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Creation of a Synthetically Useful Lipase with Higher Than Wild-Type Enantioselectivity and Maintained Catalytic Activity

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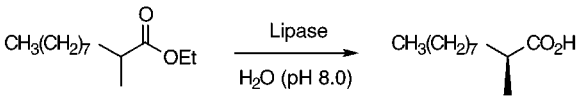
ABSTRACT



Wild-type I: 89.9% *ee* (*E* = 32)
Wild-type II: 79.8% *ee* (*E* = 10)
Lipase hybrid: 95.4% *ee* (*E* = 54)

We have found that two *Geotrichum candidum* lipase isozymes have remarkably different abilities to differentiate between enantiomers of ethyl 2-methyldecanoate. By rational recombination of selected portions of the two isozymes, we have created a novel lipase with an enantioselectivity superior to that of the best wild-type parent isozyme. Site-directed mutagenesis identified two key amino acid residues responsible for the improved enantioselectivity without compromised total activity of the reengineered enzyme.

Lipase catalysis provides one of the best routes to obtain enantiomerically pure building blocks for the synthesis of structurally complex molecules with controlled stereochemistry.[1] A main obstacle, however, encountered by synthetic chemists is the difficult task of identifying a suitable lipase for a given synthetic application. To facilitate this selection process, empirical rules have been developed for some lipases to predict the fastest-reacting enantiomer of chiral alcohols[2] and carboxylic acids.[3]

The structural basis for lipase enantiorecognition is not well enough understood to allow for the rational creation of better than wild-type enzymes. In fact, only a few examples of improved lipase/esterase enantioselectivity through protein engineering methods such as site-directed mutagenesis,[4] in vitro directed evolution,[5] or chemical modification[6] have been reported. An enzyme with an enantiomeric ratio (*E*)[7] below 15 is usually considered to be unacceptable for practical purposes, while *E* values of 15−30 are regarded as moderate, and above this value excellent.[8]

The fungus *Geotrichum candidum* produces two lipase isozymes (GCL II and I).[9] The gene codings for these

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(2) Kazlauskas, R. J.; Weissfloch, A. N. E.; Rappaport, A. T.; Cuccia, L. A. *J. Org. Chem.* **1991**, *56*, 2656−2665.

(3) Ahmed, S. N.; Kazlauskas, R. J.; Morinville, A. H.; Grochulski, P.; Schrag, J. D.; Cygler, M. *Biocatalysis* **1994**, *9*, 209−225.

(4) Holmquist, M.; Martinelle, M.; Berglund, P.; Clausen, I. G.; Patkar, S.; Svendsen, A.; Hult, K. *J. Protein Chem.* **1993**, *12*, 749−757.

(5) (a) Reetz, M. T.; Zonta, A.; Schimossek, K.; Liebeton, K.; Jaeger, K.-H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2830−2832. (b) Bornscheuer, U. T.; Altenbuchner, J.; Meyer, H. H. *Biotechnol. Bioeng.* **1998**, *58*, 554−559.

(6) (a) Gu, Q.-M.; Sih, C. J. *Biocatalysis* **1992**, *6*, 115−126. (b) Tuomi, W. V.; Kazlauskas, R. J. *J. Org. Chem.* **1999**, *64*, 2638−2647.

(7) The ability of an enzyme to distinguish between two competing enantiomeric substrates is defined by the enantiomeric ratio, *E*. This is the ratio between the specificity constants (k_{cat}/K_M) of the enzyme for the two competing enantiomers denoted *S* and *R*; $E = (k_{cat}/K_M)_S/(k_{cat}/K_M)_R$.

(8) Reference 1b, pp 39−40.

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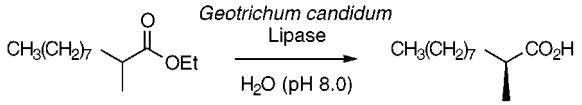
enzymes were cloned and expressed in yeast *Pichia pastoris* as previously described.[10] This expression system allows for the production of recombinant GCLs in high yield and purity.[10,11] The enantiomeric ratios, *E* values, of the purified recombinant GCLs were measured toward emulsified racemic ethyl 2-methyldecanoate[12] (Scheme 1). The free acid produced in this model reaction belongs to a class of 2-methyl-branched carboxylic acids that are versatile building blocks for the synthesis of chiral drugs,[13] pheromones,[14] and liquid crystals.[15]

Scheme 1



Enzyme activities were measured with a pH-stat instrument (TIM900 Titration Manager, Radiometer, Copenhagen, Denmark) at pH 8.0 and 25 °C. Reactions were run under nitrogen to no more than 40% conversion, which allows for the most accurate *E* value determination. The enantiomeric excess (ee) of the released 2-methyldecanoic acid was then determined by gas chromatography[16] after derivatization to the corresponding diastereomeric phenylethyl amides obtained from reaction with enantiomerically pure (*R*)-1-phenylethylamine as previously described.[17] The *E* values were calculated from the ee of the product (ee_p) and the extent of conversion (*c*) according to $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.[18]

From these experiments, we discovered that the GCL isozymes have distinctively different enantioselectivities in our model reaction despite their high (86%) amino acid sequence identity of the 544 residues constituting the full-length lipases. While GCL II showed a moderate enantiomeric ratio of $E = 10$, GCL I exhibited a significantly higher enantioselectivity of $E = 32$. The latter represents, to the best of our knowledge, one of the highest reported enantiomeric ratios of a lipase catalyzing the hydrolysis of a 2-methyldecanoic acid ester. As a comparison, the closely structurally related *Candida rugosa* lipase shows a very low enatiomeric ratio, $E = 1.4$, for the same substrate.[19]

To create a GCL with better than wild-type enantioselectivity, we have explored a method based on swapping homologous regions between related enzymes to yield a protein with modified functionality compared to the parental enzymes.[20] Selected segments of the two isozyme genes were recombined, fractional isozyme recombination, to create full-length genes coding for a series of novel lipase variants. A schematic representation of the constructed and characterized lipases are shown in Figure 1.

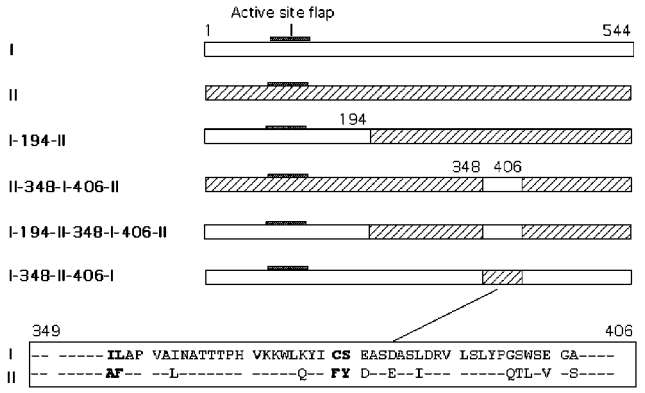


Figure 1. Top: Schematic representation of *G. candidum* lipases used for the analysis of enantioselectivity determinants. The names of the constructs are listed to the left, and numbers indicate the amino acid residue at the N-terminal side of the junction between the lipase isoenzymes. Bottom: Comparison of amino acid residues within positions 349−406 in *G. candidum* lipases I and II. Residues targeted by site-directed mutagenesis (357, 358, 379, and 380) are shown in bold.

We focused on two regions in the GCL molecule: (i) the flap (residues 61−105) believed to regulate substrate access to the active site and involved in conformational rearrangements during interfacial activation[21] and (ii) a segment (residues 349−406) known to control fatty acyl chain length specificity (FAS) of the enzyme.[11] These regions, intimately connected to lipase function and substrate differentiation, seemed to be suitable target segments to exchange between the isozymes.

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(11) Holmquist, M.; Tessier, D. C.; Cygler, M. *Biochemistry* **1997**, *36*, 15019−15025.

(12) For the synthesis of racemic 2-methyldecanoic acid, see: (a) Allen, C. F.; Kalm, M. J. In *Organic Synthesis*; Rabjohn, R., Ed.; Wiley: New York, 1963; Vol. 4, Note 2, p 618. For the synthesis and characterization of racemic ethyl 2-methyldecanoate, see: (b) Berglund, P.; Holmquist, M.; Hedenström, E.; Hult, K.; Högberg, H.-E. *Tetrahedron: Asymmetry* **1993**, *4*, 1869−1878.

(13) See, for example: Sheldon, R. A. *Chirotechnology: Industrial Synthesis of Optically Active Compounds*; Marcel Dekker: New York, 1993.

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(15) Bydén, M.; Edlund, H.; Berglund, P.; Lindström, B. *Prog. Colloid. Polym. Sci.* **1997**, *105*, 360−364.

(16) Perkin-Elmer 8420 gas chromatograph equipped with a CPSIL-5CB capillary column, 25 m × 0.32 mm i.d., df = 1.2 μm (Chrompack) using helium as carrier gas (1.7 mL/min, split 1:20).

(17) (a) Sonnet, P. E. *J. Org. Chem.* **1987**, *52*, 3477−3479. (b) Reference 12b.

(18) Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299.

(19) Berglund, P.; Holmquist, M.; Hult, K. *J. Mol. Catal. B: Enzym.* **1998**, 283−287.

(20) For a recent review see: Nixon, A. E.; Ostermeier, M.; Benkovic, S. B. *Trends Biotechnol.* **1998**, *16*, 258−264.

(21) Schrag, J. D.; Cygler, M. *J. Mol. Biol.* **1993**, *230*, 575−591.

(22) (a) Plasmid YpDC429 was created by: Bertolini, M. C.; Schrag, J. D.; Cygler, M.; Ziomek, E.; Thomas, D. Y.; Vernet, T. *Eur. J. Biochem.* **1995**, *228*, 863−869. (b) The gene coding for GCL I-194-II was created by cloning the SfiI-Eco91I DNA fragment (1035 bp) from plasmid YpDC429 into plasmid YpDC523 to yield plasmid YpMH429 (9846 bp).

The GCL I-194-II lipase hybrid was created.[22] This lipase variant contains the 194 N-terminal residues of GCL I, including the flap, fused to residues 195−544 of GCL II (Figure 1). The GCL I-194-II construct showed an enantioselectivity of $E = 14$, somewhat higher than that of GCL II (Table 1). The introduction of the FAS segment (residues 349−406) from GCL I into GCL II resulted in the GCL II-348-I-406-II lipase.[11] That lipase displayed an enantiomeric ratio of $E = 16$, slightly higher than that of GCL I-194-II. In GCL II, the segments spanning residues 1−194 (including the flap) and 349−406 were then exchanged for the corresponding residues of GCL I.[23] The enzymatic characterization of this lipase hybrid, GCL I-194-II-348-I-406-II, resulted in an enantiomeric ratio of $E = 20$ intermediate to that of the two wild-type GCLs. Its overall enzymatic activity toward the racemic model substrate was 50% and 30% of GCL I and II, respectively. These experiments showed that the different enantioselectivities of the wild-type GCLs could not be explained only by amino acid differences within the exchanged segments. The introduction of the flap segment, the FAS segment, or both of these segments from GCL I into the GCL II scaffold led to lipase variants with *E* values and total activities intermediate to those of the wild-type parent isozymes. The exchange of residues between homologous regions of related enzymes usually results in enzymes with intermediate properties of the parental proteins.[20] Contrasting this general behavior of reengineered enzymes, we found that the GCL I-348-II-406-I hybrid[11] had a chiral recognition ability of $E = 54$, surpassing that of the best wild-type parent isozyme ($E = 32$).

Table 1. Enantioselectivities (*E*) and Initial Rates of Recombinant *G. candidum* Lipase Variants in the Hydrolysis of Emulsified Racemic Ethyl 2-Methyldecanoate at pH 8.0 and 25 °C

lipase	ee_p (%)	conversion (%)	initial rate (μmol min^{-1} mg^{-1})[a]	E^a
wild types				
I	89.8	37	0.19	32
II	79.8	15	0.27	10
hybrids				
I-194-II	85.9	5.7	0.18	14
II-348-I-406-II	88.6	5.6	0.14	16
I-194-II-348-I-406-II	88.6	17	0.10	20
I-348-II-406-I	95.4	20	0.10	54
site-directed mutants				
I Ile357Ala/Leu358Phe/ Cys379Phe/Ser380Tyr	95.6	9.0	0.14	49
I Cys379Phe/Ser380Tyr	91.7	10	0.08	26
I Ile357Ala/Leu358Phe	95.5	19	0.18	54

[a] *E* was calculated from $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$[18] from ee of the product (ee_p) and the extent of conversion (*c*) given in the table, i.e., end point analysis.[24] The coefficients of variation of the *E* values and initial rates are estimated to be 10% based on duplicate experiments performed with the II-348-I-406-II construct.

Within the identified sequence portion (residues 349−406), 14 amino acid residues differ between isoenzymes I and II (Figure 1). Most of them are located at the surface of the lipase molecule.[21] Three of these residues (357, 358, and 379) presumably contribute directly to the formation of the substrate binding cavity of the enzyme. We focused our attention on GCL I and determined the entioselectivity of several site-directed mutants (Table 1). Within the identified segment, the Ile357Ala/Leu358Phe/Cys379Phe/ Ser380Tyr[11] mutations were introduced in GCL I. These substitutions had the same effect on enantioselectivity as exchanging the whole FAS segment in GCL I. Importantly, this mutated lipase retained a higher overall activity (74% of wild type) compared to the GCL I-348-II-406-I hybrid (50% of wild type). The subsequently created site-directed mutant containing the Cys379Phe/Ser380Tyr[11] replacements at the bottom of the active site cavity of GCL I lowered the overall activity of the enzyme to 40% of wild type. Yet, the enantioselectivity remained unaffected at a level of $E \approx 30$. By contrast, the Ile357Ala/Leu358Phe[11] mutations at the active site entrance resulted in a raised enantiomeric ratio from $E = 32$ to $E = 54$, thus fully accounting for the improved enantioselectivity seen in the GCL I-348-II-406-I construct. From the experimentally determined *E* value and the initial rate in the resolution (Table 1), one can calculate the rates for the individual enantiomers. The Ile357Ala/Leu358Phe mutations reduced the reaction rate for the slow-reacting *R*-enantiomer only, while the *S* enantiomer hydrolysis rate remained the same as in the wild-type lipase. We had thus improved the *E* value of GCL I with essentially preserved total activity.

In conclusion, we have discovered that the two GCL isozymes have different enantioselectivities. We have shown that by introducing a segment, corresponding to 14 point mutations (Figure 1), from one isozyme into the structural environment of the other can create a lipase with a superior enantioselectivity compared to the best wild-type parent enzyme. It is evident that a gene-shuffling strategy guided by the 3D structure of the protein is a powerful means for the fast molecular adaptation of a lipase for improved specificity toward a nonnatural substrate. We have furthermore identified key residues at two spatially remote positions in the substrate binding site of GCL I that control, independently, the enantioselectivity and the overall catalytic activity of the lipase. Besides highlighting important structural features and their relation to the function of the lipase, we anticipate that the knowledge emerging from this study will serve as a valuable guide for further region- or site-directed protein engineering efforts aiming at creating lipases better than those offered by nature.

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(23) The gene coding for GCL I-194-II-348-I-406-II was created from plasmids YpDC429 and YpDC566. The SfiI-BstEII fragment (1035 bp) from YpDC429 was cloned into the SfiI-BstEII-digested plasmid YpDC566 yielding plasmid YpMH614 (9849 bp).

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