Journal of Molecular Catalysis B: Enzymatic 85–86 (2013) 99–104

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Lipase catalyzed kinetic resolution for the production of (*S*)-3-[5-(4-fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one: An intermediate for the synthesis of ezetimibe

Amit Singh, Yogesh Goel, Amit Kumar Rai, U.C. Banerjee *

Department of Pharmaceutical Technology (Biotechnology), National Institute of Pharmaceutical Education and Research, Sector-67, S.A.S. Nagar 160062, Punjab, India

ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form 22 August 2012
Accepted 22 August 2012
Available online 29 August 2012

Keywords:
Transesterification
Ester hydrolysis
Candida rugosa lipase
Ezetimibe

ABSTRACT

Efficient enzymatic methods were developed for the synthesis of (*S*)-3-[5-(4-fluoro-phenyl)-5-hydroxy-pentanoyl]-4-phenyl-oxazolidin-2-one, by transesterification of (*RS*)-3-[5-(4-fluorophenyl)-5-hydroxypentanoyl]-4(*S*)-4-phenyl-1,3-oxazolidin-2-one [(*R*,*S*)-FOP alcohol] and hydrolysis of (*RS*)-1-(4-fluorophenyl)-5-oxo-5-[(*S*)-2-oxo-4-phenyloxazolidin-3-yl] pentyl acetate [(*R*,*S*)-FOP acetate] using lipase as enzyme source. The synthesized *S*-diastereomer is an intermediate for the potent cholesterol absorption inhibitor, ezetimibe. Among various lipases tried, *Candida rugosa* lipase in diisopropyl ether was best for both the reactions. Vinyl acetate was found as suitable acyl donor in transesterification reaction. A higher amount of enzyme (500 mg) was required for the transesterification of 10 mM substrate; it may be due to the enzyme denaturation by acetaldehyde formed in the reaction. The ester hydrolysis reaction worked well, excellent conversion and *de* were obtained at 40 °C, pH 7. The 300 mg enzyme hydrolyzed 120 mg (*R*,*S*)-FOP acetate with 50% conversion and 99.5% *de*.

1. Introduction

Microbial enzymes are routinely used as green alternatives for the synthesis of chiral pharmaceuticals with required enantiomeric purity. Today, drug regulatory agencies prefer maximum enantiomeric purity level in the drug substances to avoid variations in pharmacological responses elicited by racemates. Lipase has been a preferred and popular choice as the biocatalyst, especially due to its ability to work at aqueous/organic interphase, overcoming the solubility issue of hydrophobic compounds. Resolution of racemic alcohols using lipases has been the major tool for the synthesis of chiral alcohols. Lipases can catalyze selective esterification or transesterification of the racemic alcohol to yield an acylated alcohol, leaving other enantiomer unaffected [1–6]. Alternatively, the racemic ester may be prepared first by chemical means and then selectively hydrolyzed to alcohol by lipase, leaving other ester unaffected [7–10]. Thus enantiopure ester and alcohol may be recovered from the reaction mixture by simple chromatographic methods, since, the ester formed and alcohol left exhibit marked difference in physical and chemical properties. Ezetimibe, the first marketed drug of ACAT (acyl-CoA-cholesterol acyl transferase) inhibitor suppresses cholesterol absorption, storage and transport from the intestine, mainly by antagonizing Niemann-Pick C1-like protein 1 (NPC1L1) action [11–13]. The synthesis of ezetimibe requires enantiomerically pure alcohol, 3-[5-(4-fluorophenyl)-5(*S*)-hydroxypentanoyl]-4(*S*)-4-phenyl-1,3-oxazolidin-2-one [(*S*)-FOP alcohol], as a crucial intermediate. This is generally synthesized by the reduction of the corresponding ketone, 1-(4-fluorophenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione [FOP dione] using various chemical catalysts (Fig. 1). Fu et al. used Corey–Bakshi–Shibata catalyst [(*R*)-MeCBS] with borane tetrahydrofuran complex (BTHF) and obtained 92–96% *de* with <1% diol formation [14]. An improvement of this process was provided by Bertrand et al. [15] using (*R*)-MeCBS with borane diethylaniline complex (BDEA) as the reducing agent. The *de* was up to 98% with <1% diol formation. (−)-β-Chloro diisopinocampheyl borane ((−)-DIP) and (*R*)-diphenylprolinol have also been used as catalyst for this purpose [16,17]. All the above mentioned catalysts produce diol byproduct in the reduction process and the synthesized (*S*)-FOP alcohol is not chirally pure (<98% *de*). Moreover, the boron used in these catalysts is non degradable, may be hazardous for the environment. Only one microbial catalyst (*Schizosaccharomyces octosporus* ATCC 2479) has been reported for the reduction of FOP dione with 33–34% product yield and 100% *de* [18]. There is no report yet for the production of (*S*)-FOP alcohol using lipase as catalyst.

The aim of this work was to develop the efficient enzymatic method for the synthesis of pure (*S*)-FOP alcohol. Both, transesterification and hydrolysis reactions were tried for this purpose.

* Corresponding author. Tel.: +91 172 2214682/687x2142; fax: +91 172 2214692.
E-mail address: ucbanerjee@niper.ac.in (U.C. Banerjee).

1381-1177/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2012.08.014

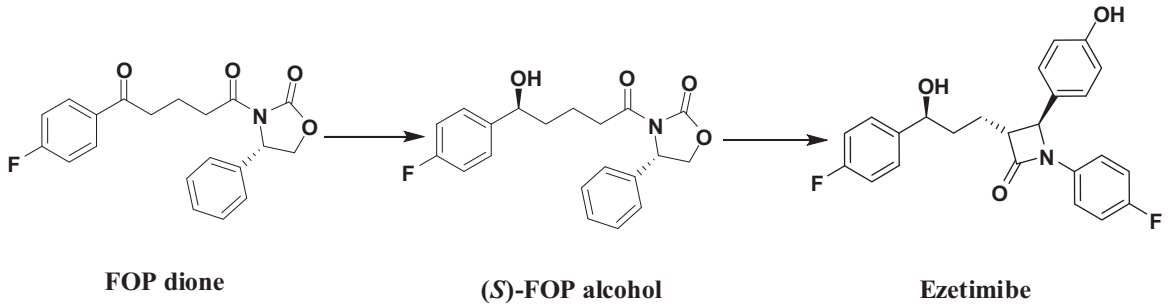

Fig. 1. Reduction of FOP dione to (*S*)-FOP alcohol, intermediate of ezetimibe, by chemical catalysts.

2. Materials and methods

2.1. Chemicals

1-(4-Fluorophenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione (FOP dione) was a kind gift from Ind-Swift Pharmaceutical Ltd. (Chandigarh, India). Lipases were purchased from Sigma–Aldrich (Germany). Borontetrahydrofuran (BTHF) and dimethylamino-pyridine (DMAP) were obtained from Aldrich Ltd (Germany). Various solvents used for HPLC analysis were purchased from J.T. Baker (Phillipsburg, NJ, USA), Rankem (Mumbai, India) and Merck Ltd (Whitehouse Station, NJ, USA), and were of HPLC grade. Solvents for synthesis and reaction were of the highest analytical grade and were dried over molecular sieves (3 A) before use. Buffer salts were obtained from Qualigens Inc. (Mumbai, India).

2.2. Analytical methods

FOP alcohol and ester formed (both by chemical and enzymatic methods) were analyzed using chiral reversed phase column (Lux 5μ Amylose, 4.6 mm × 250 mm, Phenomenex) in Shimadzu HPLC system consisting of LC-10AT pump, SPD-10A UV–VIS detector. The corresponding chiral alcohol and esters were eluted using ACN:water (55:45) as mobile phase at a flow rate of 0.5 mL/min and absorbance was measured at 215 nm with retention time 11.90 and 12.94 min for (*S*)- and (*R*)-alcohol, and retention time 16.80 and 17.92 min for (*R*)- and (*S*)-acetate ester, respectively.

The structure was confirmed by ^{1}H NMR (300 MHz) and ^{13}C NMR (100 MHz) using $CDCl_3$ as solvent (Bruker DPX 300 spectrometer; Billerica, MA, USA). Chemical shifts were recorded in parts per million, with tetramethylsilane as internal standard. Thin-layer chromatography was performed using pre-coated silica gel plates (Merck 60 F254; Merck, Darmstadt, Germany). Column chromatography was performed on Silica Gel 60 (0.040–0.063 mm, 230–400 mesh; Merck), employing hexane and ethyl acetate as developing solvents.

2.3. Preparation of racemic substrates

2.3.1. Synthesis of (RS)-FOP alcohol

FOP dione (5 g, 14 mmol) was dissolved in dry DCM (previously dried by distillation with P_2O_5) at room temperature and then THF:BORANE complex (13.95 mL) was added to reaction mixture at 0–5 °C with stirring (300 rpm). The reaction was subjected to nitrogen atmosphere to maintain the reducing condition. Reaction was monitored for 6 h using TLC (3:1 hexane:ethyl acetate). Solvent was evaporated and purified using column chromatography on silica gel (5:1 hexane:ethyl acetate), yielded 3 g (8.4 mmol, 70%). ^{1}H NMR: δ 7.32–7.42 (m, 4H), 7.23–7.28 (m, 3H), 6.99 (t, J = 8.1 Hz, 2H), 5.40 (dd, J = 8.6 Hz, 3.5 Hz, 1H), 4.59–4.77 (m, 2H), 4.26 (dd, J = 8.0 Hz, 3.6 Hz, 1H), 2.89–3.00 (m, 2H), 2.17 (s, 1H), 1.52–2.04 (m, 4H). ^{13}C NMR: δ 172.55, 163.32, 153.76, 140.21, 140.18, 139.39, 139.04, 129.24, 129.21, 128.92, 128.76, 115.31, 115.10, 73.28, 70.02, 57.56, 38.25, 35.17, 20.27.

2.3.2. Synthesis of (RS)-FOP acetate

(*RS*)-FOP alcohol (2 g, 5.6 mmol) was dissolved in DCM and reaction mixture was cooled to 0 °C. Triethylamine (1.16 mL) was added followed by acetic anhydride (0.53 mL) and DMAP (0.068 g). Reaction was monitored for 4 h by TLC. The reaction was worked up by diluting the reaction mixture with DCM, washing it with 1 N HCl and at last with brine solution to remove DMAP. The product was isolated and then purified using column chromatography on silica gel (5:1 hexane:ethyl acetate), yielded 1.9 g (4.75 mmol, 95%). ^{1}H NMR: δ 7.32–7.39 (m, 3H), 7.25–7.30 (m, 4H), 6.97–7.01 (m, 2H), 5.65–5.68 (m, 1H), 5.37–5.41 (m, 1H), 4.67 (t, J = 8.1 Hz, 1H), 4.27 (dd, J = 7.9 Hz, 3.6 Hz, 1H), 2.92–2.98 (m, 2H), 2.04 (d, J = 2.44 Hz, 2H), 1.86–1.91 (m, 1H), 1.53–1.79 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.09, 21.05, 35.11, 57.56, 60.40, 70.02, 74.95, 115.23, 115.44, 125.91, 128.19, 128.27, 128.76, 129.21, 136.14, 139.05, 153.71, 161.09, 165.53, 170.26, 171.18, 172.15.

Similarly, (*R*,*S*)-FOP butyrate/isobutyrate were synthesized by esterification of (*R*,*S*)-FOP alcohol with butyric anhydride and isobutyric anhydride, respectively.

2.3.3. Synthesis of FOP dione

Pyridinium chlorochromate (151 mg, 0.7 mmol) was dissolved in DCM, placed on the magnetic stirrer and equipped with condenser. (*R*)-FOP alcohol (250 mg, 0.7 mmol) dissolved in DCM, was added to pyridinium chlorochromate suspension with stirring and heated to 40 °C. Progress of reaction was checked for 2 h by TLC (3:1 hexane:ethyl acetate). The reaction was worked up by dilution with DCM and filtered through celite bed. The filtrate was washed with 1 M NaOH solution, followed by saturated salt solution and dried. The product was purified using column chromatography on silica gel (5:1 hexane:ethyl acetate), yielded 227 mg (0.64 mmol, 91%).

2.4. Lipase-mediated transesterification of (R,S)-FOP alcohol

Commercially available lipases from different sources were used for the resolution of (*R*,*S*)-FOP alcohol. Each lipase was used in three solvents (hexane, diisopropyl ether (DIPE) and toluene) separately and vinyl acetate was used as acyl donor. The substrate (*R*,*S*)-FOP alcohol had partial, moderate and good solubility in hexane, DIPE and toluene, respectively. The reaction (5 mL) was set up with 3.6 mg substrate, 50 mg enzyme (free powder or immobilized on solid support) and 5 mM vinyl acetate. The reaction contents were stirred on magnetic stirrer (500 rpm, for lipases in powder form) or rotatory shaker (250 rpm, for immobilized lipases) at 35 °C. The samples (200 μL) from the organic layer of the reaction mixture was taken out at 24 h interval for 5 days and dried on rotavapor.

It was re-dissolved in acetonitrile (1 mL), filtered through 0.22 μM filter and analyzed through HPLC system.

2.5. Optimization of transesterification reaction

Experiments were conducted to check the effect of acylating agents, type of co-solvent and substrate and enzyme concentration on the transesterification of (*R*,*S*)-FOP alcohol.

2.5.1. Acylating agent

Different acylating agents (vinyl acetate, vinyl butyrate, benzyl acetate and acetic anhydride) were tried for the resolution of (*R*,*S*)-FOP alcohol by *C. antarctica* and *C. rugosa* lipase. Toluene was used as solvents with the *C. antarctica* lipase whereas DIPE was used with *C. rugosa* lipase. Reactions (5 mL) were set up with 3.6 mg substrate and 100 mg enzyme. The acylating agents (5 mM) were added, separately to each reaction. Samples (200 μL) were withdrawn at 24 h interval for 5 days, dried in rotavapor and analyzed through HPLC system.

2.5.2. Co-solvent

The solubility of (*R*,*S*)-FOP alcohol in DIPE was increased by the addition of various co-solvents such as tetrahydrofuran, toluene, dioxane, DCM, DMF, etc. Reactions were set up with 3.6 mg substrate, 100 mg *C. rugosa* lipase in DIPE and co-solvents (5–20%, v/v) to make the total reaction volume 5 mL. The reaction was stirred on magnetic stirrer (500 rpm) at 35 °C. The samples (200 μL) from the organic layer of the reaction mixture was taken out at 24 h interval for 5 days, dried over rotavapor and analyzed by HPLC.

2.5.3. Substrate and enzyme concentration

Reactions (5 mL) were set up containing various quantity (3.6, 9, 18 and 36 mg) of (*R*,*S*) FOP alcohol with 200 mg *C. rugosa* lipase in DIPE as solvent and vinyl acetate as acylating agent. The reaction mixture was stirred on magnetic stirrer (500 rpm) at 35 °C. The samples (200 μL) from the organic layer of the reaction mixture was taken out each day for 5 days, dried in rotavapor and analyzed through HPLC system. Various enzyme concentrations were used for the resolution of 18 mg (*R*,*S*)-FOP alcohol. Reactions (5 mL) were set up containing 200–500 mg *C. rugosa* lipase in DIPE using vinyl acetate as acyl donor and stirred on a magnetic stirrer (500 rpm) at 35 °C. The samples (200 μL) from the organic layer of the reaction mixture was taken out each day up to 6 days, dried in rotavapor and analyzed by HPLC.

2.6. Lipase-mediated hydrolysis of (R,S)-FOP acetate

Similar to the transesterification reaction, commercially available lipases from different sources were used in three solvents [hexane, DIPE and toluene], for the hydrolysis of (*R*,*S*)-FOP acetate. The reaction was set-up by dissolving the substrate, [(*R*,*S*)-FOP acetate, 10 mg] in 1.5 mL organic solvent, separately and 50 mg enzyme suspended in 3.5 mL phosphate buffer (pH 7) was added to it. The reaction was stirred on magnetic stirrer (500 rpm, for lipases in powder form) or rotatory shaker (250 rpm, for immobilized lipases) at 35 °C. The samples (200 μL) from the organic layer of the reaction mixture was taken out at 24 h interval up to 5 days and dried on rotavapor. It was again dissolved in acetonitrile (1 mL), filtered through 0.22 μM filter and analyzed by HPLC.

2.7. Optimization of ester hydrolysis

The various physico-chemical parameters such as temperature, reaction pH, type of ester and substrate and enzyme concentration on the ester hydrolysis were optimized.

2.7.1. Temperature

In order to determine the optimum temperature for the hydrolysis of (*R*,*S*)-FOP acetate by *Candida rugosa* lipase, reactions were carried out in 5 mL reaction volume having 10 mg substrate dissolved in 1.5 mL DIPE and 150 mg enzyme suspended in 3.5 mL phosphate buffer (pH 7). The reaction contents were stirred on magnetic stirrer (500 rpm) and incubated at various temperatures ranging from 25 to 50 °C. The organic layer of the reaction mixture was taken out at 24 h interval up to 5 days, dried on rotavapor and analyzed by HPLC.

2.7.2. Reaction pH

The substrate (10 mg) was dissolved in 1.5 mL organic solvent and 150 mg enzyme suspended in 3.5 mL 0.1 M potassium phosphate buffer having a pH range from 5.8 to 8, was added. The reaction contents were stirred on magnetic stirrer (500 rpm) at 40 °C. The samples (200 μL) from the organic layer of the reaction mixture was taken out at 24 h interval up to 5 days, dried on rotavapor and analyzed by HPLC.

2.7.3. Various (R,S)-FOP esters

Butyric and isobutyric esters of racemic FOP-alcohol were also tried. The hydrolysis reaction was set up with 150 mg enzyme, 10 mg esters in DIPE (1.5 mL) with 3.5 mL 0.1 M phosphate buffer, pH 7 and incubated at 40 °C for 5 days. The samples (200 μL) of the reaction mixture was taken out at 24 h interval for 5 days, dried on Rotavapor and analyzed by HPLC.

2.7.4. Alcoholysis of the (R,S)-FOP acetate

Different alcohols (20 mM) were used in place of buffer for the hydrolysis of (*R*,*S*)-FOP acetate (10 mg) by the *C. rugosa* lipase at 40 °C for 5 days. Samples (200 μL) were collected at 24 h interval up to 5 days and dried on rotavapor. The reaction contents were analyzed by HPLC.

2.7.5. Substrate and enzyme concentration

The (*R*,*S*)-FOP acetate at different quantity (10, 20, 40, 60, 90, 120, 150 and 180 mg) was hydrolyzed with varying enzyme concentrations (150, 200, 250 and 300 mg) in order to achieve maximum conversion and *de* of the product formed. The reaction was carried out in 5 mL DIPE:buffer (1.5:3.5 mL) at 40 °C, and stirred on magnetic stirrer (500 rpm). The organic layer of the reaction mixture was taken out at 24 h interval up to 5 days, dried on rotavapor and analyzed by HPLC.

3. Results and discussion

3.1. Transesterification of (R, S)-FOP alcohol

Various commercially available lipases from different sources were tried for the resolution of (*R*,*S*)-FOP alcohol using different solvents (heptanes, DIPE and toluene). Immobilized lipases on various supports (*Pseudomonas cepacia* sol–gel, *Candida antarctica* acrylic–resin and Lipozyme) and free lipases from porcine pancreatine and *Mucor miehei* showed no conversion. The lipases from *Candida cylindrica* and *Aspergillus niger* showed resolution of (*R*,*S*)-FOP alcohol in heptane only (Table 1), which was not desirable due to the partial solubility of FOP alcohol in heptane. Only *C. antarctica* lipase showed transesterification in polar solvent (toluene), however, the diastereomeric excess (*de*) was very less (69.28%). Various other polar solvents such as di chloro methane, tetrahydrofuran, dimethyl formamide and dioxane were used with *C. antarctica* lipase, no improvement in *de* was observed. The *C. rugosa* lipase in DIPE was found as best catalyst for this reaction, since 100% *de* of the product was obtained (Table 1) and DIPE provided moderate

Table 1
Screening of lipases from various commercial sources for the transesterification and hydrolysis of (*R*,*S*)-FOP alcohol and (*R*,*S*)-FOP acetate, respectively. (Substrate 2 mM, enzyme 50 mg, reaction volume 5 mL.)

Source of lipases	Solvents											
	Heptane				Diisopropyl ether				Toluene			
	Conversion (%)		*de* (%)		Conversion (%)		*de* (%)		Conversion (%)		*de* (%)	
	T	H	T	H	T	H	T	H	T	H	T	H
Candida antarctica B	44.8	10.8	39	39.7	42.2	5.2	52.2	51.1	16.4	2.1	69.3	74.3
Candida rugosa	47.5	13.1	100	97.7	12.5	8.6	100	98.5	–	–	–	–
Candida cylindrica	28.2	7.2	100	89.6	–	–	–	–	–	–	–	–
Aspergillus niger	6.5	3.2	100	76.4	–	–	–	–	–	–	–	–
Mucor miehei	–	3.9	–	94.1	–	4.2	–	89.1	–	–	–	–

T, transesterification reaction; H, ester hydrolysis reaction; "–", "not detectable".

solubility of the substrate, (*R*,*S*)-FOP alcohol. All the used lipases esterified the (*R*)-FOP alcohol to (*R*)-FOP acetate.

3.1.1. Effect of acylating agent

The effect of various acylating agents on the transesterification of alcohol by lipases are well reported in the literature [19]. Various acylating agents (vinyl acetate, vinyl butyrate, benzyl acetate and acetic anhydride) were used for the transesterification of (*R*,*S*)-FOP alcohol using *C. antarctica* and *C. rugosa* lipases. Toluene was used as solvent for the *C. antarctica* lipase as an effort to increase both the conversion and selectivity. The DIPE was used as reaction solvent with the *C. rugosa* lipase to increase the conversion. However, it was observed that in all the transesterification reactions, vinyl acetate was the best acylating agent and no reaction was observed with vinyl butyrate and acetic anhydride as the acylating agents. Transesterification reaction was carried out with benzyl acetate as acylating agent using *C. antarctica* lipase in toluene, but both the conversion (21.2%) and *de* (54.8%) were lower compared to that of carried out by vinyl acetate as acylating agent (35.9% conversion and 58.4% *de*). Excellent selectivity (100%) and good conversion (23.48%) was achieved with *C. rugosa* lipase with vinyl acetate using DIPE as solvent. All the subsequent experiments were carried out with *C. rugosa* lipase with DIPE as a solvent and vinyl acetate as acylating agent.

3.1.2. Effect of co-solvent

Since, the substrate was moderately soluble in DIPE, various polar solvents such as tetrahydrofuran, toluene, dioxane, DCM, DMF, etc. were added as co-solvent (5–20%, v/v) in order to make (*R*,*S*)-FOP alcohol more soluble in the reaction mixture. Marked decrease in conversion was observed with the increased concentration of all the co-solvents tried (data not shown). This may be due to the detrimental effect of polar solvents on the *C. rugosa* lipase activity.

3.1.3. Effect of substrate and enzyme concentration

The substrate concentration was increased from 3.6 to 36 mg in the reaction mixture. Maximum conversion (48%) was achieved with 3.6 mg substrate, the conversion decreased to 36 and 24% at 18 and 36 mg substrate concentration, respectively. It may be due to the inhibition of lipase activity at higher substrate concentrations. However, *de* remained unchanged with the increased concentration of substrate. Resolution of 18 mg (*R*,*S*)-FOP alcohol was tried with 200–500 mg *C. rugosa* lipase, complete conversion (50%) with 100% *de* was achieved with 500 mg enzyme. It was observed that at lower enzyme concentration (200 mg), conversion had decreased to 33% (Fig. 2). The decrease in conversion at lower enzyme concentration was probably due to the denaturation of lipase by acetaldehyde formed during longer incubation period (5–6 days), whereas, only 2–3 days were required to achieve highest conversion at higher enzyme concentration. In transesterification reaction, vinyl acetate is converted to vinyl alcohol which tautomerizes to acetaldehyde. The enzyme denaturation during transesterification reaction using vinyl acetate as acylating agent [20,21], specially denaturation of *C. rugosa* lipase, is well reported [22,23] in literature.

3.2. Enantioselective hydrolysis of (R,S)-FOP acetate

The transesterification reaction for the resolution of (*R*,*S*)-FOP alcohol resulted in low yield. Another approach, hydrolysis of the corresponding ester (*R*,*S*)-FOP acetate to (*S*)-FOP alcohol, was tried. All the lipases tried for the transesterification reaction were also used for hydrolysis reaction. Here also, *Pseudomonas cepacia* sol–gel, Porcine pancreatine and *C. antarctica* acrylic–resin lipases did not show any conversion while lipase from *M. miehei* showed very less conversion. More or less, similar pattern as seen in the case of transesterification reaction, was observed in the hydrolysis of (*R*,*S*)-FOP acetate. No conversion was shown by the immobilized lipases and only *C. antarctica* free lipase showed conversion in the polar (toluene) solvent with very less *de*. Lipases from *Candida cylindracea* and *A. niger* showed very less conversion with lower selectivity, when heptane was used as a solvent, however, there was no conversion of the (*R*,*S*)-FOP acetate by *Candida cylindracea* and *A. niger* lipases when the hydrolysis reactions were carried out in DIPE and toluene as reaction medium. Good *de* was obtained with *C. rugosa* lipase (98.54%) in DIPE as reaction medium with less (8.61%) conversion (Table 1). Here also, all the enzymes preferentially act on the (*R*) isomer i.e. hydrolyzed the (*R*)-FOP acetate to (*R*)-FOP alcohol, thus followed the Kazlauskas rule [24].

Organic solvents with higher polarity such as tetrahydrofuran, acetone, acetonitrile, pyridine, dichloromethane, 1,4-dioxane, etc.

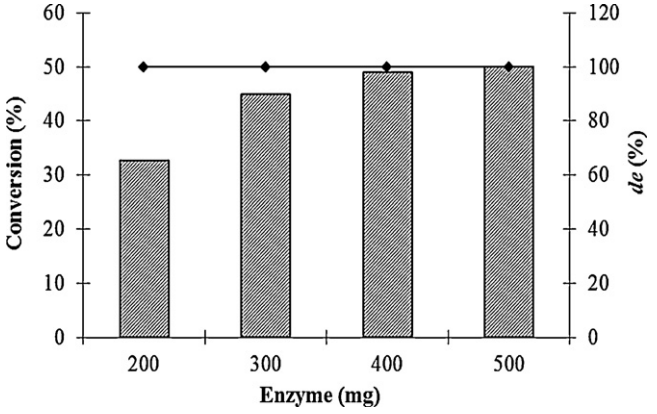

Fig. 2. Effect of enzyme concentration on the transesterification of (*R*,*S*)-FOP alcohol by *C. rugosa* lipase (substrate 18 mg, DIPE 5 mL, vinyl acetate 25 mM). The bar and line graphs represent conversion and *de* of the product formed, respectively.

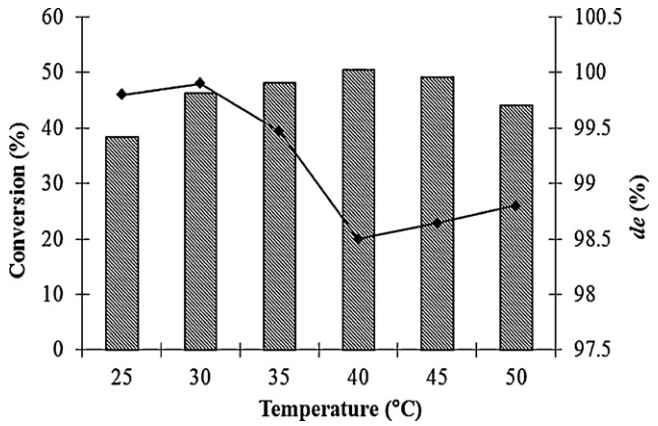

Fig. 3. Effect of temperature on the hydrolysis of (*R*,*S*)-FOP acetate by *C. rugosa* lipase (substrate 10 mg, enzyme 150 mg, DIPE 1.5 mL, buffer 3.5 mL). The bar and line graphs represent conversion and *de* of the product formed, respectively.

were tried in place of DIPE for the hydrolysis of (*R*,*S*)-FOP acetate, however, there was no conversion or very less conversion with low *de* was obtained. Usually solvents with low log P values are more hydrophilic and they reduce the catalytic activity of enzymes [25]. Heptane was not selected as reaction medium because of low solubility of the substrate, although conversion was higher (13.08%) with comparable *de* (97.66%). Hence, DIPE was selected as reaction medium. It may be mentioned that ester group imparted higher solubility to the FOP acetate, making it well soluble in DIPE.

3.2.1. Effect of temperature and pH

The effect of temperature on the hydrolysis of (*R*,*S*)-FOP acetate by *C. rugosa* lipase was studied at various temperatures ranging from 25 to 50 °C. Increased conversion was observed at higher temperature and it reached to maximum (50%) at 40 °C, and decreased thereafter. However, the *de* values decreased slightly with the increased conversion (Fig. 3). The (*R*)-FOP acetate was hydrolyzed to (*R*)-FOP alcohol by *C. rugosa* lipase, leaving (*S*)-FOP acetate as such which was the product of interest. The higher conversion was also an important criterion of the reaction, where a slightly decreased *de* may be compromised. Hence, 40 °C reaction temperature was selected for the hydrolysis of (*R*,*S*)-FOP acetate by *C. rugosa* lipase. There was no variation of concentration when the pH of reaction buffer was varied between 5.8 and 8 (data not shown).

3.2.2. Effect of different (R,S)-FOP esters

The effect of ester groups on the hydrolysis reaction by *C. rugosa* lipase was studied by replacing acetate with the butyric and isobutyric esters. Lipase from *C. rugosa* could not selectively hydrolyze the butyrate and isobutyrate esters of (*R*,*S*)-FOP alcohol, the conversion was very high (95%), however, the *de* was totally lost (data not shown). Hence, (*R*,*S*)-FOP acetate was the most suitable substrate for the *C. rugosa* lipase.

3.2.3. Effect of substrate and enzyme concentration

After the optimization of all the necessary parameters for the *C. rugosa* lipase-mediated hydrolysis reaction at 10 mg substrate level, studies were carried out under optimized condition with the increasing quantities of both the enzyme (150, 200, 250, 300 mg) and the substrate (10, 20, 40, 60, 90, 120, 150 and 180 mg). Table 2 represents the various concentrations of *C. rugosa* lipase and the substrate resolved. It was found that 300 mg enzyme was suitable for the resolution of 120 mg substrate completely. Preparative gram scale reaction was setup with 1 g of (*R*,*S*)-FOP acetate and pure (*S*)-ester was obtained with >99% *de*.

Table 2
Effect of enzyme and substrate concentration on the resolution of (*R*,*S*)-FOP acetate by *C. rugosa* lipase (solvent DIPE + water, 40 °C, pH 6.2, water content 70%).

Serial no	Enzyme used (mg)	(*R*,*S*)-FOP acetate (mg)	Conversion (%)	*de* (%)
1	150	10	50	99.2
		20	51	98.6.
		40	36	97.5
2	200	40	50	99.3
		60	51	99.2
		90	36	98.2
3	250	90	51	99.5
		120	37	98.8
		150	32	97.7
4	300	120	50	99.5
		150	35	97.9
		180	30	97.8

Table 3
Alcoholysis of (*R*,*S*)-FOP acetate by *C. rugosa* lipase. (Substrate 5 mM, enzyme 150 mg, solvent DIPE, 40 °C.)

Serial no.	Alcohol	Conversion (%)	*de* (%)	Time (days)
1	Methanol	21.4	>95%	20
2	Propanol	26.6	>95%	20
3	Butanol	24.4	>95%	20
4	Isobutyl alcohol	27.3	>95%	20
5	Hexanol	23.9	>95%	20
6	Octanol	17.9	>95%	20

3.3. Alcoholysis of the (R,S)-FOP acetate

Use of alcohols in place of water for the hydrolysis reaction is well known to improve the selectivity of the reaction [26–28]. Various alcohols were tried for alcholysis of (*R*,*S*)-FOP acetate using *C. rugosa* lipase. It was observed that rate of alcholysis of (*R*,*S*)-FOP acetate was very less along with decreased stereoselectivity, as compared to hydrolysis reaction (Table 3). Percentage conversion was very less with lower *de*, moreover, all the reactions took a longer incubation time (20 days) for conversion.

4. Conclusion

For the synthesis of (*S*)-FOP alcohol, both transesterification and hydrolysis reactions were studied. For both the reactions, *C. rugosa* lipase in DIPE as solvent gave best results. The ester hydrolysis reaction was more efficient as transesterification reaction was associated with acetaldehyde-mediated enzyme denaturation. Maximum ester hydrolysis was obtained at 40 °C (pH 7) with 300 mg enzyme and 120 mg (*R*,*S*)-FOP acetate as substrate. The undesired (*R*)-FOP alcohol was oxidized to FOP dione using Pyridinium chlorochromate, thus formed FOP dione may be recycled.

Acknowledgements

AS, YG and AKR are grateful for the fellowships provided by CSIR and DBT, Govt. of India, for carrying out this work.

References

[1] Z.G. Chen, R.X. Tan, M. Huang, Process Biochem. 45 (2010) 415–418.
[2] P. Hara, J.P. Mikkola, D.Y. Murzin, L.T. Kanerva, J. Mol. Catal. B: Enzym. 67 (2010) 129–134.
[3] E. Husson, C. Humeau, C. Harscoat, X. Framboisier, C. Paris, E. Dubreucq, I. Marc, I. Chevalot, Process Biochem. 46 (2011) 945–952.
[4] D. Sebrão, M.M. Sá, M.D.G. Nascimento, Process Biochem. 46 (2010) 551–556.
[5] T. Storz, J. Gu, B. Wilk, E. Olsen, Tetrahedron Lett. 51 (2010) 5511–5515.
[6] Y. Cao, Y. Zhuang, C. Yao, B. Wu, B. He, Biochem. Eng. J. 64 (2012) 55–60.
[7] P.B. Juhl, K. Doderer, F. Hollmann, O. Thum, J. Pleiss, J. Biotechnol. 150 (2010) 474–480.
[8] G. Tasnádi, E. Forró, F. Fülöp, Tetrahedron: Asymm. 20 (2009) 1771–1777.

[9] E. Yilmaz, K. Can, M. Sezgin, M. Yilmaz, Bioresour. Technol. 102 (2011) 499–506.
[10] A.C. Wu, P.Y. Wang, K.J. Chen, S.W. Tsai, J. Mol. Catal. B: Enzym. 74 (2012) 41–47.
[11] B.M. Domagala, M. Leady, D.S. Streetman, Pharm. Technol. 28 (2003) 191–206.
[12] G.C. Margarita, L. JeanMarie, G.B. Herbert, E.H. Brian, A.B. Duane, P.B. Matthew, Proc. Natl. Acad. Sci. USA 102 (2005) 8132–8137.
[13] S.B. Rosenblum, T. Huynh, A. Afonso, H.R. Davis, N. Yumibe, J.W. Clader, D.A. Burnett, J. Med. Chem. 41 (1998) 973–980.
[14] X. Fu, T.L. McAllister, T.K. Thiruvengadam, C.H. Tann, D. Su, Tetrahedron Lett. 44 (2003) 801–804.
[15] B. Bertrand, S. Durassier, S. Frein, A. Burgos, Tetrahedron Lett. 48 (2007) 2123–2125.
[16] S. Reddy, S., Reddy, WO/2008/032338.
[17] Y. Kumar, H.N.P.N., Meeran, S.K., Singh, P.D., Rathod, K.K., Ganagakhedkar, P., Bose, P., Kumar, WO/2005/066120.
[18] M.J. Homann, E. Previte, US/1997/5618707.
[19] A. Chojnacka, R. Obarab, C. Wawrzeńczyk, Tetrahedron: Asymm. 18 (2007) 101–107.
[20] M. Deguil-Casting, B. De Jeso, S. Drouillard, B. Maillard, Tetrahedron Lett. 28 (1987) 953–954.
[21] Y.F. Wang, J.J. Lalonde, M. Momongan, D.E. Bergbreiter, C.H. Wong, J. Am. Chem. Soc. 110 (1988) 7200–7205.
[22] H.K. Weber, K. Faber, Methods Enzymol. 206 (1997) 509–518.
[23] H.K. Weber, J. Zuegg, K. Faber, J. Pleiss, J. Mol. Catal. B: Enzym. 3 (1997) 131–138.
[24] R.J. Kazlauskas, A.N.E. Weissfloch, A.T. Rappaport, L.A. Cuccia, J. Org. Chem. 56 (1991) 2656–2665.
[25] A. Zaks, A.M. Klibanov, J. Am. Chem. Soc. 108 (1986) 2767–2768.
[26] J.D. Moseley, J. Staunton, Tetrahedron: Asymm. 11 (2000) 3197–3209.
[27] P. Ferraboschi, M.D. Mieri, L. Ragonesi, Tetrahedron Lett. 49 (2008) 4610–4612.
[28] E. Santaniello, S. Casati, P. Ciuffreda, L. Gamberoni, Tetrahedron: Asymm. 16 (2005) 1705–1708.