Evaluation of the Residual Activity of Immobilized and Free Lipases Treated with Supercritical Carbon Dioxide

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ABSTRACT

This work investigates the influence of temperature, pressure, exposure time and depressurization steps on the residual activity of four commercial lipases treated with supercritical carbon dioxide (SC-CO_2). A high-pressure cell was employed in the experiences, varying the temperature from 35 to 70 ºC, in the pressure range of 100 - 250 bar, exposure time from 1 to 6 h, and different number of depressurization steps (1 - 3). Activity losses were verified for immobilized enzymes (Lipozyme RM IM and Lipozyme 435) in all experiences. On the other hand, mild treatment conditions promoted the enhancement of the free enzyme activity (Palatase 20000 L and Lipozyme CALB L), while the highest temperature and pressure and the longest exposure time assayed led to activity losses. Additional qualitative studies were performed to investigate the effect of $SCCO_2$ on the commercial enzyme preparations. Scanning electron micrographs showed morphological alterations in the immobilization supports when compared to the untreated enzyme. Fluorescence spectra showed changes in the conformational structure of both two free enzymes when treated under SC-CO_2. These changes could explain the changes observed in the enzymatic activity.

INTRODUCTION

The utilization of enzymes as biocatalysts in many processes, either in their free or immobilized form, has become an increasingly important research field in recent years. Considerable attention has been paid to lipases, due to their high specificity in lipid biomodification [1]. To cope with the environmental drawbacks of organic solvents, biochemical catalysis with lipases can be conducted in supercritical carbon dioxide (SC-CO_2), a nontoxic, readily available, inexpensive, and easily removable solvent. In addition, the singular properties of supercritical fluids, such as liquid-like solvating power and gas-like viscosity, diffusivity and superficial tension, have a positive effect on the reaction rates and selectivities [2].

On the other hand, activity changes can be observed when enzymes are submitted to SC-CO_2. In the case of immobilized enzymes, enzyme stability is related to the nature and source of the enzyme and the immobilization method. Essential water in the enzyme microenvironment can be removed as a result of unfavorable water partitioning between the support and the solvent, causing enzyme inactivation [3–5]. Other parameters during SC-CO_2 exposure, such as high pressure, high temperature, and depressurization steps can also affect negatively the enzyme activity [6].

Some researchers reported that free enzymes are easily inactivated when exposed to $SCCO_2$ because of a pH decrease [7]. Conversely, other researchers demonstrated that free enzyme activities can be considerably enhanced (up to 760% residual activity) after incubation in SC-

CO_2 [8]. The activity changes of free enzymes in the presence of SC-CO_2 could be explained as a change in the conformational structure of the enzyme [9–12].
This work investigates the influence of temperature, pressure, exposure times and depressurization steps on the activity of four commercial lipases: immobilized Lipozyme RM IM and Lipozyme 435, and free Palatase 20000 L and Lipozyme CALB L treated with SC-CO_2. This study helps to understand the effect of $SCCO_2$ exposure in the activity of different commercial enzymes, as well as to help in the selection of the best conditions to carry out further enzymatic reactions under SC-CO_2. The analysis includes studies of the possible chemical, morphological and conformational modifications caused by $SCCO_2$ treatment. Infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM) were used to evaluate possible alterations in the immobilized enzymes. Fluorescence spectroscopy was also applied to evaluate possible changes in the conformational structure of the free enzymes.

MATERIALS AND METHODS

Materials

Commercial lipase Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on a macroporus hydrophilic resin), Lipozyme 435 (*Candida antarctica* lipase B immobilized on a macroporous, hydrophobic resin), Palatase 20000 L (free *C. antarctica* lipase B expressed on *Aspergillus niger*) and Lipozyme CALB L (free *R. miehei* lipase expressed in *A. oryzae*) were kindly donated by Novozymes (Bagsvaerd, Denmark). Carbon dioxide (99.9%) was supplied by Carburos Metálicos S.A. (Spain). All other chemicals used were of analytical grade.

Enzyme treatment under SC-CO_2

A schematic diagram of the experimental apparatus used in the enzyme treatment is showed in Figure 1. It consist in a solvent reservoir, a high pressure syringe pump equipped with a pressure controller (ISCO 260 D) and a high pressure cell equipped with a liquid retention system.
In a typical experiment, the enzyme preparation was charged into the high pressure cell, which was placed in a thermostatic water bath at the temperature established. Afterwards, the system was pressurized and maintained at constant temperature and pressure for a pre-established exposure time. Typically, the duration of the pressurization step was less than 0.5 min and accordingly was not included in the pressure holding time. Depressurization steps were performed at a decompression rate of 240 kg m^{-3} min^{-1}. Experiments were performed in the temperature (T) range of 35 - 75 ºC, pressure (p) from 100 to 250 bar and exposure time (t) from 1 to 6 h for all enzymes. Several depressurization steps (1 -3) were carried out in the case of immobilized enzymes (see Table 1).

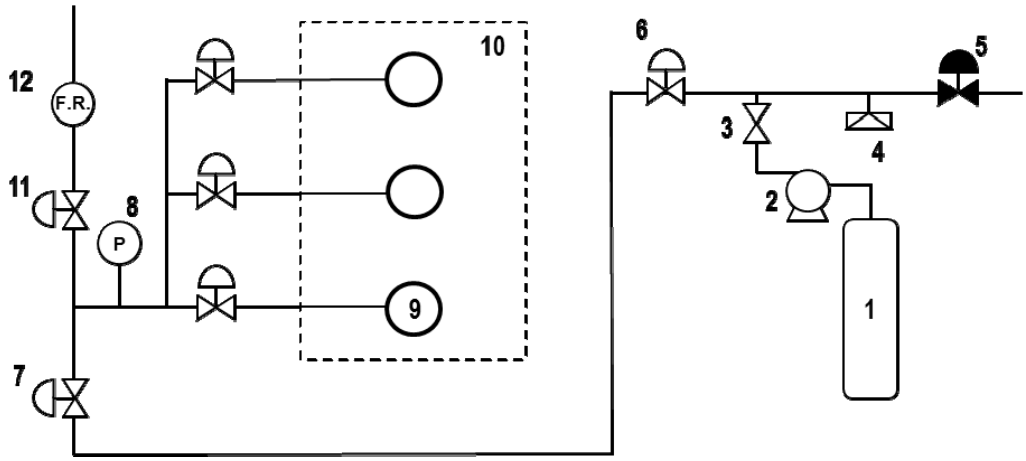

Figure 1 : Experimental apparatus for enzyme treatment under SC-CO_2. 1 : CO_2 cylinder ; 2 : Pump ; 3 : Back Pressure regulator ; 4 : Rupture disk ; 5 : Vent valve ; 6,7 : Inlet valves ; 8 : Pressure gauge ; 9 : High pressure cell (x3) ; 10 : Thermostatic water bath ; 11 : Depressurization valve ; 12 : Coriolis mass flowmeter .

Table 1 : Experimental conditions used in the enzyme treatments under SC-CO_2.

exp.	p (bar)	T (ºC)	t (min)	dep. steps
1	100			
2	150	50	180	1
3	250			
4	150	35	180	1
5		70		
6	150	50	60	1
7			360	
8	150	50	180	2
9				3

Analysis of residual activity

The enzyme activity of immobilized enzymes was determined as the initial rate in the esterification reaction of lauric acid with propanol at a molar ratio of 3:1, 60 % wt. hexane as reaction medium, and enzyme concentration of 5 % wt. in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and propanol were collected and the lauric acid content was determined by titration with KOH 0.1N in ethanol. After the addition of the enzyme to the substrates, the mixture was kept at 50 ºC for 15 min. Then, the lauric acid consumption was determined by the same experimental procedure.

The enzyme activity of free enzymes was determined as the initial rate in the hydrolysis reaction of the olive oil triglycerydes . 1 ml of enzyme preparation was added to the assay substrate, consisting on 4 ml of 10 % homogeinized olive oil and 5 ml of 50 mM phosphate

buffer pH = 7.0. The reaction was carried out at 50 ºC for 15 min. After incubation, 15 ml of ethanol:acetone (1:1) was added to terminate the reaction. Liberated fatty acids were tritated with KOH 0.1 N in ethanol. As a blank control, the reaction mixture without the enzyme was titrated in the same way.
In all cases, residual activity was calculated as the relationship between the enzyme activity after SC-CO_2 exposure and the initial enzyme activity, expressed as percentage. All enzymatic activity determinations were performed at least in triplicate.

Instrumentation

Infrared spectroscopy analyses were made to follow possible chemical modifications as a function of the treatment. FT-IR spectra were obtained in the 4000 - 400 cm^{-1} range using a Thermo-Nicolet Nexus 670 FTIR spectrophotometer. Scanning electron microscopy (SEM) was also performed to check possible changes in the morphological properties. Micrographs of the immobilized enzymes were obtained using a variable-pressure scanning electron microscope JEOL JSM-6460LV.
The tertiary structure of the free enzymes was measured by fluorescence spectra using a Varian Cary Eclipse spectrofluorimeter (Agilent Technologies) thermostated at 25 ºC. The excitation wavelength was 280 nm, and the emission wavelength was read at 290 - 450 nm. All the spectra were scanned continuously with five replicates.

RESULTS

The experimental results obtained from the residual activity determination of the four enzymes are showed in Table 2 and Figures 2-5.

Table 2 : Residual activities (% of initial activity) observed in the four commercial enzymes after SC-CO_2 treatment at the established conditions in each experience.

	Residual activity (%)								
Experiment	1	2	3	4	5	6	7	8	9
Lipozyme RM IM	100.2	92.1	89.2	96.0	76.5	94.4	87.4	91.6	88.1
Lipozyme 435	96.5	82.2	86.2	95.1	73.8	89.2	79.7	77.7	78.2
Palatase 20000 L	126.0	121.1	96.1	134.1	86.3	134.8	111.9	-	-
Lipozyme CALB L	104.1	107.5	97.3	112.3	80.9	109.6	96.2	-	-

SC-CO_2 treatment resulted in activity loss of immobilized enzymes in all experiencies. Lipozyme 435 losses were found to be slightly higher, which could be explained by considering the hydrophobic character of the immobilization support. As some authors suggest [5], the unfavorable water partitioning between the support and SC-CO_2 could be traduced in a strip away effect of the essential water from the enzyme microenvironment, thus, causing enzyme deactivation.

In both immobilized and free enzyme preparations, the highest activity losses could be attributed to thermal deactivation (exp. 5).
An increase in residual activity was observed for free lipases treated with SC-CO_2 at mild conditions (exps. 1, 2 ,4 and 6). Treatments at the highest pressure (exp. 3) resulted in low activity losses in both free enzymes. Prolongated exposure times (exp. 7) increased Palatase 20000L activity and slightly decreased Lipozyme CALB L activity. Activity improvement could be explained by considering that SC-CO_2 may interact with hydrophobic residues in the enzyme structure, promoting conformational changes that led to a more accessible active site. This hypothesis will be later confirmed by the corresponding fluorescence spectra.
Results obtained in Lipozyme CALB L residual activity determination are in agreement to those reported in the literature [11], although the activity increases at this work conditions were higher. Activity improvement were higher in Palatase 20000 L, of which no references were found in the available literature.

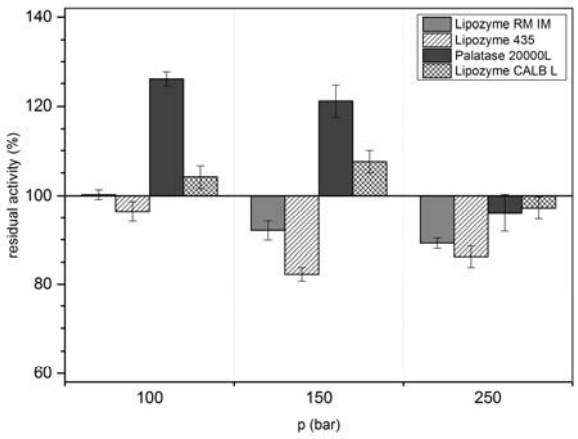

Figure 2. Enzyme activities after SC-CO_2 treatment at different pressure conditions (100-250 bar).

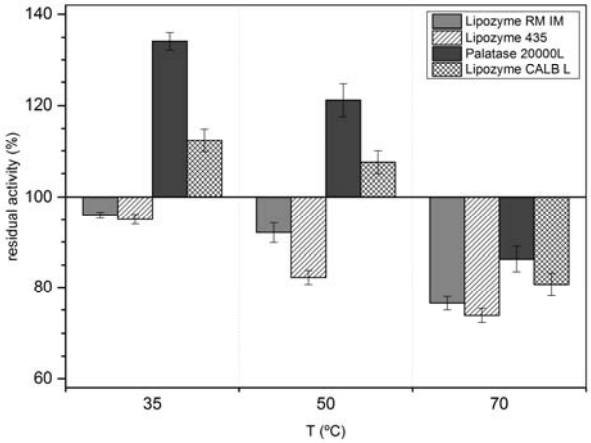

Figure 3. Enzyme activities after SC-CO_2 treatment at different temperature conditions (35-70 ºC).

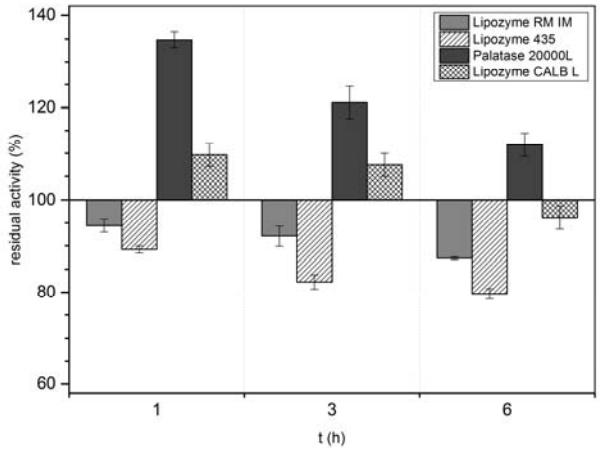

Figure 4. Enzyme activities after SC-CO_2 treatment with different exposure times (1-6 h).

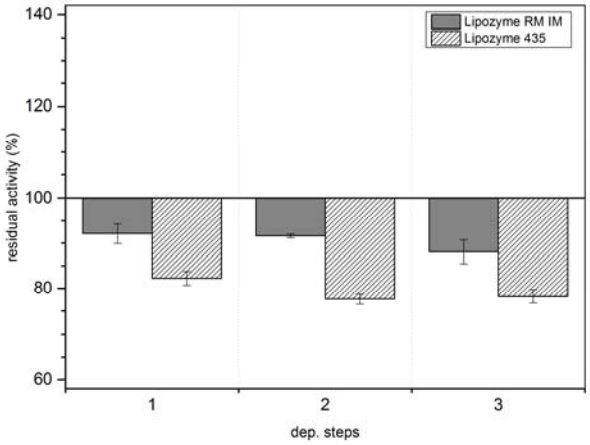

Figure 5. Enzyme activities after SC-CO_2 treatment with different depressurization steps (1-3).

SEM micrographs (images not included in this proceeding) showed that immobilized enzymes treatment under SC-CO_2 leads to changes of the enzyme-support structure. The original shape of Lipozyme 435 support was affected, becoming slightly deformed. The same could not be observed in Lipozyme RM IM as a result of the irregular shape of the support. In greater magnifications (10,000x) of both immobilized enzymes, a rough and cracked surface with an apparent increase of porosity can be appreciated after SC-CO2 treatment. The same was previously observed by Oliveira et al. on Lipozyme 435 [5].

FT-IR spectra of the immobilized enzymes after treatment under SC-CO_2 (exp. 2) are presented in Figure 6. As it can be observed from this figure, SC-CO_2 treatment has not promoted significant changes in the characteristic peaks of both materials when compared to its respective untreated enzyme spectra. These results showed that the treated immobilized enzymes did not present chemical modifications at the established experimental conditions.

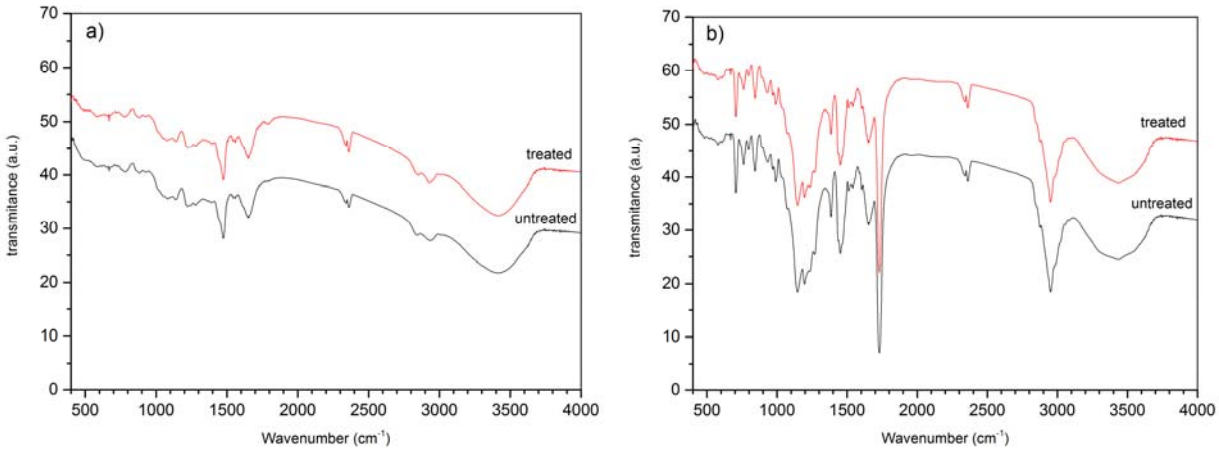

Figure 6. FT-IR spectra of a) Lipozyme RM IM and b) Lipozyme 435 before and after SC-CO_2 treatment. In both cases, treated samples from exp. 2.

Fluorescence spectroscopy is also an available means of studying the conformational structure of free enzymes. Differences in the intrinsic fluorescence intensity can indicate changes on the conformational structure [10]. As a consequence of the treatment, weak interactions between CO_2 and tryptophan and tyrosine residues could take place, inducing structural changes which led the enzyme to adopt an open conformation, with higher intrinsic fluorescence intensity. In these conditions, enzyme substrates easily reach the active site and the enzymatic activity is enhanced. This is what possibly happened in exps. 2 and 4 from Palatase 20000 L and exp. 4 from Lipozyme CALB L, where mild conditions are used.

On the other hand, some authors [10] have suggested that, when a pressure threshold that depends on the experimental conditions is reached, SC-CO_2 interactions with tryptophan and tyrosine become stronger, affecting negatively the free enzyme activity as it can be observed in exps. 5 and 7 from both free enzymes.

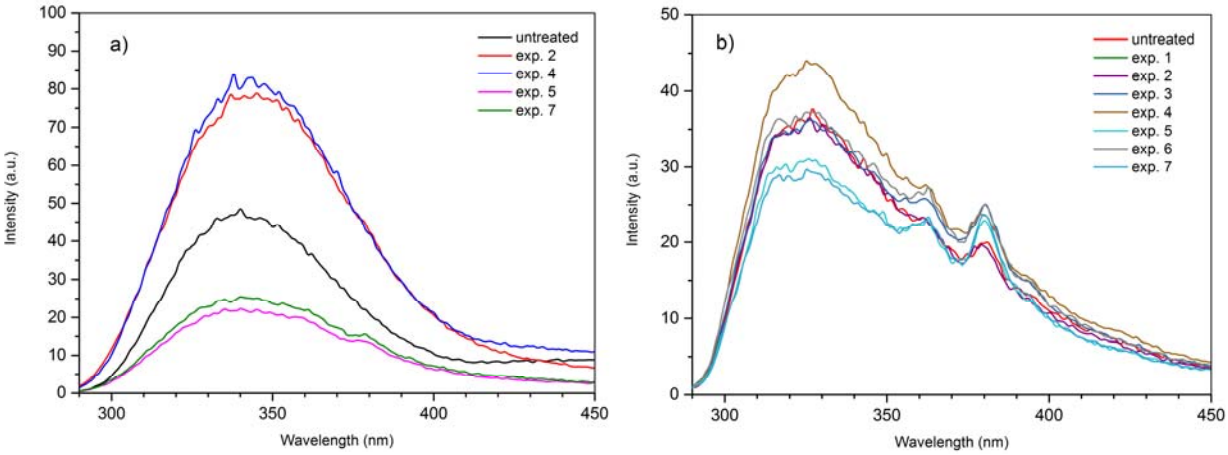

Figure 7. Fluorescence spectra of a) Palatase 20000L and b) Lipozyme CALB L before and after SC-CO_2 treatment under different experimental conditions.

CONCLUSIONS

In the present work, four commercial enzymes were submitted to several SC-CO_2 treatments under different conditions. Based on the results obtained, it can be concluded that the effect of pressure, temperature, decompression rate, and exposure time needed to affect the lipase activity strongly depends on the nature and the source of the enzyme and, mainly, whether the enzyme is presented in a free or immobilized form.
The results obtained in this work may help with the purpose of selecting appropriate operation conditions in order to use lipases with the lowest activity loss, or, when possible, the highest activity improvement at the typical reactions conducted in the biotransformation of lipid raw materials under SC-CO_2.

ACKNOWLEDGEMENTS

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