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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%