biocatalysts in the preparation of enantiopure α-amino acids,[41] which makes the activity discovered for the CAL-A preparations of distinct scientific interest.

Bulk-purified commercial enzymes such as the available CAL-A preparations are not primarily prepared for analytical or research purposes, and may contain catalytically active enzyme impurities which originate from the expression host strain.[34,37] To analyze the possible role of impurities in relation to the observed activities, one of our target CAL-A preparations (lyophilized Cat#ICR-112, Codexis) was selected as a representative for a detailed fractionation study. The other CAL-A preparations, except Chirazyme L5, were covalently immobilized and thus not suitable for the purpose. As for the activities of interest, Cat#ICR-112 catalyzed the amide bond hydrolysis of various α- and β-amino acid derivatives (Tables 2 and 3), and the corresponding immobilized derivative catalyzed the *N*-acylation of β-amino acid derivative ethyl 3-aminobutanoate *rac*-**1a** in butyl butanoate (Table 1; entry 2). To investigate whether the activities could be separated from one another, and whether they overlap with the CAL-A elution fraction instead of any possible impurity fractions, the preparation was subjected to preparative size exclusion chromatography, SDS-PAGE analysis with sequence-based identification and activity characterization.

SDS-PAGE and LC-MS/MS characterization

SDS-PAGE analysis of the CAL-A preparation Cat#ICR-112 revealed multiple protein bands of varying intensities ranging from less than 30 kDa to above 60 kDa in size (Fig. 2). Five distinct bands of potential interest migrating between ~30–55 kDa were selected as targets for in-gel digestion and LC-MS/MS analysis

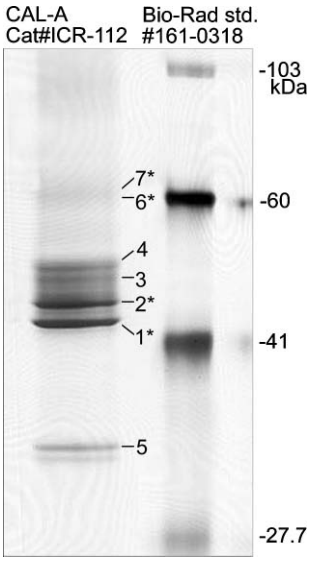


Fig. 2 SDS-PAGE analysis (Bio-Rad Criterion 10% Bis-Tris/silver stained) of the commercial CAL-A preparation (Cat#ICR-112 from Codexis) against Bio-Rad standard #161-0318. The bands submitted for in-gel digestion, LC-MS/MS analysis and sequence comparison are labeled 1–7. The best sequence hits: bands 1–2) CAL-A; bands 3–4) α-amylase (*Aspergillus shirousami*); band 5) CAL-A N-terminal half; band 6) maltase glucoamylase (*Aspergillus*); band 7) maltase glucoamylase/serine carboxypeptidase (*Aspergillus*). Bands marked with * were only or additionally analyzed from fractionated samples.

and compared against the CAL-A sequence[34] and Sprot Trembl database sequences for possible identification.

The two most intense bands on SDS-PAGE (bands 1–2 in Fig. 2) migrated a few kDa apart from one another, near the calculated molecular weight of the mature CAL-A (46129 Da). In fragment LC-MS/MS analysis and MASCOT sequence comparison both these bands shared over 40% coverage (with >10 distinct identified peptides for each) with the CAL-A sequence. To avoid cross-contamination between the bands, Cat#ICR-112 was subsequently fractionated using preparative gel filtration and re-analyzed (see the next section).

The smallest analyzed band (band 5 in Fig. 2), migrating above 30 kDa on SDS-PAGE gel, was identified as a degradation product of CAL-A. The sequence coverage was 22% with 6 distinct identified peptides exclusively at the N-terminal end of the target protein sequence.

The two analyzed fragments migrating around 55 kDa (bands 3–4 in Fig. 2) were clearly larger in size than that expected for CAL-A. In sequence-based identification these bands matched with a good score value and sequence coverage with α-amylase (best hit 54.8 kDa from *Aspergillus shirousami*). Although some peptides of lipases were identified in these two samples probably due to sample smearing during electrophoresis, apparently these proteins are not related to CAL-A, but they are contaminants originating from the expression host organism.

Gel filtration fractionation and further analysis

Cat#ICR-112 was fractionated using preparative gel filtration size-exclusion chromatography to study the distribution of different proteins in more detail. Consistent with the heterogeneity visualized in SDS-PAGE, the runs resulted in multiple eluting peaks; two distinct, partially overlapping main peaks (one with a clear shoulder) together with a few minor peaks (Fig. 3). Despite attempts to optimize the run conditions the resolution remained incomplete.

SDS-PAGE analysis of the gel filtration fractions (Fig. 4) showed that the two proteins preliminarily identified as

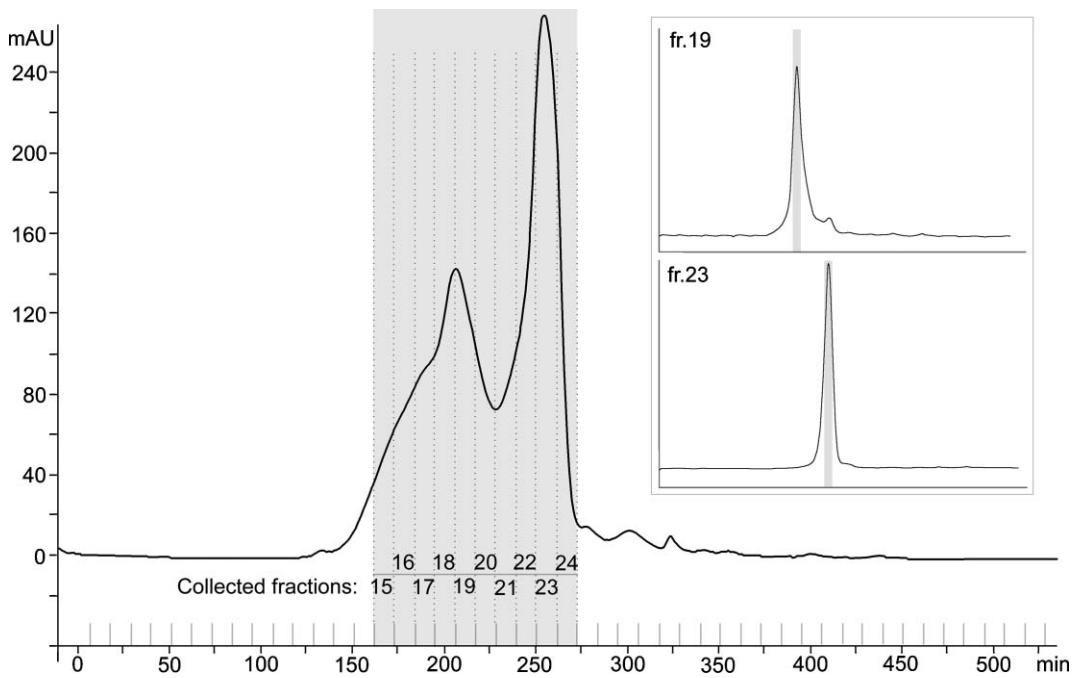


Fig. 3 UV–vis chromatogram of FPLC gel filtration fractionation of the CAL-A Cat#ICR-112. The fractions collected for analysis are highlighted. The smaller chromatograms show the peak fractions 19 and 23 after second round of gel filtration.

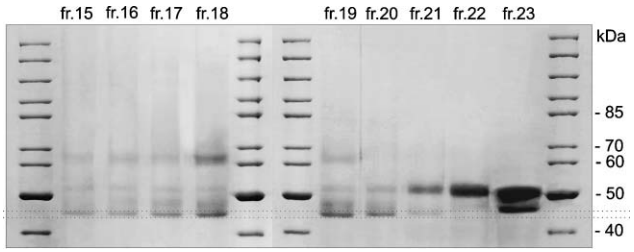


Fig. 4 SDS-PAGE analysis of concentrated gel filtration fractions 15–23 of CAL-A Cat#ICR-112 against Fermentas Pageruler standard #SM0661 (self-casted 8% gel/Coomassie Brilliant Blue stained). The two dotted horizontal lines between 40 kDa and 50 kDa show the migration fronts of the two CAL-A proteins. The collected fraction 24 is missing from the image.

CAL-A eluted throughout the two main peaks. The smaller protein (band 1 in Fig. 2) was distributed over the first main peak in consecutive fractions 15–21 (Fig. 3), whereas the larger protein (band 2 in Fig. 2) was mainly observed in the second main peak fractions 23–24. In repeated rounds of preparative gel filtration the two proteins were sufficiently separated from one another (Fig. 3), digested and characterized by LC-MS/MS as before. The total sequence coverage of the larger band was 47% in relation to the mature CAL-A sequence (the number of different identified peptides 12), and peptides corresponding to both the C-terminal end and N-terminal end were identified. The sequence coverage of the smaller band was 35% (the number of different identified peptides 10) and notably without any identified peptides spanning the C-terminal region of the target sequence. This suggests that the smaller band (>45 kDa) may be a degradation product of CAL-A which lacks a fragment of up to several kDa from the C-terminus. The number of the missing amino acids could not, however, be determined by protein mass spectrometry due to remaining protein contaminants in the sample. Inspection of the CAL-A crystal structure[18] reveals that the C-terminal residues 425–435 form an antiparallel β-sheet secondary structure loop (B10/B11) which resides on the surface of the protein, near the active site cavity. The loop functions as an "active-site flap" which restricts access to the active site in the inactive form, and it is turned away upon activation to allow substrate binding. To exclude any possible effect of non-uniform post-translational glycosylation on the separation of the two CAL-A bands, the preparation was also subjected to enzymatic *N*-glycosidase treatment. This, however, had no apparent effect on the SDS-PAGE migration profile of the bands, further supporting protein cleavage as the primary cause for the observed size difference. The native N-terminal signal peptide (21aa) or the propeptide (10aa) specific to the immature form of CAL-A[34] were not recognized in any of the runs for the protein fragment characterization.

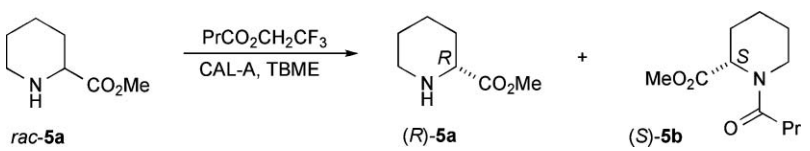
The two CAL-A species eluted in rather broad fronts in gel filtration and were no longer the most abundant proteins in the apex peak fractions. In fraction 23 the most intense band (~55 kDa) appeared to be composed of the putative α-amylase contaminants identified earlier in protein fragment characterization. In fraction 18, the concentration procedure revealed two new bands around 60 kDa, which were only barely visible and ignored in earlier SDS-PAGE runs (bands 6–7 in Fig. 2). In the subsequent protein fragment LC-MS/MS analysis and comparison against the Sprot Trembl database, the bands were best matched with *Aspergillus* maltase glucoamylase (sequence coverage 25% and number of distinct peptides 24/sequence coverage 19% and number of distinct peptides 14). Some peptides in the larger band also matched with *Aspergillus* serine carboxypeptidase sequence (4th hit; sequence coverage 13% and number of distinct peptides identified 7).

Activity in the gel filtration fractions

Each gel filtration fraction was individually analyzed for three activities corresponding to those earlier measured for the different CAL-A preparations: *N*-acylation and two amide bond hydrolytic reactions. The first monitored reaction was *N*-acylation of methyl pipecolinate *rac*-**5a** in *tert*-butyl methyl ether (TBME) (Scheme 5). CAL-A has been shown to catalyze this reaction with high enantioselectivity,[30,31] whereas acylase I-type enzymes and most lipases do not generally accept the bulky **5a** as their substrate for the *N*-acylation. This is contrary to the less-specific substrate ethyl 3-aminobutanoate *rac*-**1a** (Scheme 2) which is known to be converted by many different enzymes.[42] Hence it was rejected for this screening. The second test reaction was the hydrolysis of ethyl 3-butanamidobutanoate *rac*-**1c** (Scheme 3). The activity of interest, amide hydrolysis of β-amino acid-derivatives, is not common to known amidases, although the corresponding ester hydrolysis could be expected to be catalyzed by many related enzymes. The third studied reaction was the hydrolysis of *N*-acetylmethionine *rac*-**2a**, a catalytic activity characteristic to acylase I-type enzymes and less typical to lipases (Scheme 4).

***N*-Acylation of methyl pipecolinate *rac*-5a.** This activity was found to be distributed over the entire elution front and, as seen in quantitative comparison of relative activities (Table 4; Fig. 5a), the highest values centered around the two main peaks, fractions 18–19 and 23. In comparison to the corresponding band intensities on the SDS-PAGE gel (Fig. 4), the relative activities appeared directly proportional to the quantity of CAL-A in each fraction.

Hydrolysis of ethyl 3-butanamidobutanoate *rac*-1c. The main reaction was clearly the ester hydrolysis from *rac*-**1c** to yield (*S*)-**1b** (Table 4). However, formation of the alternate product (*R*)-**1a** confirmed that also the amide bond of the β-amino acid derivative *rac*-**1c** was hydrolyzed (Table 4; Fig. 5b). The amide hydrolytic activity was observed in both main gel filtration elution peaks and centered in fractions 18–19 and 23, overlapping almost precisely



Scheme 5 CAL-A-catalyzed *N*-acylation of methyl pipecolinate *rac*-**5a**.

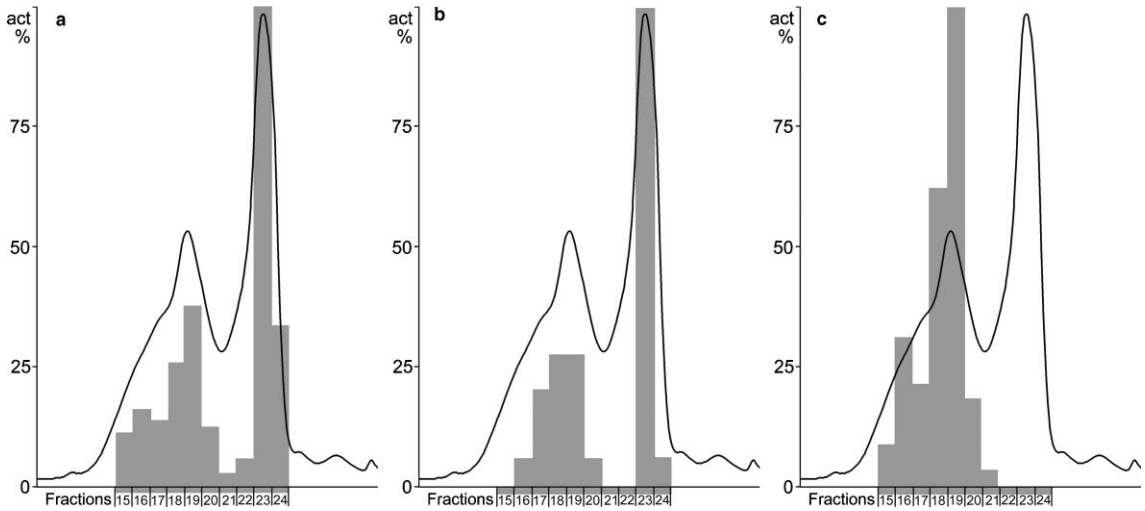


Fig. 5 Distribution of the relative activities (a) *N*-acylation of methyl pipecolinate *rac*-**5a**, (b) hydrolysis of ethyl 3-butanamidobutanoate *rac*-**1c**, and (c) hydrolysis of *N*-acetylmethionine *rac*-**2a** in the CAL-A Cat#ICR-112 gel filtration fractions. Activities shown as % in relation to the most active fraction.

Table 4 Analysis of the gel filtration peak fractions 15–24 (Fig. 3) by *N*-acylation of methyl pipecolinate *rac*-**5a** with trifluoroethyl butanoate (A), hydrolysis of ethyl 3-butanamidobutanoate *rac*-**1c** (B) and by hydrolysis of *N*-acetylmethionine *rac*-**2a** (C)

	A	B		C
Gel filtration fraction number	*c* [%]	[**1b**]/%	[**1a**]/%	*c* [%]
15	0.8	10	—	3
16	1.1	44	1	10
17	1.0	43	3	7
18	1.7	49	4	20
19	2.5	49	4	35
20	0.9	54	1	6
21	0.3	57	traces	1
22	0.5	42	traces	< 1
23	6.9	12	14	< 1
24	2.2	5	1	< 1

with the relative methyl pipecolinate *N*-acylation activity profile described above (Fig. 5a,b).

Although these two monitored activities are not characteristic to lipases, the results suggest that the two forms of CAL-A identified in the commercial preparation (bands 1–2 in Fig. 2) may both be correctly folded functional polypeptides: the lack of the C-terminal end, as expected for the smaller CAL-A fragment, does not necessarily abolish the catalytic activity of the protein.

Hydrolysis of *N*-acetylmethionine *rac*-2a; acylase I-type activity. The activity was shown to be confined to the first gel filtration elution peak, with the highest relative activities in fractions 18–19 (Table 4, Fig. 5c). Within the elution peak, the distribution of the relative hydrolytic activity and the amount of the smaller CAL-A fragment as seen in SDS-PAGE seemed to perfectly coincide (Fig. 4). The CAL-A fragment could not, however, be further isolated from the co-eluting protein impurities to directly demonstrate the function of the lipase fragment towards the substrate. One of the trace impurities present in the fraction was identified as a member of the serine carboxypeptidase family (EC 3.4.16).[43] These enzymes catalyze the cleavage of the C-terminal residues from polypeptides and participate in various reactions of intracellular protein turnover and specific protein maturation reactions. Although the protein could potentially have activity towards the provided amide substrates, the effect of this (or any other) impurity on the hydrolysis of *N*-acetylmethionine remains unclear.

Unlike observed for the *N*-acylation of methyl pipecolinate *rac*-**5a** and hydrolysis of ethyl 3-butanamidobutanoate *rac*-**1c**, hydrolysis of *N*-acetylmethionine *rac*-**2a** was not detected in the second gel filtration elution peak. This demonstrated that this source of activity is absent in fractions 22–24 and that the reaction is not catalyzed by the native form of CAL-A. In analogy to this observation, the preparations Cat#NZL-101-IMB and CAL-A-CLEA were shown to catalyze the *N*-acylation of *rac*-**1a** (Table 1), but they did not hydrolyze *N*-acetyl-α-amino acids (Table 3).

Conclusion

In this paper we demonstrate that commercial lyophilized and immobilized preparations of lipase A from *Candida antarctica* (CAL-A) have distinct hydrolytic activity toward the amide bond of various *N*-acyl-α- and β-amino acid derivatives. This property is generally uncommon to carboxyester hydrolases, especially classical lipases which naturally hydrolyze fatty acid glycerol esters. The observed reactions catalyzed by the selected CAL-A preparations (Chirazyme L5, lyo.; Cat#ICR-112, lyo.; Immozyme CALA-T2-150, Cat#NZL-101-IMB; CAL-A-CLEA) were rather slow but moderately or highly enantioselective depending on the substrate structure. Although most of the eight amide substrates were hydrolyzed, the results revealed clear differences in substrate preference between the CAL-A preparations tested.

To study the distribution of the observed activities of CAL-A, a representative of the commercial lyophilized products (Cat#ICR-112) was subjected to fractionation, sequence-based identification and functional comparison. In addition to several proteins present at low relative concentrations, SDS-PAGE and preparative gel filtration revealed two main protein species of about equal abundance. The two proteins migrated a few kDa apart, around the ~46 kDa size range expected for CAL-A. The larger protein

was identified by digestion/peptide LC-MS/MS analysis as the mature CAL-A, whereas the smaller fragment appeared to be a truncated form of the enzyme, lacking the C-terminal region. Isolated gel filtration fractions of the two forms of CAL-A were shown to have distinct catalytic properties: both peak fractions catalyzed the *N*-acylation of methyl pipecolinate *rac*-**5a** with trifluoroethyl butanoate and hydrolysis of the ester and amide bonds of ethyl 3-butanamidobutanoate *rac*-**1c**. However, only the fractions containing the truncated CAL-A catalyzed the highly enantioselective hydrolysis of *N*-acetylmethionine *rac*-**2a**. Based on the crystal structure of CAL-A, the C-terminal region constitutes a motile antiparallel β-sheet structure which may function as an "active-site flap". This structure has been proposed to affect the substrate binding properties of the enzyme in different conformational states. Accordingly, lack of the C-terminal end would leave the binding pocket more exposed, which could in turn allow the enzyme to accept substrate molecules which cannot be efficiently accommodated in the active site of the wild type enzyme.

Analysis of the commercial preparation also uncovered many minor protein contaminants originating from the expression host strain. One of these was identified as a serine carboxypeptidase which co-eluted in the main fraction together with the smaller CAL-A protein. This contaminant was present in trace quantities in the original preparation and remained undetected until fractionation and sample concentration. Although any direct effect of the carboxypeptidase on the amide bond hydrolysis could not be verified, the confirmed presence of protein impurities cannot be ignored as a potential source for the activity.

As a conclusion, preparations of CAL-A show preliminary potential to be used as an alternative for specific amidase-catalyzed reactions. The amidase activity of lipases studied here is of particular interest, because the reaction could be used for instance as an alternative mild deprotection method for amides in organic synthesis or for resolving racemic amides. CAL-A has several desirable features for commercial use: exceptional stability in low pHs, high temperature and organic solvents, together with an unusually broad tolerance for various unnatural substrates. This makes CAL-A a promising candidate for protein engineering to alter its catalytic properties or substrate preference, for instance toward different amide precursors.

Experimental section

Materials and methods

Various forms of lipase A from *Candida antarctica* were the products of Roche (Chirazyme L-5, lyo.), Fluka (CAL-A-CLEA), ChiralVision (Immozyme CALA-T2-150), ViaZym [ViaKit containing 18 immobilized CAL-As on macroporous supports (VZ1030- and VZ1031-codes), silica gel (VZ101-codes and VZ1011-1) and Celite (VZ102-codes)] and Biocatalytics (now Codexis; both free (Cat#ICR-112) and immobilized (Cat#NZL-101-IMB) forms). Acylase I from *Aspergillus* genus was obtained from Tokyo Kasei Chemicals (TCI). Reagents were purchased from Sigma, Aldrich, Fluka, Bachem and Acros. Solvents of the highest analytical grade were obtained from Aldrich, J. T. Baker and Lab-Scan.

The determination of E was based on the equation $E = \ln[(1 - c)(1 - ee_S)]/\ln[(1 - c)(1 + ee_S)]$.[44] Using linear regression E was achieved as the slope of a line. Unless otherwise stated, the calculation of conversion was based on the equation $c = ee_S/(ee_S + ee_P)$.

N-Acylation of **1a** (0.100 M) in butyl butanoate (1 cm^3) was started by adding the enzyme (40 mg cm^{-3}). The amounts of the enzyme preparations were kept constant, because the real protein content in the preparations was unknown. Five samples (0.1 cm^3) were taken at intervals and derivatized with acetic anhydride. Hydrolysis of **1b**, **1c**, **2a–c**, **3a** and **4a** (0.050 M) was carried out in phosphate buffer (pH 7.5; 0.100 M) at room temperature, the amount of the enzyme being 5 mg cm^{-3} (except for enzymes of ViaKit 20 mg cm^{-3} and other exceptions described in Tables 2 and 3). In all hydrolyses, the progress of the reactions was followed by taking samples (400–1000 μl) and filtering off the enzyme with 10 K Nanosep centrifugal tubes. Only with **2c** did we observe a background chemical reaction which stayed negligible in 2 h, when the reaction samples were analyzed.

The enantiomers of **2a–d**, **3a**, **3d**, **4a** and **4d** were analyzed by HPLC using Phenomenex D-penicillamine-column. Hydrolyses of **1b** into **1d**, and **1c** into **1a/1b/1d**, were followed by GC using Chirasil-DEX-CB (Varian) as a column. Before analysis water was evaporated from the samples (400–1000 μL), and the starting material and the product were derivatized into amido esters by adding acetic or butanoic anhydride (100 μL) and after five minutes MeOH (200 μL). The relative proportions of **1a**, **1b**, **1c** and **1d** were determined from the peak areas by taking into account the peak responses which were determined by making a solution containing all the four compounds in equal amounts.

Absolute configurations were determined based on our previous works.[38,42,45]

Preparation of the racemic starting materials

1d, **2a–d**, **3a**, **3d**, **4a** and **4d** were commercially available. Preparation of **1a** and **1c** has been previously described.[38] **1b** as a sodium salt (611 mg, yield 90%, mp 120 °C) was prepared by hydrolyzing the corresponding ethyl ester **1c** (700 mg, 3.48 mmol) with sodium hydroxide (139.1 mg; 3.48 mmol) dissolved in water (40 cm^3).

rac-**1b** (sodium salt): ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.05 (d, 1 H, *J* 8.0, N*H*), 3.95 (m, 1 H, *J* 6.8 and 14.4, C*H*), 2.06 (br m, 1 H, *J* 4.4 and 14.4, CH_2CO$_2$), 1.97 (m, 2 H, *J* 7.2 and 14.4, CH_2CONH), 1.96 (br m, 1 H, *J* 7.6, CH_2CO$_2$), 1.45 (m, 2 H, *J* 7.6, CH_2CH$_3$), 1.01 (d, 3H, *J* 6.8, CHCH_3), 0.83 (m, 3H, *J* 7.6, CH$_2$CH_3).

HRMS: MH^+ = 196.0950, $C_8H_{15}NNaO_3$ requires MH^+ = 196.0950.

Fractionation and analysis of the commercial CAL-A preparation

SDS-PAGE analysis. Samples of CAL-A were analyzed on various SDS-PAGE systems, including PhastSystem (Amersham Pharmacia Biotech) gradient 8-25 gels and 8% self-casted SDS-PAGE gels stained with Coomassie Brilliant Blue dye, and Criterion 10% Bis-Tris gels (Biorad) stained with Silver dye. The bands were compared against commercial protein standard ladders (Biorad pre-stained broad range SDS ladder 161-0318 and Fermentas PageRuler™ ladders).

Preparative gel filtration. 5–75 mg of the solubilized samples of the CAL-A preparation (lyophilized Cat#ICR-112, Codexis) were fractionated using ÄKTA FLPC chromatography system

(Amersham Biosciences) and Superdex 200 Hiload 26/60 gel filtration columns (Amersham Biosciences). The samples were injected in 5 cm^3 volume of 100 mM Tris-HCl buffer pH 7.5 used for the run, and 10 cm^3 fractions were collected for subsequent steps. CentriPrep YM-10 centrifugal filter devices (Biorad) were used for fraction concentration.

Removal of glycosyl groups. Removal of potential N-linked sugars was accomplished using commercial glycosidase PNGase F (New England Biolabs) which cleaves between the innermost GlNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. The procedure was carried out as instructed by the manufacturer.

Protein LC-MS/MS analysis for identification. The analysis and sequence comparison was carried out at Proteomics Facility (Turku Centre for Biotechnology/University of Turku and Åbo Akademi University). Proteins in the provided CAL-A samples were separated on BioRad's precast Criterion XT gel (10% Bis-Tris with MOPS buffer), cut from the silver stained gel and in-gel digested[46] for the LC-MS/MS analysis.

Tryptic peptides were analyzed using a nanoflow LC system (Famos, Switchos, and UltimatePlus; LC Packings - Dionex Corporation, Sunnyvale, CA, USA) coupled to a QSTAR Pulsar i ESI-hybrid Q-TOF tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). Peptides were concentrated and desalted on a precolumn (0.3 × 5 mm C18 PepMap100, LC Packings), and eluted at 200 $ndm^3\ min^{-1}$ by increasing the concentration of acetonitrile onto a C18 reverse phase column (75 µm i.d. × 15 cm, Magic 5 µm 100 Å C18, Michrom BioResources Inc.). A linear 25 min gradient from 98% solvent A (97.9% water, 2% acetonitrile, and 0.1% formic acid) to 65% solvent B (95% acetonitrile, 4.9% water, and 0.1% formic acid) was used.

Sequence comparison and protein identification. Data dependent acquisition was performed in positive ion mode. Two of the most intense doubly or triply charged precursor ions were selected from m/z 250 to m/z 1500. Data from LC-MS/MS runs were converted to peak list files with the Analyst QS software (version 1.1) and searched using an in-house Mascot search engine (version 2.2, Matrix Sciences, Boston, MA, USA). The following parameters were used: (1) database Swiss-Prot/Tremb (versions 51.5/34.5), taxonomy all, enzyme trypsin with up to one missed cleavages, variable modifications carbamidomethyl and methionine oxidation, and a mass accuracy 0.6 Da for both precursor ions and MS/MS data or (2) database containing only lipase A sequence,[34] but other parameters same as above.

Analysis of the test reactions. Hydrolysis of *N*-acetylmethionine **2a** was detected by adding 1 cm^3 of each fraction to *N*-acetylmethionine (1 cm^3, 0.100 M). Hydrolysis of ethyl 3-butanamidobutanoate **1c** was started by adding 1 cm^3 of each fraction to **1c** (10.1 mg; 0.050 M). The reactions were analyzed as described in Materials and Methods. For *N*-acylation of methyl pipecolinate *rac*-**5a** (Scheme 5), Celite (15.6 mg) and sucrose (2.4 mg) were dissolved in each fraction (2 cm^3) and the water was left to evaporate in a fume hood. Adsorped enzyme preparation was then added into methyl pipecolinate (0.025 M) and trifluoroethyl butanoate (0.100 M) in TBME. The reactions were analyzed by GC equipped with a chiral a column (Varian permethylated β-cyclodextrin).

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