Lipase Activity Enhancement by SC-CO_2 Treatment

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Dedicated to Professor Gérard Demazeau on the occasion of his 65th birthday

The activity of lipases from porcine pancreas, *Candida antartica* recombinant from *Aspergillus oryzae*, *Candida cylindracea* (immobilized), *Penicilium roqueforti*, *Aspergillus niger*, *Rhizopus arrhizus*, *Mucor miehei* (two types of immobilization), and *Pseudomonas cepacia* (two types of immobilization) was studied after using them as biocatalysts of blackcurrant oil hydrolysis under SC-CO_2 conditions. The reaction was performed at 40 °C and 15 MPa in a continuous-flow reactor. Increased relative activity of all used lipases after the hydrolytic reaction was observed. The most remarkable increase in the activity was noted for the lipase from *Rhizopus arrhizus* which was increased by more than 50 times. The highest activity was shown by Lipozyme®, lipase from *Mucor miehei* immobilized on macroporous resin. Both treated and untreated Lipozyme® were used as biocatalysts in hydrolytic resolution of the racemic *cis*- or *trans*-isomers of 2-(4-methoxybenzyl)cyclohexyl acetates. Satisfactory reaction yields (40 %) and excellent enantiomeric purity of the products (E = 472) were obtained when hydrolysis of the *trans*-isomer of 2-(4-methoxybenzyl)cyclohexyl acetate was catalyzed by Lipozyme® treated with SC-CO_2.

Key words: Lipase Activity, Supercritical Carbon Dioxide, Enantioselectivity

Introduction

Hydrolytic enzymes such as lipases (triacylglycerol ester hydrolases, EC 3.1.1.3.) catalyze hydrolysis of ester linkages in lipids resulting in a release of the constituent alcohol and acid moieties [1]. In organic solvents they catalyze the reverse reaction – the synthesis of esters. Environmentally harmful conventional organic solvents can be replaced by a "green" solvent, supercritical carbon dioxide (SC-CO_2) as a reaction medium with several further advantages [2, 3]: (a) the properties of SC-CO_2 including its solvent power can be varied to a large extent by changes in pressure and temperature; (b) due to the low kinematic viscosity of supercritical fluids, mass transfer of solute in the solvent is much faster than in conventional liquid solvents. Different types of enzymes as lipases, several phosphatases, dehydrogenases, oxidases, amylases and others are well suited for reactions in SC-CO_2. The enzymes used in SC-CO_2 medium are preferably in immobilized form.

The stability and activity of enzymes exposed to carbon dioxide under high pressure depend on the nature of the enzyme species, the water content in the solution, and the pressure and temperature of the reaction system. The most important factor that may cause enzyme activity loss is probably the depressurization step; in a long-term application, enzyme activity decreases with increasing number of depressurizations [4]. An increase in enzyme activity in SC-CO_2 medium was observed only exceptionally: the activity of crude α-amylase mixed with *Escherichia coli*

or *Saccharomyces cerevisiae* increased by 21 % and 35 %, respectively, during 2 h of a sterilization step at 20 MPa and 35 °C [5]. The stability of enzymes under high pressure and the effect of the medium on enzyme activity are of interest both theoretically and in practical applications. The spatial structure of enzymes may be significantly altered under extreme conditions, causing its denaturation and concomitant loss of activity. If the conditions are less adverse, the protein structure may largely be retained. Minor structural changes may induce an alternative active protein state with altered enzyme activity, specificity and stability [6]. Zagrobelny and Bright [7] showed by fluorescence spectroscopic studies with trypsin that changes in protein conformation caused by pressurization and depressurization steps are unavoidable in a high-pressure batch system. Contrary to the batch reactors, continuous-flow reactors for SC-CO_2 do not require depressurization to introduce substrates in the reactor or to recover products, and another advantage over the batch reactors is a better process economy [8].

The effect of enzyme incubation in SC-CO_2 on its activity at ambient pressure has been examined only in a few recent studies. Giessauf and Gamse [9] reported an as high as several-fold increase in the activity of lipase from porcine pancreas after its exposure to carbon dioxide at 15 MPa and 75 °C for 24 h. The activity was measured using 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester (DGGR) as substrate, and the maximum achieved increase in the activity was 760 %. The influence of temperature, pressure and humidity of SC-CO_2 and of the number of pressurization and depressurization steps on the catalytic activity and stability of enzymes was examined in the next paper [10]. The treated enzymes were crude and purified preparations of esterase EP10 from *Burkholderia gladioli*, lipase from *Candida rugosa* and esterase from porcine liver. An increase in enzyme activity was observed only for the crude preparation from *B. gladioli*; after 30 pressurization/depressurization cycles its activity increased by 20 %. The increase might be connected with enzyme purity; the impurities that decreased the stability of the crude enzyme were extracted with CO_2 that was introduced into the vessel containing the enzyme in each pressurization step. Fluorescence spectra indicated no conformational change before and after treatment with SC-CO_2.

Bauer *et al.* [11] studied the effects of humid SC-CO_2 on the activity of crude porcine pancreas lipase. Different substrates such as 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid-resorufin ester (DGGR), triolein, tributyrin and triacetin were used in the enzyme assay. The treated preparations were found to be more active with the long chain triglyceride, *e. g.*, triolein (the maximum residual activity was 675 %) and DGGR, while there was a loss of activity towards the short chain triglycerides, *e. g.*, tributyrin and triacetin.

Independently, Yan *et al.* [12] measured the hydrolytic activity of lipase from *Candida rugosa* treated with SC-CO_2. The activity increased approximately 2.5 times after the incubation for 1 h at 35 °C and 19.6 MPa. Changes in the content of proteins in the lipase and in the size of the lipase were investigated using fluorescence emission spectroscopy and scanning electron microscopy, respectively. Both size reduction of lipase particles and the purification of enzyme might be the main reasons for the increase in the lipase activity.

In this work, the changes in enzyme activity were measured after applications in the biocatalytic process. Hydrolysis of blackcurrant oil in SC-CO_2 medium was catalyzed by lipases from tissue (porcine pancreas), yeasts (*Candida antartica*, *Candida cylindracea*), moulds (*Penicilium roqueforti*, *Aspergillus niger*, *Rhizopus arrhizus*, *Mucor miehei*) and bacteria (*Pseudomonas cepacia*). Free enzymes and their immobilized forms were tested for activity before and after exposure to SC-CO_2 using triolein as a substrate.

In addition, Lipozyme® both treated with SC-CO_2 and untreated was applied to catalyze hydrolysis of *cis*- or *trans*-isomers of 2-(4-methoxybenzyl) cyclohexyl acetates. These substrates were chosen because of their close structural relationship with the insect juvenile hormone bioanalogs of the 2-(4-hydroxybenzyl)cyclo hexanone series, showing promising biological activity on several non-related insect species [13, 14].

Results and Discussion

Effect of SC-CO_2 on the enzyme activity

The hydrolytic reaction was carried out with lipases from different sources, in free or immobilized form, to produce polyunsaturated fatty acids from blackcurrant oil in SC-CO_2. The use of immobilized lipases is becoming important since enzymes in immobilized form have been reported to be more stable against pressure [15] and temperature than free ones [16], and consequently, it is more suitable for a continuous flow reactor.

Table 1. Activity of lipases (U g^{-1}) before and after their hydrolytic reaction in SC-CO_2 and during the reaction.

Lipase	Activity before the reaction[a]	Activity after the reaction[a]	Activity in SC-CO_2[b]
PPL	2.61	15.26	18.00
LB	15.00	15.90	34.00
LA	0.32	5.97	21.00
CCL	0.37	0.50	1.00
PRL	0.02	0.48	0.47
ANL	0.17	0.49	1.10
RAL	0.22	11.12	0.60
MML	3.40	14.62	2.30
L	7.75	35.65	52.00
PCLa	1.29	9.48	0.70
PCLb	3.70	37.00	2.00

[a] Determined by titration, substrate triolein; [b] determined by the colorimetric method, substrate blackcurrant seed oil.

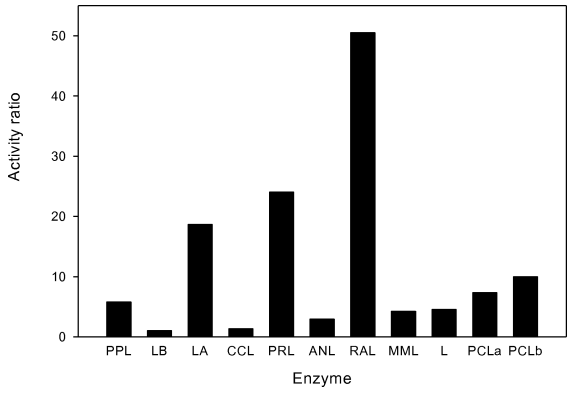


Fig. 1. Ratio of enzyme activity before and after the hydrolytic reaction in SC-CO_2 for all examined enzymes.

Enzyme	Immobilized	Substrate	Product	pH	T, °C	Activity, U g^{-1}
PPL	no	olive oil	fatty acid	8.0	37	2400
LB	no	triacetin	acetic acid	7.0	37	470
LA	no	triolein	oleic acid	8.0	40	260
CCL	in sol-gel-AK	triolein	oleic acid	7.5	37	13
PRL	no	olive oil	fatty acid	8.0	37	1.8
ANL	no	triacetin	acetic acid	7.4	40	4
RAL	in sol-gel-AK	tributyrin	butyric acid	8.0	40	2
MML	in sol-gel-AK	triolein	oleic acid	8.0	40	8.3
L	on macroporous resin	triolein	oleic acid	8.0	40	40
PCLa	in sol-gel-AK on sintered glass	for unit definition see paragraph Enzymes				11.8
PCLb	in sol-gel-AK	for unit definition see paragraph Enzymes				63

Table 2. Enzymes and their activity as declared by Fluka.[a]

[a] Unit def.: 1 U corresponds to 1 μmol product per minute.

Prior to our experiments the lipase activity of all used enzymes was determined by a titric method. Triolein was used as a substrate (Table 1). The activity assays of commercial enzymes declared by the supplier were different concerning substrate, pH and temperature (Table 2).

Among the eleven commercial lipases (2U – according to the titric method) tested in the flow reactor at 15 MPa and 40 °C in the SC-CO_2 system, Lipozyme® showed the most remarkable production of free fatty acids (52 U g^{-1}) followed by Lipase B covalently linked to a carrier from microorganisms (34 U g^{-1}) (Table 1). The difference in yield could be due to different intrinsic enzyme specificity towards the substrates. The amount of free fatty acids after the hydrolytic reaction in SC-CO_2 was determined by the colorimetric method. The method gives results comparable with the ones obtained by the titric method.

The evaluated activities of the enzymes are summarized in Table 1, and the ratio of each enzyme activity before and after its hydrolytic reaction in SC-CO_2 is depicted in Fig. 1. Increase in activity after the reaction is reported for all tested enzymes regardless of their source and of the type of their immobilization. The most remarkable increase (50 times) was observed for the lipase from *Rhizopus arrhizus* immobilized in Sol-Gel-AK. The highest increase in activity of a free enzyme (24 times) was observed in the case of lipase from *Penicilium roqueforti*. The maximum activity among the tested enzymes was exhibited by Lipozyme®, the immobilized lipase from *Mucor miehei*.

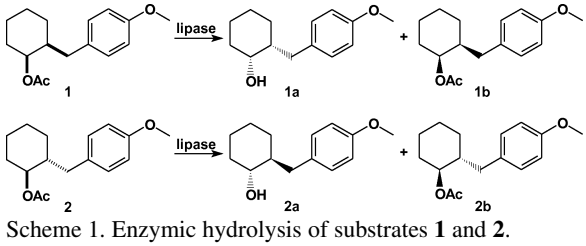
According to the analysis of samples taken in succession during the hydrolysis in SC-CO_2, the activity of enzymes in SC-CO_2 was stable except for the enzymes PPL, LA and LB where it was monotonously decreasing. The activity in SC-CO_2 was similar to the activity determined after the treatment for enzymes PPL, LA, LB, CCL, PRL, ANL and L, but it was much lower for the lipase from *Rhizopus arrhizus*, lipase from *Mucor miehei* in sol-gel-AK and lipase from *Pseudomonas cepacia* PCLa and PCLb, which seem to be less suitable for oil reactions in SC-CO_2.

Conclusions drawn from a comparison of the enzyme activity after the reaction in SC-CO_2 with the activity declared by the enzyme supplier are only tenta-

Table 3. Hydrolysis of **1** or **2** catalyzed by Lipozyme® untreated and treated with SC-CO_2.[a]

Lipozyme®	Substrate **1**					Substrate **2**				
	chem. yield, %	ee_p, %	ee_s, %	c	E	chem. yield, %	ee_p, %	ee_s, %	c	E
Untreated	5	> 99	5	0.048	209	8	> 99	8	0.074	214
Treated	10	79	8	0.091	9	40	> 99	78	0.44	472

[a] ee_p: enantiomeric purity of the product **1a** or **2a**; ee_s: enantiomeric purity of the product **1b** or **2b**; c: conversion, $c = ee_s\,(ee_s + ee_p)^{-1}$; E: enantiomeric ratio calculated according to the formula $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.



Scheme 1. Enzymic hydrolysis of substrates **1** and **2**.

tive because of the different methods used for the activity assessment. Nevertheless, it is evident that the enzyme activities after the reaction, though higher than before the reaction, are comparable or lower than the declared activities of the fresh enzymes.

The right lecture key of these successful results in terms of an increase of the lipase activity should be found in the choice of condensed CO_2 as a rinsing agent. By rinsing with CO_2, the biocatalyst's thermodynamic water activity at its optimum value enables to minimize the loss in enzyme stability and retains the same reaction performance. The washing step allowed all water produced to adsorb onto the enzyme support and thus is eliminated, maintaining the biocatalyst's optimal hydration level [17]. In our experiments the washing step was skipped, however, the lipase activity of all tested enzymes after the reaction was higher in comparison to the activity before the reaction.

Effect of Lipozyme® treatment on production of enantiomers

Lipozyme®, both, as received and after usage as the biocatalyst of the hydrolytic reaction in SC-CO_2, was employed as a biocatalyst for the hydrolysis of substrates **1** and **2** (Scheme 1), and the enantiometric purity of the obtained products **1a**, **1b**, **2a** and **2b** was evaluated. Lipozyme® was selected from all tested lipases because of its high activity after the reaction in SC-CO_2. As shown in Table 3, the untreated form of Lipozyme® produced alcoholic compounds with high enantiometric purity ($ee_p > 99\,\%$), but the conversion of the substrate into the product was lower than with the SC-CO_2 treated enzyme. On the other hand, the enantiomeric purity of the product of hydrolysis of substrate **1** catalyzed by "treated" Lipozyme® was rather low. The best results with excellent enantioselectivity (E = 472) were obtained when substrate **2** was hydrolyzed by Lipozyme® treated with SC-CO_2. The spectral data of reaction products were in agreement with previously published data [18, 19].

We can conclude that the exposure of enzymes to SC-CO_2 in a continuous-flow reactor has a positive effect on the activity of the examined lipases, which is in agreement with the recent experimental results published by Giessauf and Gamse [9], Bauer *et al.* [10, 11], and Yan *et al.* [12], but opposite to those reported by Oliveira *et al.* [19].

Experimental Section

Enzymes

Eleven lipases purchased from Fluka were examined: porcine pancreas lipase (PPL, powder), Lipase A, recombinant from *Candida antarctica* (LA, beige powder), Lipase B, covalently linked to carriers from microorganisms (LB, lyophilized, slightly yellow powder), lipase from *Candida cylindracea* (CCL, immobilized in Sol-Gel-AK), lipase from *Penicillium roqueforti* (PRL, fine powder), lipase from *Aspergillus niger* (ANL, fine powder), lipase from *Rhizopus arrhizus* (RAL, fine powder), lipase from *Mucor miehei* (MML, immobilized in Sol-Gel-AK), lipase from *Mucor miehei* Lipozyme® (L, immobilized on a macroporous ion-exchange resin granular, brown), lipase from *Pseudomonas cepacia* (PCLa, immobilized in Sol-Gel-AK on sintered glass), and lipase from *Pseudomonas cepacia* (PCLb, immobilized in Sol-Gel-AK). The enzyme activity declared by the supplier is given in Table 2. Enzyme activity is defined for hydrolysis of triacylglycerols as indicated in the Table except for lipases PCLa and PCLb, where 1 U is the amount of enzyme which forms 1 % octyl laurate (GC, area percent) from 0.5 mmol lauric acid and 1.0 mmol 1-octanol in 10 mL of water-saturated isooctane in 1 h at 20 °C.

Chemicals

The lipase Substrate® was purchased from Fluka. Blackcurrant oil was extracted from blackcurrant (*Ribes nigrum*)

seeds, a waste by-product of blackcurrant juice production, with SC-CO_2 at a pressure of 27.5 MPa and a temperature of 45 °C [20]. The oil was fractionated by column chromatography, and the fraction consisting solely of triacylglycerols was used in this work. Substrates **1** and **2** were chemically synthesized as described before [13]. All solvents (light petroleum ether, absolute ethanol, benzene, pyridine) of analytical grade, 3,3,3-trifluoro-2-phenylpropanoyl chloride and 4-(dimethylamino)pyridine were purchased from Fluka.

Hydrolysis in supercritical CO_2

The reaction was carried out in the apparatus shown schematically in Fig. 2. Carbon dioxide from a cylinder was pumped by an ISCO 260 syringe pump at an operating pressure of 15 MPa and at a flow rate of 0.7 – 0.9 g min^{-1} to a series of three stainless steel columns. The first two columns of 12 mL volume each served as saturators. The first column was filled with distilled water on glass beads, the second one with blackcurrant seed oil on glass beads. The solution of both substrates in CO_2 flowed to the reactor, a column of 4 mL volume filled with enzyme (2 U) and glass beads. The oil saturator was immersed together with the reactor in a water bath maintained at 40 °C. The solution of the reaction mixture was expanded to ambient pressure in a micrometer valve and collected in a vial. The amount of reaction mixture dissolved in 1 g of supercritical solvent was determined gravimetrically as 1.4 mg g^{-1} CO_2; the amount of free fatty acids in the vial (F, μmol) was determined using the colorimetric method according to Kwon and Rhee [21]. The enzyme activity in SC-CO_2 was calculated dividing F by the amount of enzyme in the reactor and the time of collecting the sample of the reaction mixture. After initial stabilization of the concentration profile in the reactor when approximately 10 g of CO_2 were passed, three samples were collected. The apparatus was then depressurized, the enzyme was separated from glass beads and stored for its activity assessment at ambient pressure. Altogether, each enzyme was exposed to SC-CO_2 for 110 – 140 min, and the amount of SC-CO_2 passed through the enzyme was 80 – 120 g.

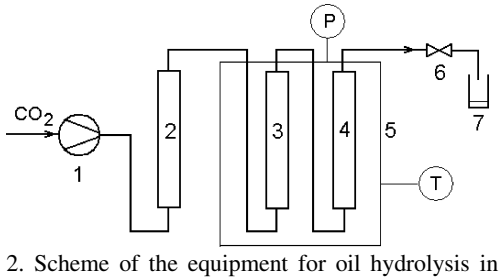


Fig. 2. Scheme of the equipment for oil hydrolysis in supercritical CO_2. (1) syringe pump, (2) saturator with water, (3) saturator with oil, (4) reactor with enzyme, (5) water bath, (6) micrometer valve, (7) vial with reaction mixture.

Enzyme activity assessment

The activity of enzymes was determined before and after their reaction in SC-CO_2. The assay mixture consisted of 15 mL 0.03 M phosphate buffer (pH = 8.0), 5 mL Lipase Substrate® (4.5 mM triolein with 1 M NaCl) and enzyme. The reaction was carried out for 30 min at 40 °C. The lipolytic activity was expressed as μmol of free fatty acids released by 1 mg of enzyme per minute. The lipase activity was quantified by titration with 10 mM NaOH in a temperature-controlled (40 °C) vessel with stirring.

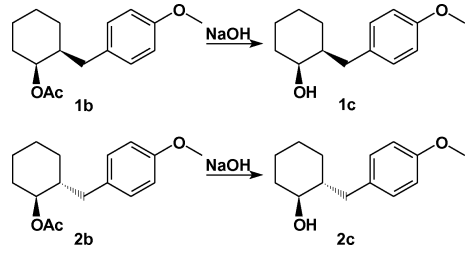
Chemical methods

a) Methods for the enantiomer synthesis

Substrate **1** was hydrolyzed to **1a** and **1b** and substrate **2** to **2a** and **2b** according to Scheme 1. Lipozyme® (1 U, unused or used in SC-CO_2) was added to a suspension of the substrate (20 mg; 0.076 mmol) in 0.2 M phosphate buffer (10 mL, pH = 7.0). The reaction was performed in stirred flasks at 25 °C for more than 6 d and monitored by TLC. The organic compounds were then extracted with diethyl ether and dried (Na_2SO_4). The solvent was evaporated, and the residue was separated chromatographically.

*b) Alkaline hydrolysis of **1b** and **2b** to enantiomers **1c** and **2c***

To determine the absolute configurations of **1b** and **2b**, these compounds were transformed to chiral alcohols **1c** and **2c** by alkaline hydrolysis (Scheme 2). 2-(4-Methoxybenzyl) cyclohexyl acetate **1b** or **2b** (5 mg, 0.019 mmol) was dissolved in a 1 M solution of sodium hydroxide in absolute ethanol (2 mL). The solution was stirred for 4 h at 25 °C, and the reaction course was followed by TLC. The solvent was then evaporated under reduced pressure. The respective chiral isomeric products **1c** and **2c** were separated and purified by column chromatography.



Scheme 2. Alkaline hydrolysis of esters **1b** and **2b**.

c) 3,3,3-Trifluoro-2-methyl-2-phenylpropanoic acid (MTPA) esters

Absolute configurations of alcohols **1a**, **1c**, **2a** and **2c** were determined on the basis of NMR analysis of their diastereoisomeric Mosher's (MTPA) esters. A general proce-

dure for the preparation of MTPA esters on a milligram scale starting from MTPA chloride is described in detail elsewhere [22]. In a typical experiment a solution of either of the enantiomers of 3,3,3-trifluoro-2-methyl-2-phenylpropanoyl chloride (0.07 mmol) in benzene (500 μL) and a solution of 4-(dimethylamino)pyridine (0.005 mmol) in pyridine (30 μL) were added to a solution of the chiral alcohol **1a**, **1c**, **2a** or **2c** (0.05 mmol) in benzene (200 μL). The mixture was stirred at ambient temperature for 3 – 5 h. Thereafter benzene was evaporated and the residue dissolved in light petroleum ether and purified by column chromatography.

Analytical methods

The ^{1}H and ^{13}C NMR spectra were recorded on a Varian UNITY 500 spectrometer (in FT mode) at 499.8 and 125.7 MHz, respectively, either in $CDCl_3$ using tetramethylsilane (δ = 0 ppm) as internal reference or in [D_6]acetone using the central line of the solvent (δ = 2.13 ppm) as internal reference. The ^{19}F NMR spectra were recorded at 470.27 MHz in $CDCl_3$ using hexafluorobenzene as external reference ($\delta = -162.9$ ppm).

Column chromatography was performed on silica gel 60 (Fluka), TLC on precoated silica gel TLC plates. A column (250 × 4 mm) filled with a Biosphere Si-100 (5 μm; Watrex, Prague, Czech Republic) was employed for HPLC analysis of the MTPA esters of **1a**, **2a**, **1c** and **2c** using light petroleum/diethyl ether (9 : 1 v/v) as mobile phase at 1 mL min^{-1}. Chiral products were separated on a chiral Nucleodex β-OH column (150 × 4 mm; Macherey-Nagel, Düren, Germany) using methanol/water (4 : 1 v/v) as mobile phase at a flow rate of 0.3 mL min^{-1}. The detection of the compounds during the HPLC analysis was at 220 and 275 nm, and the eluate was monitored from 200 to 300 nm using a diode array detector.

Acknowledgements

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