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Cydney C. Unvala April 8, 2011

Kinetic Effects of Circular Permutation and Lid Swapping Techniques on *Candida antarctica* Lipase B

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Kinetic Effects of Circular Permutation and Lid Swapping Techniques on *Candida antarctica* Lipase B

By Cydney C. Unvala

Candida antarctica lipase B (CALB) is an enzyme of the α/β-hydrolase-fold family known to catalyze a wide variety of reactions including hydrolysis, esterification, amination, transesterification, and polymerization. This catalytic versatility, along with broad substrate specificity and enantioselective properties, make CALB an attractive target for a number of industrial applications particularly in detergents, dairy processing, and pharmaceutical synthesis. Its high industrial potential has prompted increased interest in methods of enhancing CALB's versatile functions. One means of accomplishing this has been through lid swapping. Lid swapping is a protein engineering technique that identifies the lid region on one protein and replaces it with the corresponding lid region from a homologous protein in order to alter access to the protein's active site. This technique has far-reaching implications including heightened catalytic activity and improved enantioselectivity. Similar enhancements have been established through circular permutation, an alternative technique that relocates the N- and C-terminus within a protein, preserving the protein's structure while changing its local flexibility and access to its active site. I hypothesize that performing both techniques on CALB conjointly will synergistically improve its activity and enantioselective preferences without significantly compromising its structural integrity. Using the circularly permuted variant cp283Δ7 (constructed by the Lutz group) as a base, the lid region was replaced with the lid of the CALB homologue from *Gibberella zeae*. The resulting variant, cp283Δ7-GZ, has exhibited a substantial 4-fold increase in hydrolytic activity with *p*-nitrophenol butyrate in comparison to wild-type

CALB. However, the variant's activity compared to its base structure, cp283Δ7, shows decreased activity. Nevertheless, results suggest that CALB responds reasonably well to the structural alterations introduced by both techniques and encourage further investigation of their effects on the structural stability and enantioselective properties of CALB.

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Table of Contents

1. Introduction 1
    a. Lipases 1
    b. *Candida antarctica* Lipase B 5
    c. Protein Engineering 5
2. Materials and Methods 11
    a. Materials 11
    b. Protein Engineering 11
    c. Protein Expression 11
    d. Protein Purification 12
    e. Activity Assay 13
    f. Protein Immobilization 13
    g. Active-Site Titration of Immobilized Lipase 14
3. Results 16
    a. Protein Expression 16
    b. Protein Purification and Concentration 16
    c. Activity Assay 17
    d. Protein Immobilization 18
    e. Active-Site Titration 20
4. Discussion 21
5. References 23

Table of Figures

Table 1 2

Figure 1 2

Figure 2 4

Figure 3 4

Figure 4 7

Figure 5 8

Figure 6 10

Figure 7 17

Figure 8 18

Table 2 19

Figure 9 20

Introduction

Lipases

In light of recent advances in biotechnology, lipases have come to play a pivotal role in many industrial processes. One of the most commercially significant applications of lipases has been their addition to laundry detergent.[1] Lipases are able to effectively remove fatty stains by hydrolyzing the triglycerides that compose them. Furthermore, they can retain this ability while withstanding harsh washing conditions.[2] Alternatively, pharmaceutical companies take advantage of lipase's enantioselective properties to resolve racemic drug products during synthesis. This strategy has proven particularly useful in the synthesis of naproxen, an anti-inflammatory drug. Because of the many versatile functions that lipases serve, they have become popular targets for a variety of industrial applications (Table 1).[3]

There are three important properties that contribute to the versatility of lipases: their ability to catalyze multiple types of reactions, broad substrate specificity, and enantioselectivity. In biological systems, lipases play a key role in lipid metabolism by hydrolyzing ester linkages in triglycerides.[4] However, as implied by their various applications, the catalytic abilities of lipases are not limited solely to hydrolysis. In fact, they catalyze a highly diverse range of reactions including hydrolysis, esterification, amidation, and transesterification reactions (Figure 1).[5] They have even been used to catalyze polymerization reactions.[6] Further promoting the multi-faceted capabilities of lipases is their relatively broad substrate specificity. While they show a natural preference for triglycerides, lipases can also accommodate a number of structurally diverse alcohols, esters and carboxylic acids.[7] Although lipases tolerate a variety of substrates, they have exhibited enantioselective preferences with certain chiral substrates.[7] Together, these characteristics give lipase many functional dimensions.

Industry	Action	Product or Application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy Foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery Foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food Dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health Foods	Transesterifications	Health foods
Meat and Fish	Flavor development	Meat and fish products; fat removal
Fats and Oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification; hydrolysis	Specialty lipids, digestive acids
Cosmetics	Synthesis	Emulsifiers

Table 1: Various industrial applications of lipases.[3]

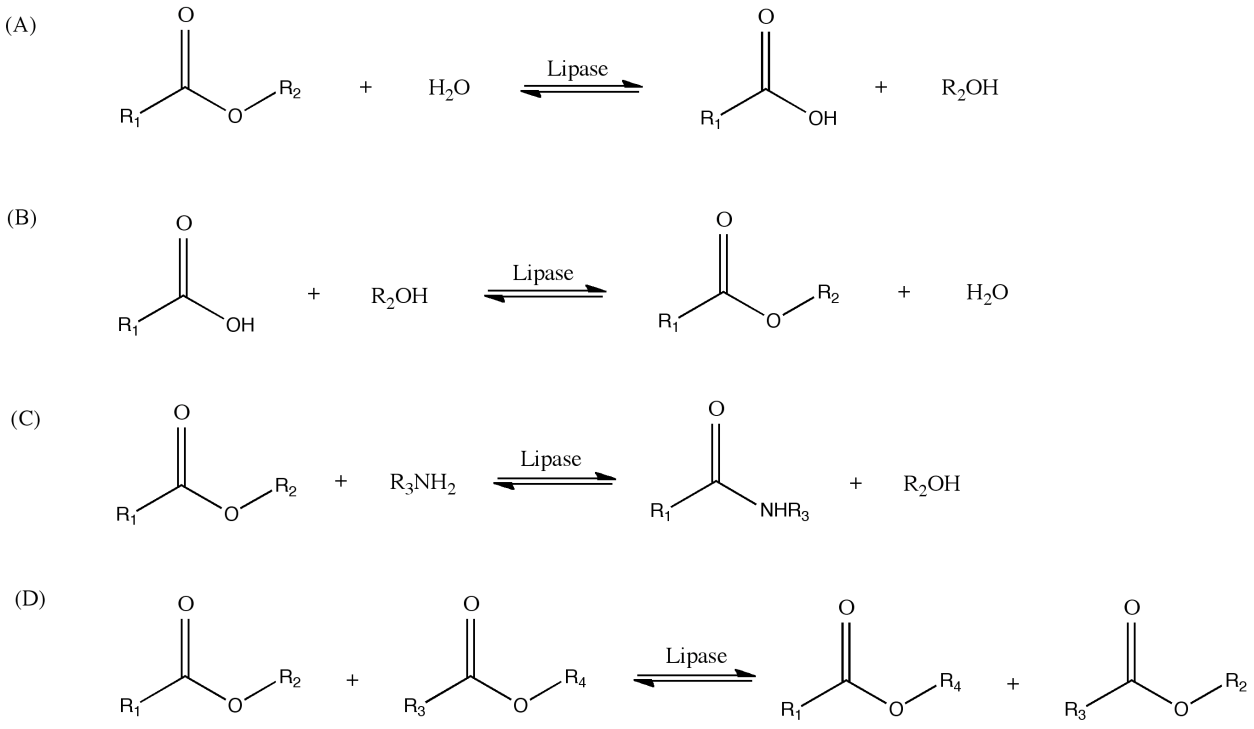


Figure 1: Lipase catalyzed reactions. (A) Hydrolysis, (B) Esterification, (C) Amination, and (D) Transesterification.[5]

Lipase's functional versatility is highly contingent on its structure. As a member of the α/β-hydrolase fold family, lipases possess a characteristic structural composition consisting of eight parallel β-sheets surrounded by α-helices on either side (Figure 2).[5] Within the protein's structural framework, these β-sheets are found to be highly twisted and bent so as to form a half-barrel shape.[8] The α/β-hydrolase fold not only provides a stable foundation for lipase structure, but also for their function – located along this fold is a nucleophile-histidine-acid catalytic triad, which is responsible for facilitating catalysis.[9,10] Lipase catalysis proceeds via a bi-bi ping-pong mechanism, which begins with the substrate acylating the enzyme. After the formation and release of the first product, water enters and hydrolyzes the acyl-enzyme intermediate. This initiates the release of the second product, preparing the enzyme for the next catalytic cycle (Figure 3).[7,11] Lipases also feature a lid region whose position and mobility influence a variety of catalytic properties. Lid regions are generally found along the carboxyl end of the α/β-hydrolase fold.[8] As this lid is strategically positioned over the enzyme's active site, conformational changes dictate substrate accessibility. Movement of the lid region from a closed arrangement to an open one is initiated by interaction at the lipid-water interface, a phenomenon known as interfacial activation.[12] Lid position additionally influences properties of the catalytic mechanism – when the lid has assumed its open confirmation, atoms forming the oxyanion become positioned for optimal stabilization of the transition state.[8] Together, the catalytic triad, lid region, and their structural proximity all contribute to the catalytic performance and high functional diversity of lipase.

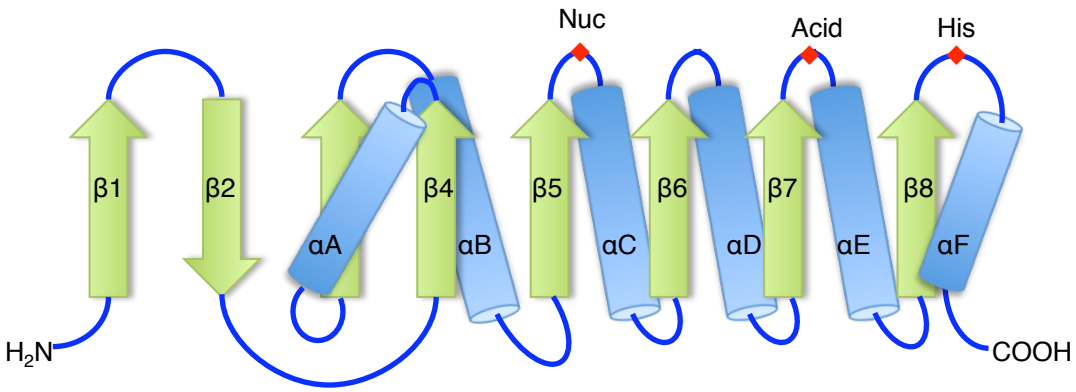


Figure 2: Diagram of the α/β-hydrolase fold. β-sheets and α-helices are indicated by green arrows and blue cylinders, respectively. "Nuc" indicates the location of a nucleophilic residue. "Acid" refers to the presence of an acidic residue. "His" marks the location of a histidine residue. Residues identified by red diamonds play an important role in lipase catalysis.[10]

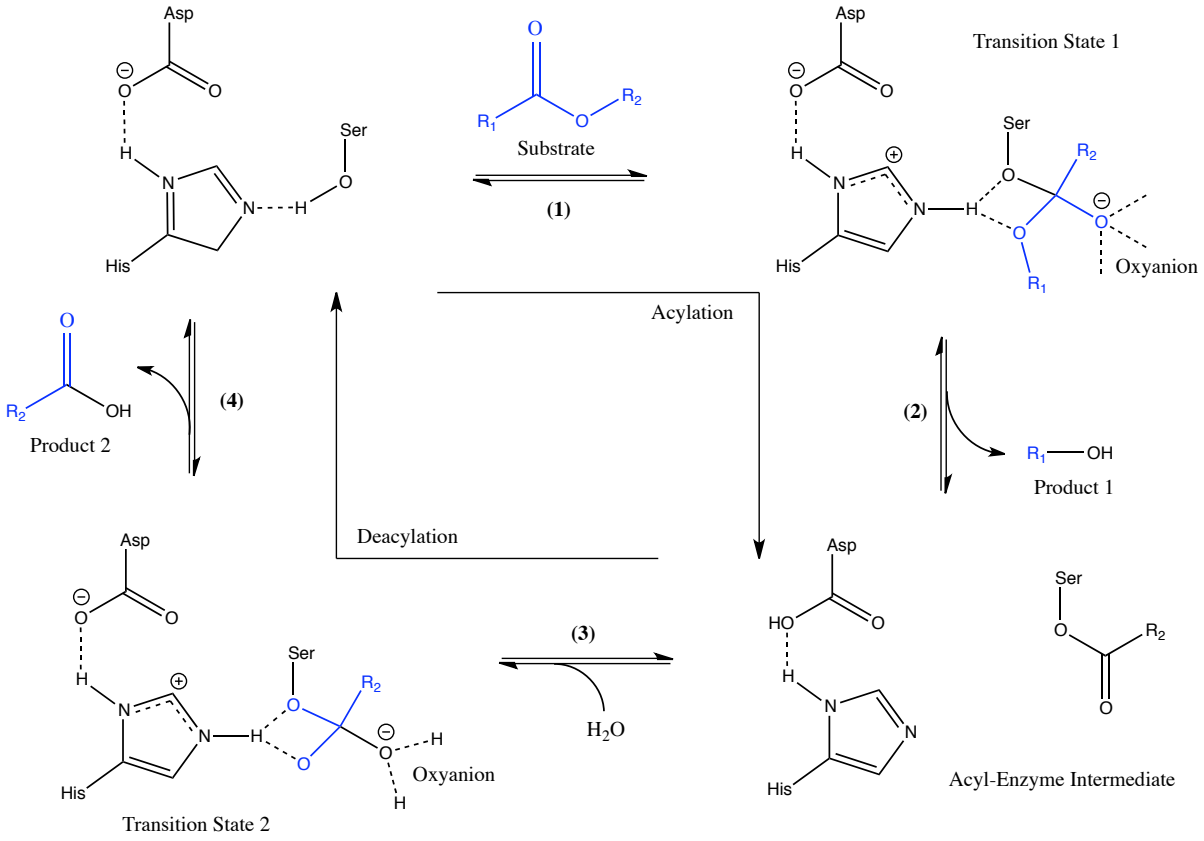


Figure 3: General reaction mechanism for lipase catalyzed hydrolysis.[8]

Candida antarctica Lipase B

A particularly important and extensively used biocatalyst is *Candida antarctica* lipase B (CALB), isolated from a strain of yeast in Antarctica for its highly robust nature.[13] Structurally, CALB exists as a monomer, comprised of 317 amino acids with a molecular weight of approximately 33 kDa.[5] CALB possesses the structurally conserved α/β-hydrolase fold along with a small lid region, identified as a short α-helix (residues 142-146) termed α5.[14]

In CALB, many of the versatile qualities expressed by lipases are observed to be significantly enhanced. CALB can maintain high catalytic performance in a wide variety of conditions. For instance, CALB can catalyze reactions in both aqueous and organic environments as well as sustain a wide range of pHs (3.5-9.5) without significant loss of activity.[13] Like other lipases, CALB accepts a wide range of substrates. However, CALB has shown poor accommodation for carboxylic acids and secondary alcohols that possess large functional groups or exhibit significant steric hindrance.[15] Tertiary alcohols are also poor substrate candidates for CALB due to their bulky nature.[16] In addition to its impressive catalytic performance and stability, CALB also expresses notable stereo- and enantioselectivity with chiral secondary alcohols and esters. CALB's enantioselective abilities are directed by the Kazlauskas rule, which predicts higher enantioselectivity for chiral substrates containing substituent groups of different size than those of similar size.[7] Overall, the qualities expressed by CALB represent the best of lipase versatility.

Protein Engineering

Because of its vast applications and high industrial potential, improving the activity, thermostability, and enantioselectivity of CALB has been a source of great interest. One way

researchers have attempted to manipulate these properties is through mutagenesis. In one study, error-prone PCR was used to introduce amino acid changes that would increase thermostability in CALB. Two resulting enzyme variants exhibited a 20-fold increase in half-life at 70 °C when compared to the wild-type.[17] Another study employed DNA family shuffling to generate mutants with various amino acid substitutions in an attempt to improve CALB's hydrolytic activity. Several mutants showed between 5- and 20-fold improvements in hydrolytic activity toward a prochiral diester substrate when compared to the wild-type.[18] However, engineering techniques that work to directly manipulate protein structure appear to be the most promising means of enhancing CALB's stability and catalytic properties.

One way to directly alter a protein's structure is through circular permutation. Circular permutation is an engineering technique that reorganizes the protein's primary sequence by covalently linking its original N- and C-termini with a peptide sequence and creating new termini elsewhere along the protein (Figure 4A).[9,10,19] This technique is particularly advantageous because it allows the protein's backbone flexibility and active site accessibility to be altered without changing the amino acid composition of the protein. In their study, the Lutz group performed random circular permutation on CALB and found that 20% of the variants generated retained hydrolytic activity.[20] One variant in particular, cp283, showed remarkable rate enhancements in hydrolytic activity of 11-fold and 175-fold for *p*-nitrophenol butyrate (*p*-NB) and 6,8-difluoro-4-methylumbelliferyl (DiFMU) octanoate, respectively.[9,10] While circular permutation has been proven to yield substantial improvements in catalytic performance of CALB, structural analysis by circular dichroism spectroscopy suggests that it also introduces substantial destabilization.[9] However, incremental truncation of the linker sequence used to connect the original N- and C-termini, which involves altering the length of the resulting loop

region (Figure 4B), was partly responsible for preserving the structural stability of the circularly permuted CALB variant. Crystallographic data revealed the formation of a cp283Δ7 homodimer as a result of the 7-residue deletion from the loop sequence.[20] Through its ability to dimerize, cp283Δ7 could retain greater structural stability. Additionally, the data indicates that the improved catalytic activity exhibited by cp283Δ7 is the result of a conformational change to the active-site binding pocket, transforming from a restrictive tunnel to a wider, more accessible crevice (Figure 5).[20]

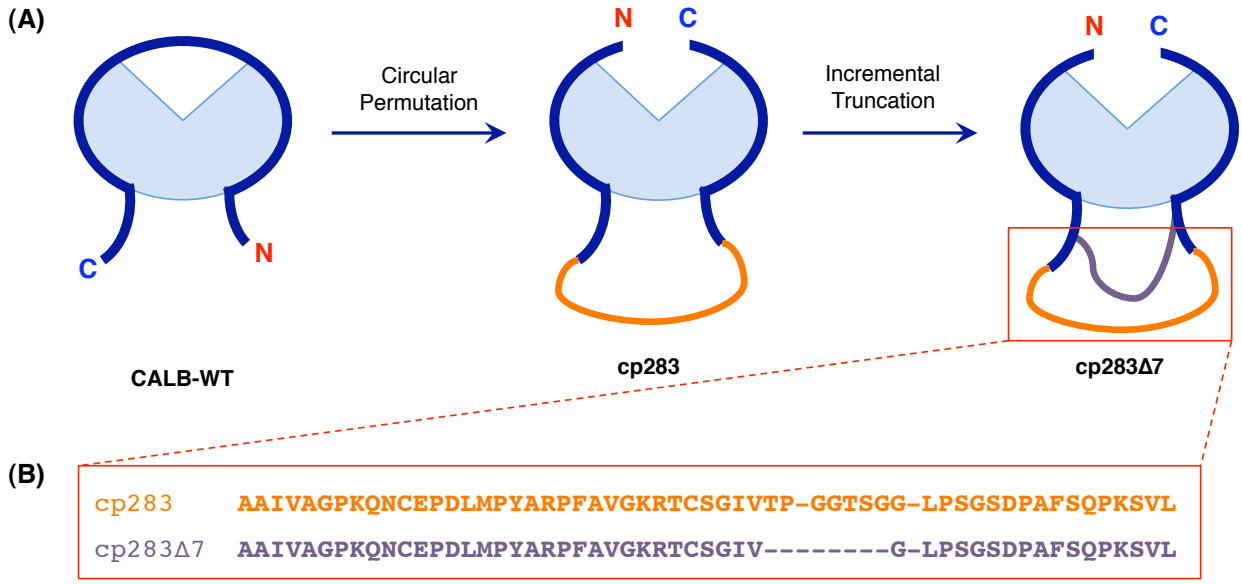


Figure 4: (A) Illustration of CALB engineering via circular permutation followed by incremental truncation on the loop region of the cp283 variant. Loop regions for cp283 and cp283Δ7 are depicted in orange and purple, respectively. (B) Amino acid sequence of loop region resulting from circular permutation for cp283 and cp283Δ7.[20]

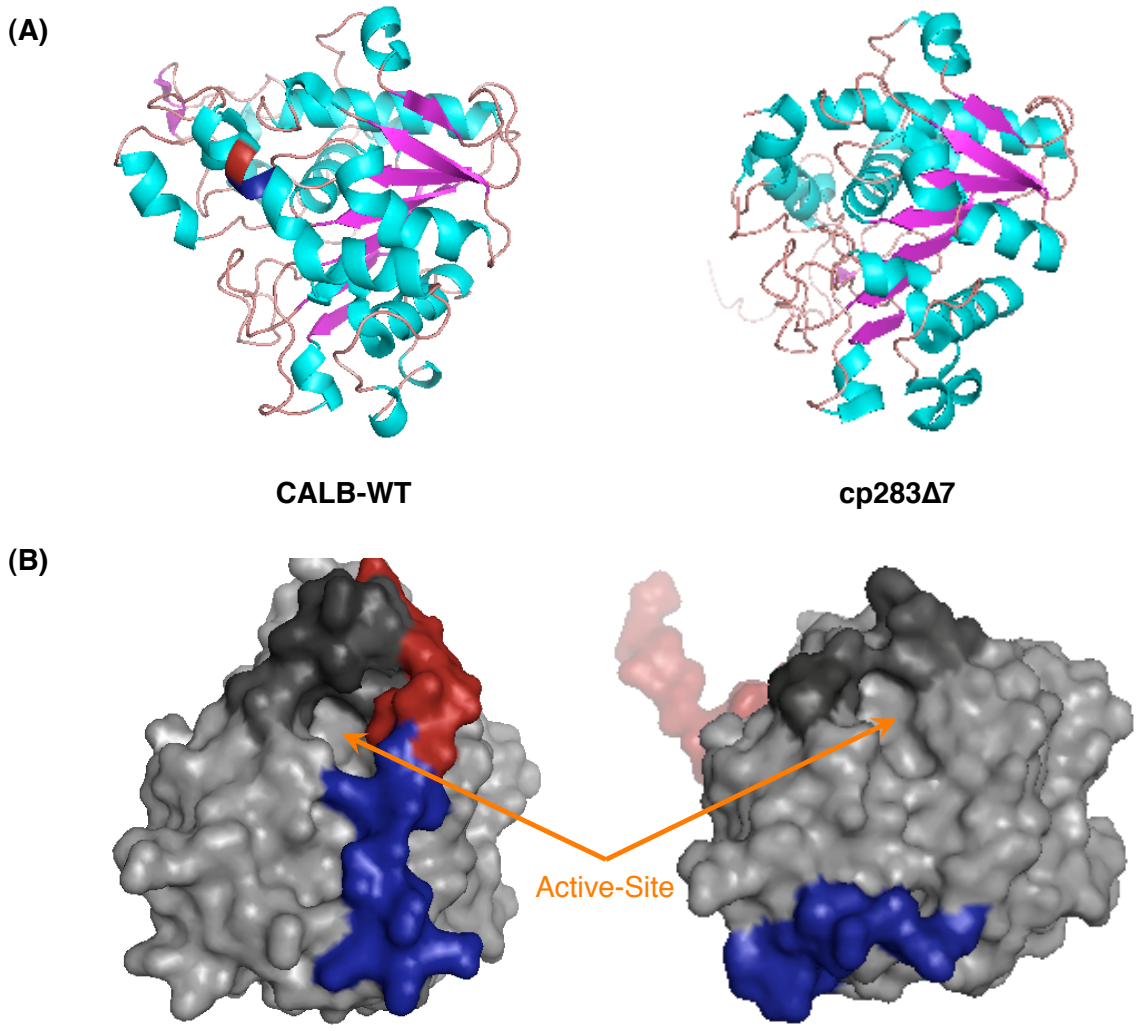


Figure 5: Image (A) depicts the ribbon structures for wild-type CALB (left) and cp283Δ7 (right). β-sheets and α-helices are colored in pink and blue, respectively. The location of new the N and C-termini observed in cp283Δ7 after circular permutation are depicted in red and blue, respectively, on wild-type CALB. Image **(B)** shows the surface structures of both wild-type CALB (left) and cp283Δ7 (right). N and C-termini regions are colored in red (N-terminus) and blue (C-terminus). The dark grey colored areas represent respective lid regions for both images. Orange arrows highlight both active-sites.[20] The images for wild-type CALB and cp283Δ7 were generated from PDB files 1TCA and 3ICW, respectively.

While circular permutation focuses on creating greater structural flexibility in order to enhance various catalytic properties, lid swapping presents an alternative engineering technique aimed at specifically altering accessibility to the protein's active site.[16] Skjøt and co-workers accomplished this by replacing CALB's lid region with those from other lipase homologues isolated from several different species (Figure 6). Of the homologues investigated, those corresponding to *Gibberella zeae* and *Neurospora crassa* generated variants that showed increased hydrolytic activity for a variety of ester substrates, particularly those containing C_α branching on the carboxylic end.[16] The improved tolerance for bulky substrates observed in these variants suggested that their active-sites had become more accessible as a result of lid swapping. Additionally, the Skjøt group reported a remarkable increase in the enantioselectivity of both variants towards the hydrolysis of racemic ethyl 2-phenylpropanoate, a core structure of the profen family.[16] Since the wild-type is known to show no enantioselective preferences towards this substrate, their findings also suggested that lid replacement with these homologues introduced highly beneficial substrate-specificity properties. Overall, this technique been proven to permit improvements in substrate selectivity and catalytic activity without compromising the enzyme's entire structure.

Figure 6: The above image depicts the amino acid sequences corresponding to lid regions (including important flanking residues) from the CALB wild-type, *G. zeae*, and and *N. crassa*. The lid sequences from *G. zeae* and *N. crassa* will replace the CALB lid sequence. Refer to Figure 5B to locate the region where the replacement occurs (depicted in dark grey). Highlighted in blue are amino acids conserved from CALB in both lid sequences. Those highlighted in green and pink represent amino acids conserved in lid regions from *N. crassa* and *G. zeae*, respectively.

Since both techniques alter protein structure and function via different routes, I predict that circular permutation performed in conjunction with lid swapping will introduce synergistic enhancements to the catalytic properties of CALB. To explore this, we replaced the lid region from cp283Δ7 with the homologue from *Gibberella zeae*. Because of its structural similarities to wild-type CALB (Figure 5A) and its dimer-enhanced stability, the cp283Δ7 variant could serve as a suitable basis for lid swapping while maintaining structural integrity and improved catalytic properties. The homologous *G. zeae* lid region appeared to be the best candidate based on kinetic data presented by the Skjøt group showing a consistent increase in hydrolytic activity relative to wild-type CALB.[16] Additionally, lid sequences from both wild-type CALB and *G. zeae* shared similar lengths and showed a generally high conservation in amino acid (Figure 6). Performing both techniques on CALB concurrently will allow us to better understand the structural features that influence various functions.

Materials and Methods

Materials

For protein purification, butyl-sepharose 4 resin was purchased from AmershamBiosciences (Piscataway, NJ). SDS-Page Molecular Weight Standard (Catalog Number 161-0317) was purchased from Bio-Rad Laboratories (Hercules, CA). Concentration of purified protein was determined on a Varian Cary Bio-100 UV-Visible Spectrometer (MD, USA). Activity assay was conducted with *p*-nitrophenol butyrate (*p*-NB), purchased from Sigma-Aldrich (St. Louis, MO), on a Synergy-HT microtiterplate reader (Bio-Tek Instruments, Winooski, VT). The Lewatit VP OC 1600 ion exchange resin used for protein immobilization was a gift from SYBRON Chemicals Inc. (Birmingham, NJ). Immobilization and active-site titration were conducted on a head-over-head shaker. Active-site titration analysis was performed on a JY-Horiba spectrofluorimeter (Edison, NJ). Methyl 4-methylumbelliferyl hexylphosphonate was synthesized by Dr. Priti Soni in the Lutz laboratory.

Protein Engineering

The cp283Δ7-GZ lid variant was constructed via overlap PCR by Ying Yu. The variant was then cloned into a pPIC9 vector between the *Xho* I and *Hind* III sites and its construction confirmed by sequencing analysis.

Protein Expression

P. pastoris cells containing the cp283Δ7-GZ lid variant were plated on MD His^- plates and incubated at 30 °C. Colonies appeared after 1-2 days. One colony was chosen for inoculation in 50 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH

6.0), 1.34% YNB, 4 x 10^{-5}% biotin, and 1% glycerol) and incubated at 30 °C until it reached an OD_{600} of 0.4-0.6. The culture was centrifuged (3,500 rpm, 4 °C, 5 min) in order to isolate the cells, which were then re-suspended in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4 x 10^{-5}% biotin, and 0.5% methanol) to an OD_{600} of 0.1 and incubated at 20 °C. Protein expression was induced by adding methanol to a final concentration of 0.5% every 24 hours for 2 days. The culture was again centrifuged (3,500 rpm, 4 °C, 10 min) and the supernatant containing the lipase variant was collected and subjected to vacuum filtration.[9]

Protein Purification

Protein purification was accomplished using hydrophobic interaction chromatography (HIC) at 4 °C. The protein culture collected from overexpression was subjected to vacuum filtration. The culture was then mixed with 4 M $(NH_4)_2SO_4$ solution and 1 M potassium phosphate buffer (pH 7.0) to a final concentration of 1 M and 50 mM, respectively. The culture was loaded on the HIC column (7 mL butyl-sepharose 4 resin [AmershamBiosciences, Piscataway, NJ]) pre-equilibrated with 1 M $(NH_4)_2SO_4$, 50 mM potassium phosphate buffer (pH 7.0). After protein loading, the column was washed with 4 volumes of pre-equilibrium buffer, followed by a stepwise reduction of $(NH_4)_2SO_4$ in the phosphate buffer (0.2 M increments, 4 column volumes per step). Lipase activity in the eluent was monitored by p-NB hydrolysis and fractions containing protein were collected and concentrated by ultrafiltration via an Amicon Ultra-15 centrifugal filter unit (Millipore, Bedford, MA: 3,800 rpm, 4 °C, 10 min).[9] To determine protein concentration, a 2 μL sample of purified protein was diluted to a volume of

100 μL with 50 mM potassium phosphate buffer and analyzed by UV-visible spectroscopy. Protein purity was verified by SDS-Page analysis. Remaining protein was stored at -80 °C.

Activity Assay

The activity of the lipase variant was determined by measuring the initial rate of hydrolysis for *p*-NB conducted at room temperature on a Synergy-HT microtiterplate reader (Bio-Tek Instruments, Winooski, VT). A 200 mM stock solution of *p*-NB was prepared in DMSO. Hydrolysis was measured in 50 mM potassium phosphate buffer (pH 7.5) with the substrate concentration varying from 0-1.6 mM at 400 nm (ε for *p*-NB = 13,260 $M^{-1}cm^{-1}$; length of the plate well was determined to be 0.66 cm).[21] Kinetic constants were determined by fitting initial hydrolysis rates to the Michaelis-Menten equation using Origin® software (version 7; OriginLab Corporation).[9]

Protein Immobilization

Two methods for lipase immobilization were explored, both of which used cation exchange resin Lewatit VP OC 1600 (SYBRON Chemicals Inc.) as the organic suspension. Both methods are described below.

Method 1:

Approximately 1.5 g resin was added to 50 ml the supernatant isolated from protein expression and was incubated in a head-over-head shaker at 4 °C until the resin had become fully saturated. Residual lipase activity in the solution was monitored using p-NB as a substrate. Once the enzyme had been fully immobilized (indicated by low residual activity in the solution), the

resin was washed several times with 50 mM potassium phosphate buffer (pH 6.0), dried under vacuum, and stored at 4 °C.[9]

Method 2:

Prior to immobilization, 4-5 g of resin was washed in 1 M potassium phosphate buffer (pH 6.4) and dried under vacuum. When dry, the resin was then stored at 4 °C. Additionally, 1 mL of purified protein, which was stored in 50 mM potassium phosphate buffer (pH 7.0), was re-suspended in 50 mM potassium phosphate buffer (pH 6.4) by buffer exchange via ultrafiltration using an Amicon Ultra-15 centrifugal filter unit (Millipore, Bedford, MA: 3,800 rpm, 4 °C, 10 min) and diluted with the exchange buffer to a volume of 10 mL. The protein suspension was added to a 15 mL centrifuge tube, containing approximately 1.5 g of washed resin and incubated in a head-over-head shaker at 4 °C until the resin had become fully saturated. Residual lipase activity in the solution was monitored using p-NB as a substrate. Once the enzyme had been fully immobilized (indicated by low residual activity in the solution), the resin was washed several times with 50 mM potassium phosphate buffer (pH 6.4) to remove any remaining soluble protein, dried under vacuum, and stored at 4 °C.[22]

Active-Site Titration of Immobilized Lipase

The amount of active lipase immobilized on resin was determined by an active site titration assay. The active-site inhibitor, methyl 4-methylumbelliferyl hexylphosphonate, was synthesized by Dr. Priti Soni. Stock solution of the inhibitor (0.3 mM) was prepared in acetonitrile and stored at -20 °C. For the titration assay, 6 different amounts of immobilized enzyme was added to a 1 mL reaction solution consisting of 30 µL inhibitor stock solution diluted in 970 µL acetonitrile. The inhibition reactions were incubated on a shaker at room

temperature for a week until the daily fluorescence reading remained unchanged. A 15 μL aliquot of the reaction mixture was taken and mixed with 135 μL Tris-HCl buffer (50 mM, pH 8.0) and the concentration of 4-MU released was determined by measuring its fluorescence intensity on a JY-Horiba spectrofluorimeter (Edison, NJ: excitation 360 nm, emission 450 nm). The amount of active lipase on the resin was calculated with reference to the linear relationship between the amount of immobilized enzyme and the amount of 4-MU released.[9]

Results

Protein Expression

Cells containing the cp283Δ7-GZ lid variant were grown in BMGY media for 18-24 hours at 30 °C. Expression of cp283Δ7-GZ lid was induced upon transferring the cells to BMMY media, which were then left to incubate for another 48 hours at 20 °C. Expression of the variant was confirmed using *p*-NB to indicate hydrolytic activity within the BMMY media.

Protein Purification and Concentration

For purification, an HIC column was used to take advantage of the hydrophobic nature of the cp283Δ7-GZ lid variant. After the protein had been loaded into the column, the eluate was tested with *p*-NB for hydrolytic activity. Negative results indicated that the protein had bound to the HIC resin. The same test was performed on the eluate from each stepwise reduction of $(NH_4)_2SO_4$ concentration in 50 mM potassium phosphate buffer (pH 7.0). No enzymatic activity was detected until a concentration of 0.2 M $(NH_4)_2SO_4$ had been reached, indicating that elution of the protein from the column had begun. Purifications thereafter were conducted using 0.3 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer to remove impurities and 50 mM potassium phosphate buffer to elute the purified protein. Elutant containing active enzyme was collected and concentrated down to a volume of 1 mL. SDS-Page analysis verified successful purification by the HIC method (Figure 7A). Purification yielded protein concentrations ranging anywhere from 0.68-5.2l mg/mL, which was determined by UV spectrometry (Figure 7B).

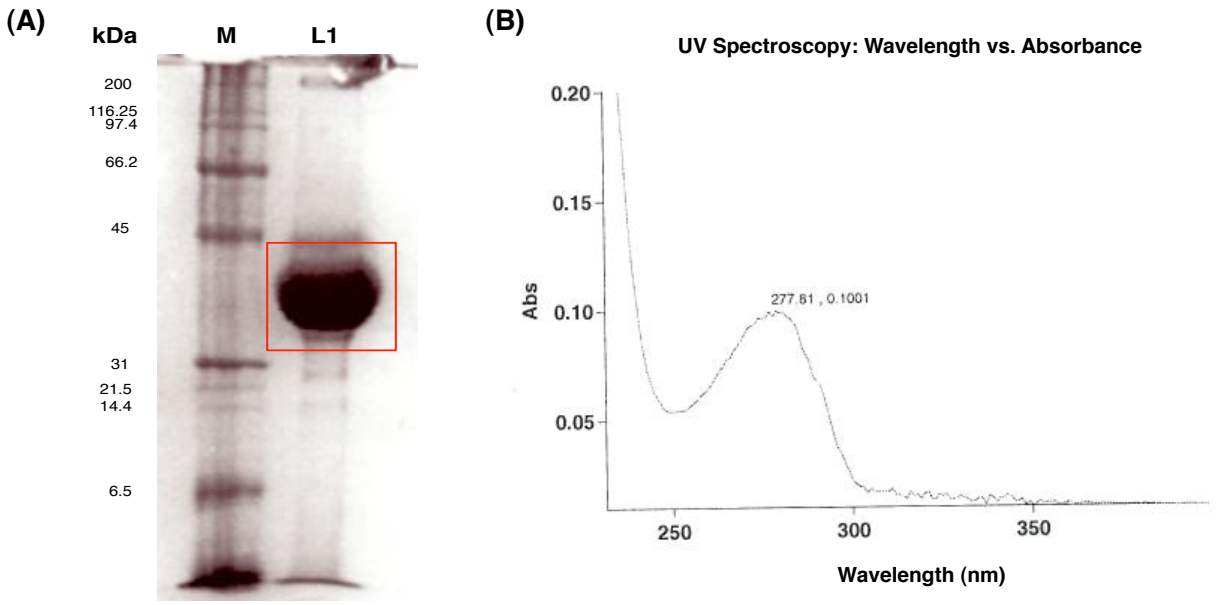


Figure 7: (A) SDS-Page analysis of protein purification. The first lane, labeled "M" contains SDS-Page molecular weight standard (catalog number 161-0317 [Bio-Rad Laboratories, Hercules, CA]). The second lane, marked "L1" contains the purified variant. The red box indicates the location of the desired protein. (B) Absorbance spectrum of purified protein. Measurements were taken in triplicate. Protein concentration was calculated using the equation,

$$C_{avg} = \frac{A_{avg}}{\varepsilon c} \text{ with } \varepsilon = 33{,}000\ M^{-1}cm^{-1}.^{23}$$

Activity Assay

The hydrolytic activity of the cp283Δ7-GZ variant was tested with *p*-NB as the substrate. The reaction assay was run three times, monitoring the change in absorbance over time with respect to various substrate concentrations. Reaction rates for each concentration were averaged and fitted to the Michaelis-Menten curve from which kinetic constants were calculated (Figure 8). Table 2 presents these results compared to those established previously for wild-type CALB, the

lid swapping variant CALB-*G.zeae* constructed by the Skjøt group, and the circularly permuted variant cp283Δ7. Kinetic results for cp283Δ7-GZ indicate a substantial 4-fold increase in hydrolytic activity compared to wild-type CALB but only a marginal increase compared to the CALB-*G.zeae* variant. Unfortunately, lid replacement on cp283Δ7 appears to have lowered its activity by 2-fold. However, these results remain questionable due to the high degree of error suggested by the significant deviation observed along the Michaelis-Menten curve in Figure 8.

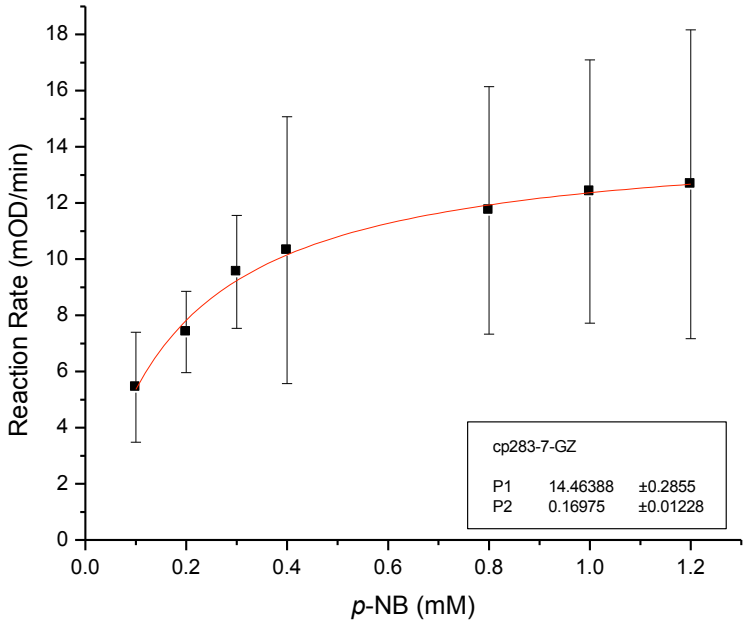


Figure 8: Michaelis-Menten curve plotting *p*-NB versus reaction rate. The absorbances were measured at λ = 400 nm.

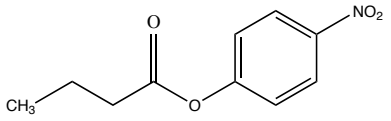
***p*-Nitrophenol butyrate (p-NB)** 				
	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($min^{-1}\mu M^{-1}$)	Relative Activity
CALB-wt [10]	410±40	305±10	0.74	1.0
CALB-*G.zeae* [16]	169±32	480±120	2.8	3.8
cp283Δ7 [20]	240±3	1400±65	5.8	7.8
cp283Δ7-GZ	169±20	541±17	3.2	4.3

Table 2: Kinetic constants for the hydrolysis of *p*-NB. Constants for cp283Δ7-GZ were calculated using the following parameters: ε = 13,260 $M^{-1}cm^{-1}$, l = 0.66 cm. The molecular weight for cp283Δ7-GZ is 32,968 g/mol.

Protein Immobilization

According to the first immobilization method, cells from the protein expression culture were discarded and the supernatant containing active enzyme was collected. The cation-exchange resin was then directly added to the supernatant. Saturation of the resin was determined using *p*-NB, which monitored decreased activity in the supernatant. However, when the resin itself was tested with *p*-NB, no hydrolytic activity was observed. Analysis of activity in the control sample (a vial of supernatant subjected to the same conditions for immobilization) suggested that the lowered activity observed in the supernatant resulted from protein denaturing.

This initial attempt was unsuccessful because the cp283Δ7-GZ lid variant is estimated to have pI = 6.776,[24] which gave the variant an overall neutral charge in the supernatant (determined to be buffered at pH 6.8). Since the resin binds by cation-exchange, cp283Δ7-GZ lid could not be immobilized. In order to correct this, the second immobilization strategy was developed to buffer the immobilization at pH 6.4 using purified protein. Analysis with *p*-NB showed only residual activity in the supernatant after only two hours. Though some hydrolytic

activity was observed on the resin, it appeared relatively low. Such low activity could result from only a small amount of enzyme being successfully immobilized on the resin or from poor enzymatic activity when buffered at pH 6.4.

Active-Site Titration

In order to determine the amount of enzyme immobilized on the resin, an active-site titration assay was conducted. The assay was performed using the inhibitor methyl 4-methylumbelliferyl hexylphosphonate. The reaction proceeds with the inhibitor inactivating the enzyme by covalently binding to the hydroxyl group of Ser105 in the active-site, thereby releasing equimolar amounts of fluorescent 4-methylumbelliferonate (4-MU) (Figure 9). Because of the linear relationship between the amount of enzyme present and the amount of 4-MU released, the amount of immobilized enzyme can be determined by measuring the concentration of 4-MU in the reaction sample. Active-site titration for the first immobilization method concluded that no protein had bound to the resin under those defined conditions. Results of the active-site titration for the second immobilization method indicated that 0.9139 mg of protein/g resin had been immobilized (Table 3, Figure 10).

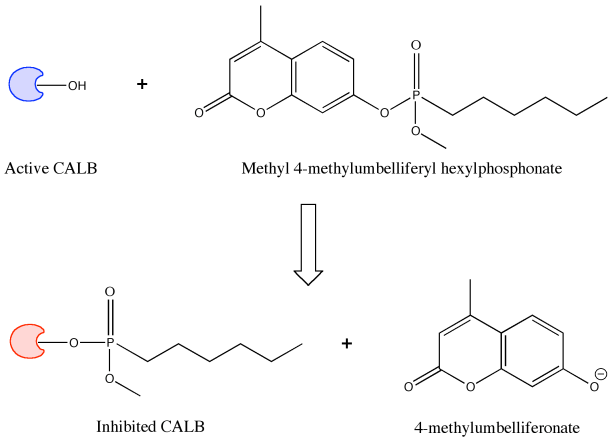


Figure 9: Schematic overview of active-site titration reaction.[25]

Active-Site Titration: cp283Δ7-GZ		
Amt Resin (mg)	**Avg Fluorescence (Intensity)**	**4-MU Released (nmol)**
3.1	462.42±105.19	0.4985±0.1134
5.2	567.42±101.01	0.6116±0.1089
7.6	594.75±99.36	0.6411±0.1071
10.1	655.75±94.82	0.7069±0.1022

Table 3: Active-site titration of immobilized cp283Δ7-GZ. Measurements were taken in triplicate at 450 nm. The amount of 4-MU released was calculated using $\varepsilon = 9{,}276.9\ M^{-1}$, which was determined by measuring the fluorescence intensity of 4-MU at different concentrations.

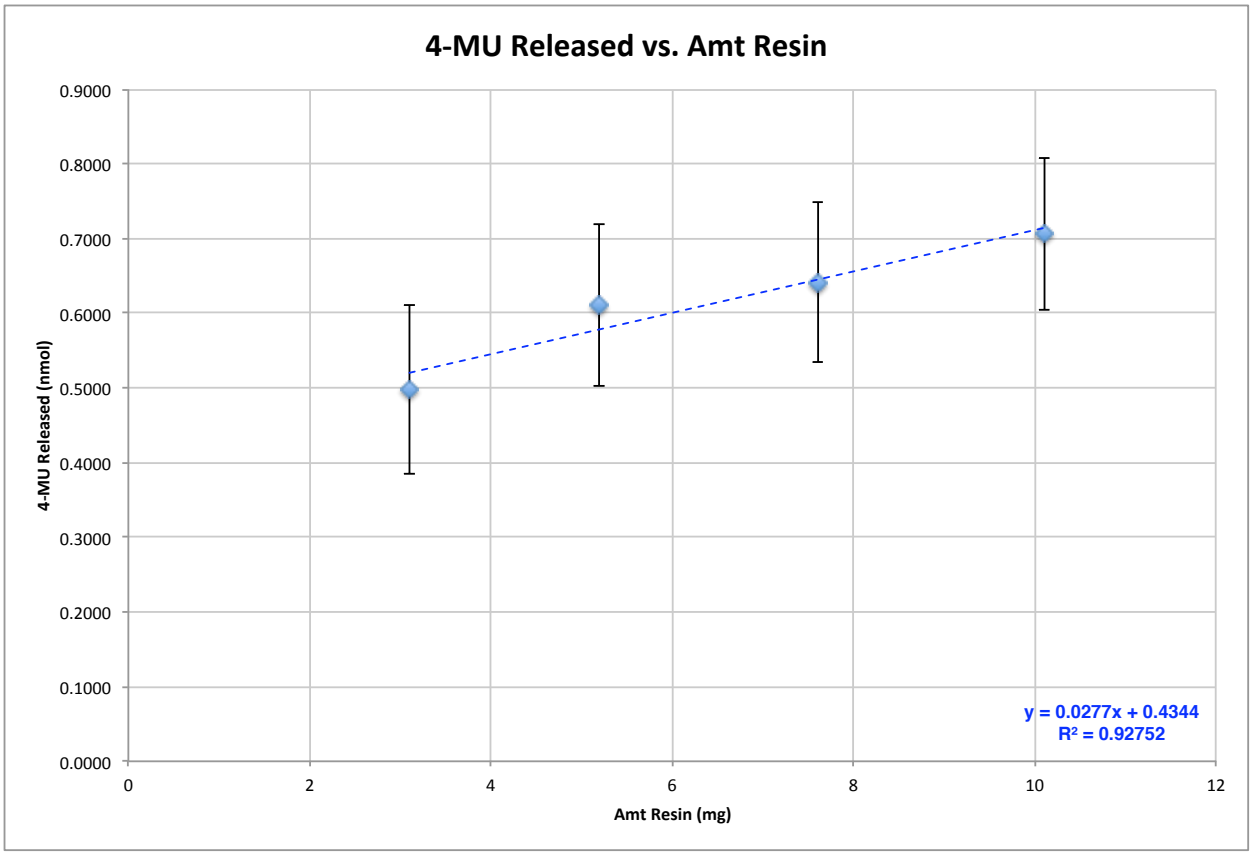


Figure 10: Graph of the total 4-MU released vs. the amount of resin used. The slope value, 0.0277, was used to calculate the amount of enzyme immobilized on the resin.

Discussion

Our data thus far suggests that the use of circular permutation and lid swapping techniques concurrently effectively enhances the hydrolytic activity of CALB, but to a lesser extent than the use of circular permutation alone. However, the reliability of these results is highly questionable due to the substantial amount of error associated with them. In fact, an activity assay conducted by Ying Yu suggests that these results highly under-represent the hydrolytic activity of cp283Δ7-GZ with *p*-NB. Her data indicated an increase in hydrolytic activity of 13-fold and 1.5-fold in comparison to wild-type CALB and cp283Δ7, respectively.[26] In retrospect, the error associated with my data most likely resulted from pipette inconsistencies. Ideally, the assay would be performed using a multiple pipette to add equal amounts of substrate and enzyme to each plate well. Since a multiple pipette was unavailable, the volumes of substrate and enzyme added probably varied substantially from well to well. The error introduced by these inconsistencies were additionally worsened by the distribution of only 2 μL of substrate to each well, which in itself has a 20% error associated with it. Though a certain amount of error will always be present without a multiple pipette, adjusting the assay design to allocate a greater percent of the total sample volume to the substrate could greatly reduce this error. The curve-fit for the kinetic data could also be improved by collecting additional data of substrate concentrations below the K_m-value.

Upon reflection, additional kinetic analysis with other substrates could be conducted. The Skjøt group had observed an increased hydrolytic tolerance for bulky substrates as a result of lid swapping. An investigation into whether this tolerance is maintained in the cp283Δ7-GZ variant could prove insightful. Two good substrates for such a study would be DiFMU octanoate[20] and lauric acid[16].

Our results also suggest the potential for future studies. With successful immobilization of the cp283Δ7-GZ variant, further kinetic studies could be conducted to analyze its catalytic activity in organic solvents. Moreover, the effects of circular permutation and lid swapping on the enantioselective preferences could be investigated. As noted earlier, the Skjøt group reported that lid swapping introduced enantioselective properties toward racemic ethyl 2-phenylpropanoate, a property not previously observed in wild-type CALB.[16] Additionally, the Lutz group reported that circular permutation had shown increased catalytic activity in their cp283 variant towards the (*R*)-enantiomer of 2-(3-fluoro-4-phenyl-phenyl)propanoic acid (flurbiprofen).[10] It would be interesting to see whether these enantioselective properties are maintained, or even enhanced, with both these substrates in the cp283Δ7-GZ variant.

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