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Glycerol acyl-transfer kinetics of a circular permutated *Candida antarctica* lipase B☆

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ABSTRACT

Triacylglycerols containing a high abundance of unusual fatty acids, such as γ-linolenic acid, or novel arylaliphatic acids, such as ferulic acid, are useful in pharmaceutical and cosmeceutical applications. *Candida antarctica* lipase B (CALB) is quite often used for non-aqueous synthesis, although the wild-type enzyme can be rather slow with bulky and sterically hindered acyl donor substrates. The catalytic performance of a circularly permutated variant of CALB, cp283, with various acyl donors and glycerol was examined. In comparison to wild-type CALB, butyl oleate and ethyl γ-linolenate glycerolysis rates were 2.2- and 4.0-fold greater, respectively. Cp283 showed substrate inhibition by glycerol, which was not the case with the wild-type version. With either ethyl ferulate or vinyl ferulate acyl donors, cp283 matched the performance of wild-type CALB. Changes in active site accessibility resulting from circular permutation led to increased catalytic rates for bulky fatty acid esters but did not overcome the steric hindrance or energetic limitations experienced by arylaliphatic esters.

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1. Introduction

Lipase B of *Candida antarctica* (CALB) is an exceedingly versatile biocatalyst for applications involving non-aqueous synthesis due to its regio- and stereoselectivity, high activity and thermostability [1]. Site-directed and random mutagenesis has been applied to the CALB sequence in an effort to enhance various attributes [2–5]. Rational protein engineering approaches have produced CALB variant proteins with improved thermostability, activity and altered enantioselectivity [6,7]. DNA family shuffling was used to create mutants with enhanced stability and hydrolysis activity toward a prochiral diester substrate [8]. These studies indicate that improvements can be made to the enzyme to suit specific applications and substrates.

Circular permutation (cp) of the CALB sequence, which introduces new C- and N-termini into the polypeptide sequence, greatly increased the catalytic efficiency of the catalyst for select substrates, particularly esters with bulky leaving groups [9,10]. Variant cp283, which has its N- and C-termini located at positions 283 and 282 of the wild-type CALB sequence, resulted in an increased hydrolytic activity for *p*-nitrophenol butyrate (11-fold) and 6,8-difluoro-4-methylumbelliferyl octanoate (175-fold) [9]. The engineered lipase further demonstrated enhanced triacylglycerol deacylation activity (5–7-fold) [11], which indicated that it may be useful for biodiesel production [12–14]. Biodiesel represents a high-volume, low-value product, for which there exists a substantial installed base of conventional catalysts that perform well [15,16]. In contrast, there are many triacylglycerol high-value transformations for which biocatalysts are ideally suited.

Structured lipids are acylglycerols refashioned by chemical or enzymatic processes to alter their fatty acid composition and/or the stereochemical positions of fatty acids on glycerol. These changes are targeted for specific metabolic effects, nutritive or therapeutic purposes, and improved physicochemical properties [17]. Structured lipids composed of polyunsaturated fatty acids such as conjugated linolenic acid, γ-linolenic acid (6,9,12-octadecatrienoic acid), and the ω-3 eicosapentaenoic acid and docosahexaenoic acids are sought in concentrated form for dietary supplementation. Beyond substitution of one fatty acid for another, a broad range of carboxyl-containing molecules can be usefully attached to the glycerol backbone. For instance, novel esters of glycerol have been investigated for their potential as lipid-soluble antioxidants [18]. Lipid derivatives of the natural dietary components ferulic and caffeic acids show strong promise in this regard [19]. Lipase-catalyzed transesterification is the preferred route for production of structured lipids containing easily oxidized/isomerized or otherwise

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readily altered substituents. However, the enzymatic processes can suffer from low reaction rates and yields when working with these unconventional fatty acid substitutes.

The present work compares the catalytic activity of wild-type CALB and cp283 in the synthesis of a variety of acyl glycerols (Figs. 1 and 2). Previous work demonstrated the superiority of cp283 for alcoholysis of vegetable oils [11]. Herein a reaction emulating the reverse course was examined to determine whether cp283 retains its performance edge in such reactions. In addition, glycerolysis reactions with γ-linolenic and ferulic acid esters, as representatives of novel or bulky acyl donors, were studied to ascertain the extent to which the molecular changes wrought by circular permutation of CALB can deliver catalytic improvements for a broad range of substrates.

2. Experimental

2.1. Reagents and materials

Novozym 435 (acrylic resin immobilized CALB) and Lewatit VP OC 1600 poly(methyl methacrylate) resin were obtained from Novozymes North America. Immobilized cp283 was prepared as previously described [9]. Methyl 4-methylumbelliferyl hexylphosphonate was prepared using a procedure modified from Qian et al. [9,20]. Ethyl γ-linolenate and mono-γ-linolenin glycerol were obtained from Nu-Chek-Prep (Elysian, MN, USA). Ethyl ferulate was provided by Ash Ingredients (Glen Rock, NJ, USA). Ferulic acid and spectroscopic grade glycerol were purchased from Sigma–Aldrich and stored in a dry box. Activated molecular sieves (3 Å) were stored under nitrogen. Vinyl acetate was purchased from Sigma–Aldrich (St. Louis, MO, USA) and stored under nitrogen at 4 °C. Mercury acetate, ethyl acetate, tetrahydrofuran (THF), hexane, and conc. sulfuric acid were purchased from Sigma–Aldrich and used as received. All other reagents were obtained from Sigma–Aldrich.

2.2. Methods

2.2.1. Active-site titration

Catalyst activity is reported as specific activity (k_{cat}) based on the amount of active enzyme determined by active site titration. The determination was conducted using methyl 4-methylumbelliferyl hexylphosphonate (4-MUHP) and a procedure adopted from Qian et al. [9,20]. Catalyst (5–20 mg) was solvated with 1 mL of acetonitrile containing 1% (v/v) water and 60 μM 4-MUHP and then reacted for one week at 23 °C. At the end of the reaction period, the solvent was sampled for fluorometric determination of 4-methylumbelliferone (4-MU) released by the single turnover of each active CALB molecule. Acetonitrile extracts and reaction solvent were combined. Aliquots were diluted into 10% acetonitrile/90% 0.1 M ammonia buffer (pH 9.5). Sample excitation was at 365 nm and fluorescence emission was measured at 448 nm (2-nm slit widths). A 4-MU linear calibration curve was constructed (15–90 nM). The concentration of 4-MU released from the lipase treatments was determined by regression. Control 4-MUHP solutions containing bare resin (Lewatit VP OC 1600) were used to correct for background hydrolysis. Analyses were performed on catalysts in triplicate, for which mean and standard deviation values are reported.

2.2.2. Enzyme reactions

Reactions were conducted using immobilized wild-type CALB (Novozym 435) and circularly-permutated CALB (cp283), solvents (when used), and substrates equilibrated to a water activity of 0.11 using a saturated LiCl solution at 23 °C in a closed container for at least one week. Reactions were conducted in 1.5-mL polypropylene microcentrifuge tubes attached to a rotator (Glas-Col, Terre Haute, IN, USA). Each reaction was conducted in triplicate. After reaction initiation by addition of enzyme, sampling (20 μL) was conducted at specified intervals. Samples were diluted into acetone, filtered (Whatman Anotop-10 0.2-μm inorganic filters), and stored at −20 °C until analyzed. Product formation was determined by HPLC using a system detailed previously [21]. A C8 reverse phase column (5 μm, 250 mm × 4.6 mm; Phenomenex, Torrance, CA, USA) was employed for separations. The column was developed isocratically (1.5 mL min^{-1}) with solvent consisting of 40% (v/v) acetone (containing 1% (v/v) acetic acid) and 60% (v/v) acetonitrile. Evaporative light scattering detector responses (70 °C drift tube, 44 °C exhaust, 1.8 SLPM N_2) for lipid products were determined from authentic standards. The concentration of feruloyl glycerol was ascertained by UV detection at 325 nm, using ethyl ferulate as the calibration standard. Initial reaction rates were determined by linear regression of production concentration as a function of time for samples in which there was less than 10% substrate consumption.

Triolein butanolysis: The reaction of triolein with 1-butanol (Fig. 1) was conducted without solvent by combining 1.0 mmol of triolein with 3.0 mmol of 1-butanol and 10 mg of catalyst (total weight of resin and support immobilized wild-type or cp283 CALB). For the reaction performed in 2-methyl-2-butanol, the triolein and 1-butanol concentrations were 0.4 and 1.2 M, respectively, and 10 mg of catalyst was dispersed in 1.0 mL of reaction medium.

Butyl oleate glycerolysis: The interesterification of butyl oleate with glycerol (Fig. 1) was performed in 2-methyl-2-butanol at 23 °C. Catalyst (5–10 mg) was dispersed in 1.0 mL of reaction medium. The butyl oleate concentration varied from 0.1 M to 1.5 M, and the glycerol concentration varied from 0.25 M to 1.0 M.

Ethyl γ-linolenate glycerolysis: The interesterification of ethyl γ-linolenate with glycerol (Fig. 1) was performed in 2-methyl-2-butanol at 23 °C. Catalyst (5–10 mg) was dispersed in 1.0 mL of reaction medium. The ethyl γ-linolenate concentration varied from 0.05 M to 1.5 M, and the glycerol concentration was 1.0 M.

Ethyl and vinyl ferulate glycerolysis: The interesterification of ethyl or vinyl ferulate with glycerol (Fig. 2) was performed in 2-methyl-2-butanol at 55 °C. Catalyst (100 mg) was combined with 1.0 mL of reaction medium. The ethyl or vinyl ferulate concentration varied from 0.25 M to 1.5 M, and the glycerol concentration was 1.0 M.

2.2.3. Vinyl ferulate synthesis

The vinyl ferulate synthesis protocol was modified from Gao et al. [22] and conducted using standard Schlenk line techniques. Ferulic acid (9.07 g, 46.7 mmol) and mercury acetate (330 mg, 1.0 mmol) were combined in a 250-mL Schlenk flask equipped with a stirbar, and then evacuated and flushed with nitrogen three times. THF (50 mL) was added via syringe with stirring followed by an excess of vinyl acetate (75 mL, 813.9 mmol). The cream colored slurry slowly dissolved into a light yellow solution upon stirring for 30 min at ambient temperature. Concentrated sulfuric acid (10 μL, 0.2 mmol) was added with a Gilson Pipetman against a nitrogen flow and the solution was heated to 40 °C and stirred for 24 h. The amber colored solution was cooled and 150 μL more conc. sulfuric acid (1.5 mmol) was added and the solution stirred at 40 °C for an additional 24 h. The reaction was terminated with the addition of sodium acetate (2.5 g, 30.8 mmol) and stirring for 1 h. The slurry was filtered through a 100-mL medium frit containing a bed of silica gel (0.05–0.2 nm, 70–325 mesh). The bed was washed with 0.5 L of 10% (v/v) ethyl acetate/hexane. The solvent was removed from the filtrate using a rotovap to produce ∼12 g of a crude, yellow, crystalline solid.

The crude vinyl ferulate was purified in ∼3.0 g portions on a 40 cm × 2 cm silica gel (0.05–0.2 nm, 70–325 mesh) column. The column was conditioned with 0.5 L of hexane followed by 0.1 L of 5% (v/v) ethyl acetate/hexane. The crude samples was dissolved in 10%

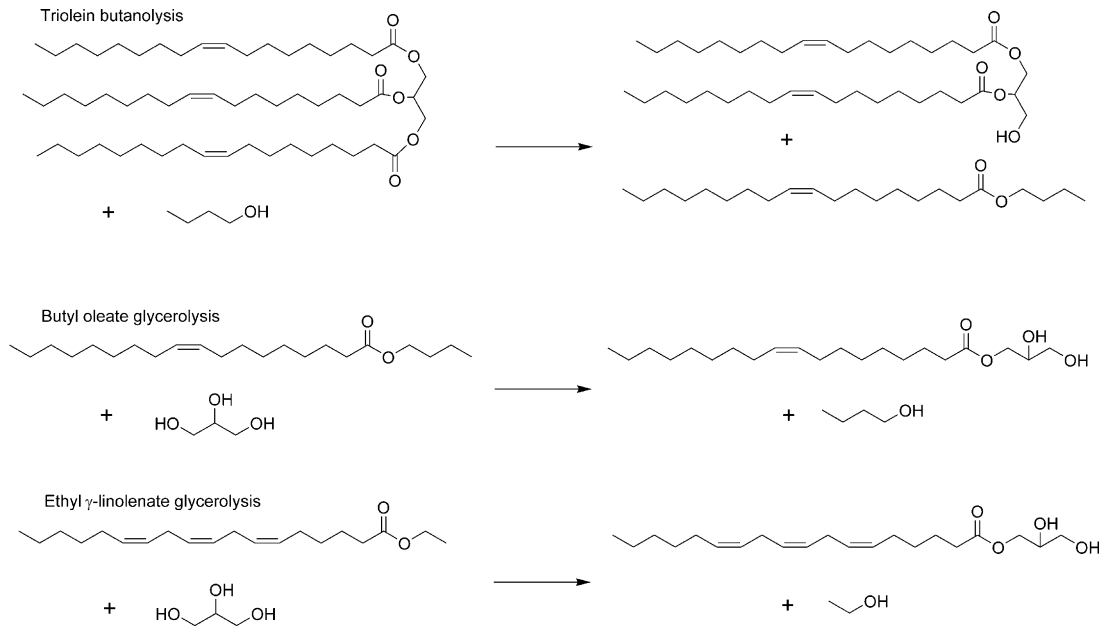

Fig. 1. Alcoholysis reactions with fatty acid esters.

(v/v) ethyl acetate/hexane and loaded onto the column. The column was developed sequentially with 1.0 L volumes of 5% (v/v) ethyl acetate/hexane, 10% (v/v) ethyl acetate/hexane, and ethyl acetate. Fractions containing the vinyl ferulate were combined, and the solvent was removed via rotovap. The resultant white powder was dried at 30 °C overnight *in vacuo* resulting in 6.08 g of vinyl ferulate with >98% purity based on ^{1}H NMR and GC–MS.

Yield: 55.3% (based on ferulic acid). ^{1}H NMR (d_6-acetone, 500 MHz): δ 8.22 (0.89 H, bs, –O*H*), 7.74 (d, J = 16.0 Hz, 1.00 H, Ar–C*H*=CH–), 7.41 (overlap of m, 2.01 H, Ar–*H* and –C(O)–O–C*H*=CH_2), 7.21 (dd, J = 2.0 Hz, JJ = 8.1 Hz, 1.08 H, Ar–*H*), 6.90 (d, J = 8.1 Hz, 1.00 H, Ar–*H*), 6.46 (d, J = 15.7 Hz, 1.05 H, Ar–CH=C*H*–), 4.92 (dd, J = 1.4 Hz, JJ = 14.0 Hz, 1.04 H, –C(O)–O–CH=CH_2), 4.62 (dd, J = 1.5 Hz, JJ = 6.4 Hz, 1.05 H, –C(O)–O–CH=CH_2), 3.94 (s, 3.19 H, Ar–O–CH_3).

3. Results

A comparison was made of the catalytic efficacy of wild-type CALB and cp283 with a variety of substrates in which glycerides serve as acyl donors, or with glycerol as an acyl acceptor (Figs. 1 and 2). Reactions were selected to elucidate differences in the kinetic attributes of the two enzymes, apart from the influence of reaction conditions (temperature, solvent). Product formation rates were determined such that initial condition changes were minimal.

3.1. Active-site titration of immobilized wild-type and cp283 CALB

Based on the amount of 4-MU released by 4-MUHP treatment of the enzyme, the active CALB content was 1.00 ± 0.03 and 0.16 ± 0.01 mmol g^{-1} of catalyst for Novozym 435 and cp283, respectively. These values were used to normalize reaction rates between the two catalysts based on active enzyme amounts.

3.2. Triolein butanolysis

Yu and Lutz [11] demonstrated that the initial transesterification rate of various vegetable oils with 1-butanol by cp283 was 4- to 7-fold faster than wild-type CALB. In the present work, butanolysis of triolein in the absence of cosolvent and at room temperature was found to be 5.3-fold faster with cp283 in comparison to wild-type CALB (Table 1), which is consistent with the prior findings. Raising the reaction temperature to 55 °C doubled the reaction rate for both enzymes, so the enzymes are similarly responsive to altered reaction temperature. Adding a cosolvent (2-methyl-2-butanol) to the reaction moderately increased (60%) the butanolysis rate of the

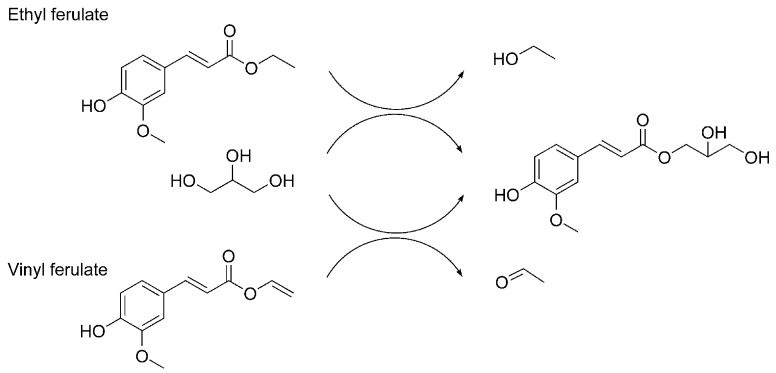

Fig. 2. Glycerolysis reaction with ethyl and vinyl ferulates.

Table 1
Butanolysis rates for wild-type and cp283 CALB.

Conditions	Initial rate (min^{-1})		Relative rate
	Wild type	cp283	
No solvent, 23 °C	63 ± 5	330 ± 10	5.3
No solvent, 55 °C	120 ± 10	610 ± 80	5.0
2M2B solvent, 23 °C	100 ± 10	300 ± 40	3.0

Relative rate: (cp283 rate)/(wild type) rate. 2M2B is 2-methyl-2-butanol. Cp283 is a circular permutated CALB variant whose N terminus starts at amino acid 283 of the wild-type sequence.

wild-type enzyme but did not significantly affect the cp283 rate, which is reflected in a lower relative reaction rate for cp283 in solvent (Table 1). The enzymes thus displayed a small difference in their response to the presence of solvent in the reaction medium. Note that addition of solvent served to lower substrate concentrations, which may have augmented or masked differences in the enzymes' response to the presence of solvent (e.g., altered substrate affinities). These data suggest however that 2-methyl-2-butanol does not adversely impact the activity of either enzyme.

3.3. Butyl oleate glycerolysis

The reaction of butyl oleate with glycerol is ostensibly the reverse of triolein butanolysis (Fig. 1); more so in the presence of solvent, which kept the substrates miscible. Butyl oleate glycerolysis rates were 5.5- and 2.4-fold greater for wild-type CALB and cp283, respectively, than their corresponding triolein butanolysis rates (with 1 M substrates at 23 °C; *cf.* Tables 1 and 2). The higher rates presumably reflect the case that butyl oleate is smaller than triolein as an acyl donor and thus more readily oriented in the active site to form the first enzyme-substrate activated intermediate of the enzyme's ping-pong bi–bi reaction pathway [23].

A more detailed study of the butyl oleate glycerolysis reaction revealed the impact of the structural changes resulting from the circular permutation of wild type CALB to cp283 (Fig. 3). Both enzymes demonstrated substrate inhibition at 1.5 M butyl oleate (data not shown). Substrate inhibition by butyl oleate observed at 1.5 M may not be due to an impact on enzyme's active site but rather result from changes to the solvent/substrate conditions at this high concentration. The butyl oleate may have been starting to form a separate immiscible liquid phase in the presence of 1 M glycerol and 2-methyl-2-butanol. In order to simplify the kinetic analysis, only butyl oleate concentrations ≤1.0 M were used for estimating kinetic constants (Table 3). The kinetic constants for the wild-type CALB (Table 3) were adequately determined by non-linear regression fitting of the experimental data to Eq. (1) for the initial rate of the reaction,

$$\frac{v_0}{[E_T]} = \frac{k_{cat}[A_0][B_0]}{K_m^B[A_0] + K_m^A[B_0] + [A_0][B_0]} \quad (1)$$

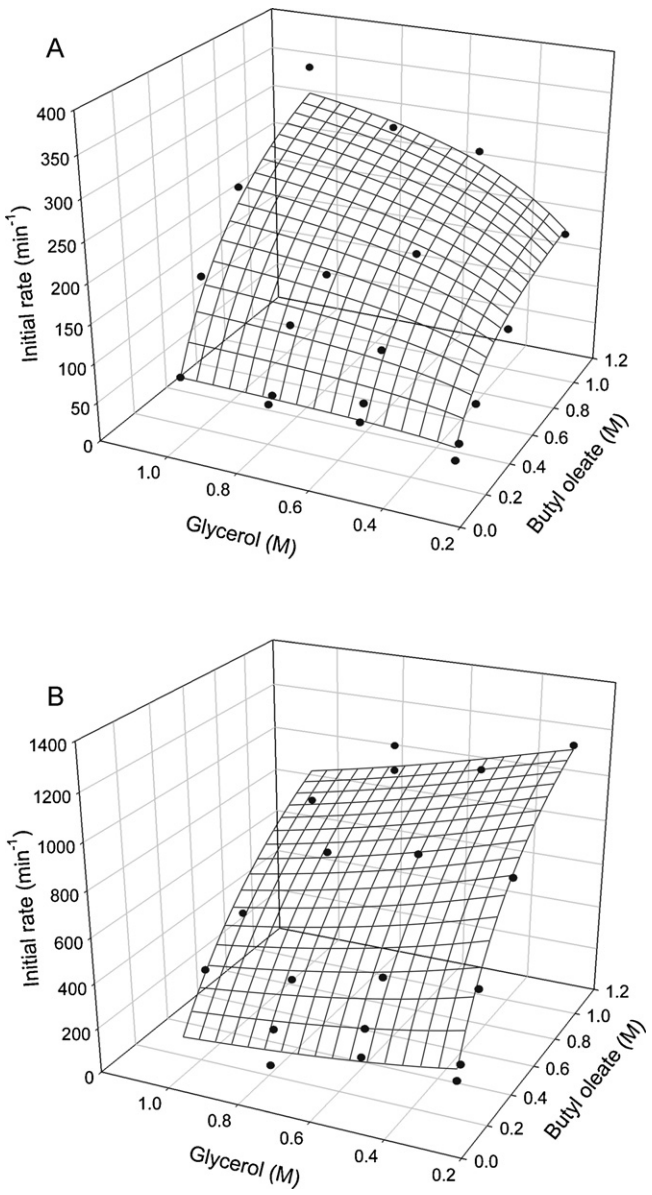

Fig. 3. Initial reaction rates of butyl linolenate glycerolysis. Wild-type CALB, panel A; cp283, panel B. Lines were drawn from Eq. (1) (wild-type) and Eq. (2) (cp283) based on the parameters given in Table 3. Reactions were conducted in 2-methyl-2-butanol at 23 °C.

where v_0 is the initial rate, $[E_T]$ is the total active enzyme concentration, k_{cat} is the first-order rate constant, $[A_0]$ and $[B_0]$ are the initial substrate concentrations of butyl oleate and glycerol, respectively, and K_m^A and K_m^B are the steady-state Michaelis constants for butyl

Table 2
Reaction rates for wild-type and cp283 CALB catalyzed acyl transfer reactions with glycerol in 2-methyl-2-butanol.

Reaction acyl donor/ temperature	Initial rate (min^{-1})		Relative rate
	Wild type	Cp283	
Butyl oleate, 23 °C	350 ± 40	790 ± 40	2.2
Ethyl γ-linolenate, 23 °C	960 ± 140	3800 ± 230	4.0
Ethyl ferulate, 55 °C	0.67 ± 0.06	0.90 ± 0.10	1.3
Vinyl ferulate, 55 °C	6.2 ± 0.6	6.0 ± 0.1	1.0

Substrate (acyl donor and glycerol) concentrations were 1.0 M. Relative rate: (cp283 rate)/(wild type) rate.

Table 3
Kinetic constants for wild-type and cp283 CALB catalyzed acyl transfer reactions with lycerol in 2-methyl-2-butanol.

Acyl donor	k_{cat} (min^{-1})		K_m^A (M)		K_m^B (M)	
	Wild type	cp283	Wild type	cp283	Wild type	cp283
Butyl oleate	670	1800	0.65	0.30	0.43	0.02
Ethyl γ-linolenate	1900	5600	0.65	0.11	–	–

Reactions were conducted at 23 °C. The K_i^B (inhibition constant for glycerol with cp283) was determined to be 0.4 M from the butyl oleate + glycerol reaction. Kinetic constants for the butyl oleate + glycerol reaction were derived from fits to the data shown in Fig. 3. Kinetic constants for the ethyl γ-linolenate + glycerol reaction were derived from fits to the data shown in Fig. 4.

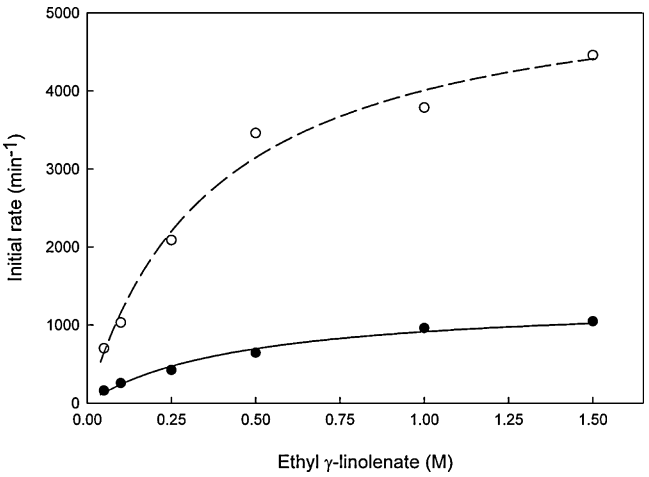

Fig. 4. Initial reaction rates of ethyl γ-linolenate glycerolysis. Wild-type CALB, filled circles; cp283, open circles. Lines (wild-type, solid; cp283, dashed) were drawn from Eq. (3) based on the parameters given in Table 3. Reactions were conducted in 2-methyl-2-butanol at 23 °C with 1 M glycerol.

oleate and glycerol, respectively. Cp283 demonstrated substrate inhibition by glycerol (Fig. 3, panel A), therefore Eq. (2) was applied to determine its kinetic constants, including K_i^B for the glycerol inhibition constant (Table 3).

$$\frac{v_0}{[E_T]} = \frac{k_{cat}[A_0][B_0]}{K_m^B[A_0] + K_m^A[B_0]\left(1 + \left[B_0/K_i^B\right]\right) + [A_0][B_0]} \quad (2)$$

The k_{cat} value of cp283 was 2.7-fold higher compared to wild-type CALB for the reaction. In addition the K_m^A (butyl oleate) was somewhat lower for cp283. Most notably, cp283 displayed dead-end substrate inhibition by glycerol ($K_i^B = 0.4$ M), while wild-type CALB did not do so under these reaction conditions. Cp283 displayed a K_m^B (glycerol) substantially lower than that of the wild-type enzyme, reflective of cp283s greater affinity for this substrate.

3.4. Ethyl γ-linolenate glycerolysis

Transesterification of the polyunsaturated γ-linolenic acid (18:3, *n*-6) with glycerol proceeded quite rapidly with either wild-type CALB or cp283. Neither enzyme displayed substrate inhibition by ethyl γ-linolenate, unlike that which was observed with butyl oleate at high concentration. Ethanol generated during the reaction would be expected to act as an inhibitor, but only at concentrations greater than that reached during initial rate determinations (>0.2 M) [24]. The reaction rate was 2.7-fold faster than the butyl oleate reaction for the wild-type enzyme (Table 2), reflecting the known preference of this lipase for polyunsaturated C18 fatty acid substrates [25]. Cp283 also demonstrated an increased reaction rate with γ-linolenate as the acyl donor such that its rate was 4-fold faster than that of wild-type CALB (Table 2).

A simple Michaelis–Menten formulation (Eq. (3)) was applied to the data (Fig. 4) to determine kinetic constants by non-linear regression (SigmaPlot 10.0):

$$\frac{v_0}{[E_T]} = \frac{k_{cat}[A_0]}{K_m^{App} + [A_0]} \quad (3)$$

Glycerol was held constant (1 M) and the acyl donor concentration was varied. K_m^{App} is the apparent value for the acyl donor. This reaction would be expected to have the same K_m^B for glycerol, as well as the same degree of glycerol inhibition for cp283 (K_i^B), as was observed for butyl oleate glycerolysis. Therefore, these parameters were not re-examined for the reaction with ethyl γ-linolenate as the acyl donor. Eq. (4) was applied to K_m^{App} to calculate K_m^A for cp283.

$$K_m^A = \frac{K_m^{App}}{1 + [I]/K_i^B} \quad (4)$$

From the determined kinetic constants (Table 3) it can be concluded that wild-type CALB did not display an affinity (K_m) difference between the butyl oleate and ethyl γ-linolenate substrates, while cp283 increased its affinity (lowered K_m) by two-thirds. Furthermore, the γ-linolenate selectivity constant (k_{cat}/K_m^A) of cp283 was 18-fold greater than what was observed with wild-type CALB. In comparison to wild-type CALB, circular permutation cp283 thus showed substantially improved catalytic performance with the polyunsaturated γ-linolenate substrate.

3.5. Ethyl and vinyl ferulate glycerolysis

The reaction of ethyl ferulate with glycerol (Fig. 2) is four orders of magnitude slower than that of the comparable reaction of butyl oleate with glycerol for wild-type CALB and cp283 (Table 2). There was a minimal difference in the initial rates between the two enzymes with this substrate. As expected [26], use of the activated ester vinyl ferulate lead to substantially higher initial reaction rates with both enzymes (~7–9-fold), but there was no preferential increase by either enzyme. Thus, the rate enhancement observed with cp283 when compared to the wild type enzyme for fatty acid alcoholysis reactions was not extended to reactions involving unsaturated arylaliphatic esters.

4. Discussion

In the present study, wild-type CALB and its circular permutation variant cp283 were examined to determine whether the circular permutant displayed greater activity than the wild-type enzyme for a range of fatty acid ester and arylaliphatic ester substrates. Glycerol was selected as the acyl acceptor for reaction with these substrates. Glycerides commonly serve as active agent derivatives for pharmaceutical and cosmeceutical applications. The reaction of butyl oleate with glycerol (Fig. 1) was selected for study because prior work had demonstrated that cp283 was particularly efficacious for butanolysis of triglycerides [11], and thus the butyl oleate reaction represents the first step in the reversal of this process. Glycerolysis of the specialty fatty acid ester ethyl γ-linolenate (Fig. 1) and arylaliphatic esters of ferulic acid (Fig. 2) represent reactions of potential commercial utility. Steric interactions of these acyl donors in most lipase active sites can be unfavorable, leading to very low reaction rates [27,28]. Variants better able to accommodate bulky acyl groups would provide utilitarian value via increased bioprocessing throughput. Relocation of the N- and C-termini to within the vicinity of the active site region is believed to lessen steric constraints in catalytic activity through an increase in chain flexibility and active site access.

In comparison to wild-type CALB, butyl oleate and ethyl γ-linolenate glycerolysis rates were 2.2- and 4.0-fold greater, respectively, with the cp283 variant. These catalytic rates are consistent with previous findings that the permutated enzyme had 2.6- to 9-fold greater relative catalytic efficiency than wild-type CALB for transesterification of triglycerides [11]. Greater active site accessibility in cp283 compared to wild-type CALB is likely to result in faster substrate binding and product release for these lipids [29].

Cp283 showed substrate inhibition by glycerol, which was not evident in wild-type CALB. Adsorption of glycerol to immobilized lipase can have an adverse impact on transesterification reactions by creating a hydrophilic diffusional barrier for hydrophobic substrates [30]. Glycerol adsorption to the support material Lewatit

VP OC 1600 used to immobilize wild-type CALB (Novozym 435) and cp283 is known [31]. There is disagreement in the literature as to whether use of a moderate polarity solvent such as *tert*-butanol overcomes this effect [31,32]. However, this influence is expected to be the same for either enzyme as they are immobilized on the same support material. The substrate inhibition demonstrated by cp283 (Fig. 3) thus likely reflects an altered sensitivity of the enzyme to glycerol rather than due to the imposition of a diffusion barrier, which would be the same for both enzymes.

The cp283 variant of CALB introduces highly polar termini into an otherwise very nonpolar α-helix (helix 17), which opens up the substrate tunnel to the active site [29]. The increased polarity of this sequence segment may account for cp283's susceptibility to substrate inhibition by glycerol, and could also contribute to the variant's lower glycerol K_m^B (Table 3). However, the commercially-sourced wild-type CALB (Novozym 435) and cp283 were not immobilized under identical conditions (i.e., differences in co-adsorbates and surface coverage by lipase), which cannot be precluded as contributing factors governing the kinetic responses of the lipases to glycerol.

With either ethyl ferulate or vinyl ferulate acyl donors, cp283 did not perform better than wild-type CALB. Changes in active site accessibility resulting from the termini relocation which led to increased catalytic rates for bulky fatty acid esters hence did not overcome the steric hindrance or energetic effects experienced by arylaliphatic acids and esters [33]. Guyot et al. [27] first pointed out the electron donating effect in cinnamic acid ester synthesis. The electron donating effects intrinsically deactivate the electrophilic carbon center of the carboxylic group for nucleophilic attack of the alcohol group of the active site serine. The use of activated esters (ethyl or vinyl leaving groups) partially counter this effect. The greater active site accessibility in cp283 compared to wild-type CALB which resulted in faster substrate binding and product release for the fatty acids failed to do so for ferulic acid esters. Given the ping-pong mechanism of the reaction, a possible reason for this lack of improvement may be that there is a difference the in rate-limiting step for the two types of substrates; the deacylation step for fast substrates such as fatty acid esters, and active site (serine) acylation for slow reacting ferulate esters [23].

5. Conclusions

A detailed kinetic analysis demonstrated that the cp283 variant, which demonstrated very high catalytic performance relative to wild-type CALB in hydrolytic reactions involving bulky leaving groups [10], also retains this advantage for reactions of fatty acid esters with glycerol. Cp283 displayed a sensitivity to substrate inhibition by glycerol that was not evident in wild-type CALB. While circular permutation has proven quite effective in identifying a CALB variant with enhanced rates of ester and triglyceride hydrolysis and synthesis, the same variant performed largely at levels similar to wild type enzyme on sterically hindered arylaliphatic esters such as ferulates. Whether these functional discrepancies result from biases in the screening of circular permuted CALB for tributyrin hydrolysis or whether these difference are a reflection of fundamental aspects related to this protein engineering strategy is not clear. Nevertheless, cp283 shows good activity for γ-linolenate which warrants further development of the engineered biocatalyst. Separately, the cause of the inhibitory effects of glycerol observed for cp283 is unclear. Spectroscopic analysis indicated no direct interaction between substrates and the termini of CP283 [9]. It could be a direct result of a change in the polar environment near the enzyme's active site, or may be related to the significantly lower enzyme immobilization load for the CALB variant. Optimization of the immobilization procedure and the support surface could eliminate the undesirable effect. In future experiments, a targeted screening of circular permutation libraries of CALB holds promise for the identification of candidates with improved activity for sterically challenged substrates and esters with unfavorable electronic effects.

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