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Increased activity of enzymatic transacylation of acrylates through rational design of lipases

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ABSTRACT

A rational design approach was used to create the mutant *Candida antarctica* lipase B (CALB, also known as *Pseudozyma antarctica* lipase B) V190A having a k_{cat} three times higher compared to that of the wild type (wt) enzyme for the transacylation of the industrially important compound methyl methacrylate. The enzymatic contribution to the transacylation of various acrylates and corresponding saturated esters was evaluated by comparing the reaction catalysed by CALB wt with the acid (H_2SO_4) catalysed reaction. The performances of CALB wt and mutants were compared to two other hydrolases, *Humicola insolens* cutinase and *Rhizomucor mihei* lipase. The low reaction rates of enzyme catalysed transacylation of acrylates were found to be caused mainly by electronic effects due to the double bond present in this class of molecules. The reduction in rate of enzyme catalysed transacylation of acrylates compared to that of the saturated ester methyl propionate was however less than what could be predicted from the energetic cost of breaking the π-system of acrylates solely. The nature and concentration of the acyl acceptor was found to have a profound effect on the reaction rate.

1. Introduction

Acrylates are industrially important compounds that have a wide range of commercial applications. The α,β-double bond present in these molecules can be used for radical polymerization to yield final products such as paints, plastics and adhesives to be used for example in the automotive industry. Traditionally, esters of acrylic acid and methacrylic acid are made chemically at elevated temperature, with polymerization inhibitors and with sulphuric acid as catalyst [1,2]. Biocatalysis is a "green" option to traditional chemistry and could provide sustainable routes to acrylic esters starting from renewable sources such as glucose and lactate [3,4]. Enzymes are attractive since they allow the use of mild reaction conditions, often displaying high activity as well as high regio-/stereo selectivity [5,6]. Especially lipases are of industrial interest due to their high stability and activity in organic media.

Lipase catalysed transacylation of acrylates has been described in the literature. In a pioneering work regioselective acrylation of sterically hindered diols was performed using a lipase from *Chromobacterium viscosum* [7]. Lipase B from *Candida antarctica* (CALB, also known as *Pseudozyma antarctica* lipase B, EC 3.1.1.3) was shown to be a superior catalyst out of 19 enzymes investigated in the enzyme catalysed transacylation of methyl acrylate with 1-undecanol [8]. The industrial interest in enzymatic acrylation is shown in the number of patents on the subject. Enzyme catalysed regioselective transacylation of acrylates with glucosides, with the aim of producing polymeric sugar acrylates, as well as monoacrylation of polyols has been described by BASF [9,10]. One limitation of enzyme catalysed acrylation is the low reaction rates compared to the rates for corresponding saturated esters. Attempts have been made to increase the reaction rate of enzymatic acrylation, mainly by the choice of solvent and by optimisation of the water activity [11,12]. The source of the low rates has to our knowledge not been discussed.

In this work we have employed a rational design approach based on computer modelling with the aim of increasing the enzymatic activity (k_{cat}) for methyl methacrylate. The analysis of the CALB mutants D134N, I189A, I189V, V190A, V190L, and I285W gave valuable insight into the problems associated with enzymatic acrylation. *Humicola insolens* cutinase (EC 3.1.1.74) and *Rhizomucor miehei* lipase (EC 3.1.1.3) were compared to CALB regarding

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performance in enzymatic acrylation. The enzymatic contribution to the transacylation of methyl acrylate and derivatives as well as to corresponding saturated esters was evaluated.

Alcohols are known to inhibit lipases by forming a dead-end complex with the enzyme [13]. The protein engineering approach to enhance enzymatic activity was compared to a reaction engineering approach. The nature and concentration of the alcohol acyl acceptor was found to have a profound effect on the reaction rate for enzyme catalysed acrylation.

2. Experimental

2.1. Chemicals and enzymes

Methyl propionate, methyl isobutyrate, methyl trimethylacetate, methyl acrylate, methyl crotonate, methyl methacrylate, 1-propanol, 2-butanol, acetonitrile (for fluorescence) and dodecane of ≥99% purity and H_2SO_4 ≥ 95% purity were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methyl α-chloro acrylate (≥99%), stabilised with hydroquinone, was from Acros Organics (Morris Plains, NJ, USA). Diisopropyl ether (≥99%) was from AppliChem (Darmstadt, Germany). 2-Ethyl-1-hexanol was from BASF (Ludwigshafen, Germany).

H. insolens cutinase wt was a kind gift from Novozymes (Bagsvaerd, Denmark). Immobilised *R. miehei* lipase was purchased from Sigma–Aldrich. Restriction enzymes were from Fermentas (St. Leon-Rot, Germany).

2.2. Preparation of C. antarctica lipase B wt and mutants

The preparation of the CALB wt (also known as *Pseudozyma antarctica* lipase B) construct in a pGAPzαβ plasmid and its transformation into the expression host *Pichia pastoris* has been described previously in detail [14]. The QuikChange® protocol from Stratagene (La Jolla, CA, USA) was used to introduce mutations. The mutagenic primers where designed to be non-overlapping in the ends [15] and were ordered from Thermo Fisher Scientific (Ulm, Germany). The DNA-sequences in the 5′–3′ direction for the forward (f) and reverse (r) primers are given below. Introduced alterations in the wt sequence are in uppercase letters for clarity.

D134N_f
5′-ggcctttgcgcccAactacaagggcaccgtcctcg-3′
D134N_r
5′-ggtgcccttgtagtTgggcgcaaaggccataagtcgatcg-3′
I189V_f
5′-cgaccgacgagGtcgttcagcctcaggtgtcc-3′
I189V_r
5′-cctgaggctgaacgaCctcgtcggtcgccg-3′
I189A_f
5′-cggcgaccgacgagGCcgttcagcctcaggtgtcc-3′
I189A_r
5′-cctgaggctgaacgGCctcgtcggtcgccgagtagagg -3′
V190A_f
5′-cgacgagatcgCtcagcctcaggtgtcc-3′
V190A_r
5′-cctgaggctgaGcgatctcgtcggtcg-3′
V190L_f
5′-cgaccgacgagatcCttcagcctcaggtgtccaactcg-3′
V190L_r
5′-cctgaggctgaaGgatctcgtcggtcgccgagtagagg-3′
I285W_f
5′-gctgcagccTGGgtggcgggtccaaagcagaactgc-3′
I285W_r
5′-ggacccgccacCCAggctgcagctgccggcgccaggagc-3′

The PCR reactions were analysed on a 1% agarose gel for the presence of products. After DpnI digestion, the mutated plasmid DNA as well as the wt plasmid DNA was transformed by heat-shock into *E. coli* XL1Blue cells according to the QuikChange protocol. All mutations were confirmed by sequencing. Prior to expression, the wt and mutated pGAPzαβ plasmids were harvested by the QIAprep Miniprep System (QIAGEN GmbH, Hilden, Germany). Around 20–40 μg plasmid DNA was linearized by Bsp HI overnight at 37 °C and concentrated by EtOH precipitation according to standard protocols. Approximately 10 μg of the linearized plasmid was electroporated into freshly made *Pichia pastoris* SMD1168H competent cells using the following settings: 1.5 kV, 400 Ω, 25 μF. Ice-cold 1 M sorbitol was immediately added after transformation. After 2 h at 30 °C, half of the transformation mixture was plated on YPDS-plates (1% yeast extract, 2% peptone, 2% dextrose (D-glucose), 1 M sorbitol, 2% agar) containing 100 μg/mL zeocin. The other half of the mixture was diluted with YPD-media (1% yeast extract, 2% peptone, 2% dextrose (D-glucose)) and was allowed to shake at 200 rpm for 3 h at 30 °C after which plating was performed (on YPDS-plates containing 100 μg/mL zeocin). Around 10 colonies were selected and grown in 10 mL YPD for approximately 36 h. After centrifugation at 4000 rpm, 4 °C, for 10 min using a Sorvall Super T21 centrifuge, the culture supernatants were analysed by SDS-gel electrophoresis (NuPAGE® 10% Bis-Tris gel, Invitrogen, Carlsbad, CA, USA) using MOPS as running buffer (MOPS 50 mM; Tris base 50 mM; SDS 0.1%; EDTA 1 mM; pH 7.7). The validity of the *C. antarctica* lipase B protein band was established by performing Western blot analysis using antibodies against CALB as previously described [14].

2.3. Protein expression and purification

Positive *Pichia pastoris* clones expressing CALB variants were grown on YPD-agar plates containing 100 μg/mL zeocin. After 2 days, colonies were picked and grown for 24 h in 5 mL YPD at 30 °C, 200 rpm. A fraction of the pre-cultures (to obtain a 1:100 dilution) were transferred to 50–500 mL BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base with ammonium sulphate without amino acids, 0.4 mg/L (4×10^{-5}%) biotin, 1% glycerol, pH 6.0). The main cultures were allowed to grow for 3–4 days at 30° at 200–260 rpm after which the optical density (OD_{600}) typically reached ≥40. The cultures were centrifuged at 4000 rpm, 4 °C for 20 min. Protein purification was performed using hydrophobic interaction chromatography (HIC) as previously described [16] with minor modifications. Ammonium acetate (9 M) was slowly added under stirring to the supernatant to a final concentration of 0.8 M. The feed-stock was filtered through a 0.2 μM bottle-top filter (Sartorius AG, Goettingen, Germany) and loaded onto an XK-16 column packed with 15 mL butyl sepharose FF and connected to an ÄKTA explorer (GE Healthcare, Uppsala, Sweden). The equilibration buffer was 50 mM potassium phosphate, 0.8 M ammonium acetate, pH 6. A linear gradient to 50 mM potassium phosphate, pH 6 was used for elution and finally milliQ was used to elute remaining protein from the column. Fractions giving raise to an A_{280}-signal were pooled and concentrated (by a factor of 10) with 50 mM potassium phosphate, pH 7.5 by using Centricon Centrifugal filters with an MWCO of 5 kDa (Millipore, MA, USA). The purity of the CALB variants was assessed by SDS polyacrylamide gel electrophoresis. Purification typically resulted in 10–20 mg pure protein/L media.

2.4. Protein immobilization and active site titration

Lipase immobilization and active site titration have previously been described by our group [17]. *C. antarctica* lipase B wt and mutants as well as *H. insolens* cutinase were immobilized on Accurel MP 1000 < 1500 μm (Accurel Systems, Sunnyvale, CA, USA). The supernatant was confirmed to be devoid of lipase activity after immobilization. The lipase-containing polypropylene beads were vacuum-dried overnight and put in a desiccator under LiCl(s) to ensure a water activity of 0.1.