Enzyme-Catalyzed Reaction of Vegetable Oil in Supercritical CO_2 in a Continuous-Flow Reactor

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This contribution contains the results of two experiments:
1. Hydrolysis of black cumin oil catalysed by immobilised enzyme Lipozyme. The activity and stability of the enzyme in supercritical carbon dioxide (SC-CO_2) was proved by preceding experiments [1]. Reaction products were analysed for the degree of oil conversion to free fatty acids. The difference in CO_2 solubility of major oil components, triacylglycerols, and that of minor components like phytosterols is small. Thus, the aim was to increase the difference in the solubility by quantitative conversion of triacylglycerols (and also di- and monoacylglycerols, if present) to more soluble free fatty acids, so that a partial fractionation of the mixture and concentration of minor components in the less soluble fraction is possible.
2. Measurement activity in SC-CO_2 of enzymes prepared in laboratory of Institute of Organic Chemistry and Biochemistry. Blackcurrant oil hydrolysis was catalysed by the tested enzymes and reaction products were analysed for the degree of conversion from which their activity was evaluated, and the composition of free fatty acids was compared to the composition of oil substrate. The enzyme activity was measured also before and after the reaction in SC-CO_2.

INTRODUCTION

Supercritical carbon dioxide (SC-CO_2) is a suitable reaction medium for non-polar substrates like vegetable oils. Compared to conventional liquid solvents, the rate of heterogeneous reactions is higher due to the good transport properties of supercritical fluids, CO_2 can be easily separated from reaction mixture by decrease in pressure, and the products are not contaminated by toxic substances. Different enzymes, including lipases, have exhibited a sufficient activity and stability in SC-CO_2, on condition that optimum moisture is maintained. This enables us to utilize the advantages of supercritical solvent and specificity of enzyme-catalyzed reactions.
Blackcurrant seed oil is rich in both α- and γ-linolenic acids which makes it a valuable source of PUFA [1]. Black cumin *(Nigella sativa)* seeds are used exclusively for edible and medical purposes [2]. The seeds contain 30-40 wt % oil, and the main fatty acids present are linoleic (58-62 wt. %), oleic (22-24 wt. %), and palmitic (13-15 wt. %) acids. The most important property of black cumin seeds is its high content of lipase enzymes [3]. In dormant seeds, lipase enzymes are generally inactive, but when the seeds are ground to obtain the oil, the lipase and oil come into contact, and enzymatic hydrolysis reactions commence immediately. Thus, grinding the sample may increase the free fatty acid content (FFA wt. %) of oil to 50 % or more, depending on the time between grinding and extraction, the storage temperature of ground seeds, the moisture content of the seeds, and the relative humidity of the storage

medium. This phenomenon can be observed also in other oil-bearing materials that contain active lipase enzymes, such as olive, palm, and rice bran [4].
The aim of the work was hydrolysis of oil triglycerides catalysed by enzymes, both commercial and prepared in our laboratory, and evaluation of the effect of enzyme exposition to supercritical carbon dioxide on their activity.

MATERIALS AND METHODS

Lipozyme®, a 1,3-specific lipase from *Mucor miehei* immobilised on a macroporous ion-exchange resin, was supplied by Fluka Chemie AG, Buchs, as well as chemicals.
Blackcurrant oil was obtained by extraction with SC-CO_2 of blackcurrant seeds (*Ribes nigrum*), which represent a waste by-product in the blackcurrant juice production in Chelcice, CR, where the plant was grown. Only fraction containing triacylglycerols was used as starting material in the hydrolytic reaction [5]. The seeds of black cumin were purchased from a local market in Istanbul (Turkey). Carbon dioxide (>99.9 %) was purchased from Linde Technoplyn, CR.

Enzymes isolated from *Geotrichum candidum*

The strain of *Geotrichum candidum* 4013 was obtained from the Culture Collection of the Department of Biochemistry and Microbiology (DBM), Institute of Chemical Technology, Prague. Culture conditions:
a) *Preparation of inoculum*
For solid culture, the strain of *Geotrichum candidum* 4013 was transferred from a culture slant. Growth medium of the following composition was used: glucose (30 g/l), corn steep (10 g/l), $MgSO_4.7H_2O$ (0.5 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (1 g/l), $NaNO_3$ (2 g/l), KCl (0.5 g/l), $FeSO_4.7H_2O$ (0.02 g/l) and agar (15 g/l). The tubes were incubated for 3 days at 25 °C and conserved at 4 °C. Liquid cultures were prepared with a medium consisting of glucose (30 g/l), corn steep (10 g/l), $MgSO_4.7H_2O$ (0.5 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (1 g/l), $NaNO_3$ (2 g/l), KCl (0.5 g/l), $FeSO_4$ $7H_2O$ (0.02 g/l) in simple Erlenmeyer flasks (250 ml) containing 100 ml of the medium and sealed with aluminium foil. The medium was sterilised for 20 min at 121 °C, inoculated with cells growing on culture slant and incubated by shaking for 24 h at 30 °C.
b) *Activation of lipases*
To determine the lipase activity (extracellular and cell-bound), the medium containing peptone (50 g/l), glucose (10 g/l), $MgSO_4.7H_2O$ (1 g/l), $NaNO_3$ (1 g/l), and olive oil (10 g/l) was used. The medium was inoculated with 10 ml of inoculum. At regular time intervals, cell growth and lipase activity were determined.
To determine the location of lipase activity in *Geotrichum candidum* 4013, lipase activity was measured in whole broth samples, supernatant and pellet samples, and whole cells that had been subjects of freezing by liquid nitrogen. The frozen cells were mechanically broken and centrifuged at 5000 x g. The lipolytic activity of both supernatant and pellet sample were determined as described bellow.
Preparation of enzymes
a) *Extracellular lipase*
Inoculated medium (5 ml) was filtered through a 0.2 μm filter to obtain a filtrate displaying extracellular lipase activity. The filtrate was lyophilized and used as biocatalyst.

b) *Cell-bound lipase*

1.- Cells were harvested by filtration of 100 ml cell suspension (inoculated medium) through a 0.2 μm filter, the remaining retentate was washed in distilled water (100 ml) and ice-chilled acetone (100 ml). Finally, acetone from the pellet was evaporated under atmospheric pressure and ambient temperature. The crude enzyme (acetone powder) was stored in refrigerator under nitrogen.

2.- Cells harvested by filtration of 100 ml cell suspension (inoculated medium) through a 0.2 μm filter were immobilized in calcium alginate by the traditional external gelation method [6]. About 20 ml of sodium alginate (3 % w/v) and 5 ml of cell suspension (5×10^6 cells per ml) were mixed well and this slurry was added drop wise to 0.2 M $CaCl_2{\cdot}2H_2O$ solution at room temperature. The beads (~4 mm) formed were then cured in a refrigerator at 4°C for l h. The beads were washed two to three times with sterile distilled water and used as source of lipase.

Hydrolysis in SC-CO_2 medium

The reaction was conducted in continuous-flow regime using home-made equipment consisting of two or three high-pressure columns in series [1]. SC-CO_2 was pumped to the first column (12 mL, i.d. 8 mm) containing water on glass beads and to the second column of identical geometry with blackcurrant seed oil on glass beads or ground black cumin seeds from where a solution of oil and water in CO2 flowed to the third column (4 mL, i.d. 8 mm) containing lipase where the reaction took place. In the case of seeds the first column with water was not necessary as they contain both oil and water. The products and remaining oil precipitated from the solution flowing out of the reactor after its expansion to ambient pressure in a heated micrometer valve and were collected in a vial. The prevailing reaction temperature was 40 °C, the pressure was 20 MPa for blackcurrant oil hydrolysis and 28 MPa in case of black cumin oil hydrolysis.

Assignment of degree of conversion and activity

The degree of conversion of oil to FFA in a sample of reaction mixture in a vial was estimated from the whole amount of the mixture determined gravimetrically, which is approximately equal to the amount of oil substrate yielding the sample, and the total amount of free fatty acids measured using the colorimetric method according to Kwon and Rhee [9] based on formation of a blue cupric acetate-fatty acid complex. The average enzyme activity was calculated from the amount of FFA in the sample, mass of enzyme in the reactor and the time of sampling. One lipase unit (U) was defined as the amount of enzyme that released 1 μmol FFA per minute.

The activity before and after the enzyme exposure to SC-CO_2 was determined with help of hydrolysis at ambient pressure. The release of yellow *p*-nitrophenol due to hydrolysis of *p*-nitrophenylpalmitate by lipase was measured. A 200-μl reaction mixture that contains 0.25 mM *p*-nitrophenylpalmitate (dissolved in ethanol), 50 mM Tris-HCl (pH 7.5), and 5 mg of crude lipase (prepared as mentioned above) was incubated at 25 °C. Since autohydrolysis of substrates produced low but significant background values at 410 nm, the absorbance in each assay was measured against a substrate-buffer mixture. After 10 min of incubation, the reaction was stopped by the addition of 2 mL of ethanol 96 %, and the *p*-nitrophenol released

was monitored spectrophotometrically at 410 nm. One lipase unit (U) was defined as the amount of enzyme that released 1 μmol *p*-nitrophenol per minute.

Analytical procedures

Preparative TLC.

The products of the hydrolytic reactions were separated into neutral lipids [remaining triacylglycerols (rTG) + diacylglycerols (DG) + monoacylglycerols (MG) and free fatty acids (FFA)] by TLC, and developed in a solvent mixture containing light petroleum-diethyl ether (4:1) mixture. The separation was made using Polygram Sil G pre-coated TLC sheeds (Macherey-Nagel, Germany) with silica gel layer (0.2 mm). Lipid bands were identified using a solution of phosphomolybdic acid (10 % in methanol). Fractions corresponding to each lipid type were extracted from the plates with freshly distilled and dry diethyl ether, evaporated and weighed to calculate yields.

Transesterification of lipid fractions.

The blackcurrant oil or lipid fractions were converted to fatty acid methyl esters according to an earlier described method [7]. The substance (1.5 mg) was dissolved in a chloroform-methanol mixture [2:3 (v/v), 250 μl], and acetyl chloride (29.4 μl) was added. The reaction mixture was heated in a sealed vial at 80 °C for 30 min. After neutralisation with silver carbonate (57.1 μg) and centrifugation of the reaction mixture, the products (fatty acid methyl esters - FAMEs) were analysed by GC.

Gas chromatography (GC) analysis of FAMEs.

The GC analyses were performed using a HP 5890 A (Hewlett-Packard, USA) gas chromatograph equipped with a HP 3393A integrator, a flame ionisation detector (FID), a split-splitless injector (split ratio 1:49) and a DB-WAX column (30 m x 0.25 mm x 0.25 μm; J and W Scientific, USA). Hydrogen was used as a carrier gas at a flow rate of 40 cm s^{-1}. The injector and detector temperatures were 240 and 250 °C, respectively. Oven temperature was 200°C. The peaks of FAMEs were identified using equivalent chain length values [8].

RESULTS

Black cumin oil hydrolysis catalysed by Lipozyme

The content of free fatty acids in the reactor effluent mixture was changing during the extraction of a batch of black cumin (Fig. 1). The oil itself contains a certain amount of FFA. As FFA are more soluble in SC-CO_2 than triacylglycerols, they are extracted preferably and in the experiment without enzyme (blue points) they were soon depleted. The hydrolysis of triacylglycerols maintains the concentration of FFA in extract (red points; the colorimetric analysis of last experimental points was already strongly influenced by extraction of colorants from seeds.) The average amount of FFA present in black cumin seeds was found 5.1 wt. %; when hydrolysis was applied, the value increased to 39.9 wt. %. The dashed line corresponds to the hydrolysis of seeds milled approximately 20 hours before the experiment. The average concentration of FFA in this effluent mixture was found 56.4 wt. %, therefore, around 16.5 wt. % of FFA was converted just by the seed enzyme. This value corresponds well to 15.6 wt. % of FFA found in the extract obtained by Soxhlet extraction of such preliminary milled seeds. Free fatty acids found in the extract had been produced by the enzymatic hydrolysis in the milled material before the high temperature of boiling hexane inactivated the enzyme.

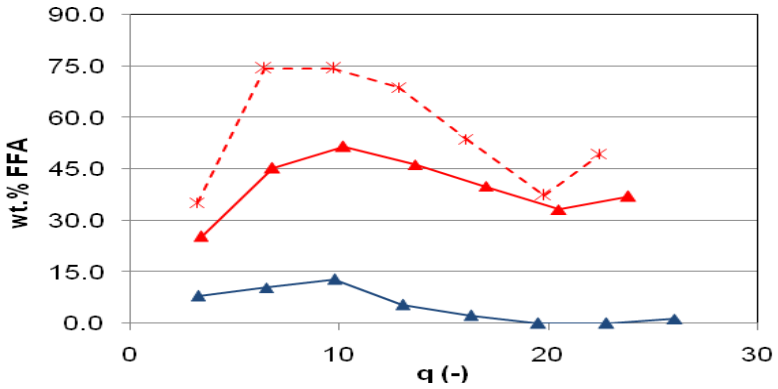

Figure 1. Evolution of concentration of FFA in the extract with dimensionless amount of solvent ($q=m_{CO2}/m_{feed}$). Blue – simple extraction, red – extraction with hydrolysis; (—) freshly milled seeds, (---) milling of seeds 20 h before extraction.

The Lipozyme activity was evaluated from the difference between the average FFA concentrations obtained from freshly milled seeds with and without the enzyme in the reactor (Table 1). An increase in the lipase activity after the reaction compared to the activity before its exposure to SC-CO_2 was observed.

Table 1. Activity of lipases from *Geotrichum candidum* 4013 and commercial Lipozyme ($U.g^{-1}$) before and after their hydrolytic reaction in SC-CO_2 and during the reaction

	Activity of lipase [$U.g^{-1}$]			
Lipase	Lipozyme®	Extracellular-lyophilized	Cell-bound-acetone powder	Cell-bound-immobilized
Substrate	Nigella seeds	Blackcurrant oil		
Before reaction[a]	0.0546	0.0668	0.0832	0.0065
After reaction[a]	0.1271	0.4256	0.1233	0.6088
In SC-CO_2[b]	0.845	2.560	3.208	6.876

[a]hydrolysis of *p*-nitrophenylpalmitate; [b]hydrolysis of oil

Blackcurrant oil hydrolysis catalysed by enzymes from *Geotrichum candidum*

As the substrate consisted only of triacylglycerols, neither its solubility in SC-CO_2 nor the degree of conversion was changing during experimental runs. The activity of the three enzymes prepared from *Geotrichum candidum* 4013, evaluated from the degree of conversion of oil to FFA, is listed in Table 1. The best results were obtained for the cell-bound immobilized lipase, which activity was eight times higher than the activity of Lipozyme in the hydrolysis of black cumin oil. A substantial increase in the activity at ambient pressure was observed for all enzymes after their exposure to SC-CO_2.

The composition of free fatty acids obtained by the enzymatic hydrolysis was compared with the initial fatty acid distribution in blackcurrant oil (Table 2). While the extracellular – lyophilized enzyme shows now specificity, as the mutual ratio of major fatty acids remains unchanged within the accuracy of analysis, the class of FFA released by the cell-bound enzymes is rich in saturated acids and oleic acid, the content of linoleic acid is strongly

reduced and linolenic acids are either absent or present at very low concentrations. This could, however, reflect fatty acid degradation, as the total amount of major fatty acids in the samples is reduced, too.

Table 2. Fatty acid composition of free fatty components (%) obtained by hydrolysis of blackcurrant oil with lipases from *Geotrichum candidum* 4013

Fatty acid	Formula	Content of FFA in blackcurrant oil [%]			
		Before hydrolysis	After hydrolysis with lipase		
			Extracellular - lyophilized	Cell-bound - acetone powder	Cell-bound - immobilized
Palmitic	16:0	6.3	1.2	17.43	10.5
Stearic	18:0	1.9	1.0	4.59	3.5
Oleic	18:1n-9	13.7	13.7	30.1	25.0
Linoleic	18:2n-6	47.4	50.3	5.36	10.23
γ-Linolenic	18:3n-6	13.0	15.2	0	2.5
α-Linolenic	18:3n-3	11.9	13.5	0	1.4
Other FFA		5.8	5.1	42.5	46.9

CONCLUSION

Lipases were prepared from the strain of *Geotrichum candidum* 4013, their activity in SC-CO_2 was determined and compared with the activity of commercial lipase Lipozyme. The activity of all enzymes has markedly increased after their exposure to SC-CO_2. In the experiments with black cumin oil the activity of enzyme present in the plant and released by milling was confirmed. The fatty acid composition of the acids liberated from oil by hydrolysis suggests a specificity of the cell-bound enzymes from *Geotrichum candidum* 4013.

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