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Enzymes for the Resolution of α-Tertiary-Substituted Carboxylic Acid Esters

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Abstract—Aromatic α-amino-α-methyl acids and α-hydrazino-α-methyl acids are known aromatic amino acid decarboxylase inhibitors. Specific derivatives such as 2-amino-2-methyl-3-(3,4-dihydroxyphenyl)propanoate, Aldomet®, and 2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl)propanoate, Lodosyn®, have been developed as therapeutic agents to treat hypertension and Parkinson's disease, respectively. We recently reported a method for the kinetic resolution of the racemic esters of such compounds using a crude preparation of a novel enzyme catalyst from the yeast *Candida lipolytica* (Yee, C.; Blythe, T. A.; McNabb, T. J.; Walts, A. E. *J. Org. Chem.* **1992**, *57*, 3525–3527). Here we report the purification and initial characterization of the active enzyme component, an enzyme given the name *Candida lipolytica* ester hydrolase (CLEH). CLEH was purified to > 95 % homogeneity by chromatography on Matrex™ Blue B resin. The enzyme was found to be a glycoprotein with M_r = 80,000–300,000. In addition to esterolytic activity, the enzyme was found to catalyze the hydrolysis of amides, anilides and peptides. Sequence analysis of internal peptides of CLEH revealed striking homology to a number of enzymes belonging to the group of serine carboxypeptidases (E.C. 3.4.16.1). One peptide aligned with the canonical serine carboxypeptidase active site sequence, GESYAG. Based on the structural relationship of CLEH to serine carboxypeptidases, three representative serine carboxypeptidases were evaluated for their utility in resolving racemic α-tertiary ester substrates and compared with the activity of CLEH. All enzymes revealed similarly high activity and enantioselectivity towards the α-hydrazino-α-methyl ester precursor of the Parkinson-drug Carbidopa. However, differences in enantioselectivity were observed with other α-tertiary-substituted ester substrates. Serine carboxypeptidase-catalyzed ester resolutions thus offer a new route to many sterically hindered homochiral α-amino, α-hydrazino and α-hydroxy carboxylic acids.

Introduction

Enzymatic methods for the production of enantiomerically pure drugs and other biologically active compounds are becoming increasingly important for the commercial manufacture of such compounds in the pharmaceutical and agrochemical industries.[1] The driving forces for the introduction of homochiral drugs include safety concerns, increasing costs of toxicity testing and in particular, documentation that affect racemates because each enantiomer needs to be evaluated as an individual compound.[2] The large number of chiral target compounds now under consideration as drugs or pharmaceutical intermediates encompasses numerous diverse molecular structures that cannot be synthesized or resolved efficiently by the limited number of enzymes that have found use in preparative organic synthesis. Practical considerations require the development of new enzyme catalysts to overcome the limitations of the currently available enzymes.

α-Amino acids are among the most commonly used chiral synthons.[3] This is largely due to their abundance in biological raw materials and the availability of synthetic methods that can be used to produce non-naturally occurring (*2R*)-stereoisomers and structural homologs. Enzyme catalysis is widely used to introduce chirality in industrial scale α-amino acid production.[4] While most enzyme catalysts are quite tolerant of sidechain structural variations and thus can be used to resolve a number of amino acid products, their activities are generally sensitive to additional substitution on the α-carbon and on the α-amino group. Examples of therapeutically important agents bearing α-tertiary-substituted amino and hydrazino acids are the drugs Aldomet® and Lodosyn.® We have previously reported an enzymatic route to the active (*2S*)-enantiomer **2** of these agents by kinetic resolution of racemic esters of the general structure **1** with a crude commercial lipase preparation (Scheme I).[5] Here we report the purification and initial characterization of the active enzyme component of the crude lipase preparation. The purified enzyme, given the name *Candida lipolytica* ester hydrolase (CLEH), revealed striking amino acid sequence homology to the known group of serine carboxypeptidases. The identification of CLEH as a new peptidase was confirmed by demonstrating its activity on peptide and anilide substrates. The practical consequence of these findings is illustrated in a series of examples that describe resolutions of ester substrates relevant for chiral drug synthesis with three commercially available serine carboxypeptidases.

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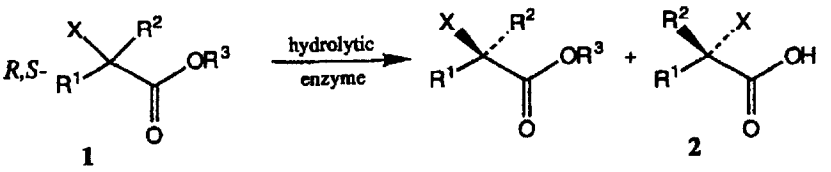


Scheme I. R^1 = arylmethyl, *i*-Bu; R^2 = Me; R^3 = Me, Et, *n*-Bu; X = amino, acylamino, hydrazino, hydroxy.

Results

Purification and characterization of C. lipolytica *ester hydrolase*

Purification of C. lipolytica *ester hydrolase.* CLEH was purified from two commercially available sources of *C. lipolytica* lipase. Because soluble proteins constituted only *ca* 1–2 % of the crude lipase preparations (Table 1), the lipase powders were first extracted with aqueous buffer and the activity of interest concentrated from the soluble extract by PEG precipitation. Purification of CLEH to > 95 % purity was accomplished by single-step chromatography on Matrex™ Blue B resin. Purified CLEH was found to migrate diffusely on SDS-polyacrylamide gels with a mobility corresponding to M_r values of 80,000–300,000 (Figure 1, lane 3). This migration pattern is often found with hyperglycosylated proteins. The species migrating with an M_r of ≈ 47,000 (Figure 1, lane 3) was only detectable by silver staining and represents a minor contaminant in the CLEH preparation. Further chromatography of the dye adsorption-purified CLEH on Mono-Q® anion exchange resin resulted in the baseline separation of two active hyperglycosylated species which eluted at slightly different ionic strengths (Figure 2; Figure 1, lanes 4 and 5). Following N-Glycanase® treatment, each active species migrated as a single sharp band corresponding to a molecular weight of 52,000 (Figure 1, lanes 6 and 7, the second band with M_r = 35,000 is N-Glycanase®). Each active form had a similar specific activity towards both benzoyl-L-tyrosine *p*-nitroanilide and thiopropyl α-methyl-*N*-acetylphenylalanate substrates and N-terminal sequence analysis for the first 20 residues yielded the same amino acid sequence for each form. The results of the purification of CLEH from 10 g crude lipase powder are summarized in Table 1.

Table 1. Summary of CLEH purification from 10 g CLL powder

	Protein [mg]	Activity [U]	Specific activity[a] [U/mg]	Purification factor	Yield [%]
Crude extract	125	2.6	0.02	1.0	
PEG Precipitation	115	2.5	0.02	1.0	99
Blue-B-column	14	1.7	0.11	5.5	65
Mono Q® peak 1	8	0.7	0.09	4.5	27[b]
Mono Q® peak 2	5	0.5	0.11	5.5	19[b]

[a]Determined with thiopropyl D,L-*N*-acetyl-α-methylphenylalanate as assay substrate.
[b]Combined yield of 46 % with 5-fold total purification.

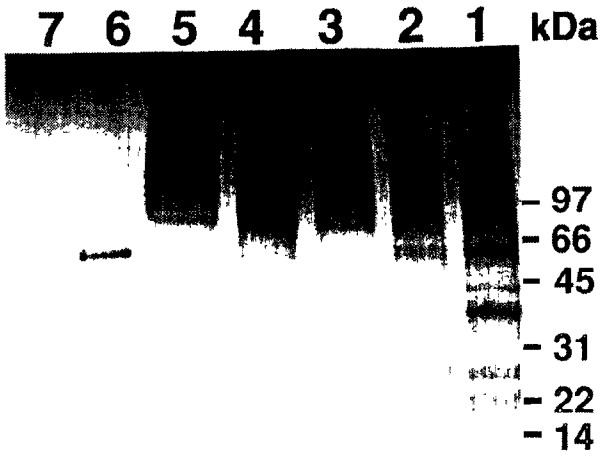


Figure 1. SDS-polyacrylamide gel electrophoretic analysis of CLEH purification. Lane 1, crude extract (3 µg protein); lane 2, PEG precipitate (3 µg protein); lane 3, Blue B-column pool (1.7 µg protein); lane 4, Mono-Q pool 1 (5 µg protein); lane 5, Mono-Q pool 2 (0.5 µg protein); lane 6, deglycosylated Mono-Q pool 1 (1 µg protein); lane 7, deglycosylated Mono-Q pool 2 (0.1 µg protein). A 4–12 % polyacrylamide gradient gel (BioRad) was used and proteins were detected by silver staining. The following molecular weight standard proteins (BioRad) were used for calibration: Hen egg white lysozyme, 14.4 kDa; soybean trypsin inhibitor, 21.5 kDa; bovine carbonic anhydrase, 31.0 kDa; hen egg white ovalbumin, 45.0 kDa; bovine serum albumin, 66.2 kDa and rabbit muscle phosphorylase B, 97.4 kDa.

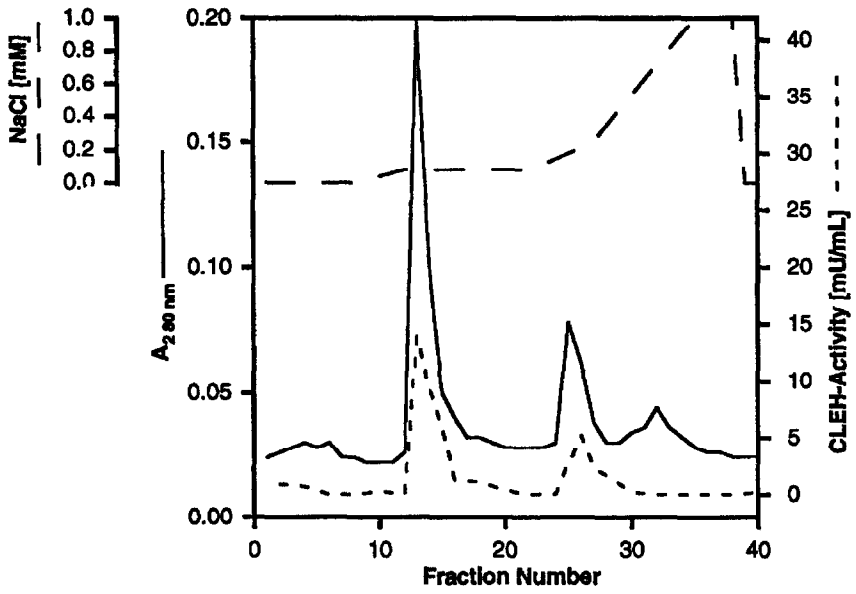


Figure 2. Mono-Q® chromatogram of CLEH-purification. The experiment was performed on a Pharmacia FPLC® system. Dye-adsorption-purified CLEH (1 mg) was loaded onto the column. Elution of proteins was followed by continuously monitoring the absorption at 280 nm and CLEH-activity was assayed in eluate fractions with thiopropyl D,L-*N*-acetyl-α-methylphenylalanate as substrate.

To determine if CLEH was produced by readily available *C. lipolytica* strains and to identify an organism for enzyme production, *C. lipolytica* strain NRRL Y-1094[6] was grown in shaking flasks and the media tested for CLEH enzyme. CLEH activity was observed in the culture medium once growth achieved stationary phase and maximum activity was achieved 2–4 days after the culture had entered stationary phase. The enzyme was subsequently purified from the broth of a 15 L fermentation. Following the clarification of the media, the CLEH activity was concentrated by hydrophobic interaction chromatography on Phenyl Sepharose® and then purified by PEG-precipitation and dye adsorption chromatography as described for the purification of CLEH from commercially available crude lipase. The enzyme isolated from the fermentation broth showed the same chromatographic and electrophoretic behaviors, N-terminal amino acid sequence and specific activity as the enzyme prepared from commercial *C. lipolytica* lipase powder.

Amino acid sequence analysis. To obtain additional primary sequence data, several peptides were generated from purified CLEH. Dye adsorption-purified CLEH was reduced with β-mercaptoethanol and alkylated on cysteine residues with 4-vinylpyridine. The pyridylethylated protein was either used directly for proteolytic digests or first deglycosylated with N-Glycanase® and then digested. Peptide fragments of CLEH were obtained by digestion with the endoproteinases Lys–C, Arg–C and Glu–C, and by chemical cleavage with cyanogen bromide. The resulting mixtures of peptides were fractionated by reversed phase chromatography. Peak fractions that appeared homogeneous by capillary zone electrophoresis were sequenced by automated Edman degradation. Sequences comprising 139 amino acids were identified through the analysis of 11 internal peptides and the N-terminus of the intact enzyme. To determine whether the primary structure of CLEH was unique, the SWISS-PROT protein sequence database (release 23.0) was searched for similar sequences using the method of Pearson and Lipman[7] implemented as FastA in the GCG program collection[8]. The majority of the CLEH sequences showed strong homology to known members of the group of serine carboxypeptidases (E.C. 3.4.16.1) with 50 % to 70 % identity for the best matches of each peptide. Alignments of these matched sequences are shown in Table 2. Only the N-terminal sequence of CLEH, S V P G D L G L D D V Q Q Y T G Y L T A, and one cyanogen bromide fragment, M I N R W I S G D Y W L V G E K H, could not be aligned to any sequence in the database.

Substrate specificity of CLEH. In addition to their nominal peptidase activities, E.C. 3.4.16.1 enzymes catalyze the hydrolytic cleavages of esters, amides and anilides of α-amino acids.[9,10] To investigate whether CLEH had similar reaction specificity, it was assayed with two standard substrates of CPY.[10,11] The synthetic peptide substrate *Z*-Phe–Ala (*N*-carbobenzoxy-L-phenylalanine-L-alanine) was hydrolyzed by CLEH at a rate of 1.2 U/mg versus 22 U/mg for CPY. The chromogenic amide substrate benzoyl-L-tyrosine *p*-nitroanilide was hydrolyzed at comparable rates by both enzymes, 0.2 U/mg for CLEH and 0.3 U/mg for CPY. Both enzymes were also equally active in hydrolyzing *N*-acetyl-α-methylphenylalanine thiopropylester with specific activities of 0.1 U/mg.

Inactivation of CLEH by serine-modifying reagents. To determine if the active site of CLEH contained an activated serine nucleophile, the sensitivity of the enzyme to inactivation by diisopropyl fluorophosphate (DFP) was examined. In similar fashion to known serine carboxypeptidases,[10,12] CLEH was rapidly inactivated by micromolar concentrations of DFP: after 5 min incubation the relative CLEH activities were approximately 0 % at 1

Table 2. Alignment of amino acid sequences of CLEH peptides with sequences of E.C. 3.4.16.1 enzymes[a] from the Swissprot database release 23.0. The active site consensus sequence is shown in bold typeface

CLEH Glu-C peptide	**ESYAG**HYIPSVGHEE
	||||||||| : |
CPY aa 256-271	**GESYAG**HYIPVFASEI
CLEH CNBr peptide	MVPHDQPKPALET
	||| ||||:|||
CP3W aa 463-474	MVPMDQPKAALEM
CLEH tryptic peptide	AWTDALP
	||||:||
CPY aa 460-466	AWTDVLP
CLEH two Glu-C peptides	EHFFYWTVESRNDPSKDPVILWLQGGPGCSSVTGLLYE
	|||:|| ||||||:||||||||:|||||||:|||:|
CPY aa 141-177	KHFFFWTFESRNDPAKDPVILWLNGGPGCSSLTGLFFE
CLEH Lys-C peptide	KVYAFLELFFQRFPQY
	|||:||||||:||:|
CPY aa 231-245	KVYNFLELFFDQFPEY
CLEH CNBr peptide	MLQXGYXVLIYAGXYDXICNW
	:|: | ||||| || ||||
CP3B aa 317-336	LLEDGINVLIYAGEYDLICNW

\| = identity : = conservative replacement[7,8]

[a]CP3W, wheat serine carboxypeptidase 3 precursor; CP3B, barley serine carboxypeptidase 3 precursor; CPY, *Saccharomyces cerevisiae* carboxypeptidase Y precursor; aa, amino acid numbers as in database.

mM DFP, 40 % at 0.1 mM DFP, 60 % at 0.01 mM DFP, 90 % at 0.001 mM DFP and 100 % in the absence of DFP. Phenylmethylsulfonyl fluoride at 1 mM also led to the rapid and complete inactivation of the enzyme.

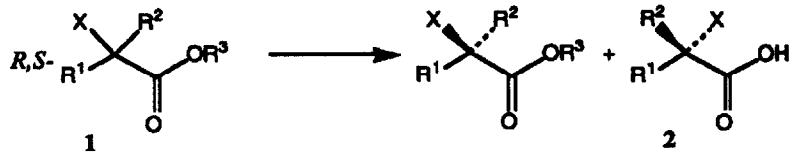
Preparative scale resolutions of α-tertiary substituted esters. To assess the usefulness of serine carboxypeptidases for preparative ester resolutions, the enantioselectivities of CLEH and the commercially available enzymes carboxypeptidase Y (CPY), carboxypeptidase W (CPW) and carboxypeptidase P (CPP) were evaluated with thirteen different α-tertiary substituted ester substrates. The results are summarized in Table 3. All substrates (**1a–g**) were hydrolyzed by each enzyme listed above and enantiomeric excesses of 90 % or better were obtained in each experiment except for the resolutions for **1c** and **1g** with CPY. The absolute configurations of products **2a,b** were determined by comparison with authentic USP standards.[5] All four enzymes examined in the present study preferentially hydrolyzed the (2*S*)-enantiomers of substrates **1a,b**. The experiments summarized in Table 3 were not optimized with respect to enzyme use, and in repeated experiments shorter reaction times or less enzyme could often be used (data not shown). The enzyme/substrate ratios indicated in Table 3 therefore do not reflect the specific activities of the enzymes and merely state conditions under which particular resolutions may be achieved.

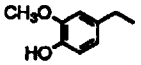
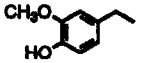
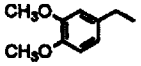
Discussion

Kinetic resolution of racemic compounds with hydrolases is still the most widely used enzymatic method for the preparation of optically active compounds both in the laboratory and in commercial manufacture.[2] As an initial approach to produce enantiomerically pure α-tertiary substituted α-amino acids (**2**), Yee *et al.*[5] evaluated a number of hydrolytic enzyme preparations commonly used in resolutions of racemic esters. Commercial preparations of lipase from *Candida lipolytica* (*Candida lipolytica* lipase) exhibited the desired activity and enantioselectivity. However, the specific activity of crude *C. lipolytica* lipase powder for hydrolyzing the target esters (**1**) was much too low for a commercially viable process. With the aim of identifying the catalytically active species, we purified and characterized the active component of *C. lipolytica* lipase.

Candida lipolytica lipase contains 1–2 % protein by weight and silver-stained SDS–PAGE showed this protein fraction to consist of at least 21 different polypeptide species (Figure 1, lane 1). After aqueous extraction of the lyophilized *C. lipolytica* lipase powder, the protein components could be concentrated by PEG-precipitation with quantitative recovery of the relevant activity. A random screening of chromatography media for protein purification led to the identification of Matrex™ Blue B resin as a highly efficient affinity adsorbent that yields esterase of > 95 % purity with 80 % recovery of activity in a single step (Figure 1). The electrophoretic mobility of the enzyme, which appears on an SDS–PAGE gel as a haze spread over a molecular weight range from 80,000 to 300,000 is indicative of a hyperglycosylated protein. Heterogeneous polymannan structures linked to asparagine residues are often found in secreted yeast proteins.[13–15] Chromatography on Mono-Q® anion exchange resin separated the activity into two peaks which migrated similarly on SDS-gels (Figure 2; Figure 1 lanes 4 and 5).

Table 3. Resolutions of α-tertiary-substituted esters with CLEH and serine carboxypeptidases



	R1	X	R2	R3	enzyme	E/S[a]	time	conv[b]	ee(ester)[c]	ee(acid)[d]
1a		-NHNH2	Me	n-Bu	CLL[e]	0.1	4h	50%	>98%	>99%
					CLEH	0.9	16h	50%	>99%	>99%
1b		-NHNH2	Me	Et	CLL	0.4	127h	50%	>98%	n.d.
					CPY	0.1	20h	46%	n.d.	>98%
					CPP	0.1	20h	50%	n.d.	>98%
1c		-OH	Me	Et	CLL	0.1	24h	62%	>99%	n.d.
					CPY	0.03	23h	91%	31%	n.d.
					CPW	0.1	8h	56%	>99%	n.d.
1d[f]		-NH2	Me	n-Bu	CLL	0.1	24h	60%	95%	n.d.
1e		-NH2	Me	Me	CPY	0.04	4h	75%	>99%	n.d.
					CPW	0.04	7h	56%	90%	n.d.
					CPP	0.03	2h	50%	>99%	n.d.
1f		-NHCOCH3	Me	Et	CLL	0.1	19d	50%	92%	n.d.
					CPY	0.04	120h	50%	>98%	n.d.
1g		-NH2	-CHF2	Me	CLL	0.2	120h	68%	97%	n.d.
					CLEH	0.03	120h	53%	94%	n.d.
					CPY	0.05	192h	54%	13%	n.d.

[a]Enzyme/substrate ratio by weight, [b]conversion of racemic ester, [c]enantiomeric excess of recovered ester substrate, [d]enantiomeric excess of acid product, [e]crude *C. lipolytica* lipase powder, [f]data from reference 5.

After treatment with N-Glycanase® to remove N-linked carbohydrate chains,[16] enzyme from both peaks migrated as single sharp bands with a mobility corresponding to a molecular weight of 52,000 (Figure 1, lanes 6 and 7). The two forms showed identical specific activities and N-terminal amino acid sequences. Taken together, these data suggest the presence of two glycoforms of CLEH. With regard to their similar sizes but differential binding to anion exchange resin, one could speculate that these glycoforms result from variations in the peripheral modification of the carbohydrate chains, such as mannose phosphorylation.[14] Furthermore, different sets of potential acceptor asparagines may be glycosylated[15] which could lead to heterogeneity in molecular shape and surface charge distribution.

The source organism of CLEH was confirmed by its purification from *C. lipolytica* strain NRRL Y-1094. The culture broth contained only low concentrations of the enzyme. Purification required an initial concentration step which was achieved by hydrophobic interaction chromatography, after which the same purification steps that were described for the isolation of CLEH from crude *C. lipolytica* lipase could be applied. The enzyme obtained from *C. lipolytica* NRRL Y-1094 cultures revealed the same catalytic and physical properties as described above. The identical N-terminal sequences are indicative of a common genetic code. The N-terminal sequence of CLEH provided no clues to the enzyme's identity or its physiological role because it lacked sequence elements sufficiently conserved to reveal similarities in sequence database searches. Only when the sequence analysis of CLEH was extended to include internal peptide sequences did it become obvious that it was genetically related to a large group of well known enzymes, serine carboxypeptidases.

Serine carboxypeptidases (E. C. 3.4.16.1) are ubiquitous enzymes in eukaryotes and have been purified and characterized from yeasts,[10,17] fungi,[9,18–21] higher plants[22–25]and animals.[22–27] It is common for one species to express simultaneously several serine carboxypeptidases to perform different functions[21], or alternatively to adjust

to environmental changes.[19,20] All serine carboxypeptidase enzymes have certain features in common, including N-linked glycosylation, a conserved active site motif and a high degree of amino acid sequence homology. However, individual members of the group also contain unique segments in their polypeptide sequences as a result of divergent evolution. The structural data determined for CLEH clearly match the general pattern typical for serine carboxypeptidases, and the partial peptide sequence ESYAG (Table 2) that represents the strictly conserved active site consensus sequence, GESYAG,[28] suggests functional similarity as well. The latter was experimentally confirmed by the amidolytic cleavage of N-protected dipeptide and anilide substrates and the irreversible inhibition by serine modifying reagents. Surprisingly, data from initial experiments aimed at studying the action of CLEH on natural polypeptide substrates suggested an endoproteolytic activity (data not shown). This is in contrast to the strictly exoproteolytic activity of most serine carboxypeptidases. A unique feature of CLEH, which could be of practical importance for its large-scale production, is the fact that it is secreted to the culture medium by *C. lipolytica* cells whereas other yeast serine carboxypeptidases are located intracellularly in vacuoles.[29]

A promising application of serine carboxypeptidases is the enzymatic preparation of (*R*)- and (*S*)-α-hydrazino acids, which has yet to be reported in the literature. Resolution of **1a** or **1b** yields homochiral (2*S*)-2-hydrazino-2-methyl-3-(3-methoxy-4-hydroxyphenyl)propanoic acid (Table 3), which can be converted to the drug Carbidopa, (2*S*)-2-hydrazino-2-methyl-3-(3,4-dihydroxyphenyl)propanoic acid, by acid hydrolysis of the methyl ether. This resolution is extremely enantioselective with *E*-values greater than 450 for all enzymes; the reaction stops at 50 % conversion and, as shown by the second example of **1a** resolution in Table 3, no hydrolysis of the (*R*)-enantiomer is detectable even after prolonged incubation with a large excess of enzyme. The remaining examples in Table 3 illustrate the structural tolerance of these enzymes for substrates with varying sizes of R^1. Also tolerated are substituents on the α-nitrogen atom (**1f**)[5] or replacement of the latter with oxygen (**1c**). The absolute configurations of α-hydrazino-α-methyl esters, e.g. **1a**, and of α-amino-α-methyl esters recovered after CLEH resolutions was determined to be 2*R*.[5] By analogy to the results with compounds **1b–f** we suggest that all enzymes used in the present study preferentially hydrolyze the (*S*)-enantiomers. The resolutions of **1c** and **1g** with CPY proceeded with low enantioselectivity, but in these rare instances good *e.e.* values could be achieved simply by using another serine carboxypeptidase. It should be noted here that an aminopeptidase from *Ochrobacterium anthropi* has been shown to be active on a similar spectrum of sterically hindered substrates, however, hydrolysis of α-hydrazino acid derivatives was not reported.[30] This novel exopeptidase was discovered by screening microorganisms and whole cells harboring the enzyme were used to resolve amides of bulky α-alkyl-α-amino, α-hydroxy and α-hydroxylamino acids.

Conclusion

Homochiral α-tertiary substituted carboxylic acid derivatives are difficult to prepare by most enzymatic methods. They cannot be obtained by asymmetric synthesis from precursors with sp^2-hybridized α-carbon atoms[31–35] and they are poor substrates for the hydrolytic enzymes commonly used in kinetic resolutions. This limitation can be overcome by the use of serine carboxypeptidases which possess high catalytic activity and enantioselectivity for kinetic resolutions of many α-tertiary substituted carboxylic acid esters. The method yields both ester substrates and acid products in high enantiomeric purity and allows considerable variations in substituent structures. The enzyme catalysts belong to a well characterized group of homologous enzymes of which several members are currently commercially available, thus providing sufficient variation in specificities and selectivities to tackle many resolution problems. In addition, large-scale processes have been developed that allow the high volume manufacture of the catalysts.[11,36] Further, several serine carboxypeptidase-encoding genes have been cloned and sequenced,[26,27,29,37–42] and a high quality X-ray structure has been determined for one member of the group.[24] The resulting thorough understanding of the biosynthesis[29,43,44] and mechanism of action[9,19,28,45–47] of serine carboxypeptidases provides an opportunity for enhanced enzyme production by recombinant expression[48,49] and to use genetic engineering to tailor catalytic properties for future requirements.[28,50]

Experimental

Materials

The strain *Candida lipolytica* NRRL Y-1094 was obtained from the United States Department of Agriculture, Peoria, IL. *Candida lipolytica* lipase was purchased either from FlukaChemie AG, Buchs, Switzerland or from Amano, Nagoya, Japan. Chiracel OD columns were obtained from J. T. Baker, Phillipsburg, NJ. Thiopropyl D,L-*N*-acetyl-α-methylphenylalanate was prepared following a published procedure.[51] Other chemicals or enzymes were purchased from Aldrich or Sigma.

Assay of hydrolase activities

Routine activity assays for carboxypeptidases were carried out with the chromogenic substrate benzoyl-L-tyrosine *p*-nitroanilide as described by Johansen *et al.*[11] The assay mixture for determination of α-tertiary ester hydrolase activity contained 50 mM HEPES buffer pH 7.5, 1.0 mM EDTA, 2.0 mM thiopropyl D,L-*N*-acetyl-α-methylphenylalanate, 1.0 mM DTNB and 10 % DMF. The release of propanethiol was followed by monitoring the absorption at 412 nm. One unit of activity is defined as the amount of enzyme required to release 1 μmol of propanethiol per minute at 25 °C. The *p*-nitroanilide and the thiopropyl ester substrates were both used to monitor

the purification of CLEH. Peptidase activity was assayed with Z-Phe–Ala according to Hayashi.[10]

Fermentation of Candida lipolytica

C. lipolytica was grown at 30 °C on medium containing 0.3 % malt extract, 0.3 % yeast extract, 1.0 % glucose, 0.5 % peptone, either in shaking flasks or in a 20 L fermentation vessel. For fermentor culture 10 mM potassium phosphate was added to the medium and the pH was maintained at 7.0 by addition of HCl and NaOH. The fermentor was agitated at 800 rpm with an aeration rate of 0.5 vvm. Foaming was controlled by the addition of Pluronic™ 25R-2. After 72 h, when no further increase in esterase activity was detected, the fermentation was terminated and the broth was clarified by centrifugation at 4500 × *g*.

Purification of C. lipolytica *ester hydrolase*

C. lipolytica ester hydrolase was isolated as homogeneous protein from both crude *C. lipolytica* lipase powder and the fermentation broth of *C. lipolytica* strain NRRL Y-1094. Buffers used in both purification schemes were as follows: Buffer A: 10 mM sodium phosphate pH 6.5 containing 1 mM EDTA; Buffer B: 10 mM Tris–HCl pH 7.8 containing 1 mM EDTA; Buffer C: 50 mM HEPES pH 7.5 containing 1.8 M ammonium sulfate and 1 mM EDTA; Buffer D: 50 mM HEPES pH 7.5 containing 1 mM EDTA. Sodium azide was added to all buffers at 0.01 % (w/v) except those used for anion exchange chromatography.

Purification of CLEH from C. lipolytica *lipase. C. lipolytica* lipase powder (10 g) was suspended in 100 mL Buffer A and stirred at room temperature for 30 min. After removal of insoluble material by low speed centrifugation, 30 g of PEG 4000 (Mallinkrodt) was added and the mixture stirred for 30 min at room temperature. Precipitated proteins were collected by 30 min centrifugation at 20,000 × *g* at 4 °C. The liquid pellet was dissolved in 10 mL Buffer A and dialyzed against 2 × 1 L of the same buffer. The dialyzed protein solution was applied under gravity flow to a column (2.6 × 17 cm) packed with Matrex™ Blue B resin (Amicon) that had been equilibrated with Buffer A. The column was washed with 300 mL Buffer A and the proteins were eluted after a step from 0 to 1 M sodium chloride in Buffer A. 13 mL Fractions were collected during the elution and the fractions containing CLEH activity were pooled. The dye adsorption-purified enzyme was dialyzed against 3 × 500 mL of Buffer B and loaded onto a Mono-Q® HR5/5 column (Pharmacia) equilibrated with Buffer B. This column was operated at a 1 mL/min constant flow rate. After washing with Buffer B, proteins were eluted with a discontinuous sodium chloride gradient in Buffer B as indicated in Figure 2.

Purification of CLEH from C. lipolytica *fermentation broth. C. lipolytica* strain NRRL Y-1094 fermentation broth (12.5 L) was adjusted to 1.8 M ammonium sulfate and 1 mM EDTA. The solution (13.5 L) was clarified by centrifugation and applied to a column containing Phenylsepharose® CL-6B Fast Flow resin (15 cm × 5 cm) that had been equilibrated with Buffer C. After washing with 1 L Buffer C, the proteins were eluted with 1.7 L Buffer D. Active fractions were purified as described above.

Enzyme characterization

Enzymatic deglycosylation. Samples of CLEH were dissolved in 6 M guanidine hydrochloride, 0.1 M Tris–HCl pH 8.5 containing 3 mM PMSF and a 100-fold molar excess of 2-mercaptoethanol over protein. After 14 h incubation under argon at room temperature the samples were diluted with water to give a final concentration of 1.5 M guanidine and 100 U N-Glycanase® were added per mg of CLEH. The deglycosylation reaction was allowed to proceed under argon for 4 h at 37 °C.

Amino acid sequence analysis. N-terminal amino acid sequences of purified homogeneous CLEH samples were determined by 20 cycles of automated Edman degradation on an Applied Biosystems 477A protein sequencer. For internal sequence analysis, native or enzymatically deglycosylated purified CLEH was treated with a 100-fold molar excess of 4-vinylpyridine over protein in the presence of 6 M guanidine. The reaction was allowed to proceed for 2 h in the dark at 22 °C under argon. The pyridylethylated protein was desalted by reversed phase chromatography on Brownlee™ RP-300 C_8 columns with acetonitrile gradient elution. Proteolytic digestions with the endoproteinases Arg-C, Glu-C and Lys-C were carried out according to the protocols supplied by the manufacturer (Boehringer Mannheim). Cyanogen bromide cleavage was achieved by 44 h incubation in 70 % formic acid with an 800-fold molar excess of the reagent over protein. The reaction was carried out with 1 mg protein per mL at 4 °C under argon atmosphere.

Inactivation with fluorophosphates. CLEH at a concentration of 0.5 mg/mL in 10 mM sodium phosphate buffer, pH 6.5, and solutions of diisopropyl fluorophosphate in isopropanol were mixed to final concentrations of 10 % (v/v) isopropanol and between 0 and 1 mM DFP. After 5 min at room temperature each mixture was assayed for hydrolase activity.

Synthesis of racemic ester substrates

The syntheses of butyl (±)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl)propanoate ((±)-**1a**) and (±)-2-acetamido-2,4-dimethylpentanoate ((±)-**1f**) have been reported previously.[5] Compound numbers refer to entries in Table 1 and Scheme I.

*Ethyl (±)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl)propanoate ((±)-**1b**).* The procedure for the preparation of butyl (±)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl)propanoate ((±)-**1a**)[5] was repeated with ethanol instead of *n*-butanol; ((±)-**1b**) was

obtained in 79 % yield after recrystallization from acetonitrile: mp 114–116 °C; HPLC analysis (YMC-pack ODS column, 4.0 × 50 mm, 3 μm; mobile phase: initially, 25 % methanol in 50 mM aqueous sodium phosphate, linear gradients to 40 % methanol at 2 min, 70 % methanol at 14 min; flow rate, 1 mL/min) showed a single peak with t_R = 5.3 min; IR ($CHCl_3$ solution) 3540, 2990, 2940, 1710, 1510 cm^{-1}; ^1H NMR (400 M Hz, $CDCl_3$) δ 6.79 (d, 7.6 Hz, 1H), 6.63 (s, 1H), 6.60 (d, 7.6 Hz, 1H), 4.14 (q, 7.1 Hz, 2H), 3.83 (s, 3H), 2.99 (d, 13.5 Hz, 1H), 2.74 (d, 13.5 Hz, 1H), 1.34 (s, 3H), 1.24 (t, 7.1 Hz, 3H).

*Ethyl (±)-2-hydroxy-2-methyl-3-(3,4-dimethoxyphenyl)-propionate ((±)-**1c**).* The starting material 1-(3,4-dimethoxyphenyl)-2-propanone was prepared according to the general procedure by Chemerda and Sletzinger[52] from vanillin. 2-Hydroxy-2-methyl-3-(3,4-dimethoxyphenyl)-propanoic acid was synthesized from 1-(3,4-dimethoxyphenyl)-2-propanone according to the general procedure of Corson *et al.*[53] This hydroxy acid was esterified according to the method described for (±)-**1a**[5] with quantitative yield of (±)-**1c** as a colorless oil. ^1H NMR ($CDCl_3$) δ TMS 6.72 (m, 3H), 4.17 (m, 2H), 3.85 (s, 6H), 3.06 (s, 1H), 3.03 (A of AB, J_{AB} = 13.6 Hz, 1H), 2.84 (B of AB, J_{AB} = 13.6 Hz, 1H), 1.48 (bs, 3H), 1.28 (t, 7.2 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ TMS 176.17, 148.58, 148.08, 128.57, 122.11, 113.43, 110.96, 75.21, 61.76, 55.85, 55.82, 45.96, 25.88, 14.23.

*(±)-α–Methyltryptophan methyl ester ((±)-**1e**).* (±)-α–Methyltryptophan (Sigma) was esterified with hydrogen chloride in methanol and isolated as the hydrochloride salt. ^1H NMR (DMSO-d_6) δ 11.2 (bs, 1H), 8.67 (bs, 3H), 7.45 (d, 8 Hz, 1H), 7.36 (d, 8 Hz, 1H), 7.25 (d, 2.4 Hz, 1H), 7.05 (dd, 8 Hz, 1H), 6.98 (dd, 8 Hz, 1H), 3.62 (s, 3H), 3.30 (s, 2H), 1.54 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 171.8, 136.4, 127.7, 126.1, 121.4, 119.0, 118.5, 111.9, 106.1, 60.3, 53.3, 33.2, 21.9.

*Methyl (±)-α-difluoromethylphenylalanate ((±)-**1g**).* This compound was prepared following the procedure of Bey *et al.*[54] and was obtained as an oil: ^1H NMR ($CDCl_3$) δ TMS 7.29 (m, 3H), 7.16 (m, 2H), 5.72 (t, 56 Hz, 1H), 3.73 (s, 3H), 3.21 (A of AB, J_{AB} = 13.4 Hz, 1H), 2.87 (B of AB, J_{AB} = 13.4 Hz, 1H), 1.68 (bs, 2H).

Enzymatic ester resolutions

Candida lipolytica lipase was used in preparative resolutions of esters **1** (Scheme I) as described previously.[5] CLEH was purified by dye adsorption chromatography and was added directly to the reaction mixes. Other serine carboxypeptidases were used as obtained from their commercial manufacturers. Butyl (*R*)-2-hydrazino-2-methyl-3-(3-methoxy-4-hydroxyphenyl)propanoate ((*R*)-**1a**), (*S*)-2-hydrazino-2-methyl-3-(3-methoxy-4-hydroxyphenyl)propanoic acid ((*S*)-**2a**) and ethyl (*R*)-2-acetamido-2,4-dimethylpentanoate ((*R*)-**1f**) were prepared by enzymatic resolution as reported previously.[5] The same reference also contains a detailed description of the methods used for determination of *e.e.* values.

*Ethyl (R)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl)propanoate ((R)-**1b**) and (S)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl)propanoic acid ((S)-**2b**).* The procedure for resolution of **1a**[5] was repeated employing ethyl (±)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl)propanoate ((±)-**1b**) rather than butyl (±)-2-hydrazino-2-methyl-3-(4-hydroxy-3-methoxyphenyl) propanoate ((±)-**1a**). Resolutions were carried out at pH 7.5 with CLEH and CPY and at pH 5.5 with CPP. The enzymatic hydrolysis was allowed to proceed to 50 % conversion and the unreacted ethyl ester was extracted with methylene chloride. The ^1H NMR spectrum was identical to that of the starting material. A portion of the ester was hydrolyzed in concentrated HCl, and then derivatized with (1*S*)-(–)-camphanic chloride. The aqueous layer, which contained the (*S*)-3-*O*-methylcarbidopa ((*S*)-**2a,b**), was buffered with sodium dihydrogen phosphate and derivatized with (1*S*)-(–)-camphanic chloride. Camphanic hydrazides derived from the unreacted ester were found to be (*R,S*)-diastereomers by HPLC analysis (YMC-pack ODS column, 4.0 × 50 mm, 3 μm; mobile phase: 35 % methanol in 50 mM sodium phosphate pH 2.7; flow rate: 1 mL/min; 280 nm detection; (*S,S*)-diastereomer t_R = 9.2 min, (*R,S*)-diastereomer t_R = 11.7 min), whereas derivatives of the acid product represented the (*S,S*)-diastereomers.

*Ethyl (R)-2-hydroxy-2-methyl-3-(3,4-dimethoxyphenyl)-propanoate ((R)-**1c**).* CLEH, CPY or CPW were added to suspensions of ethyl (±)-2-hydroxy-2-methyl-3-(3,4-dimethoxyphenyl)propionate ((±)-**1c**) (51.4–333.0 mg, 0.19–1.24 mmol) in 50 mL of 50 mM sodium phosphate buffer pH 7.5. After the reaction times indicated in Table 3, the reaction mixtures were filtered through Celite and then extracted with 2 × 25 mL ethyl acetate. The combined extracts of each reaction were treated with brine, dried with magnesium sulfate, then concentrated *in vacuo* to give ethyl (*R*)-2-hydroxy-2-methyl-3-(3,4-dimethoxyphenyl)-propionate ((*R*)-**1c**) in 12–38 % yield. The ^1H NMR spectrum was identical to that of the starting material. The enantiomeric excesses of the unreacted esters were determined by HPLC analysis (Chiracel OD column, 4.6 × 250 mm, 10 μm; mobile phase: 10 % isopropanol in *n*-hexane; 2 mL/min flow rate; 254 nm detection; (*S*)-enantiomer t_R = 6.6 min and (*R*)-enantiomer t_R = 8.3 min).

*(R)-α-Methyltryptophan methyl ester ((R)-**1e**).* To suspensions of (±)-α–methyltryptophan methyl ester ((±)-**1e**) (79.5–150.0 mg, 0.342–0.646 mmol) in 50 mL of 50 mM sodium phosphate buffer pH 7.5 were added carboxypeptidases Y, W or P. The resulting suspensions were stirred at ambient temperature. After the reaction times specified in Table 3, the reactions were each extracted with 2 × 25 mL chloroform. The combined extracts of each reaction were treated with brine, dried with magnesium sulfate and concentrated *in vacuo* to yield (*R*)-α–methyltryptophan methyl ester ((*R*)-**1e**) in 10–39 % yield. Portions of the α-methyl amino esters were derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT). HPLC analysis (YMC-pack ODS-AQ column, 4.6 × 250 mm, 5 μm; mobile phase: 55 % methanol in 50 mM sodium phosphate pH 2.7; flow rate:

1 mL/min; detector wavelength: 254 nm; (*S,S*)-diastereomer t_R = 15.9 min and (*R,S*)-diastereomer t_R = 16.8 min) indicated an excess of the (*R,S*)-diastereomer in all experiments.

Methyl (S)-α-difluoromethylphenylalanate ((S)-1g). CLEH, *C. lipolytica* lipase or CPY were added to suspensions of methyl (±)-α-difluoromethylphenylalanate ((±)-**1g**) (146–500 mg, 0.64–2.18 mmol) in 100 mL 50 mM sodium phosphate buffer pH 7.5. The resulting suspensions were stirred at 35 °C and maintained at pH 7.3 by dropwise addition of 2 N KOH as necessary for the specified times (Table 3). The progress of the reactions was monitored by reversed-phase HPLC. To each reaction was added Celite (21 g), 100 mL deionized water, and 100 mL chloroform. The resulting suspensions were filtered and the layers of the filtrates were separated. The aqueous layers were re-extracted with 1 × 100 mL chloroform. The combined extracts of each reaction were treated with a brine wash, dried over magnesium sulfate, and then concentrated *in vacuo* to afford methyl (S)-α-difluoromethylphenylalanate ((*S*)-**1g**) in 7–39 % yield. The ^{1}H NMR spectrum was identical to that of the starting material. The enantiomeric excesses of the unreacted amino esters were determined by HPLC analysis (Chiracel OD column, 4.6 × 250 mm, 10 μm; mobile phase: 2.5 % isopropanol in *n*-hexane; flow rate: 1 mL/min; 254 nm UV detection; (*S*)-enantiomer t_R = 8.3 min and (*R*)-enantiomer t_R = 9.4 min).

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