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Esterification of R/S-ketoprofen with 2-propanol as reactant and solvent catalyzed by Novozym® 435 at selected conditions

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ABSTRACT

The enzymatic esterification of R/S-ketoprofen with 2-propanol catalyzed with the commercial biocatalyst Novozym® 435 is addressed in this investigation. The low reaction rate registered in this reaction was investigated in terms of the effect of the alcohol on the physicochemical–enzymatic stability of the biocatalyst and the interaction of the substrates with the catalytic triad at a molecular level.

The effect of contacting 2-propanol:H_2O mixture on Novozym® 435 was investigated at 45 °C for an extended period of time (8 days). The mixture dissolves the polymethylmethacrylate (PMMA) that constitutes the support of the *Candida antarctica* B lipase (CALB). Additionally, the alcohol diffuses into the biocatalyst's beads remaining strongly adsorbed (the alcohol desorption is evidenced only upon heating at 187 °C) and altering the inner texture of the biocatalyst's beads. Additionally, 2-propanol modifies the secondary structure of the enzyme by decreasing the β-sheet contribution and increasing the β-turn structure. The molecular modeling of the interaction of R/S-ketoprofen and 2-propanol with the catalytic triad of the lipase provides evidences that the secondary alcohol exerts an important steric hindrance for the reaction to proceed.

1. Introduction

Among the racemic drugs, the 2-arylpropionic acids (the "profen" family) are an important group of non-steroidal anti-inflammatory pharmaceuticals (NSAIDs), widely used as racemic mixtures to control the symptoms of arthritis and related connective tissue diseases [1]. R/S-ketoprofen (2-(3-benzoylphenyl)-propionic acid), one of the most useful NSAIDs, has received attention since the past two decades and its anti-inflammatory effect is approximately 160 times the anti-inflammatory potency of aspirin on a unit weight basis [2–4]. It has analgesic, anti-inflammatory and antipyretic properties, and is effective for the treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis [5–7]. Commercially, ketoprofen is marketed and administrated as a racemic mixture of "R" and "S" enantiomers, which are equivalent on a unit weight basis. However, (S)-ketoprofen and (R)-ketoprofen display significantly different pharmacologic activities and benefits [8]. S(+)-enantiomer has the therapeutic activity of reducing inflammation and relieving pains while the R(−)-enantiomer can be used as a toothpaste additive to prevent periodontal disease [4,9]. There is always a danger that one enantiomer of a drug may possess the desired activity and the other, although inactive in producing the desired activity, may possess extraneous and even harmful pharmacologic properties [7,10]. A major reason for the use of mixtures of enantiomers remains the cost of separation of the enantiomers which exceeds the potential advantage of a possible increase in the activity. Therefore, it has been a key issue in pharmaceutical industry to obtain the pure, active form of ketoprofen [7].

An alternative enzymatic route in the preparation of enantiopure (S)-ketoprofen using lipase as biocatalyst is gaining importance because of their remarkable properties (e.g. stereo and substrate specificity), milder reaction conditions reduction in the energy requirements and the production cost [11]. Novozym® 435 produced by Novozymes Co., is the most widely tested biocatalyst in the esterification of profens. This catalyst is composed of *Candida antarctica* lipase B (CALB) physically immobilized within beads of a macroporous resin. Among the commercially available biocatalysts, there is no doubt that Novozym® 435 is the most commonly heterogeneous biocatalyst used in the enantioselective synthesis of optically active alcohols, amines and carboxylic acids. Novozym® 435 is the commercial immobilized *C. antarctica* lipase B (CALB)

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produced by submerged fermentation of a genetically modified *Aspergillus* microorganism. The lipase is adsorbed on a macroporous resin called Lewatit VP OC 1600, according to the information given by the Novozymes Co. in their website. This macroporous resin is a polymer of methacrylic acid cross-linked with divinylbenzene (DVB) [12]. The enantioselectivity of CALB toward R(−)-enantiomer is well known and has been proven in several reports. In the process of enantioselective esterification, racemic ketoprofen acid will react with an alcohol in the presence of Novozym® 435 to yield the (R)-ketoprofen ester and water [4]. Esters are generally produced in organic solvents, such as isooctane [13–20]. Although higher conversion yields in organic solvent simplify product recovery, solvent toxicity is a problem for many applications. In addition, some organic solvents employed are too expensive to allow profitable commercial scale-up [21]. The major advantages of a solvent-free system are that the absence of solvents facilitates downstream processing, since fewer components would be present in the reaction mixture at the end of the reaction; moreover, the elimination of solvents from the production step offers significant cost savings and minimizes environmental impact. In addition, it is possible to use high substrate concentrations [21,22]. There are few studies concerning lipase-catalyzed production of esters in solvent-free system [23].

In this work, the esterification of R/S-ketoprofen using 2-propanol as reactant and solvent is investigated through several aspects, such as the effect the alcohol on the physical integrity and the secondary structure of the active protein of the commercial biocatalyst Novozym® 435. Additionally, the interaction of the substrates with the active site is theoretically modeled in order to obtain insights on the reaction at a molecular level.

2. Experimental

2.1. Materials

C. antarctica lipase (CALB L) (batch LCN02102) and the commercial biocatalyst Novozym® 435 (batch LC200217) were obtained as a gift from Novozymes Brasil (Paraná, Brazil). The specific surface area (determined through the BET method) of the biocatalyst is 72 m^2/g.

Highly pure *C. antarctica* lipase (35,500 g/mol) purchased from Sigma–Aldrich Argentina (10.9 U/mg) was used as a reference for the infrared analysis purposes.

Additionally, R/S-ketoprofen (Parafarm, 99.80%, batch 030718 000928/004), 2-propanol (J.T. Baker, 99.93%) and potassium hydroxide 1 mol/l in ethanol (Riedel-de Haën) were used.

2.2. Procedures for the quantification of proteins

2.2.1. Determination of the protein loading of Novozym® 435

The total amount of protein of the starting Novozym® 435 was determined through the method described by Gross and coworkers [24] and already reported previously by some of us [25]. Novozym® 435 (0.1000 g) was contacted at 37 °C for 30 min with 3.00 ml of dimethylsulfoxide (J.T. Baker, 100%) at 200 rpm in a shaker bath. The biocatalyst's beads were separated by filtration and washed with portions of 5.00 ml of dimethylsulfoxide. Then the recovered beads were contacted for 30 min with 3.00 ml of 5% Triton X-100 solution at 37 °C, filtered afterwards, washed three times with 5.00 ml portions of the same solution and finally washed with distilled water. This procedure allowed us the recovering of the support Lewatit VP OC1600 and to quantify the total amount of enzyme of Novozym® 435 through the bicinchoninic acid assay described previously [25].

2.2.2. Quantification of the protein desorbed out of Novozym® 435

The amount of enzyme desorbed out of Novozym® 435 was determined through UV–Vis spectroscopy as reported previously [25]. In this context, the absorbance of the supernatant solution remaining after each treatment with 2-propanol was studied in the 200–400 nm range with an equipment Perkin Elmer Lambda35. Then, the absorbance at 256 nm was correlated with the concentration of CALB by means of a calibration curve performed with several solutions of known concentration of the CALB L enzyme in aqueous medium. In turn, the concentration of protein in the starting solution of CALB L was obtained through a precipitation method as described previously [25].

2.3. Procedure for the treatment of Novozym® 435 with 2-propanol–4.76% H_2O

The effect of 2-propanol on the biocatalyst was investigated by contacting 1.0000 g of the biocatalyst with 10.00 ml of a mixture of 2-propanol:4.76% (v/v) H_2O at 45 °C and 200 rpm for 8 days. The water activity of such mixture was equal to unity according to the measurement performed with a thermometer and humidity meter CEM DT-615 MIB instruments.

Then, the beads were dried in a desiccator for 8 days (to dehydrate) and further heated at 160 °C for 10 min (to desorb the 2-propanol), cooled down and weighed. This last procedure was repeated six times until constant weight. This procedure allowed establishing the total weight loss of the biocatalyst and the amount of adsorbed 2-propanol.

The solvent was allowed to dry and the remaining solid was dissolved with 1.50 ml of water, centrifuged to separate the non-soluble substances and recover the enzyme for further quantification through UV–Vis spectroscopy as described in Section 2.2.2.

2.4. Esterification of profens with 2-propanol

The reaction of R/S-ketoprofen with 2-propanol was performed at the optimum operative conditions previously determined for the esterification of profens with ethanol [26]. In this context, the reactions were performed in closed 100 ml vials, which were kept at constant temperature (45 °C) and stirring (200 rpm) in a shaker bath. In all cases 0.500 g of R/S-ketoprofen (1.966 mmol) was dissolved in the minimum volume of 2-propanol (3.125 ml; 40.95 mmol) with no need of heating. The molar ratio 2-propanol:R/S-ketoprofen correspond to 21.

The reaction medium also possesses 4.76% of water added (150 μl) that corresponds to the optimum amount of water for the biocatalyst performance. The kinetics of the esterification of R/S-ketoprofen was investigated using Novozym® 435 (160 mg) as received and in a second set of experiments, the biocatalyst was pretreated with 2-propanol (according to the methodology described in Section 2.3) before the esterification of R/S-ketoprofen.

The esterification of R/S-ibuprofen with 2-propanol was also performed at selected conditions for comparison purposes. In this context, 0.500 g of R/S-ibuprofen (2.42 mmol) was dissolved in 1.00 ml (13.01 mmol); 1.30 ml (17.39 mmol) and 3.20 ml (42.81 mmol) of 2-propanol corresponding to 2-propanol:R/S-ibuprofen molar ratios equal to 5.4; 7.2 and 21, respectively.

Chiral analysis of both enantiomers of R/S-ketoprofen and R/S-ibuprofen was conducted by chiral HPLC analysis using a Nucleodex beta-PM (Macherey-Nagel) with an UV detector operated at 230 nm. The mobile phase (metanol/0.1% TEAA pH 4.0) was operated at a flow rate of 0.7 ml/min. All samples were run in triplicate. Enantiomeric excess (ee) referred to the form S(+)-ketoprofen was

calculated according to Eq. (1) where [S] and [R] account for the concentrations of the S(+) and R(−) enantiomers respectively.

$$ee = \left[\frac{[S]-[R]}{[S]+[R]}\right] \times 100 \quad (1)$$

The conversion of the profens was also verified by titration of the final reaction mixture. Being R/S-ketoprofen or R/S-ibuprofen the only acid compounds present in the reaction mixture, titration of the samples with a basic solution of KOH in ethanol of known concentration showed to be an accurate and reliable method to determine their concentration.

2.5. Determination of the protein's secondary structure with diffuse reflectance Fourier infrared spectroscopy (DRIFT)

The secondary structure of the starting Novozym® 435 along with the biocatalyst exposed to 2-propanol for 8 days and the evolution of the secondary structure upon reaction was followed through diffuse reflectance Fourier infrared analysis (DRIFT) coupled with isotopic exchange with deuterium oxide. The isotopic exchange of water molecules by D_2O molecules allows investigating the Amide I signal (1700–1600 cm^{-1}) without the interference of the bending vibration of O—H species that typically appears at 1640 cm^{-1}. The H–D isotopic exchange of water molecules of all samples was performed by contacting the sample with 100 μl of deuterium oxide (D > 99%) under a vigorous stirring for 10 min and further incubation overnight at room temperature.

DRIFT spectra were recorded using a Harrick module with praying mantis mirrors set-up (Harrick Scientific Co.). A Nicolet 8700 FTIR spectrometer with a MCT-A cryogenic detector was used to acquire the spectra (4 cm^{-1} resolution, 100–250 scans). The spectrometer and the mirrors that direct the radiation toward the cell are continuously purged with dry air (from a Parker Balston generator) in order to eliminate the contribution of CO_2 and water vapor from the spectra.

To estimate the secondary structure, peak fitting of the Amide I band (1700–1600 cm^{-1}) by Lorentzian-shaped components was performed on the non-deconvoluted spectra. The software used with this purpose was a special peak fitting module of Origin 5.0. The positions and number of the components were determined from the second derivative analysis of the spectra. The contribution of each component to the Amide I band was evaluated by integrating the area under the curve and then normalizing to the total area of the Amide I band.

2.6. Scanning electron microscopy analysis and fractal dimension estimator calculation

The internal texture of Novozym® 435 before and after contacting with 2-propanol for 8 days, and the biocatalyst (with and without previous exposure to the alcohol) after 72 h of reaction was investigated through scanning electron microscopy analysis with an SEM Philips 505. 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 electron microscope.

Images of the samples at 8400× magnifications were taken and analyzed with the FERImage program to calculate the fractal dimension D and the d_{min} parameter by using the variogram [27–30]. The variogram used to determine parameters that characterize the surface roughness, consists of a graph of the variance of variation of heights in a surface for different steps, as a function of such steps and at logarithmic scale. The slope of the graph is related to the fractal dimension D as $D = 3 - \text{slope}/2$. The fractal dimension values ranging from $2 < D < 2.5$ indicate a persistent smooth surfaces while values $2.5 < D < 3$ are an indication of antipersistence, being a completely rough surface when D is close to 3. For many images observed with the SEM, the variogram presents a fractal behavior at low scale and a behavior that seems to have an asymptotic tendency at high scale, but, if the vertical axis was expanded variance maximums and minimums appear. This periodic region was described by two parameters: d_{min} and d_{per}. The parameters d_{min} and D will be used in this work to characterize the texture of the images corresponding to the different samples. The d_{min} parameter corresponds to the inferior end of the periodic scale region and is representative of the smallest cell size with enough statistic weight to produce periods. It is worth noticing that the D parameter determined in this work, is strictly a fractal dimension estimator since the linear range on the graph (corresponding to the sample fractal behavior) is not very extensive and moreover, the linear region includes blended information: corresponding to the own sample and to the microtome used to cut slices of the sample.

2.7. Temperature programmed desorption

Temperature programmed desorption analysis was performed over Novozym® 435 after being treated with 2-propanol for 8 days. Details of the equipment used in this investigation have been published before [25,31,32]. The samples (49.9 mg) are 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 are detected in the mass spectrometer and recorded in a computer. The following m/e ratios were employed to identify the desorbed species: 2-propanol C_3H_7OH, m/e = 45 (100), m/e = 43 (16), m/e = 27 (13) and 29 (10); acetone, m/e = 43 (100) and m/e = 58 (27); propylene, m/e = 41 (100), m/e = 39 (74) and 42 (70); H_2O, m/e = 18; CO_2, m/e = 44 and CO, m/e = 28.

2.8. Molecular modeling

A simple molecular modeling was performed using MM2 from Chem3D 5.0 Ultra in order to explore the reasons of the enantioselectivity to the S(+) form and the low catalytic activity. From the Protein data Bank (PDB) the structure of CALB was obtained and the portion of the catalytic triad and the medium and large pocket were included. 2-Propanol and R/S ketoprofen were modeled considering their steric energetic minima and later 2-propanol was located near the His and Ser (or not) from the beginning of the reaction mechanism. The structures were minimized until a cutoff of 0.1 kcal/mol was obtained between one run and the following one.

The reaction mechanism considered has several steps:

1. Adsorption of R/S ketoprofen near serine 105 in the presence or not of 2-propanol.
2. Intermediate 1 with 2-propanol present or not.
3. Acyl enzyme of R/S ketoprofen without 2-propanol present.
4. Adsorption of 2-propanol near the acyl enzyme carbonyl group and considering the distance to the N from His 229 in the case of 2-propanol not present since the beginning; H distance to N from His 224 important.
5. Intermediate 2, R/S ketoprofen coordinated with 2-propanol.
6. Adsorption of isopropyl ester of ketoprofen and regeneration of serine.

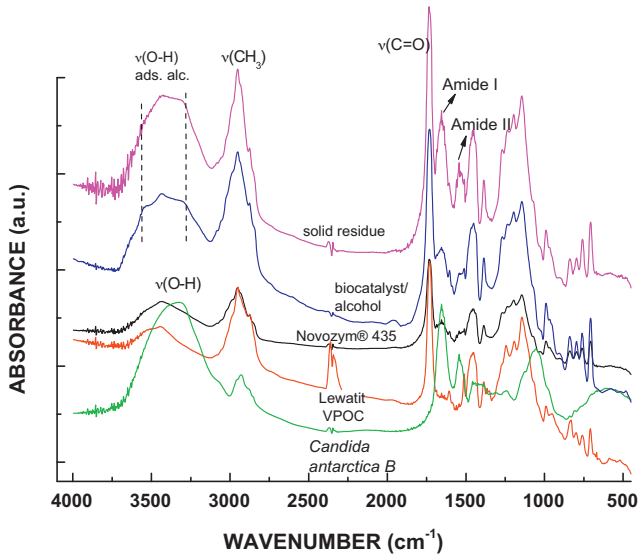

Fig. 1. Infrared spectra of highly pure CALB, Novozym® 435 before and after being in contact with 2-propanol for 8 days; the resin Lewatit VP OC1600 and the solid phase recovered after drying the liquid medium in contact with the biocatalyst.

3. Results

3.1. Degradation of Novozym® 435 upon contact with the 2-propanol–water medium

Previous investigations by some of us demonstrated that Novozym® 435 deactivates in the fourth cycle of consecutive use (48 h per cycle that corresponds to a total of 8 days) in the esterification of R/S-ibuprofen with ethanol as reactant and solvent [26]. Further investigations provided evidences of the dissolution–disaggregation of Novozym® 435 in contact with ethanol which in turn induces the loss of the surface protein (*C. antarctica* B lipase) and the cross-linked macroporous polymer polymethylmethacrylate (PMMA) (known as Lewatit VP OC1600) that forms the internal core of the biocatalyst's beads [25,31]. In this context, the biocatalyst was treated for 8 days at 45 °C with a mixture of 2-propanol containing 4.76% of added water in order to obtain insights on the effect of the alcohol on the integrity of Novozym® 435. The methodology is similar to the one applied before to investigate the effect of ethanol which allows to compare the influence of both alcohols on the physical and chemical stability of the biocatalyst.

Fig. 1 compares the infrared spectra of highly pure CALB, Novozym® 435 before and after being in contact with 2-propanol for 8 days; the resin Lewatit VP OC1600 and the solid phase recovered after drying the liquid medium in contact with the biocatalyst. Additionally, Table 1 summarizes the band's position and the assignments of the infrared signals.

The infrared spectra of *C. antarctica* lipase possesses an intense signal centered at 3337 cm^{-1} arising from the stretching vibration of the intramolecular hydrogen bonded N—H species that is superimposed with the stretching vibrations of O—H species [25,33,34]. Additionally, the lipase possesses the intense bands corresponding to the Amide I and Amide II signals centered at 1653 cm^{-1} and 1540 cm^{-1}, respectively [33]. The infrared signal known as Amide I arises from the stretching vibration of the carbonyl bond C=O of the backbone structure of the proteins. This vibration is not affected by the nature of the side chain but it is influenced by the secondary structure of the proteins. The infrared signal called Amide II is attributed to the out-of-plane combination of the in plane

Table 1
Summary of the nature of the organic groups and the corresponding infrared signals that were detected on Novozym® 435, Lewatit VPOC, *Candida antarctica* CALB lipase and the solids recovered upon contacting Novozym® 435 with 2-propanol.

	ν(O—H)	ν(C—N)	ν(CH3)	ν(C=O)	Amide I	Amide II	Aromatic ring vibration ν(C=C)	δ_{as}(C—H)	δ_s(C—H)	ω(CH2) τ(CH2)[a]	Out-of-plane δ(C—H)[b]
CALB lipase		3337 cm^{-1}	2931 cm^{-1} 2951 cm^{-1}		1653 cm^{-1}	1540 cm^{-1}					
Lewatit VPOC	3444 cm^{-1}		2874 cm^{-1} 2951 cm^{-1}	1730 cm^{-1}			1511 cm^{-1} 1456 cm^{-1}	1385 cm^{-1}	1363 cm^{-1}	1270–1090 cm^{-1}	990, 995, 834, 799, 759, 708 cm^{-1}
Novozym® 435	3438 cm^{-1}		2876 cm^{-1} 2952 cm^{-1}	1734 cm^{-1}	1654 cm^{-1}		1456 cm^{-1}	1387 cm^{-1}		1270–1090 cm^{-1}	990, 995(s), 843, 801, 762, 706 cm^{-1}
Novozym® 435/alcohol	3440 cm^{-1}	3301 cm^{-1}	2876 cm^{-1}	1731 cm^{-1}	1655 cm^{-1}			1385 cm^{-1}		1270–1090 cm^{-1}	990, 995(s), cm^{-1}
Recovered solids	3434 cm^{-1}	3297 cm^{-1}	2876 cm^{-1} 2952 cm^{-1}	1735 cm^{-1}	1655 cm^{-1}	1543 cm^{-1}	1455 cm^{-1}	1385 cm^{-1}		1270–1090 cm^{-1}	992, 995(s), 843, 798, 759, 708 cm^{-1}

[a] ω(CH_2) and τ(CH_2) wagging and twisting vibration of methylene groups.
[b] Out of plane bending of aromatic C—H groups.

bending mode of the N—H bond and the stretching vibration of the C—N bond of the protein.

The support Lewatit VP OC1600 possesses an intense signal at 1730 cm^{-1} due to the stretching vibration of the carbonyl groups of the polymethylmethacrylate (PMMA) $-(CH_2CCH_3)_n-CO-O-CH_3$ [25,31,35,36]. Additionally, the intense signals belonging to asymmetric and symmetric stretching of the methyl groups (2951 cm^{-1} and 2874 cm^{-1}, respectively) along with asymmetric and symmetric bending vibrations (of weak intensity) of the methyl groups (1387 cm^{-1} and 1361 cm^{-1}, respectively) are observed [25,36,37]. These last two signals are characteristic of the C—H vibration of methyl and methylene groups attached to a tertiary carbon atom such as the one of the PMMA resin (the expanded molecular structure of the polymer is available in Ref. [25]). The intense set of signals observed in the range from 1270 cm^{-1} to 1090 cm^{-1} are characteristic of twisting and wagging vibrations of methylene groups. The presence of divinylbenzene (used as cross-linker in PMMA) is evidenced through the doublet of medium intensity at 1511 cm^{-1} and 1456 cm^{-1} due to the skeletal vibrations involving the carbon–carbon stretching within the aromatic ring. Additionally, a set of weak signals is observed from 990 cm^{-1} to 708 cm^{-1} that is characteristic of the C—H out-of-plane bending bands of the alkyl-substituted benzene [36,37].

The PMMA resin does not have O—H species therefore, the broad signal of medium intensity at ~3465 cm^{-1} could be ascribed to adsorbed water molecules.

The commercial biocatalyst Novozym® 435 possesses both the signals belonging to CALB (a weak signal belonging to Amide I is observed at 1654 cm^{-1}) and the macroporous polymer as expected (see Fig. 1 and Table 1).

The solids recovered from the organic medium show both the CALB enzyme and the species of the macroporous matrix as can be concluded from the similarity of the infrared signals of those three systems (see Table 1). The nature of the solid phase recovered from the 2-propanol that was in contact with Novozym® 435 evidences the disaggregation of the biocatalyst exposed to the organic medium. In this context, the results are similar to those reported previously in terms of the detrimental effect of ethanol on Novozym® 435.

Additionally, the shoulders at 3289 cm^{-1} and 3541 cm^{-1} observed in the infrared spectra of the biocatalyst after being in contact with the alcohol and the recovered solids might be an evidence of 2-propanol that remains molecularly adsorbed even though the alcohol was allowed to evaporate before the analysis of the samples. These signals have been ascribed to O—H stretching vibrations of un-dissociated 2-propanol molecules adsorbed on carbon containing samples [38]. This observation was further corroborated through the temperature programmed surface desorption of the alcohol as will be shown in the following sections.

3.1.1. *Extent of the disaggregation of Novozym® 435 upon contact with the 2-propanol–water medium*

The results discussed above provide evidences of the dissolution–disaggregation of Novozym® 435 in contact with 2-propanol–4.76% water which in turn induces the loss of the surface protein (*C. antarctica* B lipase) and the polymeric matrix of the biocatalyst's beads. The amount of protein and PMMA left in the liquid medium after contacting the biocatalyst with 2-propanol–4.76% water for 8 days was quantified in order to establish the extent of the disaggregation of Novozym® 435. The biocatalyst (2.0022 g) was allowed to interact with 20.00 ml of 2-propanol–4.76% H_2O for 8 days as described in Section 2.3. After that period of time, the remaining liquid was separated by filtration with a GE nylon membrane filter (0.45 μm pore size) in order to retain the non-soluble substances as polymethylmethacrylate. The biocatalyst's beads were washed 3 times with

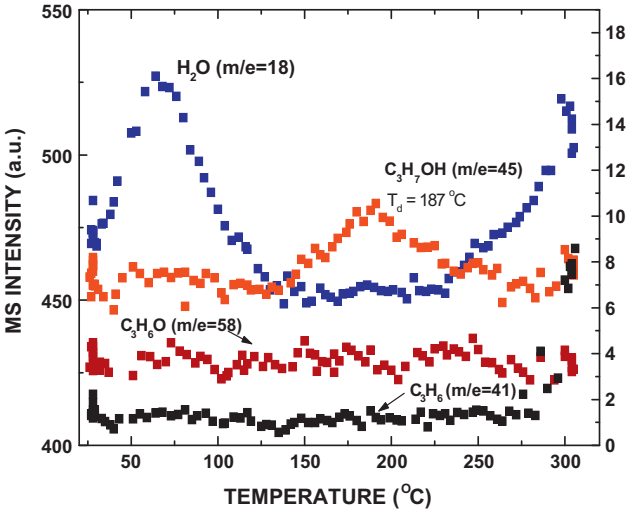

Fig. 2. In situ temperature programmed surface reaction spectra of Novozym® 435 after being in contact with 2-propanol for 8 days at 45 °C.

5.00 ml of 2-propanol and the solvent was separated by filtration (as described before) each time. The liquids remaining after each wash were added to the liquid phase initially separated after the 8 days treatment of Novozym® 435 and allowed to evaporate. The solids retained in the filters and the one precipitated from the liquid phase (after the 2-propanol was completely dried) were weighed and then suspended in a minimum amount of water (1.50 ml) and further centrifuged in order to separate the non-soluble substances. Then the aqueous medium containing the enzyme was analyzed through UV–Vis spectroscopy to quantify the amount of desorbed protein.

The biocatalyst's beads after being dehydrated in a desiccator for several days were sequentially heated at 160 °C for 10 min, cooled down and weighed (see Section 2.2 for details). The cycle was repeated until constant weight which ensures that the 2-propanol was removed from the beads. The temperature chosen for the treatment corresponds to the starting temperature of the 2-propanol desorption found in the temperature desorption analysis as will be described in the following section.

The results obtained in that series of experiments are presented in Table 2 along with the information reported before (in Ref. [25]) concerning the effect of ethanol on Novozym® 435. The experiments demonstrated that the detrimental effect of 2-propanol in the integrity of the biocatalyst is much less pronounced that the one observed with ethanol.

In fact, Novozym® 435 loses 1.2% of PMMA support, additives and protein upon contact with 2-propanol vs. 16.6% when is in contact with ethanol. The amount of irreversible adsorbed alcohol is also an order of magnitude lower (0.0368 g/g biocatalyst vs. 0.3881 g/g biocatalyst) when 2-propanol was used. In this context, the adsorption phenomenon of this alcohol on the biocatalyst is discussed in the following section.

3.2. *The irreversible interaction of 2-propanol over Novozym® 435*

The interaction of 2-propanol–4.76% H_2O with Novozym® 435 was investigated through temperature programmed desorption–reaction analysis of the species remaining adsorbed on the biocatalyst's surface/bulk after being in contact with such medium for an extended period of time as described in Section 2.3. Fig. 2 shows the desorption profile of the species with $m/e = 45$ that belongs to molecular 2-propanol. The alcohol molecularly

Table 2
Summary of the amounts of polymethylmethacrylate, additives and enzyme lost and alcohol adsorbed upon contacting Novozym® 435 with ethanol and 2-propanol. Comparison of the percentage of total weight loss, percentage of protein loss per total amount of protein of the biocatalyst and mass of alcohol adsorbed per gram of Novozym® 435.

Alcohol	Mass (g)					% weight loss	% protein loss[b]	Mass alcohol adsorbed per 1 g biocatalyst
	Starting biocatalyst	Recovered biocatalyst	Support/additives dissolved in liquid phase	Alcohol desorbed	Protein dissolved			
Ethanol[a]	1.0000	0.8340	0.1645	0.3881	1.4744×10^{-3}	16.6	2.76	0.3881
2-Propanol	2.0022	1.9778	0.0230	0.0738	1.3591×10^{-3}	1.2	1.28	0.0368

[a] The data corresponding to Novozym® 435 in contact with etanol was taken from Ref. [26].
[b] Ratio between the amount of enzyme lost due to the alcohol treatment and the total amount of enzyme of Novozym® 435.

desorbs with a maximum at 187 °C (ethanol desorbs at 184 °C according to [25]). This fairly high temperature that corresponds to a high activation energy of desorption is an evidence of a strong alcohol–biocatalyst interaction. Two desorption events of water molecules ($m/e = 18$) are observed. The first one observed at low temperature (centered at 63 °C) corresponds to the loss on constitutional water of the proteins. A second event happens when the temperature rises ($T > 239$ °C) and is accompanied with the desorption of CO and CO_2, corresponds to the decomposition of amino acids and the PMMA support [25].

The absence of acetone ($m/e = 58$) or propylene ($m/e = 41$) demonstrates that the adsorbed alcohol does not further reacts with the biocatalysts.

3.3. Esterification of R/S-ketoprofen and R/S-ibuprofen with 2-propanol as reactant and solvent

The esterification of R/S-ketoprofen with 2-propanol with 4.76% of water added was performed with Novozym® 435 with and without previous treatment with the alcohol–water mixture for 8 days. The objective of such experiments was to elucidate if the detrimental effect of the alcohol has an impact on both the conversion and the enantiomeric excess of the reaction. Fig. 3 shows the performance of the biocatalyst in the esterification of R/S-ketoprofen. Additionally, the conversion and enantiomeric excess in the esterification of R/S-ibuprofen dissolved in the minimum amount of 2-propanol (alcohol:R/S-ibuprofen molar ratio equals to 5.4) after 48 h of reaction at 45 °C is presented for comparison.

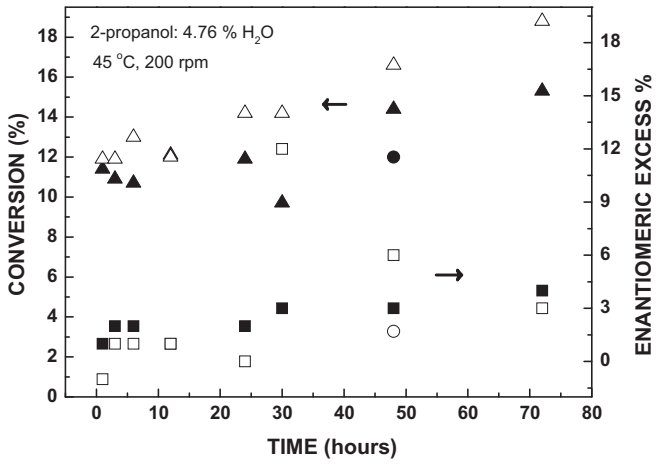

Fig. 3. Conversion and enantiomeric excess toward S(+)-ketoprofen in the esterification of R/S-ketoprofen with 2-propanol. Additionally, the conversion and enantiomeric excess in the esterification of R/S-ibuprofen dissolved in the minimum amount of 2-propanol (alcohol:R/S-ibuprofen molar ratio equals to 5.4) after 48 h of reaction at 45 °C is presented for comparison. *Symbols*: (▲) conversion of R/S-ketoprofen and (■) enantiomeric excess ee % toward S(+)-ketoprofen catalyzed with starting Novozym® 435; (△) conversion of R/S-ketoprofen and (□) enantiomeric excess ee % toward S(+)-ketoprofen catalyzed with Novozym® 435 previously treated with 2-propanol for 8 days; (●) conversion of R/S-ibuprofen and (○) enantiomeric excess ee % toward S(+)-ibuprofen in 48 h of reaction.

The results demonstrated that the conversion of R/S-ketoprofen toward the esters increases slowly from about 10% after 60 min of reaction to about 19% after 72 h regardless of the previous contact of the biocatalyst with 2-propanol or not. Although, a 12% enantiomeric excess to the S(+)-ibuprofen was observed after 30 h that value decreases rapidly. The low values of enantiomeric excess (~5%) clearly demonstrate that the biocatalyst is not enantioselective in the esterification of the profen. This observation cannot be attributed to the nature of the profen since the esterification of R/S-ibuprofen shows a similar behavior. In this context, the esterification of racemic ibuprofen with an alcohol:profen molar ratio equal to 5.4 showed a 12% conversion and 1.7% of enantiomeric excess to S(+)-ibuprofen. Even lower conversion and enantiomeric excess were observed when the amount of alcohol was increased providing evidences of the inhibitory effect of 2-propanol. In this context, alcohol:profen molar ratios equal to 7.2 and 21 provided conversions of 5.3% (ee = 0%) and 4.7% (ee = −2.1%), respectively.

3.4. Evolution of the secondary structure of the C. antarctica B lipase upon contact with 2-propanol–water medium

Novozym® 435 was also analyzed through infrared spectroscopy in order to investigate the effect of the 2-propanol–water media and the reaction conditions on the secondary structure of the protein that remained adsorbed on the beads. The beads of biocatalyst were contacted with deuterium oxide overnight in order to exchange the water molecules by D_2O. The isotopic exchange possess the advantage that the much lower absorption of D_2O solvent compared with H_2O in the 1700–1500 cm^{-1} allows a better signal-to-noise ratio along with a higher resolution spectrum [39–41]. The analysis of the infrared spectra of enzymes in the 1700–1600 cm^{-1} region (the so-called Amide I region) provides qualitative and also quantitative information on the secondary structure elements that compose the protein. The Amide I band originates from the stretching vibrations of the peptide carbonyl groups in the backbone of the protein, whose frequency depends on the hydrogen-bonding and coupling along the protein chain, and is therefore sensitive to the protein conformation.

The percentage of α-helix, β-sheet, turns and random to the structure of the starting Novozym® 435, treated with 2-propanol–water media, and the evolution of the secondary structure of the biocatalyst (with and without alcohol treatment) during the esterification of R/S-ketoprofen is shown in Tables 3 and 4. The percentage contribution of each structure was obtained through the deconvolution, integration and further normalization of the corresponding signals involved in the Amide I. In this context, 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

Table 3
Contribution of the α-helix, β-sheet, random structure and β-turns to the secondary structure of the starting Novozym® 435, after treated with 2-propanol for 8 days (at 45 °C without reaction) and after esterification of R/S-ketoprofen for 1 h toward 72 h using the starting biocatalyst without any previous contact with the alcohol.

	Percentage contribution			
	α-Helix[a]	β-Sheet[b]	Random[c]	β-Turns[d]
Starting Novozym® 435	23.6	26.9	23.7	25.8
8 days with 2-propanol	20.2	10.0	1.7	68.1
1 h of reaction	22.6	21.3	6.5	49.6
3 h	20.3	0.8	0.1	78.7
6 h	20.3	7.1	1.1	71.5
12 h	20.9	8.2	3.6	67.3
24 h	23.2	12.6	1.3	62.8
30 h	20.0	7.1	17.3	55.4
48 h	20.8	8.5	2.0	68.6
72 h	20.1	21.6	2.8	55.5

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

1631 cm^{-1}, 1637 cm^{-1} and 1686 cm^{-1} wavenumbers. Similarly, the contribution of the β-turn structure corresponds to the addition of the areas of the signals located at 1664 cm^{-1}, 1666 cm^{-1} and 1676 cm^{-1} [25,33,34,39,41].

The secondary structure of the protein of the starting Novozym® 435 is composed by 23.6% of α-helix, 26.9% of β-sheet, 23.7% of random structure and 25.8% of β-turns. The exposure to 2-propanol causes a drastic increase in the β-turn structure from 25.8% toward 68.1% and diminishes both the contribution of the β-sheet and the random structure (see Tables 3 and 4). A high content of the β-turn structure along with a ∼22% (in average) of the α-helix is maintained almost unaltered when the pretreated biocatalyst (exposed 8 days to 2-propanol) is used in the esterification of R/S-ketoprofen up to 72 h (see Table 4). This observation was further confirmed when the secondary structure of the non-treated biocatalyst used in the esterification reaction was investigated. In this context, Novozym® 435 (non previously exposed to 2-propanol) used in the esterification of R/S-ketoprofen increases the content of β-turn from 25.8% (starting sample) toward 49.6% in the first hour of reaction and reaches 78.7% after 3 h of reaction (see Table 3). Again, a content of approximately 20% of α-helix is observed all along the 72 h of reaction.

3.5. Effect of 2-propanol on the inner texture of Novozym® 435

The inner texture of the fresh Novozym® 435, after interacting with 2-propanol for 8 days, and the biocatalyst (with and without being pre-treated with 2-propanol) after 72 h of reaction was investigated with the fractal dimension estimator D and the d_{min} parameter, by using the variogram method, implemented on the FERImage program as described in Section 2.6. This program works with square images therefore, the central portion of the corresponding images, squares of typically 6.12 μm × 6.12 μm, 6.32 μm × 6.32 μm and 8.69 μm × 8.69 μm were taken. If the image is anisotropic, both parameters, D and d_{min}, will not necessarily be constant in different directions, therefore, an average value of those obtained at six different directions, between 0° and 90°, was used. Figs. 4–7 show the micrographs and the graphs of the variance vs. step of the cross section of the starting Novozym® 435, after treatment with 2-propanol for 8 days, non-pretreated and used in the esterification of R/S-ketoprofen for 72 h and pretreated and further used in the esterification of R/S-ketoprofen for 72 h, respectively. Additionally, Table 5 presents the fractal dimension estimator D and the minimum cell size d_{min} of the samples described above.

The D values of the starting Novozym® 435 and after treatment with 2-propanol–4.76% H_2O are similar (∼2.74) and indicate an anti-persistent fractal behavior ($D > 2.5$). The esterification of R/S-ketoprofen with 2-propanol–4.76% H_2O at 45 °C for 72 h affects the texture of the biocatalyst. The reaction conditions leads to a smother texture with lower D values than the samples described above (see Table 5). Although, the extended exposure of the biocatalyst to 2-propanol affects the d_{min} (from 0.0967 μm to 0.0651 μm) that parameter goes back to its initial value when this biocatalyst pretreated is used in the esterification. This observation indicates that the alcohol diffuses inside the biocatalyst's beads diminishing

Table 4
Contribution of the α-helix, β-sheet, random structure and β-turns to the secondary structure of the starting Novozym® 435, after treated with 2-propanol for 8 days (at 45 °C without reaction) and after esterification of R/S-ketoprofen for 1 h toward 72 h using the pretreated biocatalyst.

	Percentage contribution			
	α-Helix[a]	β-Sheet[b]	Random[c]	β-Turns[d]
Starting Novozym® 435	23.6	26.9	23.7	25.8
8 days with 2-propanol	20.2	10.0	1.7	68.1
1 h of reaction	16.9	0.9	2.86	79.3
3 h	20.8	0.2	0.2	78.9
6 h	17.4	9.1	3.1	70.3
12 h	27.2	3.4	7.2	62.0
24 h	25.0	3.3	9.7	61.9
30 h	26.9	5.4	2.6	65.1
48 h	25.4	0.3	3.6	70.7
72 h	17.1	0.9	1.3	80.6

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

Table 5
Fractal dimension estimator D and minimum cell size d_{min} with enough statistic weight to produce periods of starting Novozym® 435, after treated with 2-propanol for 8 days (at 45 °C without reaction), used in the esterification of R/S-ketoprofen for 72 h as received and Novozym® 435 treated with 2-propanol and used in the esterification of R/S-ketoprofen for 72 h.

Novozym® 435	D	d_{min} [μm]
Starting sample	2.744 ± 0.018	0.0967 ± 0.0051
Treated with 2-propanol for 8 days	2.727 ± 0.022	0.0651 ± 0.0032
Used as received in the esterification of R/S-ketoprofen for 72 h	2.598 ± 0.034	0.097 ± 0.010
Used in the esterification of R/S-ketoprofen for 72 h after treatment with 2-propanol for 8 days	2.698 ± 0.051	0.096 ± 0.017

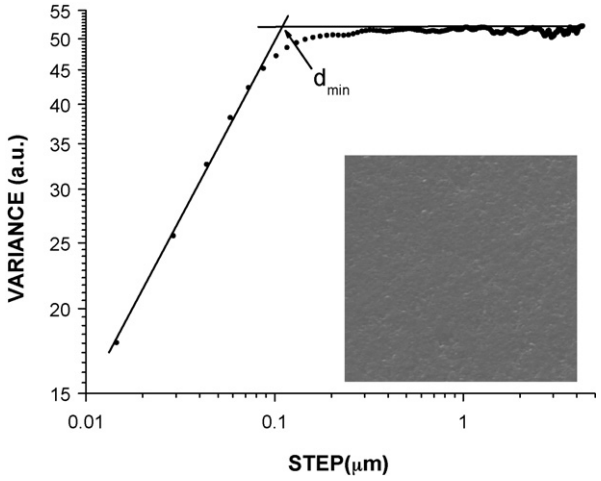

Fig. 4. Variogram corresponding to the 8.69 μm × 8.69 μm central portion of the image taken at 8400× magnification of a slice of the cross section of starting Novozym® 435. The image appears as an insert in the graph.

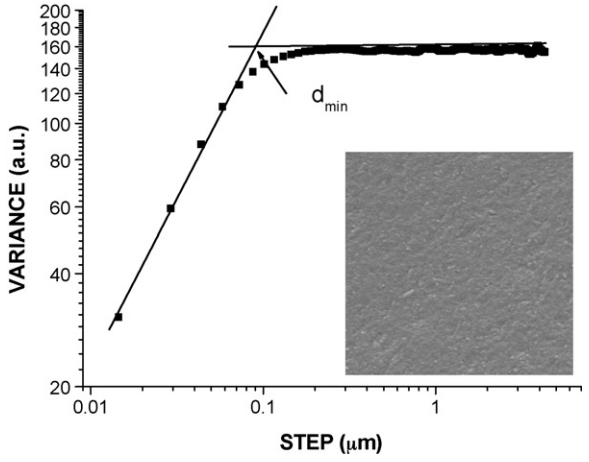

Fig. 6. Variogram corresponding to the 8.69 μm × 8.69 μm central portion of the image taken at 8400× magnification of a slice of the cross section of Novozym® 435 used in the esterification of R/S-ketoprofen for 72 h. The image appears as an insert in the graph.

the size of the SEM image texture pattern. However, the presence of 2-propanol and the R/S-ketoprofen under reaction conditions produces a pickling effect over the inner texture that conducts to a behavior that is similar to the starting biocatalyst.

3.6. Results of a simple molecular mechanics study of 2-propanol and R/S ketoprofen esterification mechanism

The ketoprofen is different from ibuprofen considering the voluminous substituent in the position 3 of phenyl moiety. When S(+)-ketoprofen is adsorbed near the serine 105 of CALB, the methyl group interacts with Thr 40, Val 154 and Ile 285, with average distances of 2.8 Å to hydrogen atoms from these amino acids. Considering R(−)-ketoprofen, the methyl group interacts with Val 154, Gln 157 and with the phenyl moiety of the benzoyl group – an interaction that is not present in the case of ibuprofen [26]. Table 6 shows the results of the calculation of the steric energy obtained for the different steps with R(−) or S(+)-ketoprofen (keto). From the table is clear that the S(+)-ketoprofen has no hindrance to produce the intermediate 1 with 2-propanol near the active catalytic triad, whereas R(−)-ketoprofen shows a barrier of near 7.5 kcal/mol (see Fig. 8). Later, the barrier to produce the intermediate 2 is near 24 kcal/mol for R(−)-keto vs. near 11 kcal/mol for S(+)-ketoprofen.

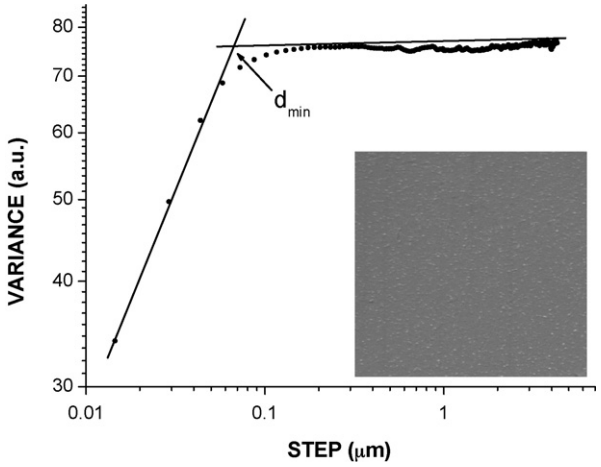

Fig. 5. Variogram corresponding to the 8.69 μm × 8.69 μm central portion of the image taken at 8400× magnification of a slice of the cross section of Novozym® 435 after contacting with 2-propanol for 8 days at 45 °C. The image appears as an insert in the graph.

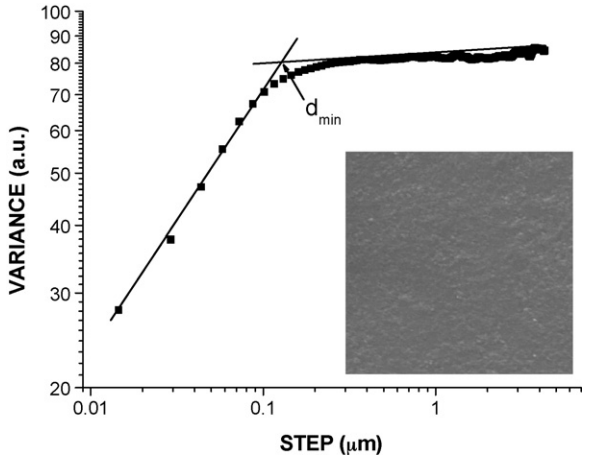

Fig. 7. Variogram corresponding to the 8.69 μm × 8.69 μm central portion of the image taken at 8400× magnification of a slice of the cross section of Novozym® 435 pretreated with 2-propanol and used in the esterification of R/S-ketoprofen for 72 h. The image appears as an insert in the graph.

Table 6
Steric energy (kcal/mol) calculated for the different steps of the mechanism of interaction of R/S-ketoprofen and 2-propanol with the lipase B of *Candida antarctica*.

Step number	Mechanism	R(−)-ketoprofen	S(+)-ketoprofen
1	Adsorption of ketoprofen near serine 105	−146.5	−143.1
	With 2-propanol near the ketoprofen	−162.3	−148.5
2	Intermediate 1	−152.1	−146.8
	Intermediate 1 + 2-propanol	−150.9	−152.4
3	Acyl enzyme without 2-propanol	−145.6	−145.8
4	Adsorption of 2-propanol near acyl enzyme	−168.1	−160.5
5	Intermediate 2	−144.5	−149.1
6	Adsorption of ester of ketoprofen	−142.3	−148.2

The comparison is done point to point (changing the R(−) to the S(+) structure) due to the known problems of the MM2 results when bonds are broken or charges are included. Therefore, the structures are similar at each step and comparisons are done between the conformation when R(−)-ketoprofen is the acid vs. when S(+)-ketoprofen is the acid. However, they are very useful in qualitative and comparative ways, especially when hydrogen bonding is considered. The steric and electronic structure of 2-propanol and its interactions with ketoprofen and the catalytic triad are completely different than in the case of ethanol [26].

When 2-propanol is analyzed in the interaction with the acyl-enzyme intermediate of R/S-ketoprofen, there are at least two

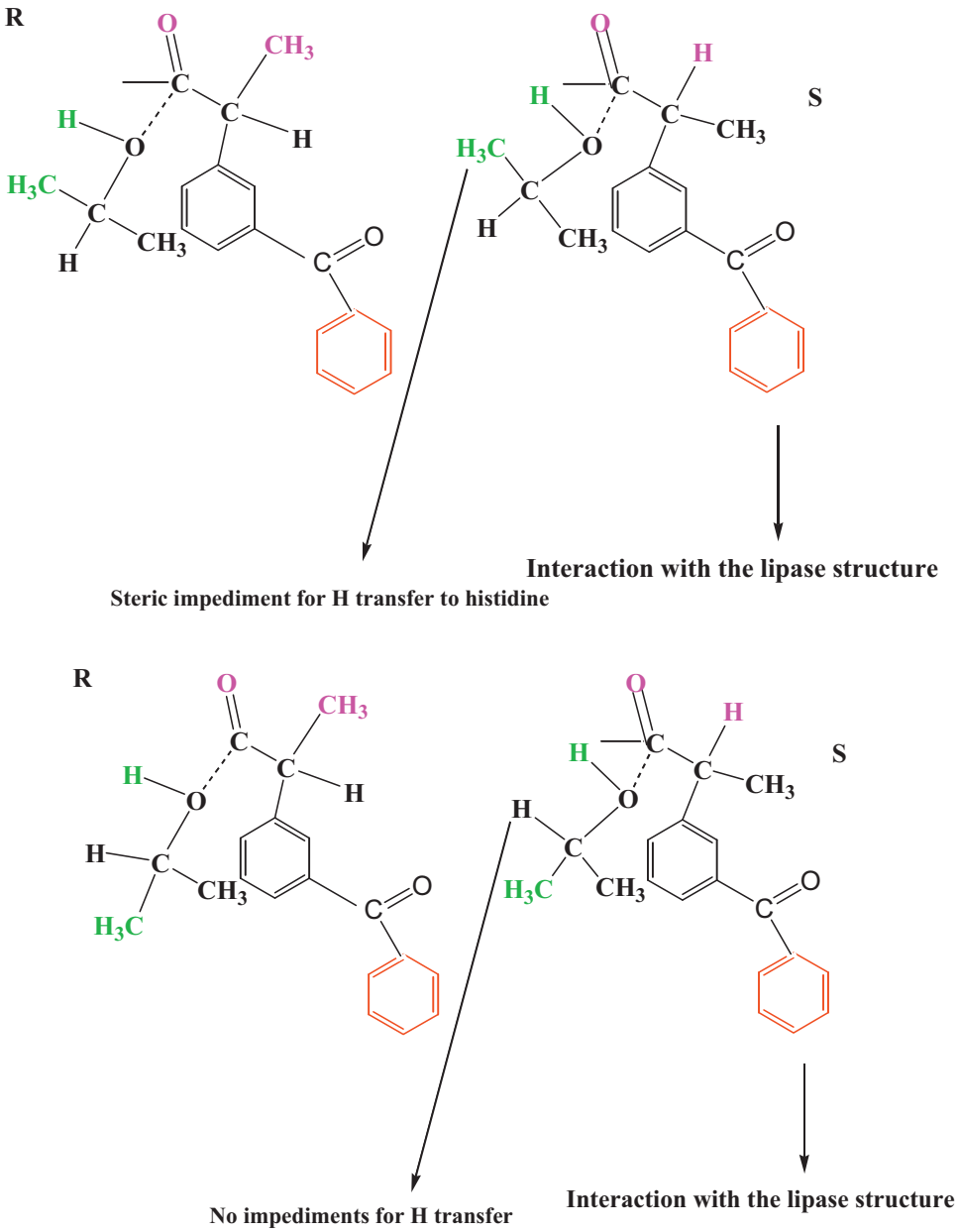

Fig. 8. Representation of the interaction of the S(+)-ketoprofen, R(−)-ketoprofen and 2-propanol with the active catalytic triad of the lipase B of *Candida antarctica*.

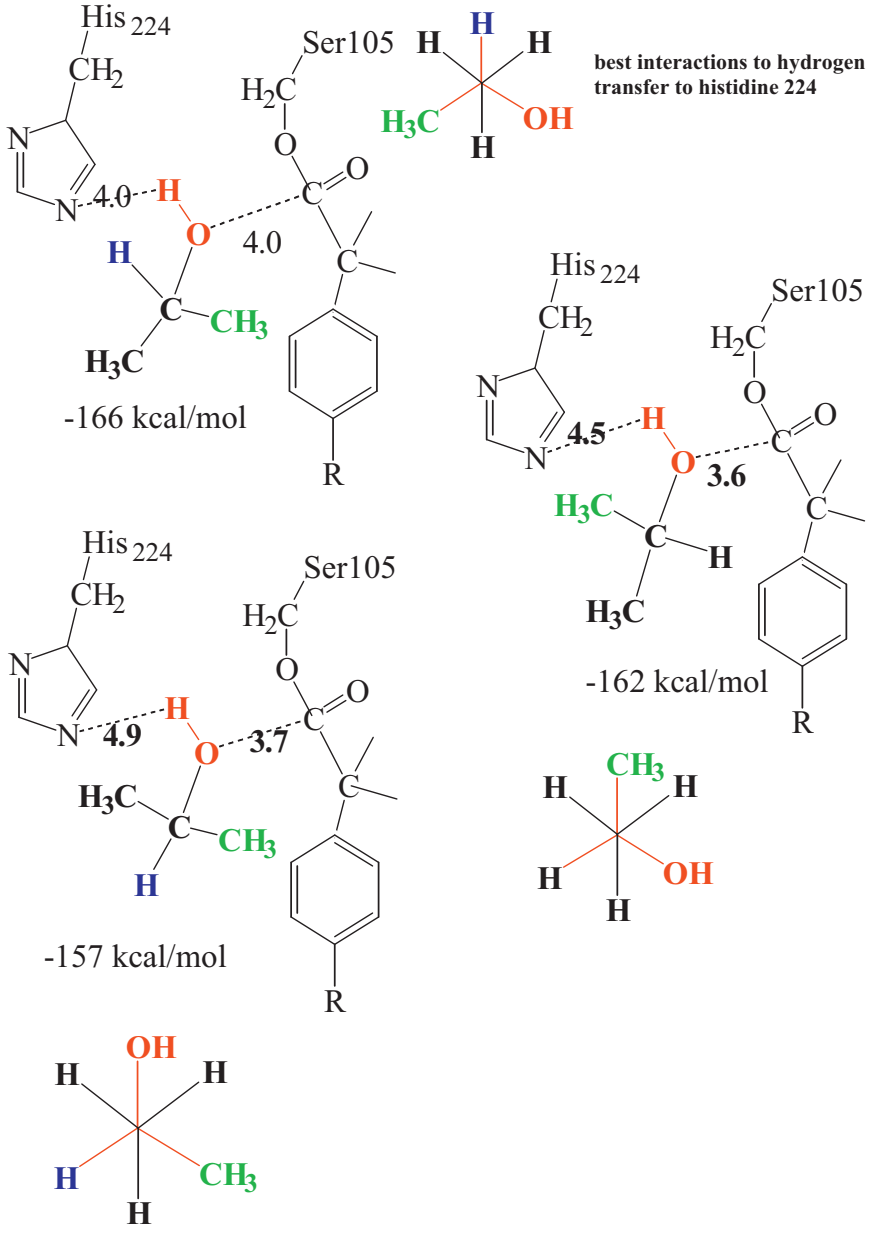

Fig. 9. Steric energies involved in the different interactions of 2-propanol with the acyl-enzyme complex.

different forms of adsorption (staggered and eclipsed) near the serine group (see Fig. 9). The form with two hydrogen atoms side to side staggered is preferred by closed to −1.5 kcal/mol. But the adsorption is possible of both forms. This implies that the hydrogen transfer from the 2-propanol to the His 224 – a key step in the mechanism – may be sterically and electronically hindered if the adsorption form of 2-propanol is staggered but the methyl group is located near the His and a change of conformation in the alcohol is needed to produce the reaction. Here, it is proposed that the conformational forms of the alcohol and the steric hindrance to hydrogen transfer to Histidine decreases the reaction rate of the profen in such a way that the reaction is controlled by the conformational change of the 2-propanol more than by the conformational distribution of substituents around the chiral C of the profen. The adsorption is however so strong that this step is very stable in comparison to the intermediate 2, generating a high steric barrier energy of near 24 kcal/mol for R(−)-keto vs. 11.4 kcal/mol for S(+)-ketoprofen for the formation of intermediate 2 for this particular alcohol. It is interesting that R(−)-ketoprofen presents a lower steric energy in general. The intermediate 2 is particularly stable and therefore difficult to react to produce the ester for the R(−) and S(+) species.

Then the problem with 2-propanol is twofold: due to intrinsic conformational possibilities in the molecule and exchange between them and due to the requirement of the hydrogen transfer to Histidine and the steric and electronic barriers that this reaction finds with 2-propanol coordination near the acyl enzyme. The low 12% enantiomeric excess to the S(+)-ketoprofen form may be explained by the relative steric interaction found for the R(−) species in the case of the produced ester and the low activity to the higher adsorption stability of 2-propanol near the acyl enzyme of R(−)-ketoprofen in the Ping Pong Bi Bi mechanism for the R(−) enantiomer. 2-Propanol adsorption is less stable in the case of the acyl enzyme of S(−)-ketoprofen than in the case of the acyl enzyme of R(−)-ketoprofen.

The R/S enantiomeric structure of ketoprofen is not the key in this slight preference by the S(+), but the distances of the oxygen atom of the OH species to the carbon atom of carbonyl in acyl enzyme and the hydrogen from 2-propanol to the N from Histidine are very important.

The adsorption of 2-propanol near the acyl enzyme in R(−)-ketoprofen produces distances near 4.0 Å to carbonyl C and near 4.0 Å to N from Histidine (see Fig. 9). The hydrogen must be directed to the N from Histidine, the approach of the 2-propanol near to Histidine (distance N—H= 3.27 Å) generates a distance O—C from carbonyl of 4.142 Å still too long. The steric energy increases near 9 kcal/mol when an approach is forced; the activation energy is probably very high to the hydrogen transfer due to the increase of the repulsive interaction with methyl groups of 2-propanol with the phenyl group of ketoprofen.

We propose that the 2-propanol increases the activation energy of the reaction to achieve the intermediate 2 and also has repulsive interactions that make only some of the conformational species more able to the reaction to proceed. The S(+)-ketoprofen could be favored only by subtle differences (less stable intermediate from the steric point of view) in the case of the intermediate 2.

4. Discussion

The present investigation is a follow up of previous studies reported by some of us regarding the effect of ethanol and ethanol–water mixtures on the commercial biocatalyst Novozym® 435. In this opportunity, the effects of a mixture of 2-propanol–4.76% water on the physical integrity of the biocatalyst's beads, the secondary structure of the active protein and the catalytic performance of Novozym® 435 are thoroughly screened. Previous studies demonstrated that ethanol (with or without water added) dissolves the polymethylmethacrylate that composes the beads of Novozym® 435. In this context, 16.6% of the biocatalyst's mass was lost and also the 2.76% of the total protein content of Novozym® 435. In contrast, the 2-propanol does not have a comparable effect with the ethanol in terms of capability of dissolve PMMA. The interaction of Novozym® 435 with 2-propanol for extended periods of time produces 1.2% weight loss along with 1.28% of protein loss. The infrared analysis of the residue left after the treatment with 2-propanol confirmed the presence of polymethylmethacrylate. The capability of methanol, ethanol, 1-propanol and 2-propanol (with and without water added) of dissolving PMMA at room temperature was reported by McEwen and coworkers [42]. The authors demonstrated a co-solvency effect that occurs when the components of the mixture can preferentially (and separately) solvate different sites on the polymer chain. In this context, PMMA possesses hydrophobic side chains (methyl groups) and hydrogen bond accepting groups (O═C—O—CH_3 ester groups) [25,43]. Schubert and coworkers demonstrated that the co-solvency effect implies the water–hydrogen bonding to the ester moieties of the polymer forming a hydration-shell around the carbonyl groups that somehow facilitates the action of the alcohol [43]. The modification of the inner texture of the beads evidences that the 2-propanol–water mixture diffuses into the biocatalyst's beads as was already demonstrated with ethanol by some of us [25,31]. The temperature programmed desorption of 2-propanol from Novozym® 435 provided evidences of an intense physical adsorption on the enzyme and/or the polymeric matrix. The alcohol molecularly desorbs at 187 °C without further reaction similarly as observed in the temperature programmed desorption of ethanol (T_d = 180 °C) from Novozym® 435. This observation leads to the conclusion that under reaction conditions (45 °C) the alcohol remains adsorbed as a spectator species most probably forming dead-ends that inhibits the esterification process [26].

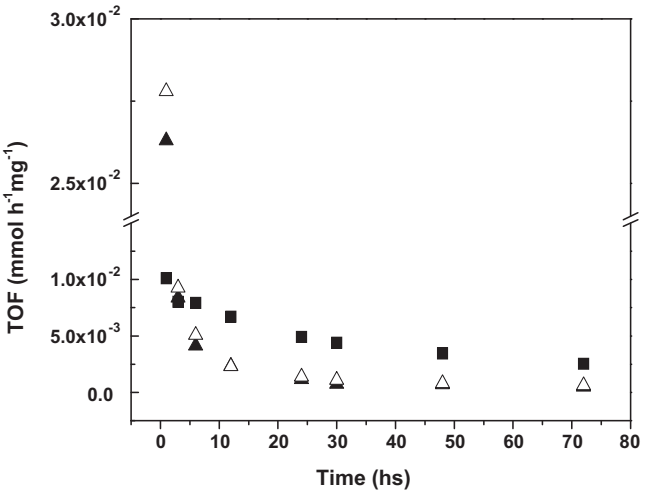

Fig. 10. Turnover frequency of the R/S-ketoprofen and R/S-ibuprofen converted to the R/S-ester per time and amount of remaining protein in the biocatalyst's beads vs. time of reaction. *Symbols*: (■) TOF of the esterification of R/S-ibuprofen with ethanol at 45 °C (data from Ref. [27]); (▲, △) TOF of the esterification of R/S-ketoprofen with 2-propanol at 45 °C catalyzed with starting and treated Novozym® 435, respectively.

The investigation of the secondary structure of the protein of Novozym® 435 in contact with 2-propanol for 8 days gave similar results than the biocatalyst used in the esterification of R/S-ketoprofen with 2-propanol (without being in contact with the alcohol). This observation indicates that the modification of the secondary structure is a consequence of the interaction with 2-propanol rather than the presence of the profen. In this context, the modification of the protein's secondary structure produced upon contacting with 2-propanol–water for extended periods of time (an increase of the β-turn contribution and a decrease in the β-sheet content) happens also after 3 h of reaction using a fresh biocatalyst not previously in contact with 2-propanol.

The results discussed up to here show similarities in terms of the effects of both ethanol and 2-propanol on the physical integrity of Novozym® 435, the formation of protein's dead ends and the intensity of the alcohol–protein interaction.

However, the catalytic performance in the esterification of R/S-ibuprofen with ethanol and R/S-ketoprofen with 2-propanol is significantly different. Fig. 10 compares the turnover frequency of the R/S-ketoprofen and R/S-ibuprofen converted to the R/S-ester per time and amount of remaining protein in the biocatalyst's beads. The comparison of the turnover frequencies allows a direct comparison of the performance of the active protein as a function of the nature of the alcohol (primary or secondary alcohol) independently of the amount of protein (active sites) and the time of reaction [44]. The comparison of the TOFs of the esterification of R/S-ketoprofen with 2-propanol catalyzed with Novozym® 435 fresh and exposed to 2-propanol, shows a similar behavior in terms of catalytic performance as discussed before. However, the TOFs values in the steady state (after 24 h) using ethanol are an order of magnitude higher ($\sim 4 \times 10^{-3}$) than the ones in 2-propanol ($\sim 6 \times 10^{-4}$). This observation evidences that the nature of the alcohol that is, primary vs. secondary alcohols is a key in the biocatalyst's performance. Earlier investigations reported by Arroyo and Sinisterra indicated that the rate of esterification of 2-arilpropionic acids catalyzed with microbial lipases is influenced by the nature of the alcohol [45]. The authors demonstrated that the highest rate of esterification of (R/S)-2-arilpropionic acids is reached with primary alcohols, diminishes with secondary alcohols and is almost null with tertiary alcohols (such as tert-butyl alcohol). In this case, the authors used isooctane as a co-solvent and the reaction was carried at 50 °C with immobilized *C. antarctica* lipase. Further

studies by Sinisterra and coworkers found no yield toward the ester when 2-propanol was used in the esterification of racemic ibuprofen catalyzed with *Rhizomucor miehei* lipase [46]. More recently, similar results were reported for the esterification of R/S-ibuprofen when *Candida* sp. was used [47]. These investigations described the enzyme's behavior attributing the causes of the poor biocatalysts' performance to a steric hindrance of the alcohol. The molecular modeling studies performed in the present investigation provides for the first time in the literature a detailed screening of the different steps of the mechanism and the steric energy involved in each of them. These and previous results demonstrated that the poor catalytic performance of the CALB enzyme cannot be attributed to the profen molecule (being either ibuprofen or ketoprofen) but to the conformation arrangements of the secondary alcohol when adsorbed on the catalytic triad. In this context, a conformational arrangement of 2-propanol with both methyl groups away from the OH species favors the transference of the hydrogen atom to the Histidine and the subsequent formation of the propyl ester of ketoprofen. In contrast, the adsorption of the alcohol in such a way that a methyl group is close to Histidine increases the activation energy to achieve the intermediate species.

5. Conclusions

The present investigation is a detailed screening of the effect of 2-propanol both on the integrity of the commercial biocatalyst Novozym® 435 and the esterification of a profen at a molecular level. Moreover, the biocatalyst's performance in the esterification of profens (R/S-ibuprofen and R/S-ketoprofen) using both ethanol and 2-propanol were compared in terms of the turnover frequencies that is a parameter not influenced by the amount of protein and time of reaction. The TOFs comparison evidences that the low reaction rate of the CALB enzyme (low conversion and enantiomeric excess toward de S (+)-ketoprofen) when using 2-propanol cannot be attributed to the nature of the profen itself but to the interaction of the secondary alcohol with the catalytic triad of the CALB enzyme. The inhibition exerted by the secondary alcohol upon interaction with the lipase B of *C. antarctica* is the key to understand the catalytic performance of Novozym® 435. In this context, the action of 2-propanol on the physical integrity of Novozym® 435 is considerably less intense than the effect of ethanol. In fact, a 16.6% vs. 1.2% loss of the bead's mass is produced when the biocatalyst was contacted with ethanol and 2-propanol, respectively. Similarly, the percentage of protein loss (2.73% vs. 1.28%) was also rather lower when using 2-propanol.

Temperature programmed desorption studies undoubtedly demonstrated that both ethanol and 2-propanol remain strongly adsorbed (most probably hydrogen bonded) to the biocatalyst's surface. The formation of dead-end complexes between the secondary alcohol and the protein (similarly to the ethanol-protein dead-ends) is another drawback in the esterification process using 2-propanol.

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