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Enzymatic kinetic resolution of aliphatic *sec*-alcohols by LipG9, a metagenomic lipase

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ABSTRACT

Bioprospection for new enantioselective enzymes for application in organic synthesis is a prominent area of investigation in biocatalysis. In this context, here we present the evaluation of an immobilized lipase isolated from a metagenomic library (LipG9) for the enzymatic kinetic resolution (EKR) of aliphatic *sec*-alcohols, which are still challenging substrates, since low enantioselectivity values are usually observed for these resolutions. LipG9 was successfully employed in EKR of aliphatic alcohols, which were resolved with satisfactory conversions (19–59%) and enantiomeric excesses for alcohols (26–88%) and esters (30–96%) by transesterification reactions, demonstrating that its performance is equal to or better than commercially available enzymes for the same reaction.

1. Introduction

Biocatalysis focused on organic synthesis is already a well-established practice in academy and industry [1–8]. For every synthetic transformation employing a conventional organic method, it is possible to propose an enzymatic approach as an alternative. In a synthetic route, the coupling between chemical and enzymatic (chemoenzymatic) processes is practicable [9,10]. The use of enzymes in organic synthesis implies using biodegradable catalysts that are isolated from renewable sources (plants, animals and microorganisms), since these are important properties for biotechnological applications. Among the enzymes that have been successfully used in biocatalysis are the lipases (glycerol ester hydrolases EC 3.1.1.3), which, in vivo can hydrolyze acyl esters in aqueous-lipid interfaces [11,12]. The great success of lipases in organic synthesis has several reasons, including no requirement for coenzymes; applications for a very broad range of synthetic substrates; and activity and stability in non-aqueous media such as organic solvents [13], ionic liquids [14,15] and supercritical fluids [16]. Furthermore, lipases usually exhibit high level of regio-, chemo- and enantioselectivity, making them valuable reagents for preparation of optically active compounds [17–22].

Kinetic resolution of racemates is the most widely used lipase-mediated reaction for preparation of optically active compounds [7]. Considering that the structural variability of synthetic substrates is much higher than that of the available commercial enzymes, bioprospection for new lipases has become a prominent area of investigation in biocatalysis. The traditional methods to isolate lipases rely on laboratory cultivation of microorganisms. This approach has a major limitation, given that only a small portion of such microorganisms (about 1%, in terms of prokaryotic genomes) can be cultivated under artificial conditions [23–25]. Therefore, the great majority of microorganisms (and consequently their enzymes) remains inaccessible and unknown. Besides this, many microorganisms can be harmful to health (e.g., pathogenic fungi and bacteria), so their manipulation requires special containment conditions.

In this context, a microorganism-independent culture technique that overcomes these limitations is a metagenomic approach [26,27]. In recent years, several enzymes have been isolated from metagenomic libraries, such as esterases [28–30], epoxyhydrolases [31], celulases [32], proteases [33], nitrilases [34], beta-glucosidases [35], decarboxylases [36], oxirredutases [37], amidases e peptidases [38], deoxyribonucleases [39] and lipases [40–43]. Although several lipases [40–46] have been isolated from metagenomic libraries, few reports describe their application in organic synthesis focusing on the preparation of optically active compounds. To the best of our knowledge, there are few enzymatic kinetic resolutions [47–51] and syntheses of esters and biodiesel [41,48] mediated by metagenomic lipases.

In this paper, we report the evaluation of an immobilized metagenomic lipase, LipG9, in EKR of aliphatic alcohols. LipG9 is

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a 55 kDa protein, which was isolated from a metagenomic library constructed from a fat-contaminated sediment collected from a wastewater treatment plant [41,47]. The immobilized form, Im-LipG9, is thermostable (up to 60 °C) and active in different organic solvents, and showed maximum specific hydrolytic activity of 66 U mg^{-1} of protein employing triolein as substrate in *n*-heptane. A previous EKR assay [47] demonstrated that LipG9 has excellent enantioselectivity ($E > 200$) in the transesterification reaction of 1-phenylethanol and the hydrolysis of 1-phenylethyl acetate, proving to be a promising biocatalyst for organic synthesis.

To increase the range of synthetic substrates, we evaluated Im-LipG9 in EKR of aliphatic *sec*-alcohols, a versatile class of organic compounds useful as synthetic intermediates in asymmetric synthesis for the preparation of anti-Alzheimer drugs [48], agrochemicals [49] and liquid crystals [50]. Inspite of their applicability in organic synthesis, the preparation of enantioenriched aliphatic alcohols by biocatalysis remains a challenge since low values of enantioselectivity are usually obtained in EKR of this class of substrates [51].

2. Experimental data

2.1. Materials

Unless otherwise stated, commercially available materials were used without further purification. All solvents were analytical grade. Analytical thin-layer chromatography (TLC) analyses were performed by using aluminum-backed silica plates coated with a 0.25 mm layer of silica gel 60 F254 (Merck, Darmstadt, HE, Germany), visualized with an ultraviolet light ($\lambda = 254$ nm), followed by exposure to a vanillin solution and mild heating. Standard chromatographic purification methods were followed using 35–70 mm (220–440 mesh) silica gel (Sigma–Aldrich, MO, USA). ^{1}H (200 MHz) and ^{13}C NMR (50 MHz) spectra were recorded with a Bruker DPX 200 spectrometer (Bruker, Massachusetts, USA). The chemical shifts of ^{1}H and ^{13}C NMR were assigned to internal $CDCl_3$ ($\delta_H = 7.26$ ppm), tetramethylsilane ($\delta_{TMS} = 0.00$ ppm) or $CDCl_3$ ($\delta_C = 77.0$ ppm). ^{1}H NMR spectra were recorded at 200 MHz frequency and the data are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sext = sextet, m = multiplet, brs = broad singlet), coupling constant (J) in Hertz and relative intensity. ^{13}C NMR spectra were recorded at 50 MHz frequency and data are reported as chemical shift in ppm (δ). Infrared spectra were recorded from KBr discs by FTIR with Bomem MB100 spectrometer (ABB Bomem, Zurich, Switzerland) with internal reference. Maximum absorption (ν_{max}) is reported in wavenumbers (cm^{-1}). Enantioselectivity parameters (ee, enantiomeric excess and E, enantioselectivity coefficient) were determined according to Chen et al. [52] by chiral GC analyses, which were performed with a GC-17A chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a hydrogen flame ionization detector and a CP Chirasil-DEX CB chiral column (25 m × 0.25 mm diameter, 0.25 μm film thickness). One micro liter of the samples was injected with a split ratio of 1:50, using N_2 as the carrier gas. The injector and detector were set at 220 °C. The temperature programming for all compounds was: 40 °C (1 min) to 100 °C—gradient rate: 2 °C min^{-1}. Aliphatic alcohols **1a–6a** did not show resolution in the chiral chromatography column used, requiring derivatization to respective propionates (**1c**–**6c**). The absolute configurations of compounds were attributed by comparison with the data available in the literature [51–55]. The retention times (t_R/min) were as follows for acetates (**1b**–**6b**) and propionates (**1c**–**6c**): (*R*)-**1b**: 9.91; (*S*)-**1b**: 8.38; (*R*)-**1c**: 13.38; (*S*)-**1c**: 12.25 (*R*)-**2b**: 15.19; (*S*)-**2b**: 12.95; (*R*)-**2c**: 18.91; (*S*)-**2c**: 17.63; (*R*)-**3b**: 20.79; (*S*)-**3b**: 18.94; (*R*)-**3c**: 25.10; (*S*)-**3c**: 23.99; (*R*)-**4b**: 27.12; (*S*)-**4b**: 25.46; (*R*)-**4c**: 31.50; (*S*)-**4c**: 30.63; (*R*)-**5b**: 11.61; (*S*)-**5b**: 10.38; (*R*)-**5c**: 15.32; (*S*)-**5c**: 14.60; (*R*)-**6b**: 13.53; (*S*)-**6b**: 12.77; (*R*)-**6c**: 17.80; (*S*)-**6c**: 17.28.

2.2. General procedure for synthesis of alcohols 1a–6a

To a solution of appropriated commercial ketone (50 mmol) in methanol (50 mL) at 0 °C, $NaBH_4$ (1.93 g, 51 mmol) was added in portions. In the sequence, the reaction was carried out under vigorous stirring at room temperature for 2–6 h. Then the methanol was removed by simple distillation, distilled water was added to the white residue and the pH was adjusted to pH 6.0 with aqueous HCl solution 1 mol L^{-1}. The organic phase was extracted with dichloromethane (3 × 20 mL), dried over anhydrous magnesium sulphate, filtered off and concentrated by simple distillation, leading to racemic alcohols **1a–6a**.

Pentan-2-ol (**1a**): Colorless liquid, bp 119 °C, yield 85%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.91–1.11 (m, 3H), 1.28 (d, $J = 6.2$ Hz, 3H), 1.39–1.53 (m, 4H), 1.80 (brs, 1H), 3.73–3.80 (m, 1H). ^{13}C NMR (50 MHz, CDCl3), δ (ppm): 13.9, 18.8, 23.4, 41.5, 67.7; IR (cm^{-1}): 3341, 2959, 2922, 1467 and 1363.

Hexan-2-ol (**2a**): Colorless liquid, bp 138 °C, yield 95%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.84–0.99 (m, 3H), 1.18 (d, $J = 6.2$ Hz, 3H), 1.24–1.52 (m, 5H), 1.89 (brs, 1H), 3.79 (sext, $J = 6.2$ Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 22.6, 23.3, 27.9, 38.9, 68.0; IR (cm^{-1}): 3348, 2966, 2922, 2856, 1467, 1371 and 1113.

Heptan-2-ol (**3a**): Colorless liquid, bp 159 °C, yield 91%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.89–1.39 (m, 14H), 1.73 (brs, 1H), 3.78 (sext, $J = 6.2$ Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 22.6, 23.4, 25.4, 31.8, 39.3, 68.1; IR (cm^{-1}): 3328, 2966, 2922, 2856, 1467 and 1371.

Octan-2-ol (**4a**): Colorless liquid, bp 174 °C, yield 89%. ^{1}H NMR (200 MHz, CDCl3, TMS), δ (ppm): 0.89 (s, 3H), 1.20 (d, $J = 6.2$ Hz, 3H), 1.22 (brs, 1H), 1.23–1.49 (m, 10H), 3.78 (sext, $J = 6.2$ Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 14.0, 22.6, 23.4, 25.7, 29.3, 31.8, 39.3, 68.1; IR (cm^{-1}): 3355, 2973, 2929, 2863, 1467, 1378 and 1327.

4-Methyl-pentan-2-ol (**5a**): Colorless liquid, bp 132 °C, yield 94%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.92 (d, $J = 6.6$ Hz, 6H), 1.20 (d, $J = 3.2$ Hz, 3H), 1.33–1.51 (m, 1H), 1.57 (brs, 1H), 1.63–1.86 (m, 1H), 3.79–3.98 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 22.3, 23.1, 23.9, 24.8, 48.6, 66.1; IR (cm^{-1}): 3319, 2959, 2871, 1467, 1392, and 1327.

Hexan-3-ol (6a): Colorless liquid, bp 135 °C, yield 92%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.92 (t, $J = 7.2$ Hz, 3H), 0.93 (t, $J = 6.9$ Hz, 3H), 1.31–1.56 (m, 6H), 1.74 (brs, 1H), 3.44–3.61 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.8, 14.1, 18.8, 30.1, 39.1, 73.0; IR (cm^{-1}): 3348, 2966, 2944, 2871, 1459 and 1319.

2.3. General procedure for synthesis of acetates 1b–6b

To a solution of appropriated alcohol **1a–6a** (25 mmol) in pyridine (15 mL), acetic anhydride (5.1 g, 50 mmol) was added. After stirring overnight at room temperature, ethyl acetate was added (20 mL) and the mixture was repeatedly washed with aqueous saturated solution of $CuSO_4$ until complete removal of pyridine. The organic phase was then isolated, dried over anhydrous magnesium sulfate, filtered off and concentrated under reduced pressure. The crude products were purified by flash column chromatography on silica gel (*n*-hexane: ethyl acetate 9:1) to give acetates **1b–6b** in 58–84% yields.

2-Pentyl acetate (**1b**): Colorless oil, bp 134 °C, yield 58%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.92 (t, $J = 7.1$ Hz, 3H), 1.21 (d, $J = 6.2$ Hz, 3H), 1.25–1.68 (m, 10H), 2.04 (s, 3H), 4.83–4.99 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.8, 18.5, 19.8, 21.2, 30.0, 70.7, 170.7; IR (cm^{-1}): 2959, 1738, 1467, 1378 and 1238.