ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 9 (2000) 57–64

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

A simple process for increasing the specific activity of porcine pancreatic lipase by supercritical carbon dioxide treatment

Andreas Gießauf *, Thomas Gamse

Institut für Thermische Verfahrenstechnik und Umwelttechnik, Inffeldgasse 25, A-8010 Graz, Austria

Received 24 February 1999; accepted 27 July 1999

Abstract

Treatment of crude porcine pancreatic lipase preparations (powders) with supercritical carbon dioxide (SC-CO_2) at 75°C and 150 bar leads to a time-dependent increase of enzyme activity (activities were measured prior and after SC-CO_2 treatment). After 24 h of incubation in SC-CO_2, a maximum activity of 860% was measured compared to the untreated enzyme (using 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester as substrate). SC-CO_2 treatment caused a weight loss of about 4%. 0.47% of the weight loss (mean of three independent experiments) were separated as an oily extract in a tube that was connected with the extractor and through which depressurization was performed. In the extract, free fatty acids were found (mainly palmitic acid, stearic acid and oleic acid). No chemical modification on the protein reactive groups were found as studied by determination of reactive free amino groups, determination of free –SH groups and by determination of the carbonyl content (as an oxidation marker). No significant difference in the protein content between the untreated and SC-CO_2 processed lipase powders were found. Tryptophan fluorescence emission spectra showed no change of the emission maximum indicating no larger protein conformational change. SC-CO_2-treated lipase showed no loss of its increased activity after storage in a freezer for 6 months.

Keywords: Lipase; Supercritical carbon dioxide; Protein reactive groups; Fatty acids; Fluorescence

1. Introduction

Lipase from porcine pancreas (PPL) is a triacylglycerol lipase (EC 3.1.1.3) with a sequence of 449 amino acid residues [1–3] and seven disulfide bonds [4]. Porcine pancreatic lipase has been the most intensively characterized pancreatic lipase [5]. Its biochemical and biophysical properties are reviewed by Gargouri et al. [6]. PPL is used as a biocatalyst in the food industry and for flavour production [7] and for organic synthesis, e.g., for regioselective esterification, for enantioselective hydrolysis of *meso*-compounds, for the regioselective acylation of hydroxy compounds, for the lipase catalyzed synthesis of sugar esters and for several other utilizations in industrial scale as reviewed in Ref. [8]. PPL has been already used as a biocatalyst for the esterification of glycidol [9,10] and for the enzymatic hydrolysis of triolein and its partial glycerides in SC-CO_2 [11]. The research activities on enzymatic catalysis

* Corresponding author. Lehrstuhl für Organische Chemie und Biochemie, Technische Universität, München, Lichtenbergstraße 4, D-85747 Garching, Germany. Fax: +49-89-289-13363

1381-1177/00/$ - see front matter
PII: S1381-1177(99)00084-3

in SC-CO_2 are reviewed by Aaltonen and Rantakylä [12], Russell et al. [13], and Kamat et al. [14]. In preceding studies, the stabilities (against pressurization/depressurization steps, the thermal stability and the long term stability) of several enzymes (lipase from *Candida rugosa* supplied by Sigma [15], lipase AY from *C. rugosa* supplied by Amano [16], lipase PS from *Pseudomonas species* from Amano [16] and esterase EP10 from *Burkholderia gladioli* [15]) have been compared. Lipase from *C. rugosa* (Sigma) [15] showed higher stability against pressurization/depressurization steps and a 24-h incubation at 75°C and 150 bar than the above-listed enzymes and esterase from porcine liver (Sigma) [16]. In this study, the unusual behaviour of porcine pancreatic lipase after treatment with dry SC-CO_2 that can be possibly used for production of highly active crude PPL powder is described.

2. Experimental

All chemicals used if not otherwise stated were purchased from Merck (Darmstadt) and were of pro-analysis quality. 1,2-*O*-Dilauryl-*rac*-glycero-3-glutaric acid resorufin ester (DGGR) and Thesit (a trademark for polyoxyethlylene-9-lauryl ether) were supplied by Boehringer Mannheim. 2,4-Dinitrophenyl-hydrazine was supplied by Sigma. Bovine serum albumin (fatty acid free, purity $>98\%$) was a kind gift of Hämosan (Graz). The carbon dioxide with a purity $>99{,}94\%$ (v/v) and a dew point lower than -60°C was purchased from Linde (Graz) and stored in a tank with a capacity of 3200 l. 2,4,6-Trinitrobenzenesulfonic acid (TNBS; purum; 1 M in water) and 5,5′-dithiobis(2-nitrobenzoic acid) ($\sim 99\%$) were supplied by Fluka (Buchs). Lipase from hog pancreas with an activity of 23.3 U/mg solid was supplied by Fluka and lipase from porcine pancreas with an activity of 20 U/mg solid from Serva (Heidelberg) (activities given by the suppliers). In this article, PPL is a lipase from pig or hog. The lipase purchased by Fluka was stored at -20°C and the lipase from Serva at $+4$°C as recommended by the suppliers.

2.1. Treatment of lipase with SC-CO_2 and determination of enzyme activity

For the first experiments, ~ 40 mg of lipase from porcine pancreas (Serva) were balanced on a filter paper and the folded filter was put inside the bottom of an enzyme reactor (140 ml).

2.1.1. Thermal stability

Lipases were incubated for 1 h at temperatures between 35°C and 105°C and 150 bar in an enzyme reactor placed in a water bath (filled with water or diethylene glycol).

2.1.2. Change of activity with incubation time

Incubation of PPL was performed up to 24 h at 75°C in SC-CO_2 (the filters were always put into a preheated reactor).

2.1.3. Stability against pressurization / depressurization steps

Enzymes were incubated for 1 h at 150 bar and 35°C before each depressurization. Immediately after pressure reached atmospheric values, pressurization was started again. During the night, lipase samples from Serva were stored at 4°C until all 30 pressurization/depressurization steps were finished. For all other studies, lipase from Fluka was used. For the study of increase of activity of the enzyme in SC-CO_2 (at 75°C/24 h and 150 bar) ~ 500 mg of lipase from porcine pancreas was weighed in a 25-ml beaker closed with a filter paper fixed with a filament. This beaker was put into an enzyme reactor and the reactor was weighed in a water bath filled with diethylene glycol.

2.2. Determination of enzyme activities

After SC-CO_2 treatment, the filters were removed from the enzyme reactor. The filters

were put in plastic tubes (closed with a screw cap) and stored under identical conditions as the untreated PPL in a refrigerator or freezer for at least 12 h before analysis.

2.2.1. Lipase assay

100 μl of a substrate solution (6 mg DGGR was dissolved in 12 ml of a 1:1 mixture of dioxan/Thesit (10% (w/v) in distilled H_2O)) was added to 900 μl of the enzyme solutions (0.1 M KH_2PO_4, pH = 6.8, enzyme concentration: 0.555 mg PPL/ml). The increase of absorbance at λ 572 nm after a lag time of 100 s was used to measure the enzyme activities. All absorbance measurements were performed at 25°C with a Shimadzu UV160A spectrophotometer equipped with a Lauda RM6 temperature control. Unit definition: One unit of lipase liberates 1.0 μmole of resorufin from DGGR per minute at pH = 6.8 and 25°C.

2.3. Protein determination

For measuring the protein content, the method described by Bradford [17] was used. In detail, the reagent solution was prepared by dissolving 100 mg SERVA Blue G in 50 ml 95% ethanol. After addition of 100 ml 85% (w/v) H_3PO_4 the solution was diluted with distilled water to a volume of 1 l. All sample solutions (and bovine serum albumin standard solutions) were prepared by dissolving the proteins in 0.15 M NaCl solution. 100 μl of the standard solutions (protein concentration 0.1–1 mg/ml) and the sample solutions (1 mg/ml) were mixed with 5 ml of the Bradford reagent solution. After 5 min, the absorbance at 595 nm was read against a blank.

2.4. Measurement of weight loss and extract analysis

For the determination of the weight loss of PPL, a weighed portion of ~500 mg (exact values see Table 1) was balanced into a 25-ml beaker closed as already described. This beaker was placed into an preheated extractor (volume 300 ml) and incubation for 24 h at 150 bar and 75°C was performed using a Spe-ed SFE instrument (from Applied Separations). The beaker plus the enzyme powder was balanced before and immediately after treatment with SC-CO_2. The extract was disengaged in a glass-tube connected with the CO_2 outlet. Before and after depressurization, the glass-tube was balanced. After depressurization, the extract was put at once into a freezer and stored at −20°C before analysis. For analysis by gas chromatography esterification of free fatty acids to fatty acid methyl esters (FAME) was performed by using BF_3/MeOH as esterification reagent with small modification as described by Sattler et al. [18] for lipoprotein extracts. To the extract, 0.5 ml of toluene and 1.0 ml BF_3/MeOH were added and esterification was performed at 110°C for 90 min with Teflon screw-capped tubes in a dryer. After cooling, 2 ml distilled water were added and extracted three times with *n*-hexane (2 ml). The extracts were brought to dryness under a stream of nitrogen at room temperature. The residuals were dissolved in 300 μl methylene chloride and used for analysis by gas chromatography.

Table 1
Increase of activity of PPL (Fluka) by incubation of 500 mg in SC-CO_2 (75°C, 150 bar)

Incubation time [h]	Activity increase [U/g]	Relative activity [%]
Untreated enzyme	0.57 ± 0.18	100
1	1.90 ± 0.21	333
8	2.53 ± 0.22	444
24	3.43 ± 0.40	602
50	3.40 ± 0.43	596

2.5. Analysis of FAME by gas chromatography

FAME were chromatographed on a 60 m × 0.323 mm i.d. DB-WAX column (J&W Scientific) with a film thickness of 0.5 μm. Analysis was performed with a Hewlett Packard HP 5890 Series II chromatograph equipped with a tem-

perature controlled autosampler (2°C) and a flame ionization detector (FID, detector temperature 300°C). Hydrogen was used as a carrier gas at a flow rate of 0.934 ml/min. Following temperature program was used: 210°C (4 min), 210°C–240°C (5°C/min) and 240°C.

2.6. Determination of moisture content of untreated / SC-CO_2-treated PPL

To determine which percentage of the weight loss after SC-CO_2 treatment (75°C/24 h per 150 bar) is caused by release of moisture from the enzyme powder to carbon dioxide, a Sartorius MA 30 moisture analyzer has been used. 495 mg untreated lipase or 500 mg SC-CO_2-treated lipase were balanced on disposable sample dishes and the moisture content was determined by heating for 7 min at 105°C. These settings were used because no colour change of the enzyme powder was observed and a shorter drying period or lower temperatures caused less weight loss.

2.7. Determination of protein reactive groups

To study if SC-CO_2 treatment (75°C/150 bar per 24 h) causes a chemical modification of reactive groups on the protein, the following methods have been used.

The free thiol (–SH groups) content of the enzyme powder has been determined by the method described by Ellman [19]. A total of 39.6 mg of 5,5′-dithiobis(2-nitrobenzoic acid) were dissolved in 10 ml 0.2 M KH_2PO_4 buffer pH = 7.0. 10 ml of PPL solutions in 0.2 M KH_2PO_4-buffer pH = 8.0 were prepared and insoluble parts were removed by filtering through 0.22 μm cellulose acetate syringe filters. 3-ml protein solution was mixed with 2 ml 0.2 M KH_2PO_4-buffer pH = 8.0 and 5 ml distilled water. 20 μl of the reagent solution were added to 3 ml of this mixture and the absorbances at 412 nm were measured after 40 min against a blank.

Determination of the content of free amino groups (ε $-NH_2$ groups of lysine residues) of the enzyme powder was performed as published in Ref. [20]. 1 ml of the PPL solutions (1 mg/ml) in 4% $NaHCO_3$ pH = 8.5 were prepared and mixed with 1 ml of the reagent solution. The blank solutions contained 1 ml of the PPL solution + 3 ml conc. HCl (37%; w/w) solutions + 1 ml reagent. This solutions were incubated for 2 h at 40°C. The samples were treated exactly in the same way as described in Ref. [20]. The absorbance at 346 nm (measured against a blank) was used to compare to number of free $-NH_2$ of untreated or SC-CO_2 PPL.

The carbonyl content of PPL was determined by the method described by Fields and Dixon [21]. A reagent solution of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl was prepared. PPL solutions with 0.25, 0.5 and 1 mg/ml in 0.2 M KH_2PO_4 pH = 6.9 were prepared and filtered through a 0.22-μm cellulose acetate syringe filter. A 0.3-ml portion of 10 M HCl was added to 2.1 ml of a PPL solution before mixing with 0.1 ml of the reagent solution. After incubation for 90 min (time until the absorbance at 370 nm reached a constant value), the solutions with PPL concentrations > 0.25 mg/ml became turbid. Therefore, the solution with the lowest protein concentration (0.25 mg/ml PPL) was used for determination of the carbonyl content.

2.8. Tryptophan fluorescence

The lipase was dissolved in the same buffer as used for the activity measurements. The emission spectra of the clear solution (after filtering through a 0.22-μm cellulose acetate syringe filter) were recorded at 30°C with a Perkin Elmer LS50B spectrofluorimeter (instrument settings: excitation wavelength 280 nm, emission wavelengths 300–420 nm, excitation/emission slit 3 nm, scan speed 100 nm/min) equipped with a temperature control. The emission spectra are the mean over five scans.

3. Results and discussion

3.1. Treatment of lipase with SC-CO_2 and determination of enzyme activity

In Fig. 1(a), the change of activity after treatment with SC-CO_2 of lipase from porcine pancreas (Serva) is shown. Incubation for 1 h at temperatures below 65°C leads to no significant loss/increase of enzyme activities as observed for several other enzymes [15,16]. Interestingly, an incubation for 1 h at 75°C and 150 bar leads to an increase of activity to a value of 242.6% of the initial activity (0.453 U/g solid). Compared to 75°C, the increase of the specific activity at temperatures between 85°C and 105°C

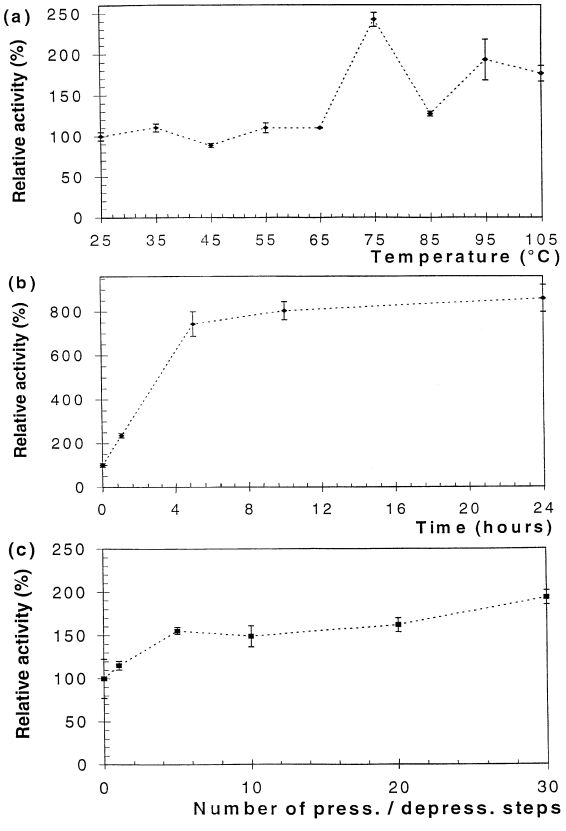

Fig. 1. Effect of treatment of 40 mg PPL (Serva) with SC-CO_2 in a 140-ml enzyme reactor: (a) activity change after 1 h incubation at a pressure of 150 bar, (b) dependency of activity increase with incubation time at 75°C and 150 bar, (c) activity change after pressurization/depressurization steps after 1 h incubation at 150 bar and 35°C.

were always lower (Fig. 1(a)). For esterase from *P. marginata* [15], lipase from *C. rugosa* (Sigma) [15], lipase from *P. species* (Amano) [16] and lipase AY from *C. rugosa* (Amano) [16], no such effect was observed. Only a decrease or no effect on enzyme activity was caused by treatment with SC-CO_2 at temperatures > 75°C. For the increase of the activity of PPL, a time-dependent effect was observed. As shown in Fig. 1(b), within the first hours of incubation of 40 mg PPL in a 140-ml reactor, a rapid increase of activity took place reaching an activity of 3.90 U/g enzyme powder (860% activity increase) after 24 h of incubation. The effect of several pressurization/depressurization steps on PPL is shown in Fig. 1(c). Even after several pressurization/depressurization steps PPL showed an increase of activity to a value of 0.88 U/g enzyme powder (191% activity increase). Kasche et al. [22] and Lozano et al. [23] showed that pressurization/depressurization steps are causing enzyme inactivation and at previous studies in our department only a loss of enzyme activity or no influence on activity after several steps [15,16] was observed. Until now, there has been only one report that by treatment with SC-CO_2, an increase of enzyme activity was observed, but this effect is very small compared to the results presented in this work: Kamihira et al. [24] showed that treatment with SC-CO_2 (200 atm, 2 h, 35°C) leads to a small increase of activity of α-amylase in presence of microorganisms (21% and 35% activity increase) under conditions as used for sterilization.

For further experiments, lipase from hog pancreas (Fluka) was used to check if this effect occurred only with the crude PPL preparation supplied by Serva. The results obtained by PPL from Fluka were similar (data not shown). Instead of ~40 mg PPL for further experiments, 500 mg of PPL (Fluka) have been used. The increase of the activity of PPL is shown in Table 1. The enzyme powder that has been incubated for 24 h at 75°C showed the highest activity increase and is used for all further

Table 2
Weight loss of lipase from porcine pancreas by SC-CO_2 treatment (75°C, 150 bar, 24 h)

Weighed portion of lipase [mg]	Tube + enzyme before SC-CO_2 treatment [g]	Tube + enzyme after SC-CO_2 treatment [g]	Weight loss [mg]	Extract [mg]
498.0	13.7710	13.7503	20.7 (4.2%[a])	1.2
504.1	13.4615	13.4423	19.2 (4.1%[a])	2.0
519.9	24.9536	24.9243	29.3 (5.6%[a])	4.1

[a]Relative loss of weight in percent compared to weighed portion balanced immediately after depressurization.

comparisons between untreated and SC-CO_2-treated PPL.

No further increase of the activity (compared to the activity after 24 h) was observed after incubation of PPL for 50 h. A similar increase of enzyme activity was observed if 500 mg enzyme powder were incubated for 24 h at 75°C and 150 bar in a 300-ml extractor (615% increase of activity) or in a 140-ml enzyme reactor (602% increase of activity). PPL was also incubated for 24 h at 75°C in a dryer (under air oxygen at atmospheric pressure) to exclude the possibility that this effect is only caused by partial thermal unfolding of the protein. Only a small increase of the activity from 0.57 U/g (untreated) to 0.91 ± 0.09 U/g (159.7% activity increase; 75°C) solid was observed compared to the activity after SC-CO_2 treatment 3.40 U/g.

3.2. Protein determination

The protein content of PPL supplied by Serva was determined with $5.7\% \pm 0.7\%$ (mean ± SD; $n = 4$; w/w) and of PPL supplied by Fluka with $15.3\% \pm 0.4\%$ ($n = 4$; w/w). PPL treated for 24 h with SC-CO_2 (75°C, 150 bar) had a protein content of $14.9\% \pm 0.5\%$ ($n = 4$; w/w). These results indicate no significant difference ($P < 0.05$) of the protein content before and after SC-CO_2 treatment. By the fact that no change of the protein content occurs, the relative activity changes of the activity in U/g solid are identical with the changes of the specific activity in U/g protein.

3.3. Weight loss and extract analysis

In Table 2, the weight loss of PPL after treatment with dry SC-CO_2 of three extractions with ~500 mg enzyme powder is listed. A mean weight loss of $4.6\% \pm 0.8\%$ (by exposure for several minutes to air humidity, this value decreases) was observed, but in the class tube connected with the outlet, only $0.47\% \pm 0.28\%$ (mean ± SD; $n = 3$) of the extract was found. After derivatisation as used for free fatty acid analysis, the fatty acids listed in Table 3 were identified in the extract. The main components found were palmitic acid, stearic acid and oleic acid. The fact that by this procedure unsaturated free fatty acids are removed leads to the speculation that the storage life of the SC-CO_2-treated PPL may increase (because unsaturated free fatty acids oxidize under air oxygen and may inactivate PPL). By using a moisture analyzer (see Section 2.6) a weight loss (moisture content) of 4.99% was observed for untreated PPL and only 3.13% for SC-CO_2-treated PPL. This means that part of the weight loss of SC-CO_2-treated PPL is water. The water content observed by us for untreated PPL (Fluka) is very similar to the value of 5.5% determined by others [10] for crude PPL (Sigma) by the Karl–Fischer titration.

Table 3
Fatty acid analysis of the extract obtained from PPL as described in Section 2.5 and analyzed by gas chromatography (see Section 2.6). The total peak area within a retention time of 16 min was set 100%

Free fatty acid	Relative peak area [%]
Myristic acid (C14:0)	3.3
Palmitic acid (C16:0)	35.3
Palmitoleic acid (C16:1)	1.6
Stearic acid (C18:0)	23.6
Oleic acid (C18:1)	18.5
Linoleic acid (C18:2)	3.6
Relative area of all identified peaks	85.9

3.4. Determination of protein reactive groups

The protein sequence of PPL (sequence obtained from the SWISS-PROT protein database) contains 14 cysteine residues (amino acid position 4, 10, 90, 101, 103, 181, 237, 261, 285, 296, 299, 304, 433, 449). Six disulfide bridges were investigated using a variety of techniques (Cys-4–Cys-10, Cys-237–Cys-261, Cys-285–Cys-296, Cys-299–Cys-304 and Cys-433–Cys-449) including an isomeric form (Cys-90–Cys-101 or Cys-90–Cys-103) [4]. By using the method described by Ellman et al. [19], no free accessible –SH groups were determined. The absorbance values at 412 nm were -0.0003 ± 0.0011 ($n = 3$) for untreated PPL and -0.0026 ± 0.00058 ($n = 3$) indicating the absence of free accessible thiol groups in the enzyme powder and no change of the number –SH groups by SC-CO_2 treatment (no free accessible –SH groups are formed by cleavage of disulfide bridges).

PPL contains 21 lysine residues (amino acid position 36, 42, 69, 80, 91, 95, 107, 136, 197, 232, 239, 295, 317, 341, 349, 350, 373, 397, 399, 419, 428). By determination of the free amino groups by the method described in detail in Section 2.7, the absorbance values at 346 nm were 1.038 ± 0.049 ($n = 6$) for untreated PPL and 1.051 ± 0.054 ($n = 6$) for SC-CO_2-treated PPL. There is no significant difference ($P < 0.05$) in the number of free $-NH_2$ and the possibility that reactive decomposition products (formed during incubation of crude PPL at 75°C) modify the free amino groups can be excluded. In case of subtilisin, it has been given a physical demonstration by laser desorption mass spectroscopy (LD-MS) that carbon dioxide covalently modify proteins [25]. Kamat et al. [25] speculate that carbon dioxide form covalent complexes with free amino groups on the surface of the enzyme.

Carbonyl formation has been used as an early marker for various oxidative modifications of amino acids in proteins [26]. The absorbance values at 370 nm are for the untreated PPL 0.116 ± 0.013 ($n = 8$) and for SC-CO_2-treated PPL 0.104 ± 0.019 ($n = 8$). These results indicate no significant modification ($P < 0.05$) of the carbonyl content prior and after SC-CO_2 treatment. Decomposition of amino acid residues or impurities under formation of carbonyl groups was not observed.

3.5. Tryptophan fluorescence

PPL contains six tryptophan residues well distributed over the whole protein sequence (amino acid position 17, 85, 116, 252, 338 and 402). Fluorescence emission maxima of different proteins were found in a large wavelength range between 308 and 352 nm [27]. The blue shift of fluorescence emission of Trp-residues in proteins compared to free Trp is interpreted as being due to the shielding of the tryptophan residues from the aqueous phase and it is not surprising that for small proteins, there is a substantial contact of the tryptophan residues with the aqueous phase [27]. The emission maxima of PPL upon excitation at 280 nm were found at 341 nm (several Trp residues are exposed to the aqueous phase) for both untreated and SC-CO_2-treated PPL and no change of the band shapes of the emission spectra occurred (data not shown). These results indicate no larger conformational change by SC-CO_2 treatment.

4. Conclusions

This is the first report that an enzyme activity increases several-fold after treatment with SC-CO_2. The procedure described here has several advantages (it is not labor-intensive, there is no loss of enzyme powder and no solvent residues remain in the PPL powders). It may be used for PPL powders to increase the specific activity of this industrially important biocatalyst but further tests have to be performed if SC-CO_2-treated PPL shows changes in its regio- or enantioselectivity.

Acknowledgements

This work was supported by a grant of the Austrian Science Fund (FWF project No. 12151 CHE). Fluorescence spectra were recorded at the Institut für Biochemie, Karl-Franzens University Graz.

References

[1] J.D. De Caro, M. Boudouard, J.J. Bonicel, A.A. Guidoni, P. Desnuelle, M. Rovery, Biochim. Biophys. Acta 671 (1981) 129.
[2] J.D. Bianchetta, J. Bidaud, A.A. Guidoni, P. Desnuelle, M. Rovery, Eur. J. Biochem. 97 (1979) 395.
[3] A.A. Guidoni, J.J. Bonicel, J.D. Bianchetta, M. Rovery, Biochimie 61 (1979) 841.
[4] F. Benkouka, A.A. Guidoni, J.D. De Caro, J.J. Bonicel, P.A. Desnuelle, M. Rovery, Eur. J. Biochem. 128 (1982) 331.
[5] R. Verger, in: B. Brogström, H.L.Brockman (Eds.), Lipases, Elsevier, Amsterdam, 1984, p. 84.
[6] Y. Gargouri, H. Moreau, R. Verger, Biochem. Biophys. Acta 1006 (1989) 255.
[7] T. Godfrey, S. West (Eds.), Industrial Enzymology, Data Index 3, 2nd edn., Macmillan, London, 1996, p. 555.
[8] R.D. Schmid, R. Verger, Angew. Chem., Int. Ed. 37 (1998) 1609.
[9] J.F. Martins, T. Corrêa de Sampaio, I. Borges de Carvalho, S. Barreiros, Biotechnol. Bioeng. 44 (1994) 119.
[10] J.F. Martins, I. Borges de Carvalho, T. Corrêa de Sampaio, S. Barreiros, Enzyme Microb. Technol. 16 (1994) 785.
[11] G. Glowacz, M. Bariszlovich, M. Linke, P. Richter, C. Fuchs, J.-T. Mörsel, Chem. Phys. Lipids 79 (1996) 101.
[12] O. Aaltonen, M. Rantakylä, CHEMTECH 21 (1991) 240.
[13] A.J. Russell, E.J. Beckman, A.K. Chaudhary, CHEMTECH 24 (1994) 33.
[14] S.V. Kamat, E.J. Beckman, A.J. Russell, Crit. Rev. Biotechnol. 15 (1995) 41.
[15] A. Gießauf, T. Gamse, E. Klingsbichel, H. Schwab, R. Marr, in: A. Ballesteros, F.J. Plou, J.L. Iborra, P.J. Halling (Eds.), Progress in Biotechnology, Vol. 15, Elsevier, Amsterdam, 1998, p. 471.
[16] A. Gießauf, W. Magor, D.J. Steinberger, R. Marr, Enzyme Microb. Technol. 24 (1999) 577.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] W. Sattler, H. Puhl, M. Hayn, G.M. Kostner, H. Esterbauer, Anal. Biochem. 198 (1991) 184.
[19] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[20] M.L. Kakade, I.E. Liener, Anal. Biochem. 72 (1969) 273.
[21] R. Fields, H.B.F. Dixon, Biochem. J. 121 (1971) 587.
[22] V. Kasche, R. Schlothauer, G. Brunner, Biotechnol. Lett. 10 (1988) 569.
[23] P. Lozano, A. Avellaneda, R. Pascual, J.L. Iborra, Biotechnol. Lett. 18 (1996) 1345.
[24] M. Kamihira, M. Taniguchi, T. Kobayashi, Agric. Biol. Chem. 51 (1987) 407.
[25] S. Kamat, G. Critchley, E.J. Beckman, A.J. Russell, Biotechnol. Bioeng. 46 (1995) 610.
[26] A.Z. Reznick, L. Packer, Methods Enzymol. 233 (1994) 357.
[27] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Chap. 11.3, Plenum, New York, 1983, p. 354.