Journal of Molecular Catalysis B: Enzymatic 118 (2015) 52–61

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Towards a green enantiomeric esterification of R/S-ketoprofen: A theoretical and experimental investigation

María Victoria Toledo[a], Carla José[a], Sebastián E. Collins[b], María Luján Ferreira[c], Laura E. Briand[a,*]

[a] Centro de Investigación y Desarrollo en Ciencias Aplicadas-Dr. Jorge J. Ronco. Universidad Nacional de La Plata, CONICET, CCT La Plata. Calle 47N 257, B1900AJK La Plata, Buenos Aires, Argentina
[b] Instituto de Desarrollo Tecnológico para la Industria Química INTEC, Universidad Nacional del Litoral, CONICET, Güemes 3450, 3000 Santa Fe, Santa Fe, Argentina
[c] Planta Piloto de Ingeniería Química – PLAPIQUI, CONICET, Universidad Nacional del Sur, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 2 May 2015
Accepted 7 May 2015
Available online 15 May 2015

Keywords:
Lipase
Novozym® 435
Alcohols
R/S-ketoprofen
Kinetic resolution

ABSTRACT

Methanol, ethanol, 1- and 2-propanol were used as reactants and solvents in the esterification of R/S-ketoprofen catalyzed with Novozym® 435. The interaction of the alcohols with Novozym® 435 was studied at a molecular level through various spectroscopic techniques and molecular modeling. The results evidenced the dissolution of the polymeric support, loss of active protein, strong adsorption of the alcohols, modification of the secondary structure of the protein and smoothing of the inner structure of the biocatalyst's beads upon extended contact with the alcohols. Nevertheless, none of those drawbacks influences the specific activity and enantiomeric excess toward S(+)-enantiomer that remain unaltered upon extended contact with ethanol, 1- and 2-propanol as acyl acceptors. However, theoretical calculations demonstrated that methanol introduces steric and electronic hindrance within the step of the coordination of the R/S-ketoprofen with the catalytic triad.

1. Introduction

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) are a family of enzymes broadly applied in organic chemistry. They have wide specificity and also the ability to react with different substrates [1]. They are able to catalyze not only hydrolytic but also synthetic reactions of esterification, transesterification, interesterification and alcoholysis among others. These enzymes are chosen for application in several industries such as, food, detergent, pharmaceutical, biodiesel, cosmetic and paper due to their high activity, substrate-, regio- and stereospecificity along with their milder reaction conditions [2–6].

Pharmaceutical industries apply the enantioselectivity of lipases for preparing different pharmaceuticals and fine chemicals containing a chiral center [1,7]. Several drugs are found in their racemic form. However, in many cases one enantiomer is responsible for the activity of interest while the other could be inactive, be an antagonist of the active enantiomer or have a different activity which could produce side effects or being toxic. In this context, the production of stereochemically pure drugs has many advantages, such as the reduction of the administered dose, improved therapeutic window, prevents side effects and a more precise estimation of dose-response relationship [8].

2-Arylpropionic acids (profens) constitute an important group of non-steroidal anti-inflammatory substances (NSAIDs). They have their pharmacological activity mainly on the (S)-enantiomer. These are widely used for relieving pain and for the treatment of inflammatory diseases, such as rheumatoid arthritis and osteoarthritis [9]. In most cases, the (R)-enantiomer often has a very poor activity, unwanted physiological side effects and toxicity [10]. Among the side effects, gastrointestinal disorders are frequently present, being ulceration and mucosal hemorrhage at different locations from the gastrointestinal tract the most frequent adverse reactions [11].

Lipases are one of the most suitable enzymes for kinetic resolution of profens due to their wide substrate specificity, their ability to recognize chirality and do not require labile cofactors [12]. Moreover, from an environmental point of view, processes that use enzymes are greener, less hazardous and friendlier because they do not contaminate as in the case of using chemical catalysts [13,14]. In this context, the investigations regarding the kinetic resolution of R/S-ketoprofen with lipases are summarized chronologically

* Corresponding author. Tel.: +54 221 4 211353/210711.
E-mail address: briand@quimica.unlp.edu.ar (L.E. Briand).

http://dx.doi.org/10.1016/j.molcatb.2015.05.003
1381-1177/

within the Supplementary Information section in Table 1S (see refs. [15-29]. This table shows the various acyl acceptors, reaction conditions (molar ratio of substrates, temperature, others), co-solvents, conversion and enantiomeric excess of the esterification of the profen with alcohols. In this regard, the immobilized lipase B of *Candida antarctica* known as Novozym® 435 is the most widely investigated (see entries 1, 2, 4–7, 10, 12, 15 and references within Table 1S). The enantiomeric esterification of racemic ketoprofen with alcohols such as methanol, ethanol, 1-propanol, 1-butanol and dodecanol between others, was assayed with various organic co-solvents (1,2 diisopropyl ether; CCl_4, hexane, diisopropyl ether, methylene dichloride, isobutyl methyl ketone, acetonitrile, *etc.*). The investigation reported by Park et al. is probably the most complete one on that topic (entry 4 in Table 1S, ref. [18]). The authors found that short chain alcohols enhanced the reaction rate in the presence of a mixture of ethylene dichloride and n-hexane 20% (v/v) as co-solvent. In particular, the use of ethanol as acyl acceptor and solvent provided 6% of profen conversion with 2% of enantiomeric excess toward the S-enantiomer at 37 °C in 75 h of reaction (see entry 4, center paragraph in Table 1S). A more recent investigation by De Crescenzo et al. reported that there was no reaction of 1-dodecanol and ketoprofen in a solventless media (entry 6, ref. [20]). However, they prepared propyl ketoprofenate by direct esterification with 1-propanol for 4 days with the solely aim of obtaining the ester for calibration purposes. The authors determined a much higher rate of reaction when using 1-propanol (900 μmol h^{-1} g^{-1} of enzyme) instead of 1-dodecanol (200 μmol h^{-1} g^{-1} of enzyme) with xylenes as co-solvents. Additionally, the rate of reaction resulted lower (48 μmol h^{-1} g^{-1} of enzyme) when solely 1-propanol was used. More recently, some of us assayed the esterification of racemic ketoprofen with 2-propanol without success (see entry 15 in Table 1S, ref. [29]).

To the knowledge of the authors there is no additional information of the solventless esterification of racemic ketoprofen besides those commented above. In this context, the kinetic resolution of ketoprofen through the esterification with solely short chain alcohols (without the addition of co-solvents) is reported for the first time in the literature in this contribution. A complete picture of the effect of the short chain alcohols on the catalytic performance of the CALB lipase in the esterification of profens is provided at molecular level (through theoretical calculations) and experimental evidences.

2. Experimental

2.1. Materials

The commercial biocatalyst Novozym® 435 (batch LC200217) was a gift of Novozymes Brasil (Paraná, Brazil). R/S-ketoprofen (Parafarm, 99.80%, batch 030718 000928/004); methanol (Tedia), ethanol (Carlo Erba) and 1-propanol (Sigma–Aldrich, ≥99.5%) and potassium hydroxide (1 mol/L) in ethanol (Riedel-de Haën) were used.

2.2. Esterification of profens with short chain alcohols

The esterification of R/S-ketoprofen with methanol, ethanol and 1-propanol as reactants and solvents catalyzed by Novozym® 435 were carried out at the optimum operative conditions previously determined for the esterification of R/S-ibuprofen with ethanol [30]. R/S-ketoprofen (0.5000 g; 1.966 mmol) was dissolved in 0.70 mL (17.28 mmol) of methanol; 1.00 mL (17.15 mmol) of ethanol; 1.9 mL (24.98 mmol) of 1-propanol with 4.76% of water added corresponding to the optimum amount of water for the performance of the biocatalyst [30]. Those volumes correspond to the minimum amount required to dissolve the profen. The molar ratio of alcohol: R/S-ketoprofen is 9 for methanol and ethanol, and 13 for 1-propanol. The esterification of R/S-ketoprofen was catalyzed with 160 mg of Novozym® 435. This commercial biocatalyst was employed as received and previously pretreated with the alcohols (according to the methodology described in Section 2.3) before the esterification reaction. All the reactions were performed in closed 100 mL vials, at a constant temperature of 45 °C in a shaker bath at 200 rpm for 72 h. The analysis of both enantiomers was conducted by chiral HPLC analysis with a Nucleodex beta-PM column (Macherey-Nagel, Germany) and an UV detector at 230 nm Previously, the samples were diluted to 30 ppm and filtered with nylon hydrophilic membrane filters (Osmonic Inc, 0.45 μm pore size, 13 mm diameter). A volume of 10 μL was injected for analysis. The mobile phase (methanol/0.1% TEAA pH 4.0 (50/50 v/v)) was operated at a flow rate of 0.700 mL/min. The retention time of S(+)-ketoprofen, R(−)-ketoprofen and esters were 11.665 min, 12.187 min and 22.844 min, respectively. The resolution factor Rs was of 0.5. All samples were run a minimum of four times with a relative error of 2.4% and standard deviation of 0.5 units. Enantiomeric excess (ee) referred to the form S(+) of the remaining ketoprofen was calculated according to the Eq. (1), where Area S(+) and Area R(−) account for the chromatographic areas of the S(+) and R(−) enantiomers, respectively.

$$\text{ee}\quad S(+)\% = \left[\frac{\text{Area}\quad S(+) - F_C * \text{Area}\quad R(-)}{\text{Area}\quad S(+) - F_C * \text{Area}\quad R(-)}\right] \times 100 \tag{1}$$

F_C is a correction factor employed to solve the difference in sensibility of the detector to each enantiomer. It was calculated as the ratio between the area of the S(+) and R(−) enantiomer (Eq. (2)) of a 30 ppm solution of R/S-ketoprofen.

$$F_c = \frac{\text{Area}\quad S(+)}{\text{Area}\quad R(-)} \tag{2}$$

The conversion of profen was determined by titration of the final reaction mixture with a basic solution of KOH in ethanol of known concentration [30]. The titrations were performed in triplicate with a relative error of 4% and a standard deviation of 3 units.

The specific activity of the biocatalyst was calculated as the ratio between the conversion of the profen to esters (in μmol) per weight (mg) of protein and the reaction time (min).

Relative errors involved in conversion, enantiomeric excess and in the specific activity are indicated with errors bars in figures presented in this investigation.

2.3. Procedure for the treatment of Novozym® 435 with the alcohols

A sample of Novozym® 435 (1.000 g) was contacted with 10.00 mL of a mixture of the alcohols with 4.76% (v/v) H_2O at 45 °C and 200 rpm for a period of time of 8 days in order to investigate the effect of the alcohol over the biocatalyst [29,31].

Then, the beads were washed 4 times with 5.00 mL of the corresponding alcohol and filtered with nylon hydrophilic membrane filters (0.45 μm pore size, 13 mm diameter) obtained from Osmonic Inc. to retain the non-soluble substances as polymethylmethacrylate. The biocatalyst was dried in a desiccator for 8 days (to dehydrate) and further heated for 10 min at a certain temperature necessary to desorb the alcohol (the desorption temperatures were determined as described in Section 2.4), and after a step of cooling down, the solid was weighed. This procedure allowed the determination of the total weight loss of the biocatalyst and the relative amount of alcohol adsorbed on the material.

The liquids remaining after each washing step were added to the liquid phase initially separated and allowed to dry. The resultant solid was dissolved with 2.00 mL of distilled water, centrifuged

to separate the non-soluble substances and recover the enzyme for further quantification through the bicinchoninic acid assay as described in Section 2.5. The biocatalyst in contact with the alcohol:H_2O mixtures and the solids retained in the filters were analyzed by infrared spectrometry.

2.4. Temperature programmed desorption spectra

Novozym® 435 previously treated with the corresponding alcohol for 8 days was studied by temperature programmed desorption TPD analysis. Details of the equipment used in this investigation have been published before [29,31,32]. The samples (49.9 mg) were heated up to 400 °C at 10 °C/min under a flow of pure helium (35 cm^3 (NTP) min^{-1}) for the temperature programmed desorption experiment. The species resulting of desorption and/or reaction of the surface species were detected in the mass spectrometer and recorded in a computer.

2.5. Quantification of the protein through the bicinchoninic acid assay

The amount of protein that was recovered from the treatment of Novozym® 435 described in Section 2.3, was quantified through the bicinchoninic acid assay. The BCA method involves the preparation of three aqueous solutions that are called A, B and C for brevity. Solution A contains 0.4000 g of BCA (Fluka 90%), 0.8000 g Na_2CO_3 (Mallinckrodt), 0.1630 g $NaHCO_3$ (Anedra), 0.1581 g NaOH (Carlo Erba 97%) and 0.0641 g sodium tartrate dibasic dihydrate (Fluka 99%) in 40 mL of distilled water. Solution B contains 4% w/v of $CuSO_4$ and solution C is a mixture of solution A and B in a 100:2 v/v ratio (in this particular case, solution C corresponds to 30.00 mL of solution A and 0.60 mL of solution B, and is prepared just before the quantification).

The calibration curve was obtained by employing highly pure *Candida antarctica* lipase B (Sigma–Aldrich Argentina 10.9 U/mg). The calibration was performed in distilled water. Initially, a CALB solution (0.0044 g) in distilled water (4.00 mL) was obtained. The actual concentration of the solution was obtained from the absorbance value at 280 nm (A^{280}) and the extinction coefficient of the enzyme (ε^{280} CALB = 41,285 M^{-1}) according to the Beer–Lambert law: $A^{280} = \varepsilon^{280} * b * c$. The concentration of this starting solution was 0.1956 mg/mL. Various calibration solutions were obtained by dilution with distilled water, resulting in nine solutions of concentrations between 0 and 200 μg/mL. The bicinchoninic acid assay involved the reaction of 170 μL of each calibration solution with 1.70 mL of solution C (working reagent) for 10 min at 60 °C. The mixtures were allowed to cool down and then the absorbance was measured at 562 nm with an equipment Perkin Elmer Lambda 35 Spectrophotometer using cuvettes Uvette® 220–1600 nm of 1 cm path length. The calibration curve obtained fits the following linear equation:

$$A^{562} = 0.0029 * [\text{protein} \quad (\mu g/mL)] - 0.0354 \quad r^2 = 0.9917 \qquad (3)$$

2.6. Determination of the protein's secondary structure with Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTs)

The secondary structure of CALB in Novozym® 435 without previous treatment along with CALB in the biocatalyst exposed to the various alcohols for 8 days and the evolution of the secondary structure upon reaction was studied through *D*iffuse *R*eflectance *I*nfrared *F*ourier *T*ransform *S*pectroscopy (DRIFTs). Previous to DRIFTs analysis, isotopic exchange of water molecules by D_2O molecules was carried out. This technique allows the investigation of the Amide I signal (1700–1600 cm^{-1}) without the interference of the bending vibration of O-H species (mainly from adsorbed water) that typically appears at 1640 cm^{-1}. The percentage contribution of each structure was obtained through the deconvolution, integration and further normalization of the corresponding signals involved in the Amide I. The methodology employed was described previously when the secondary structure of Novozym® 435 in contact with 2-propanol was studied [29].

2.7. Scanning electron microscopy (SEM) analysis and fractal dimension estimator calculation

The internal texture of Novozym® 435 before and after contacting the alcohols for 8 days and the biocatalyst (with and without previous exposure to the alcohols) after 72 h of reaction were investigated through scanning electron microscopy analysis with an environmental ESEM FEI Quanta 400 microscope. The samples were prepared as ultra thin specimens by embedding the biocatalyst in a LR White resin and further sliced with a microtome. These specimens, covered with a conductive gold layer in order to avoid electrical charges on the surface, were observed in the microscope in the high vacuum mode.

The images of the samples at 20,000× magnifications were taken and analyzed with the FERImage program to calculate the fractal dimension D and d_{min} parameters by using the variogram methodology [33–36].

2.8. Theoretical study

To explore the importance of steric interactions at the level of the active site and neighborhoods of the catalytic triad from CALB, a simple molecular mechanics study was carried out, using the same model and structures than previously reported in other manuscripts from some of us [29]. The main interest was on the alcohol therefore ketoprofen enantiomer was only of slight importance. Methanol, ethanol and 1-propanol were modeled and the initial situation, the tetrahedral intermediary 1 and the acyl enzyme were simulated.

3. Results

3.1. Esterification of R/S-ketoprofen with short chain alcohols without co-solvents added

The esterification of R/S-ketoprofen with methanol, ethanol and 1-propanol (with 4.76% of water added) as reactants and solvents was catalyzed with Novozym® 435. The biocatalyst was employed with and without previous treatment with the alcohol for 8 days (264 h) with the aim of obtaining evidences on the effect of the alcohol on the biocatalyst's performance. Fig. 1A–D shows the specific activity and the enantiomeric excess toward the S(+)-enantiomer in the esterification of R/S-ketoprofen with the alcohols. The results obtained with 2-propanol are also presented for comparison purposes [29]. Additionally, the results of the esterification in terms of conversion are presented in the supplementary information accompanying this article (see Figs. S1–S4).

The comparison of the specific activities obtained with the biocatalyst with and without previous contact with the alcohols clearly evidences that the esterification of the profen with 2-propanol is not feasible. This behavior is attributed to the steric hindrance of the secondary alcohol and is independent of the effect of the alcohol on the integrity of the biocatalyst itself [29]. In contrast, the esterification with methanol proceeds with the starting biocatalyst (with a similar specific activity as ethanol and 1-propanol) but falls down after extended contact with the alcohol. This observation is an evidence of the deleterious effect of the acyl acceptor on the biocatalyst as will be discussed in the following sections.

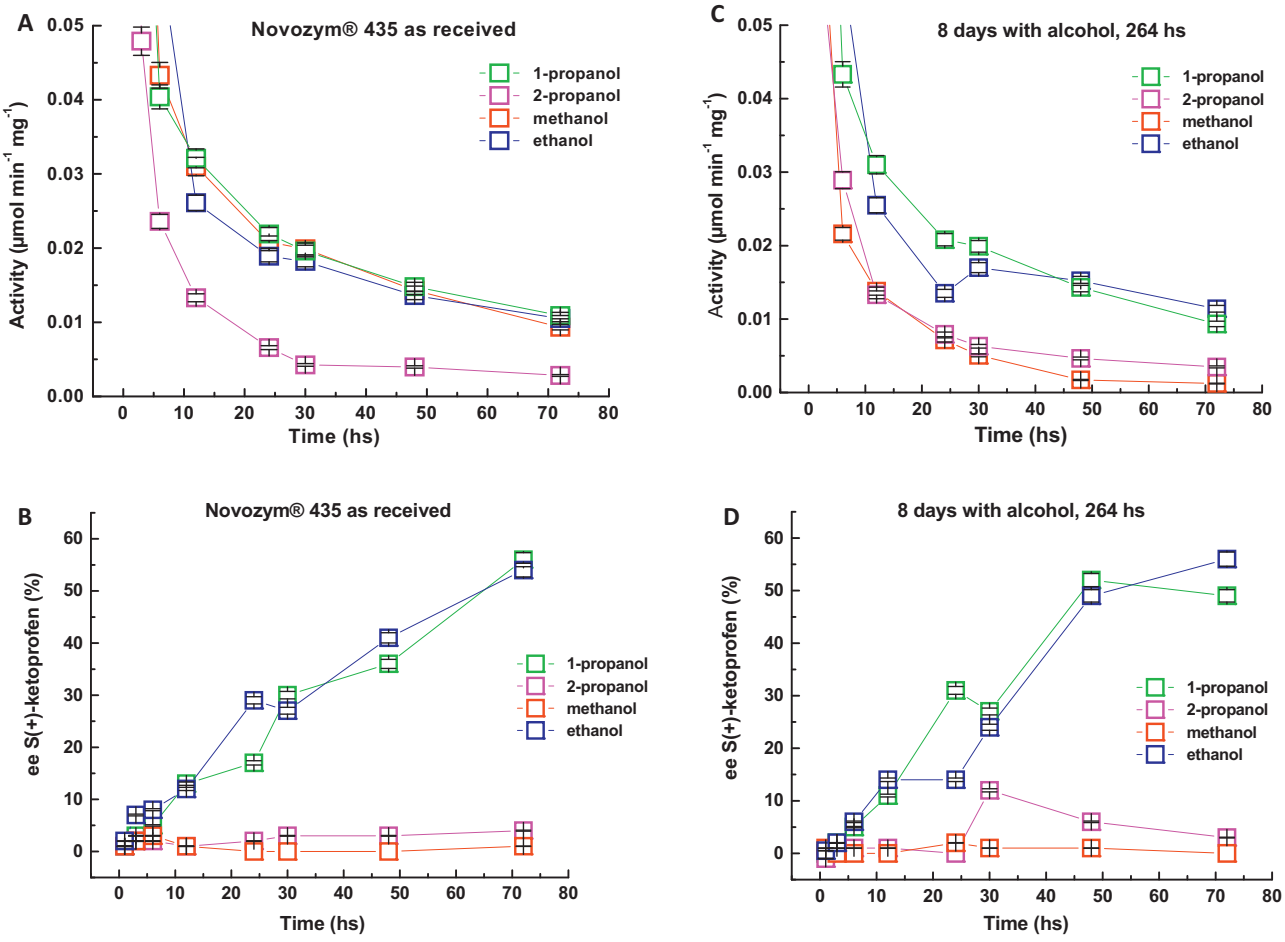

Fig. 1. Enantiomeric excess and specific activity of the esterification of (R/S)-ketoprofen with methanol, ethanol, 1- and 2-propanol catalyzed with Novozym® 435 as received (Fig. 1A and B) and previously treated with the corresponding alcohol for 8 days (Fig. 1C and D).

The esterification of R/S-ketoprofen with both ethanol and 1-propanol in a solvent-free system is a feasible process and it is not influenced by an extended contact of the biocatalyst with those alcohols. The esterification of R/S-ketoprofen reaches 50% conversion toward the methyl and propyl esters (specific activity $\sim 1.4 \times 10^{-2}$ μmol min^{-1} mg^{-1}) at 48 h of reaction regardless of the treatment of the catalytic material (see Figs. S2 and S3 in the supplementary information). A similar behavior is observed for the enantiomeric excess toward the S(+)-enantiomer that climbs to about 56% at 72 h of reaction (Fig. 1B and D).

The esterification of ibuprofen was also studied for comparison purposes. These investigations demonstrated that in contrast with the above observations, the extended contact of Novozym® 435 with the alcohols exerts a detrimental effect in the enantiomeric esterification of ibuprofen (see Figs. S5 and S6 in the supplementary information).

3.2. Stability of Novozym® 435 upon extended exposure to short chain alcohols

Novozym® 435 was contacted with a mixture of the corresponding alcohol with 4.76% (v/v) H_2O for 8 days in order to elucidate the effect on the physical integrity of the biocatalyst. Previous investigations evidenced the dissolution of Novozym® 435 in contact with ethanol and 2-propanol which in turn induces the loss of the surface protein (lipase B of *Candida antactica*) and polymethylmethacrylate (PMMA) (known as Lewatit VP OC1600) that forms the internal core of the biocatalyst's beads [29,31]. Similarly to those observations, the extended exposure to methanol and 1-propanol shows a similar trend. In fact, the solids recovered from the organic medium possess CALB enzyme and the species attributed to the macroporous matrix which proves again the disaggregation of the biocatalyst (see the infrared spectra in the Fig. S7 in the supplementary information). The quantification (see Table 1) demonstrates that the shorter is the alcohol the higher is the percentage of protein loss. The effect of 1-propanol in terms of protein desorption is much less pronounced than the one observed with the other alcohols. Again, the total weight lost with methanol and ethanol (11.6% and 16.6%, respectively) is an order of magnitude higher than with 1- and 2-propanol (5.9% and 1.2%, respectively).

The experiments of desorption at programmed temperature demonstrated that methanol and 1-propanol adsorb over the surface/bulk of the biocatalyst as already reported with other alcohols. The TPD spectra are shown in Fig. 2A to D and the temperatures of desorption and the amount of adsorbed alcohols are presented in the Table 1.

The desorption profiles show that methanol ($m/e = 31$), ethanol ($m/e = 31$), 1-propanol ($m/e = 31$) and 2-propanol ($m/e = 45$) desorb molecularly at 154 °C, 184 °C, 200 °C and 187 °C, respectively. These high temperatures of desorption indicate a strong alcohol-biocatalyst interaction that can be ascribed as a physisorption moreover considering that the alcohols desorbs as intact molecules [37–39]. However, the presence of dimethyl ether ($m/e = 45$ in Fig. 2A) and propene ($m/e = 41$ in Fig. 2C) is an evidence of a

Table 1
Summary of the percentage of weight and proteins lost; temperature of desorption of the alcohols and alcohol adsorbed upon contacting Novozym® 435 with methanol, ethanol, 2-propanol and 1-propanol.

Alcohol	Mass (g)					
	Starting biocatalyst	Recovered biocatalyst	% weight loss	% protein loss [a]	T_D [b] (°C)	Mass alcohol adsorbed/gr biocatalyst
Methanol	1.0002	0.8846	11.6	1.93	154	0.0212
Ethanol[c]	1.0000	0.8340	16.6	1.27	184	0.3881
1-Propanol	1.0005	0.9412	5.9	0.57	200	0.0404
2-Propanol[d]	1.0011	0.9889	1.2	0.79	187	0.0368

[a] ratio between the amount of protein lost (mg) due to the alcohol treatment and the total amount of protein (mg) of Novozym® 435.
[b] temperature of desorption of the alcohols obtained from the temperature programmed desorption analysis.
[c, d] The data corresponding to Novozym® 435 in contact with ethanol and 2-propanol were taken from Ref. [31] and [29], respectively.

certain degree of chemisorption of methanol and 1-propanol. The chemisorption of the alcohols generates intermediate methoxy and propoxy species that further dehydrate over active sites of acidic nature [40–42].

Two desorption events of water molecules ($m/e = 18$) are observed. The first one below 100 °C corresponds to the loss of constitutional water of the proteins. A second event happens when the temperature rises ($T > 239$ °C) and is accompanied with the

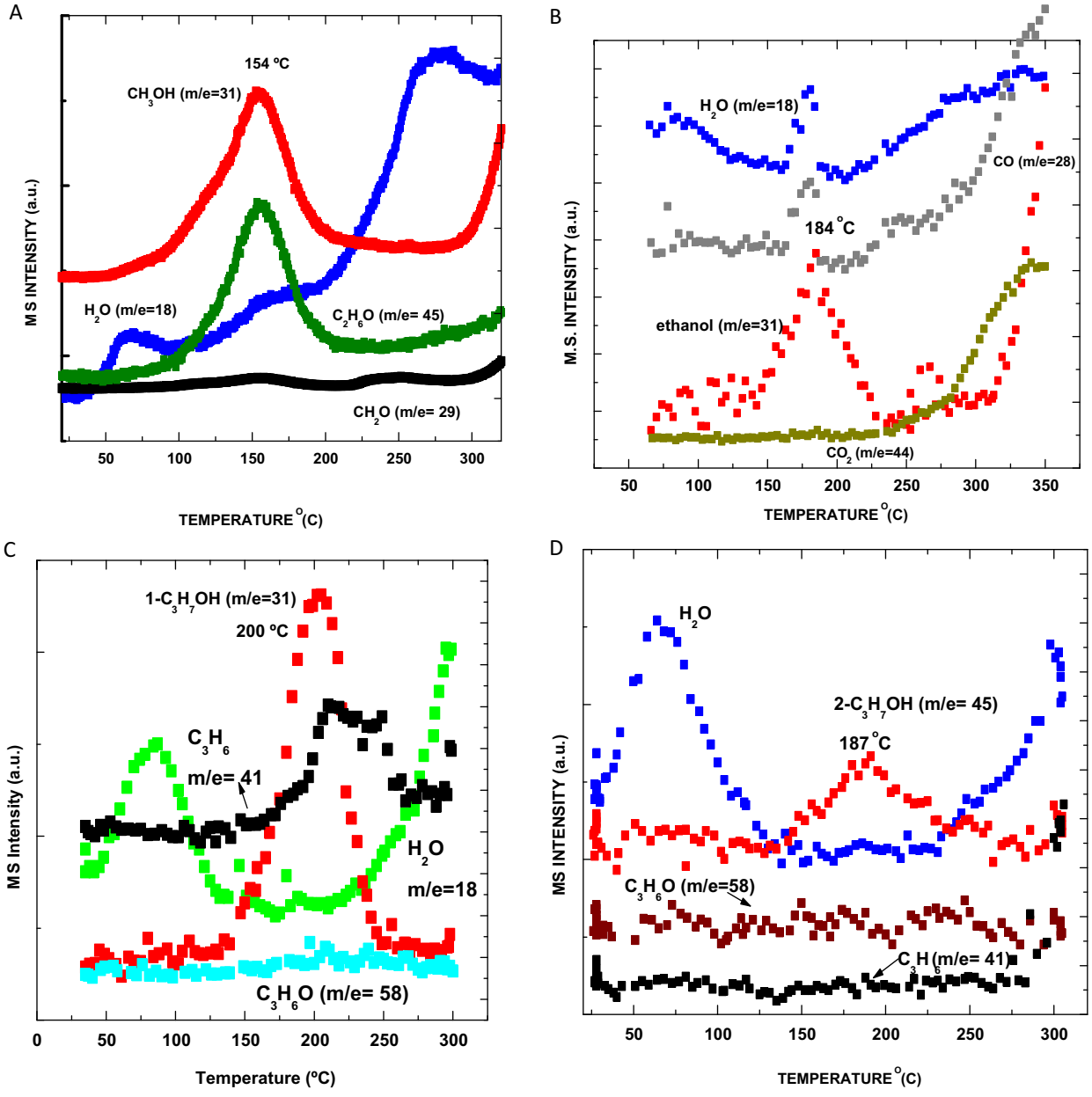

Fig. 2. *In situ* temperature programmed desorption spectra of Novozym® 435 after being in contact with methanol (A), ethanol (B), 1-propanol (C) and 2-propanol (D) for 8 days at 45 °C.

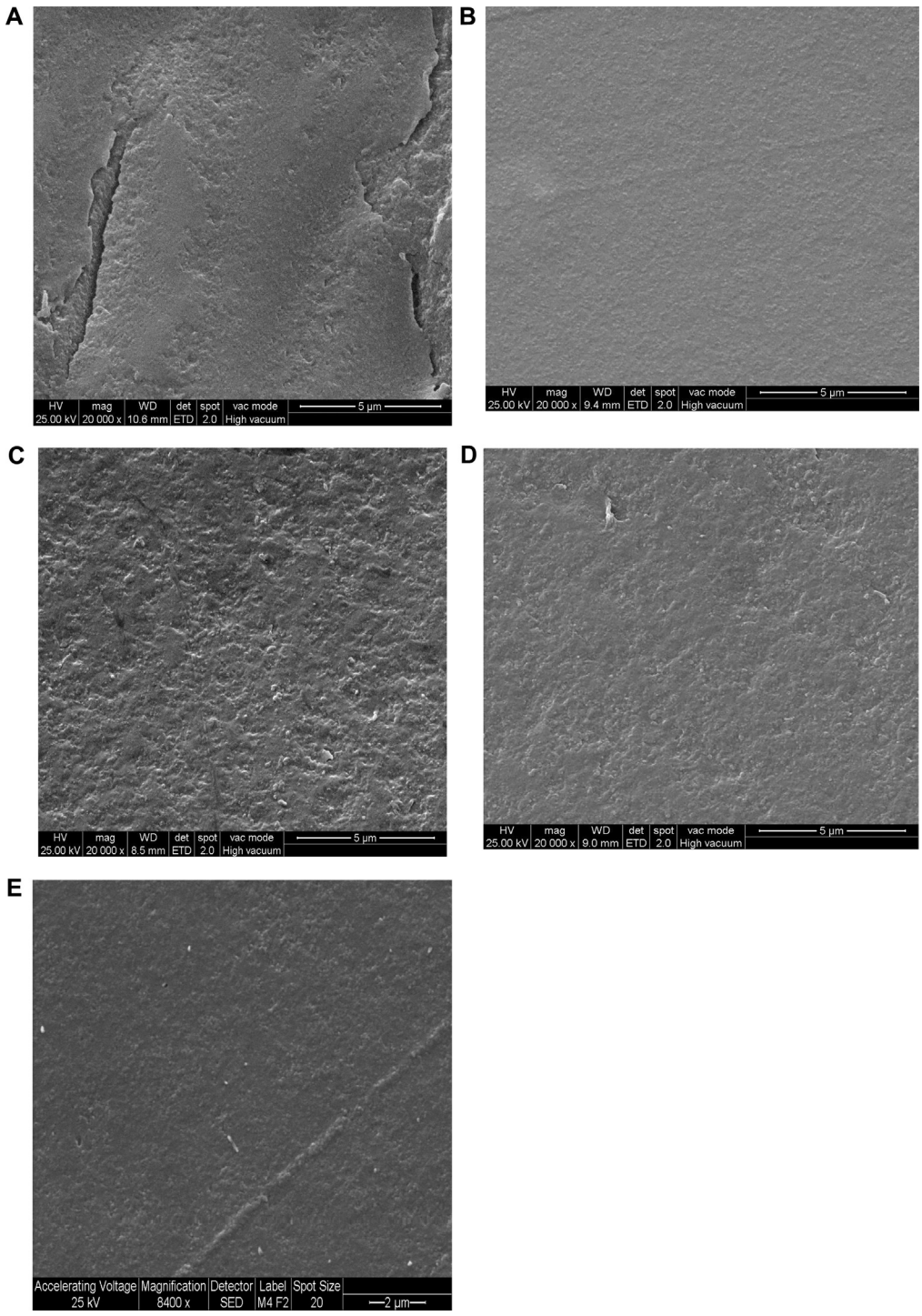

Fig. 3. Images of the cross section of the beads of Novozym® 435 as received (A) and after being in contact with methanol (B), ethanol (C), 1-propanol (D) and 2-propanol (E) for 8 days at 45 °C. The Images were taken with an environmental scanning electron microscope at 20,000×.

desorption of CO and CO_2 which corresponds to the decomposition of amino-acids and the PMMA support [31].

The adsorption of the alcohols and the degradation of the polymeric matrix do not only occur at the surface but also in the interior of the beads. The investigation of the cross section of the beads of Novozym® 435 through scanning electron microscopy and the parameters (fractal dimension and minimum cell size) that describe the surface roughness and the texture are presented in Fig. 3 and Table 2. The Fig. 3A–E shows images of the inner texture of starting Novozym® 435 and after being in contact with the alcohols for 8 days at 45 °C.

The fractal dimension values ranging from $2 < D < 2.5$ indicate a persistent smooth surface while values $2.5 < D < 3$ are an indication of anti-persistence being a completely rough surface when D is close to 3 [33,34]. The starting Novozym® 435 possesses a fractal dimension equals to 2.8347 indicating a certain rough texture and a minimum cell size d_{min} of 0.0582 µm. Nevertheless, the extended exposure to the alcohols and even under reaction conditions

Table 2
Fractal dimension estimator D and minimum cell size d_{min} of starting Novozym® 435, after being in contact with short chain alcohols for 8 days at 45 °C.

Texture estimators	Starting Novozym® 435	Treated with alcohols for 8 days			
		Methanol	Ethanol	1-Propanol	2-Propanol
Fractal dimension *D*	2.8347 ± 0.0027	2.595 ± 0.054	2.665 ± 0.004	2.460 ± 0.042	2.727 ± 0.022
d_{min} [μm]	0.0582 ± 0.0022	0.05952 ± 0.0017	0.1015 ± 0.022	0.0692 ± 0.017	0.0651 ± 0.0032

(without previous exposure to alcohols) diminishes the D parameter and increases the d_{min}. This observation certainly evidences that the alcohols diffuse into the beads and exert a smoothing action due to the degradation of the polymeric matrix as discussed before (see the descriptors of the texture of the biocatalyst after reaction in Table S2 included in the supplementary information).

3.3. Evolution of the secondary structure of the lipase B of Candida antarctica

The percentages of aggregates, α-helix, β-sheet, turns and random in the secondary structure of starting Novozym® 435 and after exposure to the alcohol-water mixtures for 8 days are presented in the Table 3. Additionally, the Tables S3–S5 (within the supplementary information) show the secondary structure of the biocatalyst during the esterification of R/S-ketoprofen with methanol, ethanol and 1-propanol (the results obtained with 2-propanol were already published in [29]). The analysis of the infrared spectra of enzymes in the 1700–1600 cm^{-1} region (Amide I region) provided qualitative and quantitative information about the secondary structure of the protein from CALB in Novozym® 435.

The percentage contribution of each structure was obtained through the deconvolution, integration and further normalization of the corresponding signals of Amide I. The contribution of the α-helix and random structures correspond to the area of the signals at 1654 cm^{-1} and 1643 cm^{-1}, respectively. The contribution of the β-sheet structure was obtained by adding the area of the signals appearing at 1631 cm^{-1}, 1637 cm^{-1} and 1686 cm^{-1} wavenumbers. Similarly, the contribution of the β-turn structure corresponded to the addition of the areas of the signals located at 1664 cm^{-1}, 1666 cm^{-1} and 1676 cm^{-1} [29,43–46]. Finally, a signal at 1619 cm^{-1} corresponds to β aggregates.

The secondary structure of the protein CALB of the starting Novozym® 435 is composed by 26.4% of α-helix, 19.4% of β-sheet, 25.3% of random structure, 28.7% of β-turns and 0.2% of aggregates. The biocatalyst exposed to the alcohol-water mixtures for a period of 8 days presents a modified secondary structure where the percentage of β-turns increases and the contribution of β-sheet and random structure diminishes although the content of α-helix remains almost unaltered. The aggregation of the protein (6%), the increase in β-sheet and the modification of the α-helix are only observed in the case of the exposure to methanol. The investigations by Fang et al. also demonstrated that methanol induces the modification of the conformation of CALB [47]. Although the authors observed the decrease in the α-helix structure (in agreement with our results) they did not detected the formation of aggregates but certainly, they correlated the inactivation of the enzyme with the protein's unfolding caused by the alcohol.

Similarly, a high content of the β-turns structure, a decrease of the contribution of β-sheet and random structure and a percentage of α-helix almost constant was observed when the biocatalyst (previously treated with the alcohols or not) was used in the esterification of R/S-ketoprofen with the short chain alcohols for 72 h.

3.4. Esterification of ketoprofen at molecular level. Theoretical calculations

Fig. 4 shows the interaction between ethanol and 1-propanol in the neighborhood of the catalytic triad of CALB, at the coordination step. Additionally, Fig. 5 shows the results for coordination, intermediary 1 and the acyl enzyme for methanol, ethanol and 1-propanol. It is important to take into account that the bonds involved are similar but the total number of atoms is not. The comparison is only qualitative. As a general rule, the most negative the steric energy, the better for the stabilization of the conformer.

The MM2 calculation detected the difference among methanol, ethanol and 1-propanol in steric energies, being always the same R/S-ketoprofen with the structure of the whole molecule at the initial step, the intermediary 1 and finally the acyl enzyme.

With methanol the ketoprofen carbonyl is located at the pocket where the alcohol has to be coordinated and interferes clearly with the formation of the tetrahedral intermediary being positioned between the ketoprofen and the Thr40. In the case of ethanol and 1-propanol the position of the acyl enzyme is correct to react with the alcohol later. The carbon 1 of propanol is not at the same plane than the carbon from the acyl group. The problem with propanol seems to be in the transition states more than in the stable intermediaries, probably due to the impact of the additional methylene group. The methanol introduces steric and electronic hindrance from the step of the coordination of the R/S-ketoprofen.

The alcohol inhibition effect seems to be related to: (a) the relative occupancy of the alcohol before the ketoprofen coordination and the steric hindrance involved, (b) how the alcohol affects

Table 3
Contribution of the aggregates, α-helix, β-sheet, random structure and β-turns to the secondary structure of the starting Novozym® 435, after treated with methanol, ethanol, 1- and 2-propanol for 8 days at 45 °C without reaction.

Novozym® 435	PERCENTAGE CONTRIBUTION				
	Aggregates[a]	α-Helix[b]	β-Sheet[c]	Random[d]	β-Turns[e]
Starting biocatalyst	0.2	26.4	19.4	25.3	28.7
Methanol	6.0	16.6	22.3	16.3	38.8
Ethanol	0.2	23.6	5.7	7.5	63.0
1-Propanol	0.4	23.8	13.2	9.3	53.3
2-Propanol	0.0	25.3	18.6	3.6	52.5

[a] Corresponds to the signal at 1619 cm^{-1}.
[b] Corresponds to the signal at 1654 cm^{-1}.
[c] Corresponds to the sum of the contribution of the signals at 1631 cm^{-1}, 1637 cm^{-1} and 1686 cm^{-1}.
[d] Corresponds to the signal at 1643 cm^{-1}.
[e] Corresponds to the sum of the signals at 1664 cm^{-1}, 1666 cm^{-1} and 1676 cm^{-1}.

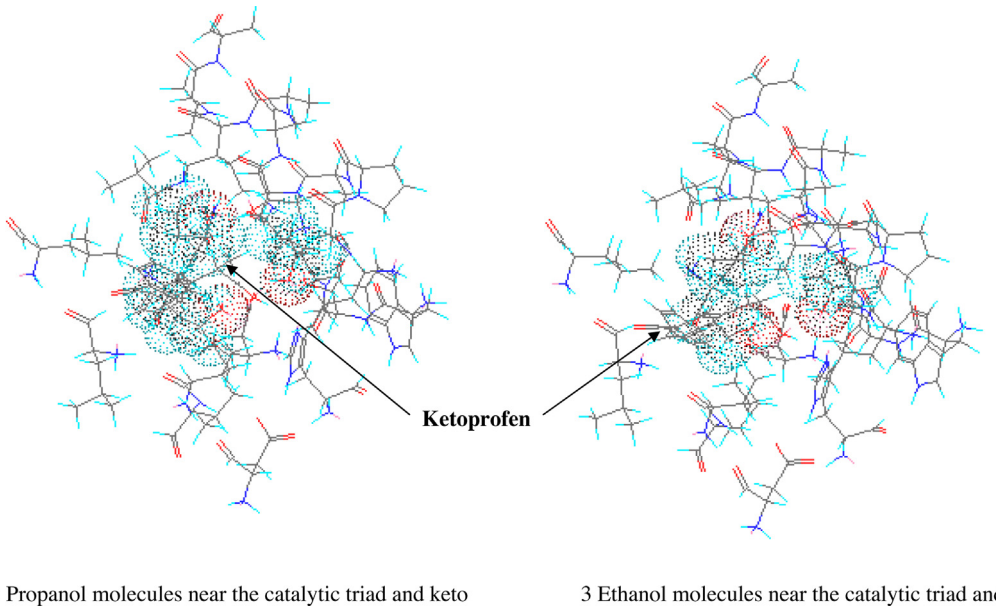

Fig. 4. Representation of the interaction of ethanol and 1-propanol with the catalytic triad of CALB and R/S-ketoprofen at the coordination step.

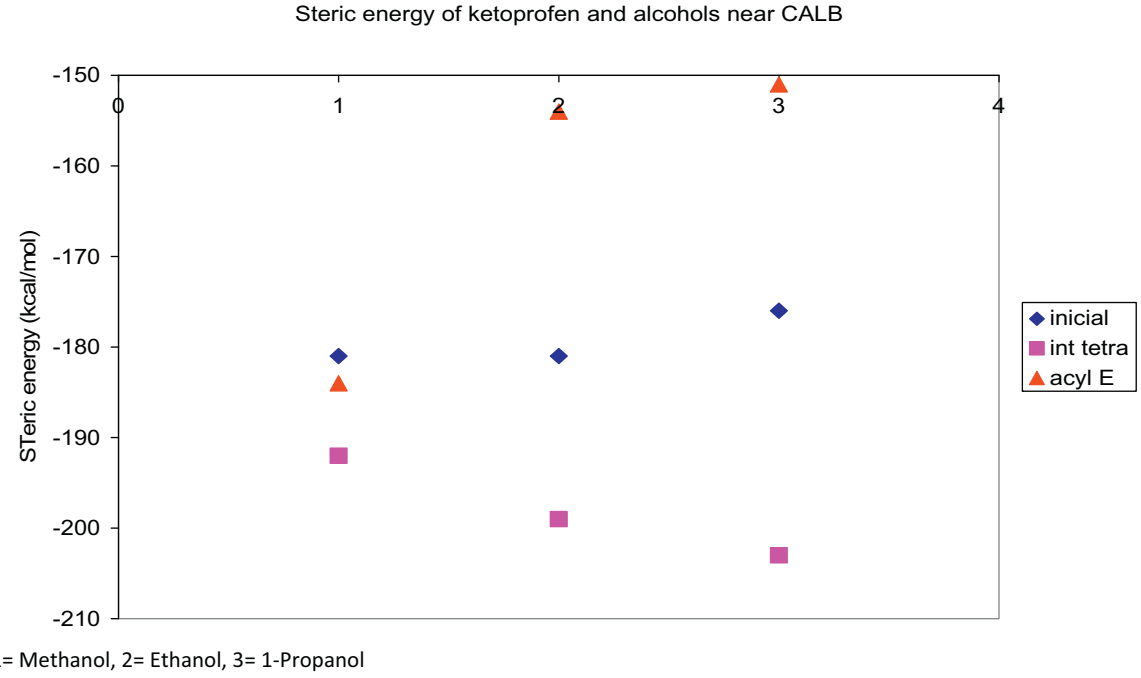

Fig. 5. Steric energy of ketoprofen and methanol, ethanol and 1-propanol near CALB. The initial step, the intermediary 1 (tetrahedral) and the acyl enzyme are considered.

the coordination of the ketoprofen, and the carboxylate group of ketoprofen with serine to produce the intermediary 1 and the stabilization through the oxyanion hole, (c) how the acyl enzyme formation is stabilized or not, and (d) proper orientation of the alcohol to produce the reaction with the acyl enzyme, once it is formed.

4. Discussion

The present investigation is a continuation of previous ones devoted to the kinetic resolution of profens (namely, ibuprofen and ketoprofen) with solely alcohols catalyzed with Novozym® 435 [29,31]. Previous investigations, using isopropyl alcohol evidenced that the secondary alcohol interacts with the lipase B of *Candida antarctica* inhibiting the catalytic performance of Novozym® 435 [29]. The evidences reported in this investigation demonstrate that the esterification of R/S-ketoprofen with ethanol and 1-propanol (without co-solvents added) at 45 °C is a feasible process and more importantly, the activity of Novozym® 435 is not influenced by the extended contact with the alcohols.

In contrast with that observation, the catalytic performance decreases when methanol is used as the acyl acceptor. This behavior is attributed to various detrimental effects of this alcohol on the physical integrity of the polymer that holds the lipase, the enzymatic structure itself and the interaction with the active site. Previous investigations by some of us demonstrated that ethanol and 2-propanol are able to diffuse inside the polymeric beads of the

biocatalyst leading to the swelling and dissolution of the PMMA [29–31,48]. The low molecular weight of methanol increases its ability to diffuse onto polymeric structures as reported by Koenig et al. [38,39]. Again this alcohol performs a smoothing action of the internal texture as discussed before.

The exposure of the protein to the alcohols affects the contribution of the β-turn structure that increases from 28.7% in the starting biocatalyst to more than 50% upon contacting with ethanol and 1- and 2-propanol for 8 days, with/without reaction with racemic ketoprofen. In contrast, methanol induces to the decrease of the contribution of the α-helix and the increase of the aggregates which evidences the denaturation of the protein.

The "real situation" many times is not considered from the theoretical and practical point of view. When the lipase is exposed to a mixture of high molar ratio short, linear alcohol: carboxylic acid, it is clear that the alcohol reaches faster the catalytic triad and it may introduce additional steric problems functioning as solvent and as substrate. From the mechanistic point of view, to have reaction, first the tetrahedral intermediary 1 has to be formed and later the acyl enzyme is generated. Later, the reaction with the alcohol takes place. The first step, the step that is required to the alcohol to react, is the formation of the acyl enzyme by the reaction of the carboxylic acid of the ketoprofen with the serine alcohol group.

1-propanol and ethanol showed different conformations and distributions than methanol around ketoprofen and induced or contributed to different conformations for carboxylic acid group in ketoprofen, around the catalytic triad of CALB. However, the initial conformer, intermediary 1 and acyl enzymes were very similar when 1-propanol and ethanol were used. The difference probably can be found in the transition states or unstable transition states and higher activation energies in the case of 1-propanol compared to ethanol. The inhibition of activity with methanol can be understood, whereas the ethanol and 1-propanol differences were based in steric interactions at unstable transition states. This proposal should have to be tested with higher levels of molecular mechanics and/or semiempirical or ab-initio calculations.

The temperature programmed desorption spectra of the alcohols from Novozym® 435 indicated an intense physical adsorption on the protein and/or the polymeric matrix similarly to previous findings with ethanol and isopropyl alcohol. The alcohols molecularly desorbed at 154 °C, 184 °C, 187 °C and 200 °C for methanol, ethanol, 2-propanol and 1-propanol, respectively. Interestingly, 1-propanol adsorbed also as alkoxy species that are intermediates species to propylene and this compound was also detected in the TPD spectra. The temperatures of desorption (T_d methanol < T_d ethanol < T_d 1-propanol) are a measure of the intensity of the interaction of the alcohol with the biocatalyst and are in accordance with the steric energy calculated through theoretical calculations.

5. Conclusions

The results discussed above allow concluding:

- The feasibility of the enzymatic kinetic resolution of racemic ketoprofen through the direct esterification with ethanol and 1-propanol (with 4.76% v/v H_2O added) as substrates and solvents without the addition of organic co-solvents. The conversion and enantiomeric excess (toward the S(+)-enantiomer) were ~48% after 48 h at 45 °C in batch reaction.
- A careful analysis of the effect of extended contact of methanol, ethanol, 1- and 2-propanol with Novozym® 435 (up to 8 days) revealed that the alcohols diffuse inside the biocatalyst's beads causing dissolution/de-aggregation of PMMA with the loss of protein. Interestingly the weight loss (accounting for polymeric matrix and protein) is higher for those alcohols that possess the smaller molecules. This observation is somehow expected considering their capability of diffusion inside the biocatalyst's beads. The alcohols irreversible adsorb on the biocatalyst as spectator species that desorbs above T_d > 100 °C. The exposure to the alcohols affects the contribution of the β-turn conformation (from 28.7% in the starting biocatalyst to ~55%) to the secondary structure of the protein as already been observed previously.
- The catalytic performance of Novozym® 435 in the enantiomeric esterification of racemic ketoprofen with ethanol and 1-propanol as acyl acceptors is not influenced by the variety of changes induced by the alcohols. However, the kinetic resolution of ibuprofen diminishes under similar conditions. This observation can not be explained at the moment.
- 1-propanol and ethanol showed different conformations and distributions than methanol around racemic ketoprofen and induced or contributed to different conformations for carboxylic acid group from ketoprofen.
- There is preliminar evidence of the importance of an extended H-bonding system in the coordination of short, linear alcohols in CALB catalytic triad and its surroundings. The coadsorption of alcohols and ketoprofen (or any other profen) has to be considered because it introduces additional steric hindrance to the profen coordination near the serine of the catalytic triad of CALB.
- Short, linear alcohols without steric hindrance are able to interact electronically and sterically with different portions of the CALB protein, not only changing the proportion of secondary structural facts, but also introducing additional steric problems to the profen coordination at the catalytic triad of the lipase.

Acknowledgments

The authors acknowledge the financial support of CONICET (project PIP 112 20130100171) and Universidad Nacional de La Plata (project 11-X626); Prof Luis A. Gambaro for the TPD experiments and Lic Mariela Theiller for the calculation of textural parameters.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.05.003

References

[1] O. Barbosa, C. Ariza, C. Ortiz, R. Torres, New Biotechnol. 27 (2010) 844–850.
[2] M. Kapoor, M.N. Grupta, Process Biochem. 47 (2012) 555–569.
[3] J. Durand, J. Lecomte, B. Baréa, G. Piombo, E. Dubreucq, P. Villeneuve, Process Biochem. 47 (2012) 2081–2089.
[4] S.K. Karmee, Appl. Microbiol. Biotechnol. 81 (2009) 1013–1022.
[5] M.L. Foresti, M. Pedernera, V. Bucalá, M.L. Ferreira, Enzyme Microb. Technol. 41 (2007) 62–70.
[6] M.D. Romero, L. Calvo, C. Alba, A. Daneshfar, H.S. Ghaziaskar, Enzyme Microb. Technol. 37 (2005) 42–48.
[7] A.C. Wu, P.Y. Wang, Y.S. Ling, M.F. Kao, J.R. Chen, J.F. Ciou, S.W. Tsai, J. Mol. Catal. B: Enzym. 62 (2010) 235–241.
[8] H. Caner, E. Groner, L. Levy, Drug Discov. Today 9 (2004) 105–110.
[9] R. Morrone, N. DíAntona, D. Lambusta, G. Nicolosi, J. Mol. Catal. B: Enzym. 65 (2010) 49–51.
[10] M.G. Kim, E.G. Lee, B.H. Chung, Process Biochem. 35 (2000) 977–982.
[11] J.F. Ciou, P.Y. Wang, A.C. Wu, S.W. Tsai, Process Biochem. 46 (2011) 960–965.
[12] J.B. Sontakke, G.D. Yadav, Ind. Eng. Chem. Res. 50 (2011) 12975–12983.
[13] L.C. Bencze, C. Paizs, M.I. Toşa, M. Trif, F.D. Irimie, Tetrahedron: Asymm. 21 (2010) 1999–2004.
[14] A. Kamal, M.A. Azhar, T. Krishnaji, M.S. Malik, S. Azeeza, Coord. Chem. Rev. 252 (2008) 569–592.
[15] M. Arroyo, J.V. Sinisterra, J. Org. Chem. 59 (1994) 4410–4417.
[16] G. Duan, C.B. Ching, E. Lim, C.H. Ang, Biotechnol. Lett. 19 (1997) 1051–1055.
[17] T. López-Belmonte, A.R. Alcántara, J.V. Sinisterra, J. Org. Chem. 62 (1997) 1831–1840.
[18] H.J. Park, W.J. Choi, E.C. Huh, E.Y. Lee, C.Y. Choi, J, Biosci. Bioeng. 87 (1999) 545–547.

[19] W. Du, M.N. Zong, W.F. Chen, Y. Guo, Q. Li, Microbiology 27 (2000) 429–432.
[20] G. De Crescenzo, A. Ducret, M. Trani, R. Lortie, J. Mol. Catal. B: Enzym. 9 (2000) 49–56.
[21] N. D'Antona, P. Lombardi, G. Nicolosi, G. Salvo, Process Biochem. 38 (2002) 373–377.
[22] A.R. Alcántara, P. Dominguez de María, M. Fernández, M.J. Hernaíz, J.M. Sánchez Montero, J.V. Sinisterra, Food Technol. Biotechnol. 42 (2004) 343–354.
[23] C.-S. Chang, C.-S. Hsu, J. Chem. Technol. Biotechnol. 80 (2005) 537–544.
[24] S. Zhu, Y. Wu, Z. Yu, Biocatal. Biotransform. 23 (2005) 97–101.
[25] A.L. Ong, A.H. Kamaruddin, S. Bathia, W.S. Long, S.T. Lim, R. Kumari, Enzyme Microb. Technol. 39 (2006) 924–929.
[26] P. De Oliveira Carvalho, F. Jares Contesini, M. Ikegaki, Braz. J. Microbiol. 37 (2006) 329–337.
[27] A.L. Ong, A.H. Kamaruddin, S. Bathia, H.Y. Aboul-Enein, J. Sep. Sci. 31 (2008) 2476–2485.
[28] X. Li, T. Liu, L. Xu, X. Gui, F. Su, Y. Yan, Biotechnol. Bioprocess. E 17 (2012) 1147–1155.
[29] M.V. Toledo, C. José, S.E. Collins, R.D. Bonetto, M.L. Ferreira, L.E. Briand, J. Mol. Catal. B: Enzym. 83 (2012) 108–119.
[30] M.L. Foresti, M. Galle, M.L. Ferreira, L.E. Briand, J. Chem. Technol. Biotechnol. 84 (2009) 1461–1473.
[31] C. José, R.D. Bonetto, L.A. Gambaro, M.P. Guauque Torres, M.L. Foresti, M.L. Ferreira, L.E. Briand, J. Mol. Catal. B: Enzym. 71 (2011) 95–107.
[32] L.A. Gambaro, L.E. Briand, Appl. Catal. A: Gen. 264 (2) (2004) 151–159.
[33] F.D. Bianchi, R.D. Bonetto, Scanning 23 (2001) 193–197.
[34] J.L. Ladaga, R.D. Bonetto, in: P.W. Hawkes (Ed.), Characterization of Texture in Scanning Electron Microscope Images. Advances in Imaging and Electron Physics, vol. 120, Academic Press, 2002, pp. 136–189.
[35] A.P. Pentland, IEEE Trans. Pattern Anal. Mach. Intell. PAMI-6 (6) (1984) 661–674.
[36] R.D. Bonetto, J.L. Ladaga, Scanning 20 (1998) 457–463.
[37] L.E. Briand, in: J.L.G. Fierro (Ed.), Metal Oxides: Chemistry and Applications, CRC Press Taylor & Francis, Florida, 2005, pp. 353–390.
[38] J. González-Benito, J.L. Koenig, Polymer 47 (2006) 3065–3072.
[39] B.A. Miller-Chou, J.L. Koenig, Prog. Polym. Sci. 28 (2003) 1223–1270.
[40] L.J. Burcham, L.E. Briand, I.E. Wachs, Langmuir 17 (2001) 6164.
[41] L.J. Burcham, L.E. Briand, I.E. Wachs, Langmuir 17 (2001) 6175.
[42] L.J. Burcham, M. Badlani, I.E. Wachs, J. Catal. 203 (2001) 104.
[43] M.L. Foresti, G.M. Valle, R.D. Bonetto, M.L. Ferreira, L.E. Briand, Appl. Surf. Sci. 256 (2010) 1624–1635.
[44] A. Barth, Biochim. Biophys. Acta 1756 (2007) 1073–1101.
[45] A. Barth, C. Zscherp, Q. Rev. Biophys. 35 (2002) 369–430.
[46] C.R. Llerena Suster, C. José, S.E. Collins, L.E. Briand, S.R. Morcelle, Process Biochem. 47 (2012) 47–56.
[47] X. Fang, Y. Zhan, J. Yang, D. Yu, J. Mol. Catal. B: Enzym. 104 (2014) 1–7.
[48] L.O. Wiemann, P. Weisshaupt, R. Nieguth, O. Thum, M.B. Ansorge-Schumacher, Org. Res. Dev. (13) (2009) 617–620.