Catalysis Science & Technology

PAPER

Cite this: *Catal. Sci. Technol.*, 2017, 7, 1953

Molecular recognition of an acyl–enzyme intermediate on the lipase B from *Candida antarctica*†

María V. Toledo,[a] Carlos R. Llerena Suster,[a] María L. Ferreira,[b] Sebastián E. Collins[c] and Laura E. Briand *[a]

Received 9th February 2017,
Accepted 24th March 2017

DOI: 10.1039/c7cy00245a

rsc.li/catalysis

This investigation provides evidence of the acyl enzyme species involved in the interaction of *R*/*S*-ketoprofen with the lipase B from *Candida antarctica*. The interaction between the profen and the enzyme was studied by *in situ* time-resolved ATR-FTIR under both static and transient conditions. Particularly, modulation excitation spectroscopy (MES) with phase sensitive detection (PSD) allowed us to univocally distinguish the signals belonging to the interaction between ketoprofen and the enzyme from the strong background signals. These experimental tools coupled with theoretical DFT analysis allowed us to propose various species derived from the interaction of ketoprofen with serine through H bonding (without reaction) and the acyl enzyme species (ester bond formation) which are the intermediates in the biocatalytic assisted esterification and hydrolysis using lipases.

1. Introduction

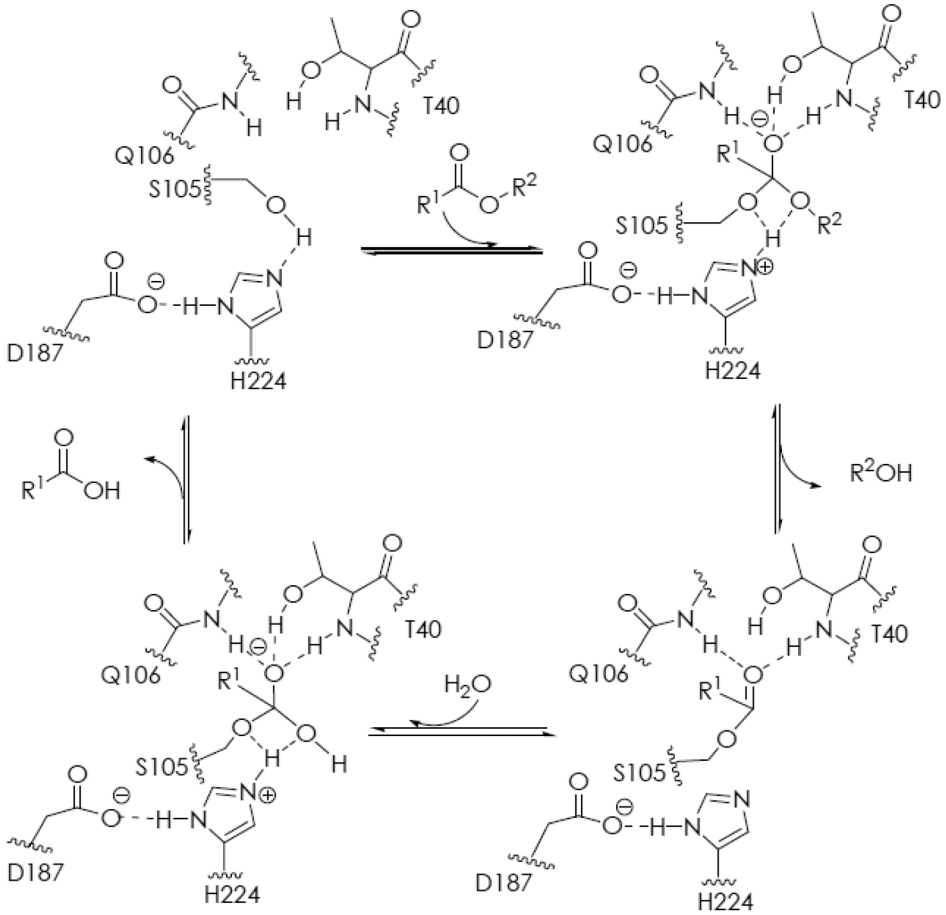
Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a family of hydrolytic enzymes whose biological function in bacteria, fungi, plants and higher animals is to catalyze the hydrolysis of triglycerides to obtain free fatty acids and glycerol. Further, these enzymes are of great utility in various reactions and numerous industrial processes due to their ability to catalyze the reverse reactions of esterification, transesterification and interesterification. Additionally, their chemo-, regio- and enantioselectivity and their ability to react with a wide variety of substrates make these enzymes the most widely used in organic chemistry.[1] The pharmaceutical industry applies the enantioselectivity property of lipases for preparing different pharmaceuticals and fine chemicals containing a chirality center. Among the racemic substances, 2-arylpropionic acid derivatives are an important group of non-steroidal anti-inflamatory pharmaceuticals (NSAIDs) widely used in the treatment of pain and inflammation associated with tissue injury. Even though they are commercially available as racemic mixtures, the pharmacological activity remains mainly on the *S*-enantiomer which also causes fewer adverse effects than the racemic form.[2,3] Therefore, in the last 20 years, lipases have been widely applied in the kinetic resolution of these racemic mixtures with the aim of obtaining optically pure *S*-2-arylpropionic acids.[4–15] Lipase B from *Candida antarctica* (CALB) has been one of the most used. CALB is composed of 317 amino acids and has a molecular weight of 33 kDa. Its active site is a catalytic triad composed of serine (S) 105, aspartic acid (D) 187 and histidine (H) 224, and an oxyanion hole formed by threonine (T) 40 and glutamine (Q) 106 is found.[16] The mechanism of action of this enzyme has been described as Ping Pong Bi Bi with the formation of two tetrahedral intermediates and an acyl–enzyme complex, and competitive substrate inhibition by the alcohols used as acyl acceptors[17] (Scheme 1). The substrate enters the active site and becomes activated and coordinated to the oxyanion hole formed by T40 and Q106.[18] The hydrogen atom of S105 is transferred to the Nε of H224 of the catalytic triad and the Oγ attacks nucleophilically the carbonyl group of the substrate to form the first tetrahedral intermediate which is stabilized by the oxyanion hole. An acyl–enzyme complex is formed when the hydrogen atom in H224 is transferred to the oxygen atom to release an alcohol from the intermediate. A second nucleophilic attack is initiated by a nucleophile (water or alcohol in hydrolysis or esterification reactions, respectively) on the carbonyl carbon of the acyl–enzyme complex to form the second

[a] *Centro de Investigación y Desarrollo en Ciencias Aplicadas-Dr. Jorge J. Ronco., Universidad Nacional de La Plata, CONICET, CCT La Plata. Calle 47 No 257, B1900AJK La Plata, Buenos Aires, Argentina. E-mail: briand@quimica.unlp.edu.ar; Tel: +54 221 4 211353/210711*

[b] *Planta Piloto de Ingeniería Química – PLAPIQUI, CONICET, Universidad Nacional del Sur, Camino La Carrindanga Km 7, 8000 Bahía Blanca, Argentina*

[c] *Instituto de Desarrollo Tecnológico para la Industria Química INTEC, Universidad Nacional del Litoral, CONICET, Güemes 3450, 3000 Santa Fe, Argentina*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7cy00245a



Scheme 1 The Ping Pong Bi Bi mechanism of *Candida antarctica* lipase B.[18]

tetrahedral intermediate. Then the product is released and the lipase is regenerated. The role of D187 in the catalytic triad is to stabilize the positive charge generated over the H224 residue in the tetrahedral intermediates.[17,19,20]

Molecular modeling studies of the mechanism of esterification of *R*/*S*-ibuprofen catalyzed by lipase B *of Candida antarctica* suggested that the enzyme prefers the *R*-enantiomer to form an acyl–enzyme complex through the amino acids S105, H224 and D187 which constitute the catalytic triad.[21] Further studies have postulated the formation of an acyl–enzyme complex between the *R*-enantiomer of ketoprofen and the lipase from *Rhizomucor miehei*.[4] The presence of the acyl–enzyme complex has been determined experimentally through infrared spectroscopic techniques in the hydrolysis of β-lactam antibiotics by a β-lactamase enzyme[22–24] and ornithine acetyl transferase (OAT)[25] and in the hydrolysis reaction of *trans*-cinnamoyl imidazole by α-chymotrypsin.[26] However, there is no experimental evidence of this intermediate species in the kinetic resolution of *R*/*S*-2-arylpropionic acids catalyzed by lipases.

In the present investigation, the molecular species involved in the interaction of *R*/*S*-ketoprofen with lipase B of *Candida antarctica* is studied by *in situ* time-resolved ATR-FTIR and molecular modeling.

2. Experimental

2.1. Materials

Highly pure *Candida antarctica* lipase B was obtained from a commercial extract of lipase B from *Candida antarctica* CALB L (Lipozyme® LC N02102) provided by Novozymes Brazil (Paraná, Brazil) according to the methodology described by Llerena-Suster *et al.*[27] *R*/*S*-ketoprofen was purchased from Parafarm (99.80%, batch 030718 000928/004) and deuterium oxide D_2O from Cambridge Isotope Laboratories (99%).

2.2. Isotopic exchange of lipase B from *Candida antarctica* with D_2O

The isotopic exchange of water with D_2O molecules allows the investigation of the amide I signal (1700–1600 cm^{-1}) without the interference of the bending vibration of O–H species (mainly from adsorbed water) that typically appears at 1640 cm^{-1}. The H–D isotopic exchange of water molecules in highly pure CALB was performed by dissolving 0.0050 g (5 mg) of the enzyme in 500 μL of D_2O. The mixture was dispersed over the ATR cell in order to prepare a homogeneous film over the crystal. Then, the cell was closed and incubated at room temperature to allow isotopic exchange to occur.

With the aim of studying the isotopic exchange evolution, the sample was analyzed once every second during the first three minutes of exchange (1 scan per spectrum), followed by one spectrum every three minutes to complete two hours (20 scans per spectrum). Finally, the cell was purged overnight with dry air in order to remove excess D_2O and remaining traces of H_2O, achieving a dry completely exchanged film. A new spectrum of completely exchanged CALB was obtained at room temperature. Then the cell was heated at 45 °C using a thermostatic water bath (Julabo) connected to the heating jacket of the cell with the aim of obtaining similar conditions to those employed in the esterification reaction, and a new spectrum was collected under these new conditions. Then 300 μL of ketoprofen in carbon tetrachloride (0.02 M) was added and the interaction was analyzed for 3 hours. Initially, spectra were recorded once every second during the first three minutes (1 scan per spectrum), followed by one spectrum every five minutes (25 scans per spectrum) until 3 hours of analysis were completed.

The secondary structure of highly pure CALB before and after the contact with *R*/*S*-ketoprofen was studied according to the methodology employed previously when the secondary structure of Novozym®435 in contact with 2-propanol was studied.[28,29]

2.3. Investigation of the intermediate species through molecular spectroscopy

2.3.1. ATR-FTIR analysis. The species generated during the interaction of *R*/*S*-ketoprofen with the CALB lipase was studied through infrared spectroscopy using a homebuilt stainless steel flow-through cell as described in detail elsewhere.[30,31] The optimized design of the ATR cell has linear-shaped entrance and exit ports close to the extremes of the cell to avoid dead-volume zones and to enable a uniform fluid velocity profile across the cell, which is fully developed immediately after the ports. The total cell volume is 60 μL. The ATR crystal used was a 45 °C trapezoidal ZnSe whose dimensions were 80 mm × 10 mm × 4 mm, providing 10 internal reflections at the liquid/crystal interface (penetration depth = 1.66 μm for n_2 = 1.4 at 1000 cm^{-1}). A film of the enzyme was prepared on the crystal described above. The cell was mounted onto an ATR attachment (Pike Technologies) inside the sample compartment of the FTIR spectrometer (Thermo-Electron, Nicolet 8700 with a cryogenic MCT detector). The bench of the spectrometer and the mirrors that direct the radiation towards 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 to the spectra. Analysis of all the spectra was performed with the software Omnic 8 and Origin 5.0.

Deconvolution of the obtained spectra was performed by peak fitting of the signal by Lorentzian-shaped components in the non-deconvoluted spectra. The software used for this purpose was a special peak fitting module of Origin 5.0. The positions and number of peaks were determined from the second derivative analysis of the spectra.

2.3.2. Modulation excitation spectroscopy with phase sensitive detection (MES-PSD). Concentration-modulation excitation spectroscopy (c-MES) experiments were performed at 35 °C, using the same set-up already described elsewhere.[30,31] Isothermal c-MES experiments in combination with phase sensitive detection (PSD) were carried out to selectively distinguish the signals belonging to the interaction between ketoprofen and the enzyme from the strong signals from the background. MES-PSD analysis of the spectra was performed according to the method developed by Baurecht and Fringeli.[32] More details on the technique can be found in the ESI.†

After recording the background spectrum, a modulation experiment was started by varying the inlet composition from carbon tetrachloride (Carlo Erba, HPLC grade) to *R*/*S*-ketoprofen (0.16 M) in CCl_4, using the desired modulation frequency. After allowing at least five modulation periods to adjust the system to the external perturbation, the recording of the spectra was started. Spectra were acquired every 3 s during each c-MES period, using reactant exchange frequencies from 1.7 to 33 mHz. The flow of reactants was achieved using a pulse-free peristaltic pump (Ismatec ICP4) located at the end of the cell. Liquids were provided from two separate glass bottles. A pneumatically actuated three-way valve controlled by software allowed switching between streams of reactants. Repetitive square-wave stimulations with flow rates from 0.5 to 2 mL min^{-1} and modulation frequencies from 50 to 200 mHz could be generated in the ATR cell.

2.4. Molecular modeling

A molecular mechanics calculation in the framework of Chem3D Cambridge Soft was performed to explore the steric interactions between ketoprofen and the serine hydroxyl in the presence of one water molecule interacting with ketoprofen through hydrogen bonding. The CALB sequence was extracted from the Protein Data Bank (1tcb; DOI:10.2210/pdb1tcb/pdb) and the Swiss PDB Viewer program was downloaded to visualize the lipase.[33] The structure of the acyl enzyme of ketoprofen and the serine molecule was modeled completely. The MM2 calculation produced a configurational minimum for the acyl enzyme structure which was isolated for the *ab initio* calculation. After a configurational MM2 minimum was obtained, a DFT study was carried out. This DFT study used the method RB3LYP with the 6-31G basis set as a frequency test in the Gaussian98W program, performed on the structure of the acyl–enzyme ketoprofen–serine with 47 atoms. The goal was to obtain by calculation the FTIR bands of the acyl–enzyme model. The complete calculation for the acyl–enzyme serine–ketoprofen involved 8 hours and 59 minutes. The calculation output reported several characteristic frequencies in the range 400 to 4000 cm^{-1}, which are presented in the following sections.

3. Results

3.1. Protein based infrared signals under isotopic exchange

It is worth noting that lipase B from *Candida antarctica* used in this investigation was thoroughly purified previously.[27] The purification involves the separation of the nonsoluble fraction of the crude extract through centrifugation. Treatment of the centrifuged sample with size exclusion chromatography (SEC) allowed us to obtain an enzymatic sample free of nucleic acids, benzoate and sorbate species. Moreover, 82% of the protein was retained with a similar specific activity to the crude extract. This enzymatic sample possesses mainly CALB with minor amounts of two other proteins of 21.5 kDa and 66 kDa. These two proteins were retained in the anionic and cationic exchange chromatography columns at pH values equal to 8.5 and 5.5, respectively.

Fig. 1 presents the spectra collected during the isotopic exchange of purified lipase B of *Candida antarctica* during the first two hours. It shows the different IR signal intensities with time in the spectral region from 1730 cm^{-1} to 1400 cm^{-1}, which includes amide I and II bands.[34–36] Meskers *et al.* described the spectral changes observed in the IR spectra of a streptavidin protein during isotopic exchange.[37] In this context, as mentioned above, the amide II band shifts by a wavenumber of 10 cm^{-1}, and it is observed in the figure that the signal at 1450 cm^{-1} increases (amide II – D_2O), while at 1550 cm^{-1} (amide II – H_2O) the signal intensity decreases over time. Meanwhile, in the amide I band, a decrease of the absorbance at high wavenumbers (1700 cm^{-1}) and an increase at low wavenumbers (1600 cm^{-1}) occur. As expected, isotopic exchange allows visualizing the amide I band without the interference of water molecules and so in general, the band between 1600 cm^{-1} and 1700 cm^{-1} increases.

Fig. 2 presents the spectra of CALB (4000–1000 cm^{-1}) completely exchanged with D_2O after overnight incubation at room temperature and 45 °C. For comparison purposes, CALB dissolved in D_2O at the beginning of the exchange is included. The band at 3378 cm^{-1} assigned to the ν(O–H) and ν(N–H) stretching vibrations (the so-called amide A and amide B) is observed. Additionally, characteristic signals of D_2O are observed in CALB dissolved in D_2O before the isotopic exchange occurs, which are the ν(O–D) stretching vibration at 2472 cm^{-1} together with the δ(O–D) bending vibration at 1206 cm^{-1}. Replacement of the hydrogen atom in the O–H species by a deuterium atom modifies the wavenumbers of the bending vibration band with a ratio of 1.37.[36]

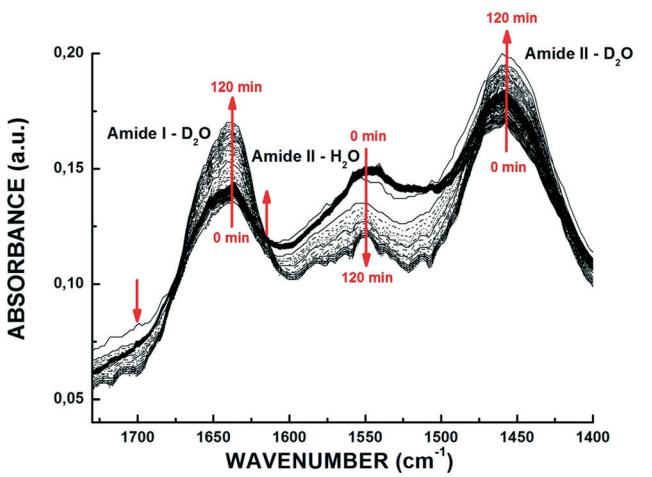


Fig. 1 Evolution of the infrared spectra of purified lipase B from *Candida antarctica* during the first two hours of isotopic exchange at room temperature.

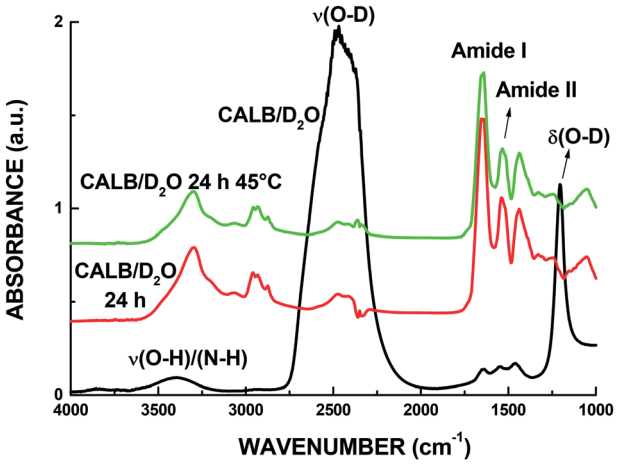


Fig. 2 Infrared spectra of CALB dissolved in D_2O at the beginning of the exchange, and completely exchanged with D_2O after overnight incubation at room temperature and 45 °C.

3.2. Investigation of the species generated in the profen–CALB interaction under static conditions

The spectra collected over 3 hours of interaction of *R*/*S*-ketoprofen with CALB (dissolved in carbon tetrachloride in the ATR cell) are shown in Fig. 3A–D.

The contribution of the spectra of CALB was subtracted; therefore the infrared signals of the new species formed *in situ* could be readily analyzed. The spectrum of pure *R*/*S*-ketoprofen obtained immediately after being dissolved in CCl_4 was included for comparison purposes. The intense signals at 1709 cm^{-1} and 1662 cm^{-1} are ascribed to the stretching mode of the carbonyl species ν(C=O) of the carboxylic and the ketone groups of the profen dissolved in carbon tetrachloride.[38]

As can be appreciated in Fig. 3A, a broad signal arises at 1740 cm^{-1} at 19 seconds of interaction and remains until three hours of analysis are completed. This new signal is ascribed to a cyclic dimer, as was also reported in the literature for ketoprofen dissolved in supercritical CO_2.[39] The species observed in the experiments performed with pure ketoprofen dissolved in carbon tetrachloride at various concentrations *i.e.* 0.02 M and 0.16 M without the presence of the enzyme are similar to the ones described above (the spectra obtained and the speciation of the profen are discussed in the ESI†). However, the interaction with the enzyme gives rise to additional signals of low intensity at 1749 cm^{-1} and 1734 cm^{-1}

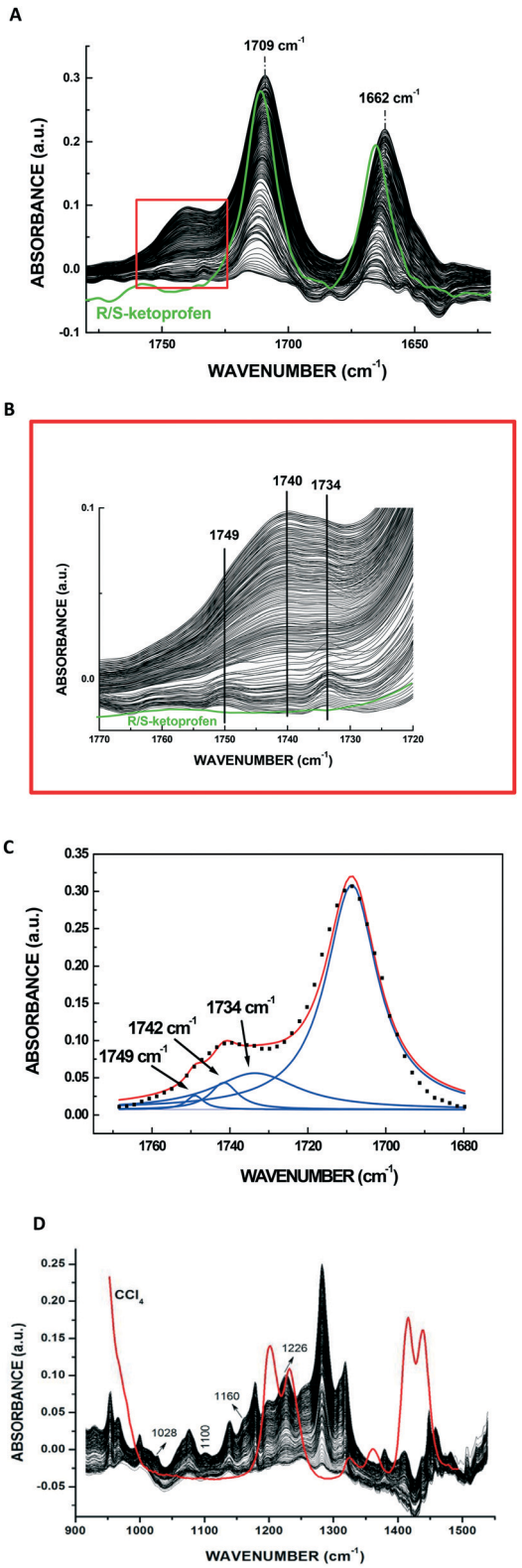


Fig. 3 (A) Time-resolved ATR-FTIR spectra collected over 3 hours of interaction of *R*/*S*-ketoprofen with CALB dissolved in carbon tetrachloride in the ATR cell and spectrum of pure *R*/*S*-ketoprofen (highlighted in green). (B) Enlarged detail of the spectra between 1770 cm^{-1} and 1720 cm^{-1}. (C) Deconvoluted spectra between 1770 cm^{-1} and 1680 cm^{-1}. (D) Time-resolved ATR-FTIR spectra in the 1300 and 1100 cm^{-1} range with the spectra of pure CCl_4 for comparison (highlighted in red).

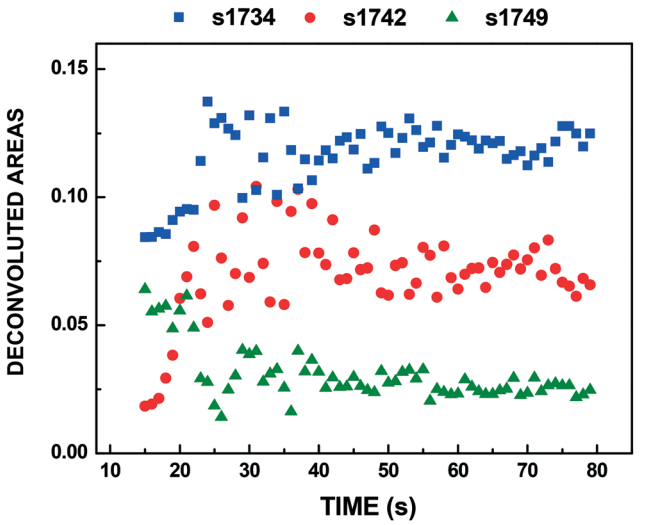


Fig. 4 Evolution of the areas of the signals at 1749 cm^{-1}, 1742 cm^{-1} and 1734 cm^{-1} up to 80 seconds of interaction of *R*/*S*-ketoprofen (0.02 M) in CCl_4 with CALB at 45 °C.

(see Fig. 3B). Moreover, these signals were further confirmed upon deconvolution of the broad signal centered at 1740 cm^{-1} (see Fig. 3C).

The progression of such signals is presented as the deconvoluted areas over time up to 80 seconds of interaction in Fig. 4. The signals at 1742 cm^{-1} and 1734 cm^{-1} grow during the first 30 s of interaction and remain without modifications. However, the behavior of the signal at 1749 cm^{-1} is somehow different since it appears immediately upon contact of the profen with the enzyme and then disappears, which evidences desorption or reaction of the species. The experiments under transient conditions will provide further evidence on this matter.

Finally Fig. 3D shows new signals in the range between 1300 and 1100 cm^{-1} which typically belong to the stretching vibrations of C–O and C–O–C bonds. New features are

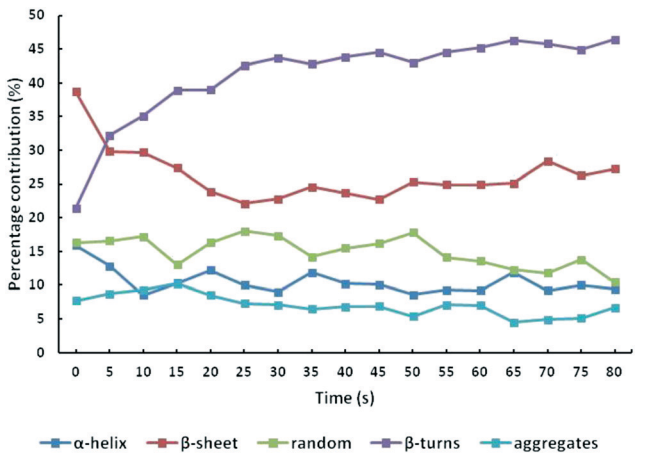


Fig. 5 Deconvoluted areas of the components of the secondary structure of the protein during the formation of the acyl–enzyme intermediate species up to 80 seconds of contact with *R*/*S*-ketoprofen (0.02 M) in CCl_4 at 45 °C.

observed at 1226 cm^{-1}, 1160 cm^{-1}, 1100 cm^{-1} and 1028 cm^{-1} which cannot be ascribed to either pure ketoprofen or carbon tetrachloride and this will be discussed in the following sections.

3.3. Influence of the acyl–enzyme intermediate on the structure of the protein

The influence of the acyl enzyme on the secondary structure of the protein was investigated through deconvolution, integration and further normalization of the corresponding signals involved in the amide I structure (from 1700 to 1600 cm^{-1}).[28,29] Fig. 5 presents the evolution of each component of the secondary structure during the first 80 seconds of interaction of *R*/*S*-ketoprofen with CALB, as described in section 3.2.

A decrease in the contribution of β-sheets and an increase in β-turns with respect to pure CALB (time 0) can be observed, while the other structures remain almost unaltered. These results are similar to those obtained in the esterification of the profen with short chain alcohols (methanol, ethanol, 1- and 2-propanol) using the commercial biocatalyst Novozym®435 (CALB immobilized on a macroporous support) reported previously.[28,29]

Structural modification of the enzyme by carboxylic acids was previously reported in the literature.[40]

After desorption of the acyl–enzyme complex from CALB through a pure carbon tetrachloride flow in the c-MES experiments (discussed in the next section), the amide I signal was analyzed in order to obtain the contribution of each component of the secondary structure. The results indicate that the decrease observed in the β-sheets and the increase in β-turns as a result of the interaction of the acyl–enzyme complex is irreversible. However, even though the α-helix and the random structure still remain unmodified, the contribution of molecular aggregates diminishes. Fang *et al.* found it difficult to explain the effect of solvent on the enzyme, but suggested that the conformation changes induced in CALB by different solvents might be different.[41] These results may be a complement to the ones reported by McCabe *et al.*, which showed that the secondary structure of CALB does not differ significantly with the change from an aqueous to an organic solvent environment.[42]

3.4. Profen–CALB reaction intermediate species are revealed through molecular spectroscopy under transient conditions

Time-resolved spectra recorded during the c-MES experiment of concentration-dependent periodic exchange of *R*/*S*-ketoprofen (from 0.16 M in CCl_4 to pure CCl_4) are presented in Fig. 6A. The strong signals belonging to the enzyme film deposited on the ATR crystal overwhelm the complete range of the spectra. Thus, the slight changes in the spectra during modulation are difficult to read. The phase-resolved spectra, after applying the PSD method, are presented in Fig. 6B. There, the static IR signals from the enzyme film are eliminated from the spectra, which allows the observation of the periodic change in the signals at 1710 and 1664 cm^{-1} due to dissolved ketoprofen being the stimulus. Additionally, some

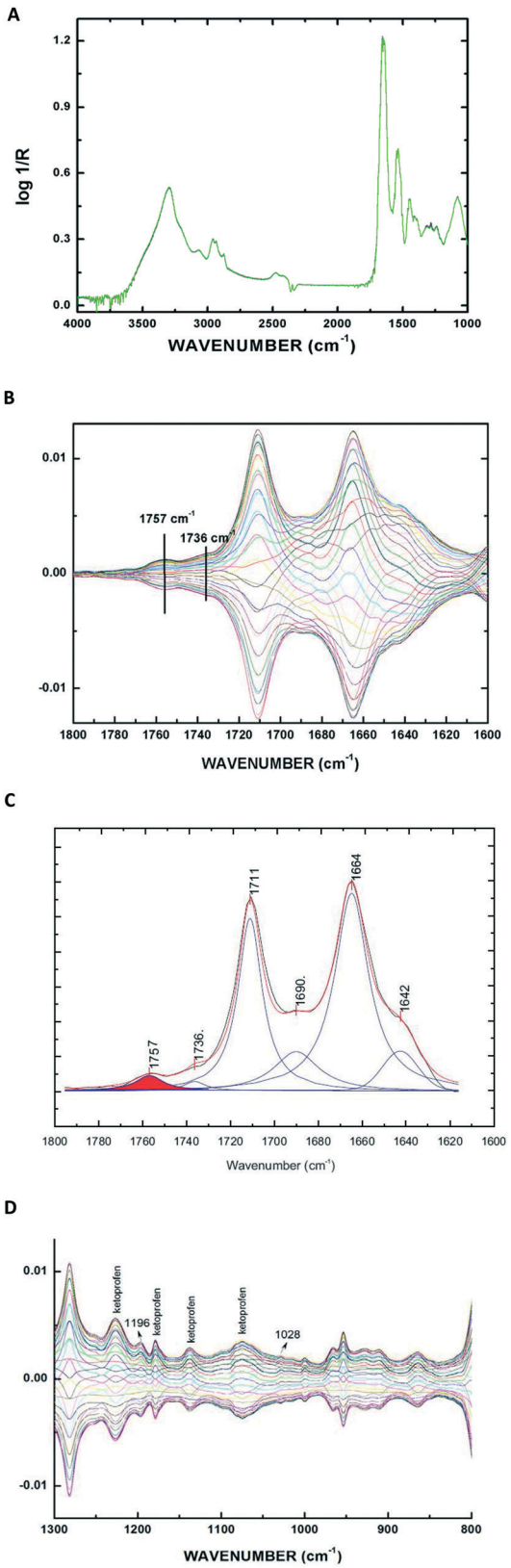


Fig. 6 (A) Time resolved ATR-FTIR spectra during a concentration MES cycle of *R*/*S*-ketoprofen (0.16 M)/CCl_4–CCl_4 over CALB (0.5 mL min^{-1}, 45 °C K, ω = 50 mHz). (B) Phase-resolved ATR-FTIR spectra after demodulation of the spectra shown before and (C) deconvoluted spectra between 1800 cm^{-1} and 1620 cm^{-1}. (D) Time-resolved ATR-FTIR spectra during a concentration MES cycle in the 1300–800 cm^{-1} range.

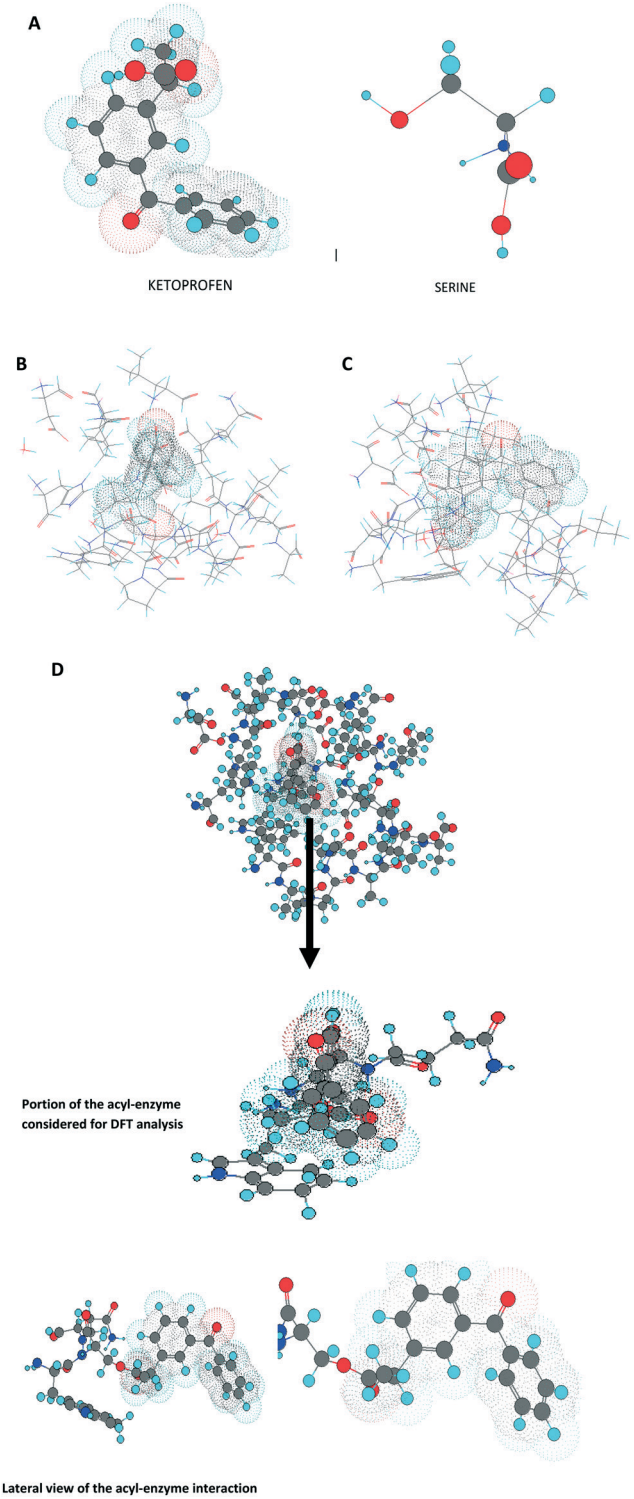


Fig. 7 (A) Molecular models of ketoprofen and serine obtained with Chem3D Cambridge soft. Front (B) and lateral (C) views of the acyl enzyme of ketoprofen in the active site; the dots represent the ketoprofen moiety. (D) Selection of the portion of the acyl enzyme after minimization, including the large and medium pockets of CALB.

perturbation on the amide I band is also observed, as indicated by the bands around 1650 cm^{-1} and as discussed in the previous section. Importantly, two bands are seen at 1757 and 1736 cm^{-1}. These signals change synchronically, but with a phase lag as the ketoprofen flows into the cell, and therefore can be ascribed as a product of the interaction with the CALB enzyme. Deconvolution of the spectra in the phase domain at the maximum intensity between 1800 cm^{-1} and 1600 cm^{-1} provides further evidence of those signals (see Fig. 6C).

It is important to note here that the c-MES experiments were performed under a flow of the reactant, that is, the maximum concentration of ketoprofen in the cell is 0.16 M in Cl_4C. Control experiments showed that IR signals due to ketoprofen dimeric species are only observed at highly concentrated solutions (see the ESI†). Then, the bands at 1757 and 1736 cm^{-1} must correspond to the molecular interaction of ketoprofen with CALB, as will be discussed next.

Additionally, Fig. 6D shows new signals at 1196 cm^{-1} and 1228 cm^{-1} which can be ascribed to the stretching vibrations of the C–O–C bond, according to DFT calculations presented in the next section.

3.5. Further evidence of the profen–enzyme surface species through DFT calculation

Fig. 7A shows the ketoprofen and serine molecules obtained with Chem3D Cambridge Soft. Additionally, Fig. 7B and C present front and lateral views of the interaction of profen with the catalytic triad including D 187, H 224 and S 105. Then, Fig. 7D presents the portion of the minimized structure of ketoprofen–serine forming the acyl–enzyme which was isolated for the *ab initio* calculation. The theoretical investigation of the interaction of ketoprofen with the serine amino acid of the active triad of CALB provided key information in terms of the infrared vibrations that would be expected. Table 1 shows the results of the DFT calculation for the infrared frequencies of serine, ketoprofen and the ester between ketoprofen and serine which is a model of the acyl–enzyme. The infrared signals of the optimized serine amino acid calculated through DFT are also compared and assigned according to the vibrations reported by Ramirez *et al.*[43] The calculated vibrations of ketoprofen and the ester formed between ibuprofen and serine (the model acyl–enzyme interaction) have been assigned according to the investigations reported by Vueba *et al.*[44] The esters are reported to present one band at 1735–1750 cm^{-1} due to the stretching vibration of the carbonyl group ν(C═O) and several more at 1000–1300 cm^{-1} due to the stretching of the C–O bond.

The theoretical end experimental investigations confirm the presence of an infrared signal at 1756 cm^{-1} due to the acyl–enzyme complex formation with simultaneous release of a water molecule (formed between the hydroxyls of the profen and serine) that is interacting with the complex through hydrogen bonds. This interaction suggests the presence of an ester between the profen and the amino acid serine of the catalytic triad. Additionally, ketoprofen might interact through three H bonds (one in the carbonyl group and two in the OH group) with the hydroxyl of serine (without reaction) and other adjacent NH and OH species of the

Table 1 Infrared wavenumbers for calculated bands (using DFT-GaussianW98) and experimental bands reported in the literature

Wavenumber (cm^{-1})						
Serine				Ketoprofen	Serine–ketoprofen ester	Assignment
This work	Ramírez *et al.*[43]					
	Calculated	Experimental	Assignment	This work	This work	Vueba *et al.*[44]
548	564	548	Skeletal bending	521		
				601	606	Bending out of plane C–O–H
631	625	600	Skeletal bending	631.8	644	Bending out of plane C–O–H
				653.9	650.8–654.4	Bending out of plane C–O–H
				660.5	668	Bending out of plane
				692.4	696	C–H bend out of plane
732	769	787	CO_2-bending vibration	721.4	721.5	C–H bend out of plane
				727.6	729.5	
					743.6	C–O–H out of plane
				754.3	756	
				775	779	CH out of plane wagging
				780	783	CH_3 rocking
837.6	815	807	CO_2-bending vibration	827	834	CH out of plane wagging
				841	840.1	C–H bend out of plane
				888	878	C–OH in plane bending
				896	898	
911	935	912			916	CH out of plane bending
941				928	928	CH out of plane bending
					962	CH_3 rock, ring deformation, CH bend
				977.6	977.6	C–O
				999.6	1000	Ring deformation
				1008	1004	
1028.4	1009	975	Skeletal stretching *ν*CC, *ν*CN, *ν*CO	1015.6	1014.8	CH in plane bending
					1038.5	C–O–C
				1041–1045	1041–1048	
				1066–1068	1061–1065	
				1073.7	1073	CH_3 rock, C–CH_3
1085	1064	1084	Ammonium rocking vibration	1088.5	1088–1092	
				1115		
					1125	C–O–C
				1138	1135	Symmetric stretch Ph–C–Ph
1141			*δ*OH	1147	1143–1147	CH phenyl in plane bending
					1155	CH phenyl in plane bending
1179	1155	1154		1176		Phenyl ring deformation
				1187	1185	
1215	1243	1238	*t*CH_2	1214.3	1212	
					1238	C–O stretching
				1251	1252	Ph–Ph ring deformation
1260.8			OH	1260.4	1260.6	
				1269	1264	
				1282	1284	
				1308		
					1321	Ph C–C stretching
1333.5	1342	1340	Symmetric stretching vibration $\nu_s CO_2$-	1337	1336	
				1372	1375–1376	C–C–H deformation
1387.5	1362	1352	Overtones	1390.8		CH_3 symm. deformation
				1399	1396–1398	
				1417.8	1416–1417	Stretching C–C en Ph
1420	1421	1423	$\bar{\omega}CH_2$	1423.6	1423	
1440.7	1457	1467	δCH_2	1434.7	1435	
					1455	CH_3 antisymm. deformation
				1490	1498	C–C stretching in phenyl
1502		1519	Bending symmetrical amine	1514	1512	NH_2
				1527	1527	
				1548	1553	
1564	1559	1559		1561	1562	
				1576–1578	1576.6	C–C stretching phenyl
				1675–1678	1674–1676	Stretching carbonyl group
				1696–1698	1696–1698	Stretching C═O in ketoprofen acid/ester

Table 1 *(continued)*

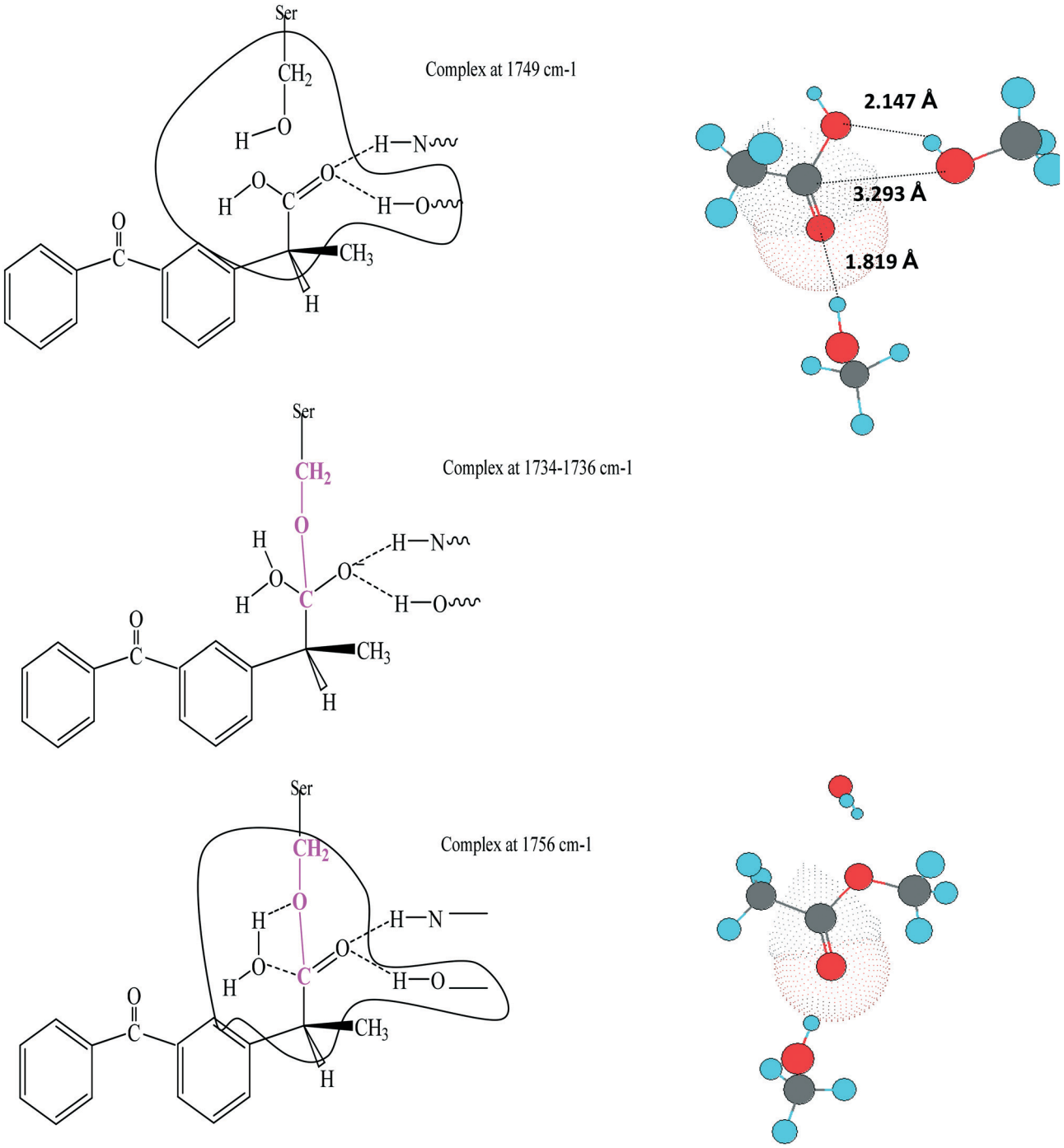
Wavenumber (cm^{-1})						
Serine				Ketoprofen	Serine–ketoprofen ester	Assignment
This work	Ramírez *et al.*[43] Calculated	Experimental	Assignment	This work	This work	Vueba *et al.*[44]
1745.8					1756	New C═O stretching in ketoprofen ester
					1832	
				1842	1842	
				1862	1862	Combination modes
1894						
					2829	
					2853	
				2873		
2888						
2901	2896	2903	Stretching CH			
2929	2964	2969	Stretching CH_2			
					2964	Symmetric stretching methyl
				3006	3010–3013	Antisymm. stretching methyl
				3053	3034	
				3085–3088	3085–3086	Stretching C–H phenyl
				3100–3105	3100–3105	
				3112–3114	3110–3114	
				3124	3124	
				3136	3136	NH
3484						
3560	3521		Stretching O–H		3577	Overtone
3745					3798–3814	
4150				4002	4004–4008	

catalytic active site. DFT investigation of this intermediate revealed a new signal at 1736 cm^{-1} which was also detected in the c-MES experiments. Moreover, the experiments show new signals in the range 1300 cm^{-1} to 1000 cm^{-1} (see Fig. 3D and 6D). According to the DFT calculations presented in Table 1, these signals provide further evidence of the ester between ketoprofen and serine. In this context, it is possible to ascribe the bands at 1028 cm^{-1} and 1100 cm^{-1} to the stretching vibrations of the C–O–C bond, the signal at 1160 cm^{-1} to the in-plane bending of the phenyl C–H group, and the one at 1226–1228 cm^{-1} to the stretching of the C–O bond.

4. Discussion

Acyl–enzyme species are the key intermediates in hydrolysis and esterification reactions catalyzed by hydrolases and lipases. Previous spectroscopic investigations concerning the formation of the acyl–enzyme intermediate used attenuated total reflection (ATR)-FTIR under static conditions. Typically, a film of the enzymatic material is placed on the crystal of the ATR cell and the substrate is allowed to interact with the active site over time. This procedure has also been applied in the present investigation with the drawback of solvent evaporation. This phenomenon might change the nature of the substrate in contact with the enzyme (formation of dimers was observed in the particular case of carboxylic acids such as ketoprofen) and also lead to the modification of the intensity of the infrared bands due to the increase in the concentration of the substrate over the crystal.[23,26] Another device used for spectroscopic investigations of the acyl enzyme is an *in situ* IR cuvette having CaF_2 windows and a path length of 50 μm where the substrate and the enzyme are mixed for analysis.[22,24,25]

The first studies started approximately 25 years ago when Swedberg *et al.* reported an acyl–enzyme intermediate by ATR-FTIR analysis. They found that the *trans*-cinnamoyl-α-chymotrypsin acyl–enzyme intermediate provided a new ester carbonyl bond that vibrates at 1723 cm^{-1} and 1710 cm^{-1}. These infrared bands suggest the existence of two different conformations of the substrate in the active site.[26] Moreover, Iqbal *et al.* studied ornithine acetyl transferase 2 (OAT2) from *Streptomyces clavuligerus* involved in the biosynthesis of the β-lactamase inhibitor clavulanic acid, which forms an acyl–enzyme complex with the substrate C-*N*-α-acetyl-L-glutamate. The acyl–enzyme showed a broad band from 1691 cm^{-1} to 1710 cm^{-1} composed of two absorption bands at 1691 cm^{-1} and 1702 cm^{-1}, with the latter appearing as a shoulder on the former, suggesting the formation of two complexes in equilibrium.[25] Further, Hokenson *et al.* reported an acyl–enzyme complex formed by the enzyme β-lactamase and different substrates positioned at 1755 ± 2 cm^{-1}, representing an average of 13 cm^{-1} decrease in frequency compared to the unbound substrates.[23] Lastly, in the study of the hydrolysis of methicillin catalyzed by a β-lactamase enzyme, Wilkinson *et al.* assigned the signals at 1742 cm^{-1}, 1728 cm^{-1} and 1707 cm^{-1} to the acyl–enzyme complex.[22] Additionally, these



Scheme 2 Complexes between ketoprofen and serine interacting with the surroundings, the acyl–enzyme intermediate with one molecule of water and their infrared signals. The assignation of 1734–1736 cm^{-1} and 1756 cm^{-1} is not related to C–O–C. The purple color in C–O–C shows the new bond.

authors reported at least four conformations which coexist for the ester carbonyl group of the stable acyl–enzyme complex species formed between the antibiotic aztreonam and the class C β-lactamase from *Citrobacter freundii*. Absorption bands assigned to these complexes were at 1746 cm^{-1}, 1727 cm^{-1}, 1708 cm^{-1} and 1690 cm^{-1}.[24]

In the present study, the interaction between ketoprofen and lipase B from *Candida antarctica* was investigated through infrared spectroscopy under both static and dynamic conditions. In the former case, the time-resolved IR was strongly influenced by the speciation of ketoprofen due to the increased concentration of the profen in contact with the enzyme during the course of the experiment. An experiment comprising a solution of ketoprofen in carbon tetrachloride (both starting from 0.02 M or 0.16 M) in the ATR cell for 68 minutes gives rise to characteristic infrared signals at 1792

cm^{-1}, 1772 cm^{-1} and 1742 cm^{-1} ascribed to the stretching vibration of the carbonyl group of ketoprofen in its monomeric, linear and cyclic dimeric forms, respectively.[39] The signal of the dimeric form of ketoprofen (~1740 cm^{-1}) overlaps with two other signals at 1749 cm^{-1} and 1734 cm^{-1} when ketoprofen is in contact with the lipase in the static kind of experiment. According to the DFT calculations, those signals could be ascribed to interactions of ketoprofen with serine and the surroundings through hydrogen bonds without reaction, such as the ones shown in Scheme 2. In fact, the complex formed between the monomer of ketoprofen and serine (1749 cm^{-1}) vanishes upon dimerization of the profen molecules. This observation somehow indicates that the dimer of ketoprofen is not a suitable conformation to generate a stable complex with serine most probably due to steric hindrance with the oxyanion hole formed by the backbone amide protons of T40 and Q106 and the side chain of T40.[16] In fact, structural analyses have revealed that nucleophilic enzymes that catalyze reactions involving carbonyl groups bind the oxygen of the carbonyl group in the oxyanion hole. This oxyanion hole involves hydrogen bonding from two main-chain amide NH groups to the two lone pairs of the carbonyl group. This interaction with the oxyanion hole polarizes the carbonyl group, activating it for nucleophilic attack and locates the substrate productively in the active site.[45]

The spectra in the phase domain show signals at 1757 cm^{-1} and 1736 cm^{-1} without the interference of the broad band of the dimeric form of ketoprofen observed in the previous time-dependent experiments. Molecular modeling plus DFT study support the assignation of the signal at 1757 cm^{-1} to the ester ketoprofen–serine, the so-called acyl–enzyme intermediate (see Scheme 2). The signal at 1734–1736 cm^{-1} was detected both in the blank experiments (without the lipase) and in the presence of lipase B from *Candida antarctica* in either the static mode or the c-MES experiments. The assignation of this signal is somehow doubtful since it could arise from the interaction of the carboxylic acid group of ketoprofen with the hydrogen from a network including the hydroxyl of serine, according to the theoretical calculations described previously. Nevertheless, the investigation reported by George *et al.* demonstrated that the complex formed through a hydrogen bond between formic acid and one molecule of water exhibits an infrared band at 1736.7 cm^{-1} due to the perturbed stretching of the carbonyl group.[46] The presence of trace amounts of water in lipase B from *Candida antarctica* might persist even after the isotopic exchange performed in the present investigation. Moreover, the calculated infrared signal of the acyl–enzyme involves a molecule of water. In this context, the interaction of ketoprofen with water cannot be ruled out as the genesis of the signal observed at 1734–1736 cm^{-1}. It is important to highlight that the main change among the ketoprofen dimer, the ketoprofen adsorbed near the CALB catalytic triad and the acyl enzyme is the formation of a new C–O–C in the acyl enzyme. It is clear that the ketoprofen travels to the catalytic triad as a monomeric molecule, due to steric hindrance. However, carbonyl groups near hydroxyl groups and important hydrogen bonding in C═O–H–O are present in the three situations. Besides, there are hydroxyl groups near each other in all these structures. The difficulty of signal assignation is clear. Even so, changes in the spectra, even if subtle, are detectable among ketoprofen, CALB and ketoprofen plus CALB in CCl_4, and therefore the possibility of multiple assignation of a band to several sources should be carefully taken into account.

5. Conclusions

Serine amino acid has been proposed as the key active site of hydrolytic enzymes since the report of Wlodawer *et al.* in 1998.[47] The authors gathered a series of investigations that evidenced the formation of acyl–enzyme species *via* nucleophilic attack by a serine residue. At that time, those observations were based on the structure of several enzymes (proteases, esterases, asparaginase, and penicillin acylase, among others) provided solely by X-ray diffraction. Later on, more information on the acyl–enzyme intermediate was obtained through infrared spectroscopy, as discussed in the previous section. The present investigation is the first report about the experimental identification through *in situ* infrared spectroscopic techniques coupled with molecular modeling and DFT calculations of the species formed between *R*/*S*-ketoprofen and the catalytic triad of lipase B from *Candida antarctica*. These tools provided key information on the various types of interactions of ketoprofen in its monomeric form with serine and residues surrounding the catalytic triad of the lipase. Scheme 2 depicts the complexes formed due to the hydrogen bonding of the carboxyl group of the profen with the hydroxyl group of serine and the oxyanion hole of the lipase, which is proposed as the genesis of the active acyl–enzyme intermediate. Additionally, the corresponding infrared signals are presented. It is worth noting that the complex exhibiting a signal at 1749 cm^{-1} was observed at concentrations of ketoprofen in carbon tetrachloride above 0.16 M and competes with the dimers that are formed upon speciation of the ketoprofen. The complex that vibrates at 1734–1736 cm^{-1} arises from the interaction through H-bonding with serine and two other residues. However, this species might also be the product of the interaction of the profen with one water molecule. Nevertheless, there is undoubtful experimental and theoretical evidence that the acyl–enzyme species vibrates at 1756 cm^{-1}. This intermediate is formed through an ester bond between ketoprofen and the serine amino acid of the catalytic triad with the simultaneous release of one molecule of water. According to the DFT calculations, this molecule of water continues to interact with the acyl–enzyme through H-bonding.

Acknowledgements

The authors acknowledge the financial support of Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET

(projects PIP 112 201301 00171) and Universidad Nacional de La Plata (project 11-X745).

References

1 M. T. Reetz, *Curr. Opin. Chem. Biol.*, 2002, **6**, 145–150.
2 J. N. Jin, S. H. Lee and S. B. Lee, *J. Mol. Catal. B: Enzym.*, 2003, **26**, 209–216.
3 S. Sjövall, M. Kokki, A. Nokela, L. Halinen, M. Turunen and H. Kokki, *J. Clin. Pharm. Ther.*, 2015, **40**, 431–435.
4 M. Arroyo and J. V. Sinisterra, *J. Org. Chem.*, 1994, **59**, 4410–4417.
5 M. T. López-Belmonte, A. R. Alcántara and J. V. Sinisterra, *J. Org. Chem.*, 1997, **62**, 1831–1840.
6 H. J. Park, W. J. Choi, E. C. Huh, E. Y. Lee and C. Y. Choi, *J. Biosci. Bioeng.*, 1999, **87**, 545–547.
7 G. De Crescenzo, A. Ducret, M. Trani and R. Lortie, *J. Mol. Catal. B: Enzym.*, 2000, **9**, 49–56.
8 A. R. Alcántara, P. Domínguez de María, M. Fernández, M. J. Hernaíz, J. M. Sanchez-Montero and J. V. Sinisterra, *Food Technol. Biotechnol.*, 2004, **42**, 343–354.
9 Y.-Y. Liu, J.-H. Xu, H.-Y. Wu and D. Shen, *J. Biotechnol.*, 2004, **110**, 209–217.
10 C.-S. Chang and C.-S. Hsu, *J. Chem. Technol. Biotechnol.*, 2005, **80**, 537–544.
11 P. O. Carvalho, F. J. Contesini and M. Ikegaki, *Braz. J. Microbiol.*, 2006, **37**, 329–337.
12 Z.-D. Long, J.-H. Xu, L.-L. Zhao, J. Pan, S. Yang and L. Hua, *J. Mol. Catal. B: Enzym.*, 2007, **47**, 105–110.
13 A. L. Ong, A. H. Kamaruddin, S. Bhatia and H. Y. Aboul-Enein, *J. Sep. Sci.*, 2008, **31**, 2476–2485.
14 Y.-Y. Zhang and J.-H. Liu, *Biochem. Eng. J.*, 2011, **54**, 40–46.
15 X. Li, T. Liu, L. Xu, X. Gui, F. Su and Y. Yan, *Biotechnol. Bioprocess Eng.*, 2012, **17**, 1147–1155.
16 J. Uppenberg, M. T. Hansen, S. Patkar and T. A. Jones, *Structure*, 1994, **2**, 293–308.
17 M. Martinelle and K. Hult, *Biochim. Biophys. Acta*, 1995, **1251**, 191–197.
18 U. T. Bornscheuer and R. J. Kazlauskas, *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*, Wiley-VCH, 2nd edn, 2006.
19 A. L. Paiva, V. M. Balcão and F. X. Malcata, *Enzyme Microb. Technol.*, 2000, **27**, 187–204.
20 M. Arroyo, *An. R. Soc. Esp. Quim.*, 2000, **1**, 19–24.
21 M. L. Foresti, M. Galle, M. L. Ferreir and L. E. Briand, *J. Chem. Technol. Biotechnol.*, 2009, **84**, 1461–1473.
22 A.-S. Wilkinson, S. Ward, M. Kania, M. G. P. Page and C. W. Wharton, *Biochemistry*, 1999, **38**, 3851–3856.
23 M. J. Hokenson, G. A. Cope, E. R. Lewis, K. A. Oberg and A. L. Fink, *Biochemistry*, 2000, **39**, 6538–6545.
24 A.-S. Wilkinson, P. K. Bryant, S. O. Meroueh, M. G. P. Page, S. Mobashery and C. W. Wharton, *Biochemistry*, 2003, **42**, 1950–1957.
25 A. Iqbal, I. J. Clifton, M. Bagonis, N. J. Kershaw, C. Domene, T. D. W. Claridge, C. W. Wharton and C. J. Schofield, *J. Am. Chem. Soc.*, 2009, **131**, 749–757.
26 S. A. Swedberg, J. J. Pesek and A. L. Fink, *Anal. Biochem.*, 1990, **186**, 153–158.
27 C. R. Llerena-Suster, L. E. Briand and S. R. Morcelle, *Colloids Surf., B*, 2014, **121**, 11–20.
28 M. V. Toledo, C. José, S. E. Collins, M. L. Ferreira and L. E. Briand, *J. Mol. Catal. B: Enzym.*, 2015, **118**, 52–61.
29 M. V. Toledo, C. José, S. E. Collins, R. D. Boneto, M. L. Ferreira and L. E. Briand, *J. Mol. Catal. B: Enzym.*, 2012, **83**, 108–119.
30 A. Aguirre, P. A. Kler, C. L. A. Berli and S. E. Collins, *Chem. Eng. J.*, 2014, **243**, 197–206.
31 A. Aguirre, C. L. A. Berli and S. E. Collins, *Catal. Today*, 2016, **283**, 127–133.
32 D. Baurecht and U. P. Fringeli, *Rev. Sci. Instrum.*, 2001, **72**(10), 3782–3792.
33 E. D. Gudiño, L. E. Iglesias and M. L. Ferreira, *Biochim. Biophys. Acta, Proteins Proteomics*, 2012, **1824**, 627–636.
34 J. Kong and S. Yu, *Acta Biochim. Biophys. Sin.*, 2007, **39**, 549–559.
35 A. Barth, *Biochim. Biophys. Acta*, 2007, **1767**, 1073–1101.
36 C. R. Llerena-Suster, C. José, S. E. Collins, L. E. Briand and S. R. Morcelle, *Process Biochem.*, 2012, **47**, 47–56.
37 S. Meskers, J.-M. Ruysschaert and E. Goormaghtigh, *J. Am. Chem. Soc.*, 1999, **121**, 5115–5122.
38 M. J. Sánchez-Dasi, S. Garrigues, M. L. Cervera and M. de La Guardia, *Anal. Chim. Acta*, 1998, **361**, 253–260.
39 M. Champeau, J.-M. Thomassin, C. Jérôme and T. Tassaing, *J. Chem. Eng. Data*, 2016, **61**(2), 968–978.
40 A. Szabó, M. Kotormán, I. Laczkó and L. M. Simon, *Process Biochem.*, 2009, **44**, 199–204.
41 X. Fang, Y. Zhan, J. Yang and D. Yu, *J. Mol. Catal. B: Enzym.*, 2014, **104**, 1–7.
42 R. W. McCabe, A. Rodger and A. Taylor, *Enzyme Microb. Technol.*, 2005, **36**, 70–74.
43 F. J. Ramirez, I. Tuñon and E. Silla, *Chem. Phys.*, 2004, **303**, 85–96.
44 M. L. Vueba, M. E. Pina, F. Veiga, J. J. Sousa and L. A. E. Batista de Carvalho, *Int. J. Pharm.*, 2006, **307**(1), 56–65.
45 M. Fischer and J. Pleiss, *Nucleic Acids Res.*, 2003, **31**(1), 319–321.
46 L. George and W. Sander, *Spectrochim. Acta, Part A*, 2004, **60**, 3225–3232.
47 G. Dodson and A. Wlodawer, *Trends Biochem. Sci.*, 1998, **23**, 347–352.

