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Catalytic and molecular insights of the esterification of ibuprofen and ketoprofen with glycerol

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ABSTRACT

The esterification of *rac*-ibuprofen and *rac*-ketoprofen with glycerol catalyzed with the commercial biocatalyst Novozym® 435 was investigated at 45 °C with various profen: glycerol molar ratios using 2-propanol as co-solvent in a batch type reaction. The conversion of *rac*-ibuprofen reached 46%, with an enantiomeric excess towards the *S*-enantiomer of 42%. When 1:4 ibuprofen:glycerol molar ratio was assayed, 75% of the *R*-ibuprofen reacted with glycerol towards the monoglyceride with 99% selectivity, which is highly relevant in the field of prodrugs synthesis. The conversion of *rac*-ketoprofen was lower, 17 % vs. 46 % of *rac*-ibuprofen, and the esterification afforded both the monoglyceride (70%) and diglyceride (30%) regardless of the ketoprofen:glycerol molar ratio. Investigations of the esterification at molecular level through concentration-modulated infrared spectroscopy, static ATR-FTIR and *in situ* Raman spectroscopy showed the continuous decay of the species belonging to *rac*-ibuprofen and glycerol providing further evidences of the reaction. Moreover, the interaction of CALB with ibuprofen modifies the contribution of the ordered structures of the lipase, which might be related with the improved catalytic performance in the esterification of that profen.

1. Introduction

Analgesics are the most widely used drugs worldwide, and 90% of them belong to the group of non-steroidal anti-inflammatory drugs (NSAIDs), a group of drugs with various chemical structures with proven anti-inflammatory, analgesic and antipyretic activity [1].

Due to their proven effectiveness, NSAIDs are used chronically in various pathologies such as rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis, among others [2]. Epidemiological and experimental studies have demonstrated beneficial effects of chronic use of NSAIDs in cardiovascular disorders, colon-rectal cancer and Alzheimer's disease [1,3,4]. In addition, case-control studies demonstrate the inverse association between NSAIDs usage and diseases such as between ibuprofen and glioblastoma multiforme that is the most common and lethal glioma [5], or several types of cancer in humans [6]. In clinical practice, however, the use of this medicinal agents is restricted owing to objectionable parameters such as adverse effects, physicochemical properties or bioavailability. In this sense, the chronic use of NSAIDs by oral administration has a high frequency of undesirable effects in the gastrointestinal (GI) tract, with hemorrhages, ulcers and perforation of the gastric mucosa being reported as the most frequent [7]. Therefore, their chronic use is limited by their significant toxicity [5]. The *S*-enantiomer of the racemic NSAIDs inhibits the enzyme cyclo-oxygenase (COX-1 and/or COX-2 isoforms), resulting in a decrease in the production of prostaglandins and thromboxanes from arachidonic acid, which are known as cellular mediators in inflammatory processes [1].

The progression of adverse GI effects of NSAIDs is mainly attributed to the direct contact of the *R*-enantiomer acidic group with the gastric mucosa [8], and hence it can be reduced or prevented by their enrichment in the *S*-enantiomer. Moreover, prodrugs (which are bioreversible derivatives) have been generated by blocking/masking the carboxyl group of NSAIDs in prodrugs, which are bioreversible derivatives as an

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alternative to reduce or eliminate undesirable GI effects. For instance, Cabrera *et al.* reported a reduction of adverse GI effects by improvements in absorption, distribution, metabolization and excretion of NSAIDs prodrugs, which carboxyl group had been masked [9]. Several investigations regarding the design of NSAID prodrugs involve the use of ibuprofen, one of the most widely prescribed NSAIDs and considered essential by the World Health Organization [10]. Lolli et al., for instance, reported the synthesis of an ibuprofen derivative through modification of the carboxyl group with nitric oxide that showed less gastro-toxicity than the unmodified profen [11]. Recently, Bartels *et al.* demonstrated the protective effect against human glioma cells of the phospho-glycerol-ibuprofen-amide derivative [5].

Esterification catalyzed by enzymes is an attractive strategy for the green synthesis of NSAIDs prodrugs because esters are chemically stable in vivo [12]. For instance, Chen et al. developed an ibuprofen ester prodrug by esterification catalyzed with *Candida rugose* lipase [13]. Later, polyester prodrugs of ketoprofen were synthesized by enzyme-catalyzed polycondensation using lipase B from *Candida antarctica* [14]. The *in vitro* studies performed by the authors showed a slow drug release from the polyester under physiological conditions, which suggested this way of prodrug synthesis was promising. Recently, with the aim of enhancing drug bioavailability, ibuprofen-sorbitol ester was produced by direct enzymatic esterification with a porcine pancreas lipase [15]. Lipases (triacylglycerol hydrolases EC 3.1.13) have been widely used in enzymatic kinetic resolution of NSAIDs and since the 1990s their use is gaining importance due to their activity and stability in unconventional media, broad spectrum of substrates, mild operating conditions and no need of cofactors. In this sense, the investigations reported by some of us proved that commercial lipase Novozym® 435 (the lipase B from *Candida antarctica* adsorbed on polymethylmethacrylate) selectively catalyzes the esterification of *R*-ibuprofen and *R*-ketoprofen, enabling the concentration of the pharmacologically active *S*-enantiomer [16–19]. This contribution further investigates the enzymatic esterification of *rac*-ibuprofen and *rac*-ketoprofen catalyzed with Novozym® 435 to develop potential prodrugs with the *R*-enantiomer blocked by the reaction with glycerol. This polyalcohol is a non-toxic, non-irritating, biodegradable compound, widely used in chemicals, pharmaceuticals, cosmetics and food. Moreover, the boom in biodiesel production in the last decade has led to a continuous drop in the market price of glycerol, which is now considered a waste [20], because 10 kg of glycerol are generated as by-product per 100 kg of biodiesel [21]. Therefore, its use in the esterification of profens will contribute to its valorization. Besides batch experiments at different concentrations of profen, glycerol and co-solvent, transient and steady state *in situ* infrared studies are performed to understand the process.

2. Experimental

2.1. Materials

The commercial biocatalyst Novozym® 435 (batch LC200217), obtained as a gift from Novozymes Brazil, R/S-ketoprofen (Parafarm, 99.80 %, batch 030718 000928/004), R/S-ibuprofen (Parafarm, 99.23 %), 2-propanol (J. T. Baker, 99.93 %), glycerol (Anedra, 99.5 %), and potassium hydroxide in ethanol 1M (Riedel-de Haën) were used for batch esterification reactions.

For *in situ* analyses, lipase B from *Candida antarctica* was purified from a commercial extract provided by Novozymes Brazil following the methodology previously detailed by Llerena Suster *et al.* [22]. Deuterium oxide D_2O from Cambridge Isotope Laboratories (99%) was employed.

2.2. Batch esterification experiments of rac-ibuprofen and rac-ketoprofen

2.2.1. Reaction set-up

The batch esterification experiments of *rac*-ibuprofen and *rac*-ketoprofen with glycerol were performed for 48 h in closed 100 mL vials in a shaker bath at a constant temperature of 45 °C, stirring at 200 rpm, and with 2-propanol as co-solvent. Although some authors have performed a similar reaction in a solventless system [23], profens were poorly soluble in glycerol at the reaction temperature used in this work, which optimizes the activity of Novozym® 435 in the esterification of racemic ibuprofen [24]. The solvent was selected according to the key information provided in previous investigations by some of us on the interaction of several short chain alcohols with CALB lipase [18,19]. Those investigations proved that the enzymatic kinetic resolution of *rac*-ketoprofen and *rac*-ibuprofen through the esterification with ethanol and 1-propanol in the absence of organic co-solvents (solely with 4.76 % v/v H_2O added) was a feasible process. The catalytic performance decreased when methanol was used as the acyl acceptor. This behavior was ascribed to various deleterious effects of this alcohol on the enzymatic structure, on the physical integrity of the polymer that supports the lipase, and on the association with the active site [18,19,25,26]. In contrast, the esterification with 2-propanol was not feasible, because although the secondary alcohol did not affect the integrity of the biocatalyst, it exerted a steric hindrance in the active site, inhibiting the catalytic activity [18]. Additionally, it is worth noticing that the U.S. Food and Drug Administration (FDA) classifies 2-propanol as a chemical of low toxicity and low risk to human health. Moreover, this alcohol is within the class 3 of substances that may be present as residual solvents in the manufacture of pharmaceuticals. Therefore, 2-propanol was chosen as solvent to study the esterification of profens with glycerol as nucleophile, because it does not interfere in the activity, and, especially, because *rac*-ibuprofen and *rac*-ketoprofen are soluble in this alcohol. In fact, at 25 °C 0.500 g of *rac*-ibuprofen dissolves in 1.300 mL of 2-propanol and the same amount of *rac*-ketoprofen requires 3.125 mL.

Thus, in a typical experiment 1.300 mL or 3.125 mL of 2-propanol were used to dissolve 0.500 g of ibuprofen or ketoprofen, respectively. 160 mg of the commercial biocatalyst Novozym® 435 were used as received to catalyze the esterification with glycerol, and 4.76 % (v/v) of water was added, as it has been demonstrated that it is essential to keep the enzyme active in alcoholic reaction media [24]. The reaction was studied varying glycerol concentration at constant concentration of profen (1:1, 1:2, 1:3, 1:4, 1:5, and 1:6 profen:glycerol molar ratios) and varying the amount of the profens keeping glycerol constant (1:1, 2:1, and 3:1 profen:glycerol molar ratios). Blank tests were also performed to establish the conversion and enantiomeric excess of the non-catalyzed reaction.

2.2.2. Quantitative analysis of profens

The conversion of *rac*-ketoprofen and *rac*-ibuprofen towards the esters was determined through the titration of the unreacted profen with a standard solution of potassium hydroxide in ethanol [24]. The non-catalyzed esterification showed 8 % conversion of ketoprofen and 5.5 % conversion of ibuprofen.

The *R* and *S* enantiomers of each profen (in their carboxylic acid form) were analyzed through high performance liquid chromatography (HPLC) employing a chiral column Nucleodex Beta-PM (Macherey-Nagel, Germany) coupled with an UV detector at 230 nm. A sample of 50 μL was extracted from the reaction media and it was diluted in 20.00 mL of methanol (Carlo Erba 99.8 %) and tetraethylammonium acetate (TEAA) buffer 0.1 % v/v (Fluka) at a 60:40 ratio and pH 4.

The enantiomeric excess of *S*-profen (*eeS* %) was estimated as it is stated in Eq. (1), where [S] and [R] are the concentrations of the *S*- and *R*-profen, respectively [27,28].

$$eeS\ \% = \frac{[S]-[R]}{[S]+[R]} * 100 \quad (1)$$

Furthermore, the enantiomeric ratio (E) was computed as it is stated in Eq. (2) [27,28].

$$E = \frac{[\ln(1 - X)(1 - eeS)]}{[\ln(1 - X)(1 + eeS)]} \quad (2)$$

2.2.3. Semi-quantitative analysis of mono, di- and tri-glycerides trough UHPLC-MS/MS

The distribution of mono, di- and tri-glycerides was determined through ultra-high performance liquid chromatography tandem mass spectrometry UHPLC–MS/MS.

UHPLC analysis was performed employing a Acquity UPLC system (Waters) coupled with a binary solvent and a sample manager. The chromatographic separation was analyzed with an Acquity UPLC BEH C18 column (1.7 μm, 100 × 2.1 mm) (Waters) equipped with an Acquity VanGuard BEH C18 pre-column (1.7 μm, 5 × 2.1 mm) (Waters). The column was kept at 35°C and the sample manager at 5°C. The flow rate of the mobile phase was 0.3 mL/min and it consisted of organic-free water altered with ammonium acetate 0.1 mM and formic acid 0.01% (phase A) and methanol (phase B). A sample volume of 20 μL was injected in the UHPLC system. A tandem quadrupole (TQD) mass spectrometer along with an orthogonal Z-spray electrospray interface (Waters) was used for UHPLC analysis. Drying and nebulizing gas was nitrogen, which was produced from pressurized air in a N_2 LC–MS. The desolvation and cone gas flows were adjusted at 2 L/h and 849 L/h flow, respectively. To manipulate in MS/MS mode, Argon 99.995 % was the collision gas pressurized at 4.04×10^{-3} mbar in the T-Wave cell. Positive ionization mode was carried out using capillary voltage of 3.5 kV. Interface and source temperature were set at 450 and 120 °C, respectively. Dwell times of 20 ms were chosen. For UHPLC analysis, Masslynx NT v 4.1 software (Waters) was employed to analyze the spectra. The glycerides of ketoprofen and ibuprofen are not commercial and, therefore, no standard substances are available for calibration to quantify the glycerides obtained in the reaction. Instead, percentages of those substances were determined through the area of the signals obtained in the UHPLC-MS/MS.

2.3. In situ ATR-FTIR studies of purified CALB

Infrared analyses of CALB were performed in a Fourier transform infrared spectrometer with an attenuated total reflectance cell (*ATR-FTIR)*. The ATR cell used for this purpose was a homebuilt stainless steel flow-through cell as it was detailed in previous research [29–31]. The cell was disposed onto an ATR supplement (Pike Technologies) in the FTIR spectrometer sample section (Thermo-Electron, Nicolet 8700, MCT cryogenic detector). The spectrometer bench and the mirrors directing the radiation against the cell are steadily purged with dry air (generator from Paker Balston) with the aim of removing the signals belonging to CO_2 and water vapor from the spectra. Omnic 8 was the software chosen for analysis of the spectra.

2.3.1. Transient in situ studies of the esterification reaction by modulation excitation spectroscopy with phase sensitive detection (MES-PSD)

Concentration-modulation excitation spectroscopy (c-MES) experiments were realized at 45°C, employing the same arrangement previously described by some of us [29,30]. Isothermal c-MES experiments associated with phase sensitive detection (PSD) were performed to discriminate the absorption bands that belong to the interaction between 2-propanol and glycerol with the enzyme CALB from the background signals. MES-PSD spectra were analyzed as stated by Bauretch and Fringeli [32], which technique is more detailed in our previous article [31].

In the first experiment, the liquid streams were flown over the pristine ATR crystal. After the background spectrum was registered, a modulation experiment started by variating the input composition from 2-propanol to glycerol. Iterative square-wave stimulations with 0.5 mL min^{-1} flow rate and 50 mHz modulation frequency were achieved in the ATR cell. Five modulation periods were performed to adapt the system

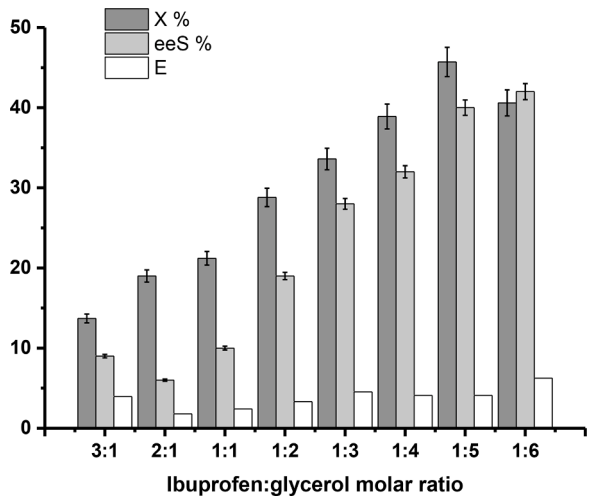


Fig. 1. Conversion (X), enantiomeric excess towards the *S*-enantiomer (eeS), and enantiomeric ratio (E) of the esterification of *rac*-ibuprofen with glycerol for 48 h at 45 °C as a function of the profen: glycerol molar ratio.

to the external perturbation, then spectra were acquired every 3 s. The liquid flow was provided by a pulse-free peristaltic pump (Ismatec ICP4) situated at the end of the ATR cell. A pneumatically actuated three-way valve connected to two separate glass bottles and controlled by software enabled the exchange of the two alcohols.

The experiment was repeated but over a film of CALB in the ATR cell. To avoid the interference of the O-H species bending vibration in amide I signal of proteins (1700-1600 cm^{-1}), the isotopic exchange of water molecules by D_2O is required [33]. For this purpose, 0.0050 g of purified CALB was dissolved in 500 μL of D_2O. The mixture was scattered in the ATR cell with the aim of preparing a homogeneous film above the crystal. Later, the cell was closed and purged with dry air overnight at 25°C to eliminate D_2O in excess and water remains, obtaining a dried and absolutely exchanged film of the enzyme. Then the modulation experiment varying 2-propanol and glycerol was started over this film of enzyme.

2.3.2. Steady state in situ studies of relevant molecular interactions

The study of ibuprofen and ketoprofen interaction with the mixture glycerol-2-propanol and CALB enzyme was performed by *in situ* ATR-FTIR in static conditions, as in transient conditions the interaction time is too short.

The film of CALB was prepared as it is detailed above. Then, the interaction of CALB with 2-propanol, glycerol in 2-propanol (3.84 M), ibuprofen in 2-propanol (1.84 M), and ibuprofen in the glycerol-2-propanol mixture (0.1000 g in 300 μl of 2propanol and 245 μl of glycerol) was studied. Similarly, the contact of CALB with ketoprofen in 2-propanol (0.63 M) and ketoprofen in the glycerol-2-propanol mixture (0.0800 g in 500 μl of 2-propanol and 140 μl of glycerol) was analyzed. The molar rates employed in these assays were the optimal values found by the authors in esterification reactions of ibuprofen and ketoprofen with glycerol as will be described in the Section 3.1.

Collected spectra were analyzed through deconvolution via signal peak fitting by Lorentzian-shaped components in the non-deconvoluted spectra. A special peak fitting module of Origin 5.0 was employed for this purpose. Second derivative analysis was performed over the spectra to determine the position and number of peaks.

Secondary structure information of the enzyme after contact with reactants and reaction mixtures was obtained from deconvolution of amide I signal. The contribution of each component was obtained by deconvolution, integration and further normalization as it was described previously [18,19].

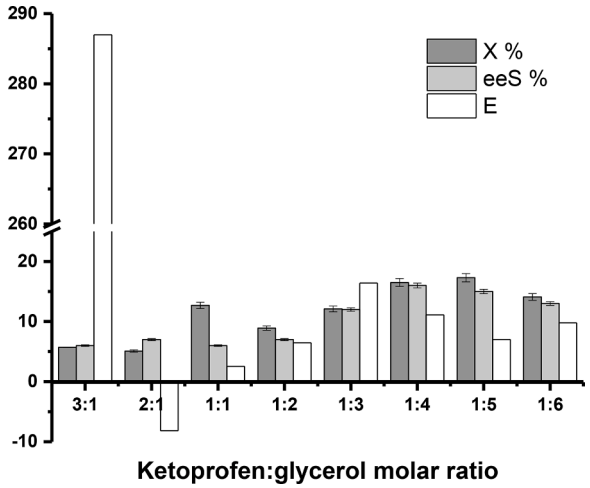


Fig. 2. Conversion (X), enantiomeric excess towards the *S*-enantiomer (eeS), and enantiomeric ratio (E) of the esterification of *rac*-ketoprofen with glycerol for 48 h at 45 °C as a function of the profen: glycerol molar ratio.

3. Results and discussion

3.1. Influence of the molar ratio of substrates in the esterification and nature of glycerides

Fig. 1 shows the conversion, enantiomeric excess towards *S*-ibuprofen and enantiomeric ratio E of the esterification of the *rac*-ibuprofen with glycerol at various ibuprofen: glycerol molar ratios. An increase in the ibuprofen to glycerol molar ratio diminishes the conversion (compare the 1:1, 2:1 and 3:1 ratio in Fig. 1), indicating that the polyol is the limiting reactant. Further proof of this observation is the continuous increase of *rac*-ibuprofen conversion and the enantiomeric excess towards the *S*-enantiomer upon increase of the amount of glycerol (see from 1:2 to 1:6 ratio in Fig. 1). The best results of ibuprofen to esters conversion (45.7 %), enantiomeric excess (42.0 %) and enantiomeric ratio E (6.3) after 48 h of reaction are obtained in excess of glycerol (1:5 and 1:6 ibuprofen:glycerol molar ratios). Nevertheless, the enantiomeric ratio is fairly low regardless of the reaction conditions, in line with our previous investigations on the esterification of *rac*-ibuprofen with an excess of ethanol, which found that after 48 h of reaction at the optimum conditions (ethanol:profen molar ratio equal to 7 without the addition of a co-solvent) an enantiomeric excess of 50 % towards the *S*-ibuprofen and 55 % of conversion were reached with an enantiomeric ratio of 3.80 [22]. Somehow these observations evidence that the esterification of ibuprofen is a kinetically controlled synthesis of condensation products, as described Kasche et al. [34]. The yield of condensation product is ruled by the formation of the acyl-enzyme intermediate followed by the interaction with the nucleophile. In fact, some of us demonstrated through spectroscopic and molecular modelling studies that the emergence of the acyl-enzyme species between the *R*-enantiomers of the NSAIDs and the catalytic triad of the lipase B from *Candida antarctica* is an energetically feasible process [31]. The shifting of the equilibrium towards the ester upon increasing glycerol amounts cannot be neglected since the enzyme catalyzed esterification is a reversible process [35].

Fig. 2 presents the conversion, enantiomeric excess towards *S*-ketoprofen, and enantiomeric ratio E of the esterification of the *rac*-ketoprofen with glycerol at various ketoprofen:glycerol molar ratios. Again, the highest catalytic activity is reached under excess of the polyol. However, after 48 h of reaction both the conversion (17%) and enantiomeric excess (16%) are significantly lower than the ones obtained with ibuprofen (46 % and 42 %, respectively). Regarding the enantiomeric ratio E, it rises to 287 when ketoprofen is in excess in the reaction medium (ketoprofen:glycerol molar ratio of 3:1). According to the literature, this high E value is out of the accuracy limit of this parameter, because above 200 even an extremely small variation in the enantiomeric excess causes a significant change in the enantiomeric ratio [36]. These results are in accordance with previous investigations by some of us regarding the esterification of *rac*-ketoprofen with various alcohols [18,19]. The fact that the lipase catalytic performance in the esterification of ibuprofen is significantly higher than for ketoprofen is somehow related to either the profens'structure, the formation of esters with new chiral centers or the co-solvent. The literature states that the kinetic resolution of racemic chiral carboxylic acids and esters with lipases is greatly influenced by the structure of the substrate that might lead to a complete switch in the enantioselectivity of the enzyme due to different binding of the chiral substrate to the catalytic active site [35]. Early investigations reported by Heinsman *et al.* also evidenced that the transesterification of racemic branched chain fatty acid esters catalyzed by *Candida antarctica* lipase was influenced by the size of the methyl and butyl group within the chiral center [37]. Therefore, the influence of the co-solvent (2-propanol) on the biocatalytic performance is another factor that should be accounted for the differences observed in the conversion of ibuprofen and ketoprofen.

Table 1 collects the conversion and the products distribution obtained in the esterification of ibuprofen and ketoprofen with glycerol at various profen:glycerol molar ratios. The esterification of ibuprofen is highly selective towards MAG (m/z = 281) at a 1:4 profen:glycerol molar ratio, and this product is composed by a racemic mixture containing 75 % of the *R*-enantiomer. At higher amounts of glycerol the selectivity to MAG drops, as more complex glycerides are progressively formed. Nevertheless, the racemic mixture of the esters of ibuprofen is still enriched in the *R*-enantiomer in a similar proportion. The analysis through UHPLC-MS/MS demonstrates that MAG and DAG, the latter in a lower proportion, are the major esterification products of *rac*-ketoprofen at 1:4, 1:5 and 1:6 profen:glycerol molar ratios, mostly in the form of the *R*-enantiomer (90 %) (see Table 1). Moreover, diglycerides and triglycerides of ketoprofen (preferentially of the *R*-enantiomer) are observed regardless of the molar ratio of substrates, in contrast with the results obtained with ibuprofen.

It could be assumed that the larger structure of ketoprofen with respect to ibuprofen prevents a proper coupling with the enzyme active site. This leads to low enzyme activity and also to changes in

Table 1
Type of glycerides and stereoisomers produced as a function of the type or profen and the profen:glycerol molar ratio: distribution of the monoacylglycerol (MAG), diacylglycerol (DAG) and triacylglycerol (TAG) obtained with the UHPLC-MS/MS analysis and distribution of the *R* and *S*-enantiomers of the esters.

profen	Profen: glycerol molar ratio		Type of glyceride (%)			Type of stereoisomer of the ester (%)	
			MAG	DAG	TAG	*R*-enantiomer	*S*-enantiomer
			m/z = 281	m/z = 469	m/z = 657		
rac-ibuprofen	1:4	38.9	99.0	0.98	—	75.1	24.9
	1:5	45.7	71.7	28.3	—	73.8	26.2
	1:6	40.6	70.4	29.0	0.6	80.7	19.3
			m/z = 329	m/z = 559	m/z = 801		
rac-ketoprofen	1:4	16.5	71.1	28.9	—	90.5	9.5
	1:5	17.3	63.9	30.9	5.1	85.9	14.1
	1:6	14.1	82.1	10.8	7.1	89.6	10.4

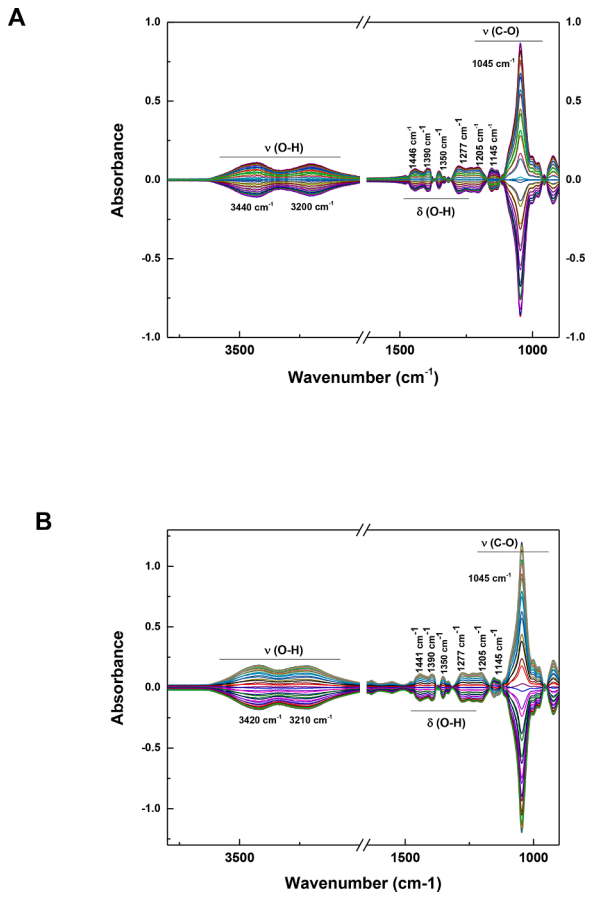


Fig. 3. Phase-resolved ATR-FTIR spectra from c-MES experiment for exchange of glycerol and 2-propanol without (A) and with (B) CALB over the ATR crystal.

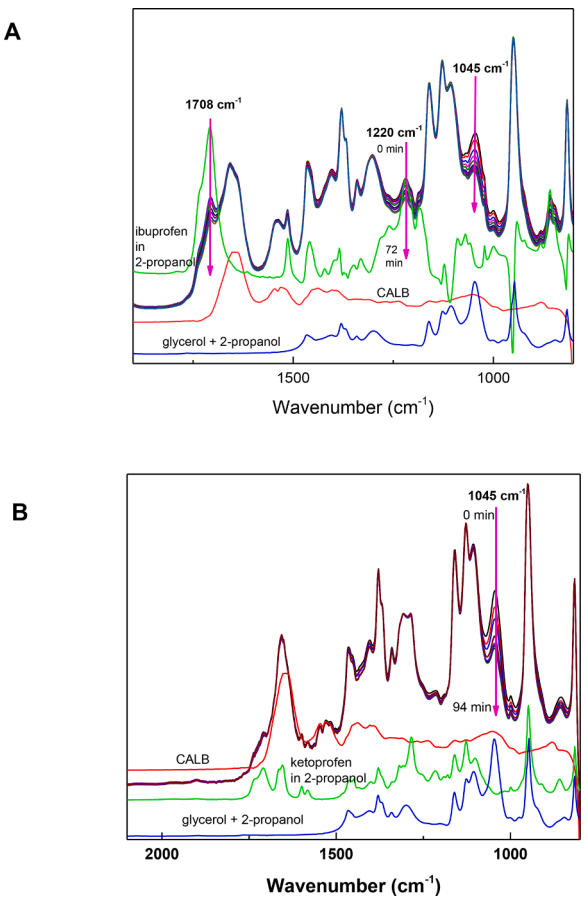


Fig. 4. Time-resolved ATR-FTIR spectra collected *in situ* during the esterification of rac-ibuprofen (A) and rac-ketoprofen (B) with CALB and glycerol in 2-propanol as co-solvent. Reference spectra of CALB and of 2-propanol solutions of ketoprofen and glycerol are included.

regioselectivity. Rivero-Pino *et al.* [38] confirm that the chemical structure of the substrate affect the regioselectivity of Novozym® 435, attributing this behavior to the substrate flexibility and steric hindrance. Furthermore, the enzyme flexibility could be influenced by the bigger and more complex structure of ketoprofen. As some of us reported in previous investigations, this feature is closely related to enzymatic activity and enantioselectivity [39].

In general, the aim of the synthesis of prodrugs through conjugation to an esterifying reagent is the selective reaction towards monoglycerides, as they remain intact in the intestines before absorption [40]. The ibuprofen and ketoprofen monoglycerides are expected to be 1-MAG, according to the reports of Ravelo et al. and Lerin et al. [23,41,42], who investigated the synthesis of ibuprofen monoglyceride and 1-glyceryl benzoate, respectively, catalyzed with free CALB and Novozym® 435. Their results demonstrated that the esterification is carried out through the primary hydroxyl groups of the polyol. Previous investigations of Duan *et al.* regarding the influence of co-solvents with a broad range of log P (from -0.23 to 4.5) on the positional selectivity of Novozym® 435 in the esterification of oleic acid with glycerol revealed that at increasing log *P* of the solvent the probability of a second acylation was higher, but with lower selectivity to 1-position, and that after one 1-position was acylated, the preference for the other 1-position over the 2-position would be strongly enhanced regardless of the co-solvent [32]. In this context, the esterification of NSAIDs with glycerol using a hydrophilic co-solvent such as 2-propanol (log P = 0.05) would also lead to the formation of 1,3-diglycerides of ibuprofen and ketoprofen. Nevertheless, the acyl migration of 1,2-DAG to 1,3-DAG and 2-monoglyceride to 1-monoglyceride that is often observed in reactions catalyzed with lipases with (1,3) positional specificity, and thus cannot be ruled out, although Li *et al.* in a study of the methanolysis of triglycerides reported that the acyl migration was favored by a higher polarity of the solvent [43], in line with the observations of Duan et al. [44]. Ferreira et al. demonstrated that 2-monoglycerides are favored by long reaction times and high biocatalyst loading in the transesterification of short to medium-chain triglycerides with ethanol catalyzed with Novozym® 435 [45]. More recently, these authors showed that acyl migration was also favoured at lower concentration of glycerol in its esterification with fatty acids catalyzed by immobilized *Rhizomucor miehei* lipase [46].

3.2. Molecular insights of the biocatalyzed esterification of rac-ibuprofen and rac-ketoprofen with glycerol

In situ infrared spectroscopy transient studies consisting of concentration-dependent experiments were performed in an ATR-FT-IR cell in order to obtain detailed information of the esterification reaction at a molecular level. It is worth noticing that the solely CALB enzyme was used in these studies in order to avoid the interference of the polymeric support of Novozym® 435. The polymethylmethacrylate support possesses an intense infrared signal owing to the stretching vibration of carbonyl species at 1731 cm^{-1} that would interfere with the typical signal of the carbonyl group stretching vibration of the acyl-enzyme species [25,31]. The lipase CALB was obtained after removal of the insoluble fraction, nucleic acids, sorbate and benzoate species of the crude extract Lipozyme® through a tailored methodology published before [22]. The purified lipase retains the hydrolytic activity of the starting crude extract (2.9 vs 2.1 IU/mg for the purified and crude extract, respectively) as it was demonstrated through the reaction of p-nitrophenyl dodecanoate towards p-nitrophenol. In this context, it is

possible to ascertain that the fundamental investigations through molecular spectroscopy using the purified CALB are reliable and somehow are the closest situation to mimic the commercial biocatalyst used under reaction conditions.

Phase-resolved spectra presented in Fig. 3 were obtained after applying the PSD method to the time-resolved spectra registered in the c-MES experiment of exchange between glycerol and 2-propanol. Fig. 3A exhibits a blank experiment without the presence of the enzyme, where the interaction of the alcohols is studied, whilst Fig. 3B shows the interaction of glycerol and 2-propanol with a CALB enzyme film over the ATR crystal. The MES-PSD technique enables to clearly distinguish the signal that belongs to the interaction between reagents from the strong background signals. In both experiments the infrared absorption bands detected are the same and correspond to the hydroxyl group (ν(O-H) and δ(O-H)) vibrations and the carbonyl group (ν(C-O)) stretching vibrations for both alcohols. As no new signals appear, it can be concluded that there was interaction neither between the alcohols nor of the alcohols with the CALB enzyme.

In addition to the c-MES experiments, static *in situ* experiments were performed with the longer times required to observe interactions, in order to study the species generated in the esterification of ibuprofen and ketoprofen with glycerol catalyzed by the enzyme CALB. In a first instance, the interaction of simple mixtures with the enzyme was analyzed, to then study the contact with more complex systems. At the conditions of the experiment, the interaction of the enzyme with the mixtures NSAID-2-propanol was not evidenced, since no new signals were detected with regard to the blank experiments (data not shown). Fig. 4 shows the infrared spectra obtained during the biocatalyzed esterification of *rac*-ibuprofen and *rac*-ketoprofen with glycerol in 2-propanol. Spectra from CALB, the mixture glycerol-2-propanol and the solution of the corresponding NSAID in 2-propanol are included as reference for comparison in each figure. Previous reports used the decay of the absorption bands over time to study the reaction yield [47]. As it may be appreciated in Fig. 4A, the signals corresponding to the stretching vibration of ketone species ν(C=O) of ibuprofen (1708 cm^{-1} and 1220 cm^{-1}) and the stretching vibration of the carbonyl group ν(C-O) from glycerol (1045 cm^{-1}) diminish over time, but not the signals related to 2-propanol, employed as solvent in this research. This confirms that ibuprofen is reacting mainly with the polyol. With regard to the esterification of ketoprofen (Fig. 4B), the only signal that diminishes over time is the one corresponding to the stretching of the carbonyl group from glycerol, but the stretching vibration of ketone species ν(C=O) corresponding to ketoprofen (1710 cm^{-1} and 1660 cm^{-1}) remains constant. This observation might indicate that the reaction with glycerol is taking place in a lesser extent with ketoprofen than with ibuprofen (in accordance with the conversion values discussed in the previous section) and therefore, a modification of the signals is not detectable.

Further investigations of the reaction of ibuprofen with glycerol through *in situ* Raman spectroscopy with an immersion probe provided remote insights in this matter (see Supplementary Information).

3.3. Modification of the secondary structure of CALB upon reaction

As it is usually recognized that conformational structure is crucial for preserving enzymes' activity, the modification of the secondary structure of CALB after the contact with the reagents and reaction mixtures was analyzed from ATR-FTIR spectra obtained for the pure CALB film (C), and for the interaction of this enzyme with 2-propanol (CI), with the mixture of glycerol and 2-propanol (CIG), with ketoprofen in 2-propanol (CIK), with ketoprofen in the mixture of glycerol and 2-propanol (CIKG), with ibuprofen in 2-propanol (CIB), and with ibuprofen in glycerol-2-propanol (CIBG). The amide I region of the infrared spectra (between 1700–1600 cm^{-1}) supplied qualitative and quantitative data of the secondary structure elements that compose the enzymes to assess the conformational changes. As D_2O solvent presents a much lower

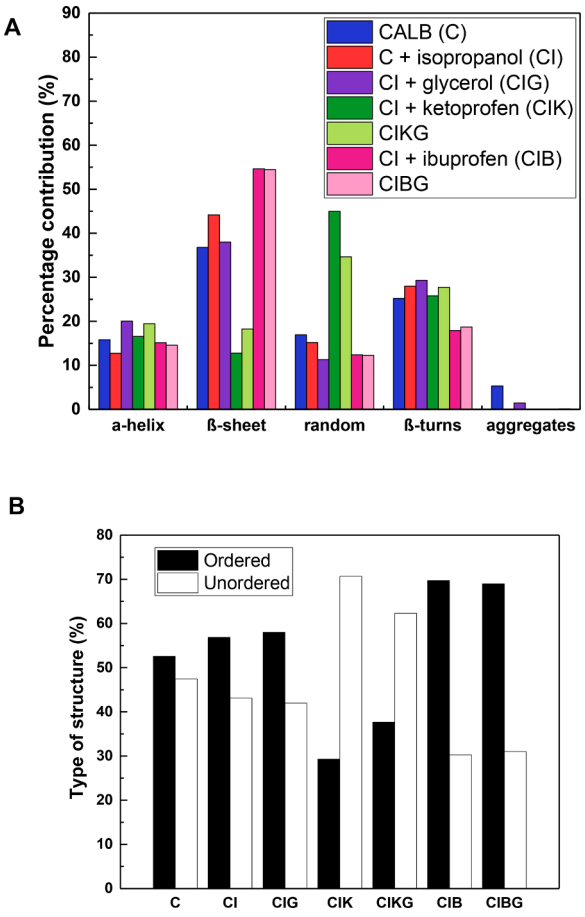
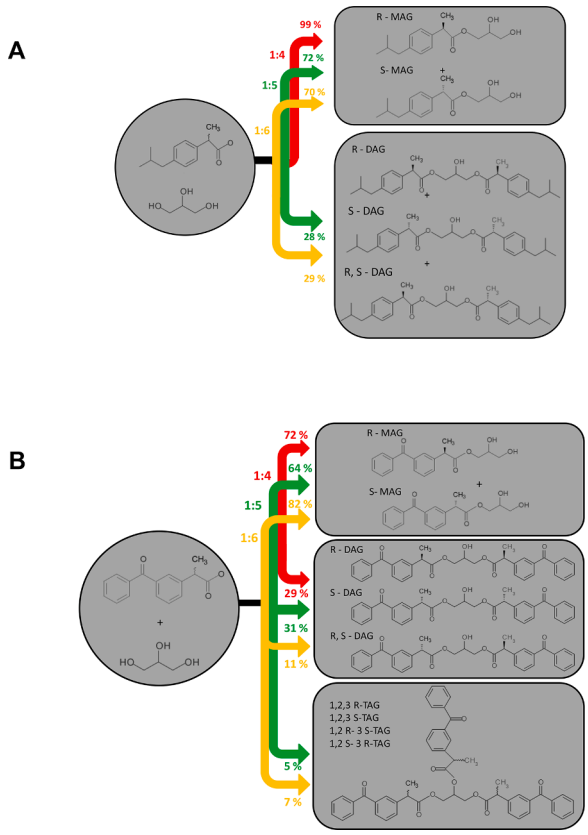


Fig. 5. Percentage contribution of each component of secondary structure (A) and of each type of them (B) for pure CALB and for CALB after contact with the reaction components and their mixtures. Nomenclature: CALB (C), 2-propanol (I), glycerol (G), ketoprofen (K), ibuprofen (B).

absorption than H_2O in the 1700–1500 cm^{-1} region, an isotopic exchange was performed to increase the signal-to-noise ratio and resolution of the spectra. The results of CALB secondary structure analysis are presented in Fig. 5. The contribution of aggregates, random structures and α-helix are conformed by the area of the signals at 1616 cm^{-1}, 1643 cm^{-1} and 1654 cm-1, respectively. The contribution of the β-sheet structure was calculated by adding the area of the signals appearing a 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 bands located at 1664 cm^{-1}, 1666 cm^{-1} and 1676 cm^{-1} [18]. According to Fig. 5A, the enzyme CALB possesses a 5.3 % of molecular aggregates. However, this contribution disappears when it is exposed to the alcohols and reaction mixtures. Previous investigations found a relation between molecular aggregates increment induced by an alcohol and the inactivation of this enzyme [19]. This suggests that somehow the contact of the reaction mixtures with the enzyme modifies the secondary structure, contributing to reverse protein aggregation and, therefore, increasing enzyme availability for catalysis, which leads to higher enzymatic activity. The contribution of α-helix remains almost unaltered upon exposure to all mixtures studied. This is a signal of the correct enzyme folding, as the reduction of this secondary structure element commonly ends up in enzyme inactivation [48]. Nevertheless, the contact with ketoprofen (CIK and CIKG in Fig. 5A) increases the contribution of random structures and decreases the percentage of β-sheet, whereas the interaction of ibuprofen with CALB (CIB and CIBG in Fig. 5A) enhances in a large extent the β-sheet contribution and diminishes β-turns. These different structural changes of the enzyme CALB



Scheme 1. Molecular representation and percentage of the various products obtained in the esterification of *rac*-ibuprofen (A) and *rac*-ketoprofen (B) with glycerol at 1:4, 1:5 and 1:6 profen:glycerol molar ratios.

might be related to the different enzymatic activity observed in the esterification reaction of both NSAIDs, as discussed previously.

The components of the secondary structure may be classified in ordered (α-helix plus β-sheet) and unordered (aggregates, random structure and β-turns) structures [49]. The content of both types of structural components was kept almost constant when the enzyme CALB was exposed to 2-propanol and the mixture glycerol-2-propanol (CI and CIG in Fig. 5B, respectively). However, the percentage of unordered structures increased after the contact of CALB with ketoprofen (CIK and CIKG in Fig. 5B), and diminishes with the contact with ibuprofen (CIB and CIBG in Fig. 5B). Therefore, the observed enzyme activity is strongly related to conformational changes in the secondary structure. In this sense, it could be supposed a correlation between the ordered structures observed in CALB after the interaction with ibuprofen and the increased catalytic activity. Changes observed in β-sheet contribution might be related to a loss of hydrogen-bonding between water molecules at the surface of the protein [49]. The increment of this structure observed after the exposure of CALB to ketoprofen leads to a more flexible structure and lower enzymatic activity. Conversely, a more rigid arrangement is induced by the contact with ibuprofen, and hence a more active enzyme [50]. These assumptions are in agreement with our recent report, which establishes that lipase flexibility is crucial for enzymatic activity [39]. Scheme 1 summarizes the results obtained in the esterification of *rac*-ibuprofen and *rac*-ketoprofen with glycerol in 2-propanol as co-solvent catalyzed by CALB. The scheme shows the products distribution as a function of the profen-to-glycerol molar ratio.

4. Conclusions

The biocatalyzed esterification of *rac*-ibuprofen and, in a lesser extent, *rac*-ketoprofen with glycerol is feasible with 2-propanol as co-solvent and Novozym® 435. It is relevant in the field of prodrugs synthesis that, under certain conditions, 75% of the *R*-ibuprofen reacted with glycerol solely towards the monoglyceride.

The analysis of the conformational structure of CALB in contact with the different reaction mixtures indicates that the secondary structure of the enzyme is altered as a consequence of the interaction with the profens rather than with the alcohols. Ibuprofen favors the ordered structures while ketoprofen raises the proportion of unordered structures, which strengthens the crucial role of the type of profen structure in the conversion and selectivity of the reaction with glycerol.

CRediT authorship contribution statement

María Victoria Toledo: Conceptualization, Investigation, Writing – original draft, Visualization. **Carla José:** Investigation, Visualization. **Carlos R. Llerena Suster:** Investigation, Visualization. **Sebastián E. Collins:** Methodology, Investigation, Writing – original draft, Visualization. **Raquel Portela:** Methodology, Investigation, Writing – original draft. **Miguel A. Bañares:** Resources, Funding acquisition. **Laura E. Briand:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mcat.2021.111811.

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