INTERNATIONAL PROJECTS

ENZYMIC TRANSFORMATIONS OF BLACKCURRANT OIL: ENRICHMENT WITH γ-LINOLENIC ACID AND α-LINOLENIC ACID

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Received 19.6.02, in revised form 10.2.03, accepted 20.2.03.

Keywords: *Candida cylindracea*, *Mucor miehei*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, lipase, blackcurrant oil, enzymic hydrolysis, enzymic esterification, γ-linolenic acid/α-linolenic acid ratio

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1. Introduction

The biological importance of γ-linolenic acid [(6*Z*,9*Z*,12*Z*)-Octadeca-6,9,12-trienoic acid, 18:3*n*-6] has been well documented[1–2]. γ-Linolenic acid is known to play a crucial role in the generation of prostaglandin derivatives[3–5]. In higher plants γ-linolenic acid is biosynthesized *in vivo* from linoleic acid [(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid, 18:2*n*-6] under the action of δ6-desaturase[6] (Fig. 1). Under normal physiological conditions in humans, γ-linolenic acid results from the hepatic bioconversion of linoleic acid, the major essential fatty acid for humans. The transformation of linoleic acid to more unsaturated γ-linolenic acid also requires the activation of liver δ6-desaturase[7] (Fig. 1). As shown in Figure 1, higher polyunsaturated fatty acids are direct precursors of prostaglandins and leukotrienes[3–5], and they also have direct impact on the correct function of cell walls. Natural plant sources of γ-linolenic acid contain variable quantities of this acid[8]. Among those natural sources, a special attention should be paid to blackcurrant (*Ribes nigrum*). The oil, isolated from the plant seeds, contains also another important polyunsaturated fatty acid, α-linolenic acid [(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid, 18:3*n*-3], which is considered to be one of the most important polyunsaturated fatty acids[9–10]. Its biosynthesis *in vivo* from linoleic acid requires activation of δ15-desaturase[6]. Both linolenic acids are natural sources for their subsequent transformation into higher polyunsaturated fatty acids *in vivo* in humans and animals[6] (Fig. 1).

The dietary requirements for linoleic acid are estimated to be around 2.7 % of the total caloric intake equivalent in children and around 3–5 g per day in adults[11]. The required amount of essential fatty acids is usually supplied by a well-balanced diet. Biochemical or clinical symptoms of essential fatty acid deficiency are extremely rare, provided that the endogenous conversion of linoleic acid into γ-linolenic acid and subsequent compounds proceeds normally. On the other hand, it is known that fat-free parental diet very rapidly exhausts the endogenous essential fatty acid resources, leading to biochemical clinical abnormalities[12–13]. The dietary ratio of γ-linolenic acid to α-linolenic acid displays different physiological effects[14–15]. It has also been reported[14] that simultaneous supplementation of γ-linolenic acid and α-linolenic acid in animal diet could have an important icosanoid-mediated physiological effect.

A number of reports suggest that the normal transformation of linoleic acid into other essential fatty acids may be suppressed under several stressful conditions[16–19], most probably as a result of the δ6-desaturase deactivation. Critically ill patients thus become at risk of developing essential fatty acid deficient status, even in the case of appropriate linoleic acid delivery. Therefore, attention has been focused on an economically available lipid source, blackcurrant oil (BCO), which could be of clinical importance in situations caused by the enzyme deficiency.

Blackcurrant seeds are a waste product in the production of blackcurrant in the Czech Republic. This product is available in relatively large quantities as a residue from the production of jams, jellies, and juice drinks. BCO was obtained by an effective extraction of blackcurrant seeds (*Ribes nigrum*) in a supercritical carbon dioxide recycling reactor[20–21]. The average fatty acid composition of the extracted BCO is shown in Table I.

Many attempts have been made to produce concentrates of polyunsaturated fatty acids from naturally occurring triacylglycerols[6,8]. Various chemical and biochemical techniques have been developed including separation on zeolites and lipase-catalyzed reactions both in water and in organic solvents[22–24]. Employing lipases offers several advantages in comparison with chemical methods. (*a*) The catalytic efficiency of lipases is high, and it results in a low quantity of the

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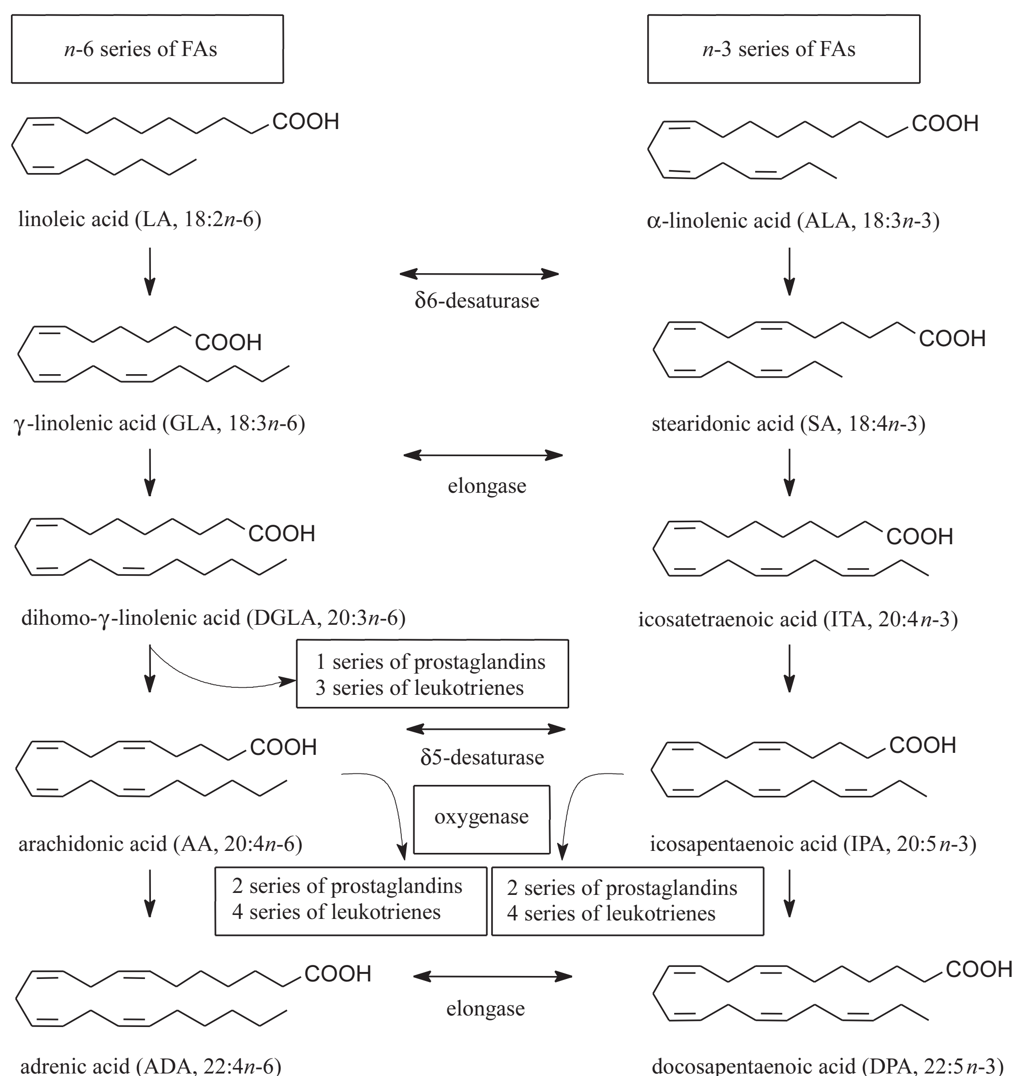

Fig. 1. **Metabolic pathways of transformations of linoleic acid into polyunsaturated fatty acids and icosanoids**

Table I
Fatty acid composition of blackcurrant oil from Chelčice, Czech Republic

Fatty Acid	IUPAC Name	Content [%]
Palmitic acid (16:0)	Hexadecanoic acid	6.3
Palmitoleic acid (16:1*n*-7)	(9*Z*)-Hexadec-9-enoic acid	0.1
Stearic acid (18:0)	Octadecanoic acid	1.9
Oleic acid (18:1*n*-9)	(9*Z*)-Octadec-9-enoic acid	13.7
cis-Vaccenic acid (18:1*n*-7)	(11*Z*)-Octadec-11-enoic acid	0.7
Linoleic acid (18:2*n*-6)	(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid	47.4
γ-Linolenic acid (18:3*n*-6)[a]	(6*Z*,9*Z*,12*Z*)-Octadeca-6,9,12-trienoic acid	13.0
α-Linolenic acid (18:3*n*-3)[a]	(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid	11.9
Stearidonic acid (18:4*n*-3)	(6*Z*,9*Z*,12*Z*,15*Z*)-Octadeca-6,9,12,15-tetraenoic acid	2.0
Gondoic acid (20:1*n*-9)	(11*Z*)-Icos-11-enoic acid	0.9
(*Z*,*Z*)-11,14-Icosadienoic acid (20:2*n*-6)	(11*Z*,14*Z*)-Icosa-11,14-dienoic acid	0.2
Unidentified	–	1.9

[a] The ratio (18:3*n*-6)/(18:3*n*-3) = 1.10

enzyme required. (*b*) High fatty acid selectivity of lipases has been well known and it is of priority importance for the intended application. (*c*) Mild reaction conditions that lipases offer in terms of pH and temperature are also important in processes that involve highly labile polyunsaturated fatty acids. The all-*Z* structure of polyunsaturated fatty acids of the natural origin is prone to partial destruction by oxidation, *Z/E* isomerization, double bond migration and polymerization.

In this study, which appeared partly in the recently published original papers[20,25], several lipases (triacylglycerol alkylhydrolases, EC 3.1.1.3) have been subjected to the investigation. The immobilized lipases from *Candida cylindracea*, *Mucor miehei* and *Pseudomonas cepacia*, and Lipozyme® (also the lipase from *M. miehei*, immobilized in a different way), and the non-immobilized (free) lipases from *M. miehei* and *P. fluorescens* were used to mediate the hydrolysis of the blackcurrant oil aimed at designing enzymic processes of enrichment with γ-linolenic acid and α-linolenic acid. Attention was also focused on investigation of selective preferences of the hydrolytic enzymes towards those polyunsaturated fatty acids. The same immobilized lipases were employed in the process of enrichment with γ-linolenic acid and α-linolenic acid contents during the enzymic esterification of free fatty acids, obtained from BCO by chemical means, with butan-1-ol.

2. Technical evaluation of the processes

Blackcurrant oil (BCO) was obtained by effective extraction of blackcurrant seeds (*Ribes nigrum*) in a supercritical carbon dioxide recycling reactor[20–21]. The main area for the production of the blackcurrant seeds is located in Chelčice (South Bohemia, Czech Republic). The fatty acid composition of the BCO of the above-described origin is shown in Table I.

Non-immobilized (free) lipase from *M. miehei* (6440 U/mg) and from *P. fluorescens* (42.5 U/mg) were employed together with the lipase from *C. cylindracea* immobilized on macroporous acrylic beads (1020 U/g), the lipase from *M. miehei* immobilized on Sol-Gel-AK (8.9 U/g), the lipase from *P. cepacia* also immobilized on Sol-Gel-AK (63 U/g), and Lipozyme® (62 U/g), i.e. the lipase from *M. miehei* immobilized on macroporous ion-exchange resin.

Triacylglycerols, diacylglycerols, monoacylglycerols, fatty acid butyl esters or free fatty acids were separated from the reaction mixtures by column chromatography techniques. These compounds were modified subsequently by chemical transesterification reactions according to the described method[26], and the obtained fatty acid methyl esters were dissolved in hexane. The GC analyses were performed with a HP 5890A gas chromatograph (Hewlett-Packard, USA), equipped with a flame ionization detector (FID) and split-splitless injector (split ratio 1:49). The injector and FID temperatures were 240 ˚C and 250 ºC, respectively, oven temperature program was set as follows: 200 ˚C (20 min), 5 ˚C.min^{-1} to 230 ˚C (15 min). A DB-WAX column (30 m×0.25 mm×0.25 µm; J&W Scientific) and hydrogen as carrier gas (average linear velocity 40 cm.s^{-1}) were used. Data were collected with a HP 3393A integrator. The peaks of respective fatty acid methyl esters were identified using commercially available standards of fatty acid methyl esters.

TLC was performed on Silufol precoated silica gel plates (Kavalier, Czech Republic). A mixture of diethyl ether/light petroleum/acetic acid (40:80:1.6 v/v/v) was used as eluent. The products were detected by spraying the developed TLC plates with a solution of phosphomolybdic acid in methanol.

Column chromatography purifications were performed on a silica gel (Hermann, Köln-Ehrenfeld, Germany), particle size 0.04–0.063 mm. The size of the column was chosen to enable the sample/silica gel ratio 1:50–1:70 (w/w). The compounds were eluted with mixtures of diethyl ether with light petroleum, in which the ratio of both eluents was adjusted to the individual mixture of compounds to be separated.

Preparation of free fatty acids was performed by alkaline hydrolysis of a BCO sample (1 g) using a 1 M solution of potassium hydroxide in 90 % aqueous ethanol (6 ml) under heating to 80 ˚C and stirring under argon for 90 min at 500 min^{-1}. The mixture was cooled to the room temperature, and deionized water (6 ml) and 6 M solution of hydrochloric acid (2 ml) were added. The obtained mixture of free fatty acids was extracted with diethyl ether. The combined extracts were dried over anhydrous sodium sulfate and evaporated under vacuum at 32 ˚C. The products (free fatty acids; 100 mg) were dissolved in isooctane (2,2,4-trimethylpentane; 3 ml) and stored at –18 ˚C. No other impurity in this product was detected by GC analysis, which was repeatedly performed before using.

The enzymic hydrolysis in aqueous media was performed at 40 ˚C in 2 ml vials under stirring. BCO (100 mg) and water (100 µl) were mixed and equilibrated at experimental temperature. The reaction was started by addition of lipase (18 U), allowed to proceed for 24 h, and then stopped by filtering off the enzymes. The products were extracted from the reaction mixture with diethyl ether and separated by column chromatography on silica gel.

When using a two-phase system for the enzymic hydrolysis, a solution of BCO (300 mg) in isooctane (2 ml) was added to a phosphate buffer (1 ml, 0.1 M, pH 7.0) containing an immobilized lipase (4.5 U). The suspension was incubated at 30 ˚C for 4 h, and then the process was stopped by filtering off the enzyme. The products were extracted from the reaction mixture with diethyl ether and dried over anhydrous sodium sulfate. The solvent was evaporated and the products were separated and purified by column chromatography on silica gel.

Enzymic esterification under conventional heating was performed using addition of lipase (1U) to a solution of free fatty acids (100 µl) and butan-1-ol (100 mg) in isooctane (3 ml). The mixture was heated to 30 ˚C and stirred at 500 min^{-1} for 2 h using a Unimax 1010 incubator (Heidolph, Germany), and then filtered to separate the enzyme, which was washed twice with diethyl ether. After evaporation of the solvents, the products were separated by column chromatography.

Application of microwave irradiation represented another modification of the enzymic esterification. The reaction mixture was prepared in the same way as described before. It was irradiated to 30 ˚C for 2 h using a Synthewave S 402 microwave reactor (Prolabo, France) in a monomode system. The reaction conditions were controlled by an algorithm, which allows a control the reaction temperature at the required value by varying power up to 20 W in operation under electromag-

netic field[27–28]. After 2-h reaction, the enzyme was separated from the reaction mixture by filtration, and washed twice with diethyl ether to collect the products. After evaporation of the solvents the products were purified by column chromatography.

3. Results and discussion

3.1. Enzymic hydrolysis

Screening of four selected lipases in their six forms for performing hydrolysis of BCO was studied[20]. The enzymes used were the immobilized lipases from *C. cylindracea* (immobilized on macroporous acrylic beads), *M. miehei* (immobilized on Sol-Gel-AK) and *P. cepacia* (immobilized on Sol-Gel-AK), and Lipozyme® (the lipase from *M. miehei* immobilized on macroporous ion exchange resin), and the non-immobilized (free) lipases from *M. miehei* and *P. fluorescens*. The experiments were performed using two modifications of the hydrolytic procedure. The hydrolysis of BCO mediated by selected lipases gave mixtures of diacylglycerols, monoacylglycerols, free fatty acids and unreacted triacylglycerols, which was in accordance with the expected reaction course. After isolation from the reaction mixture, the respective products were separated by column chromatography into several fractions. The isolated products were subjected to transesterification[26], and the resulting fatty acid methyl esters were analyzed by GC. In particular experiments, when the quantity of diacylglycerols and monoacylglycerols was low (those mediated by *C. cylindracea*), separation of fatty acids from the fraction of monoacylglycerols and diacylglycerols failed, and these groups of compounds had to be analyzed together as one individual fraction of the products. Monoacylglycerols and diacylglycerols, however, were also analyzed together. Work-up of the enzymic hydrolysis[20] using the Method I was performed during a 24-h period, and all selected lipases, i.e., the immobilized lipases from *C. cylindracea*, *M. miehei* (immobilized on Sol-Gel-AK) and *P. cepacia*, and Lipozyme® (the lipase from *M. miehei* immobilized on macroporous ion exchange resin), and the non-immobilized (free) lipases from *M. miehei* and *P. fluorescens*, were subjected to the screening procedure (Table II). In general, the rate of enzymic hydrolysis of triacylglycerols isolated from BCO beforehand corresponds generally to the quantity of the BCO hydrolyzed to diacylglycerols, monoacylglycerols and free fatty acids. Natural BCO contains ~95 % of triacylglycerols. The most satisfactory rates of hydrolysis of BCO by Method I were obtained with immobilized lipase from *P. cepacia* and Lipozyme® employed as biocatalysts. However, certain enrichment with γ-linolenic acid was observed in the collective fractions of diacylglycerols and monoacylglycerols in the transformations of BCO mediated by *M. miehei* immobilized on Sol-Gel-AK (Table II). Enrichment with both, γ-linolenic acid and α-linolenic acid, was observed in the transformations of BCO mediated by non-immobilized *P. fluorescens* (Table II). An effort was made to study the substrate regiospecificity of the lipases as regards the position of the acyl group in triacylglycerols. Two types of specific enzymes were employed: *sn*-1,3-regiospecific (*M. miehei*) and nonspecific enzymes (*C. cylindracea*, *P. cepacia* and *P. fluorescens*). The reason for using both types of lipases has reflected the fact that the most frequent positions of α-linolenic acid and γ-linolenic acid in triacylglycerols of BCO are not known yet. Selectivity of the lipases was observed only when the reaction was catalyzed by lipase from *M. miehei* and from *P. fluorescens* under the conditions of Method I. The content of γ-linolenic acid increased to 16.8 % in the collective fractions of monoacylglycerols and diacylglycerols (Table II). An increase in the ratio of γ-linolenic acid to α-linolenic acid in the same fractions of products was calculated for the products of hydrolysis of BCO mediated by *M. miehei* in all three forms subjected to the screening (Table II). Compared with the original ratio of these two polyunsaturated fatty acids (1.10; Table I), discrimination of α-linolenic acid was observed in the collective fractions of diacylglycerols and monoacylglycerols in the hydrolysis of BCO mediated by the lipase from *M. miehei* immobilized on Sol-Gel-AK (1.75), Lipozyme® (2.00), and in the fraction of free fatty acids obtained in the hydrolysis of BCO mediated by the non-immobilized lipase from *M. miehei* (2.11). Both types of the immobilized lipase from *M. miehei* discriminated γ-linolenic acid in the fractions of free fatty acids. Comparing the content of linoleic and oleic acids, the fractions of products after hydrolysis of BCO mediated by the lipases, an increase in the linoleic acid content was accompanied by a decrease in the oleic acid content in the same fraction (Table II). A decrease in the linoleic acid content was always observed, when the ratio of γ-linolenic acid/α-linolenic acid was lower than in the original BCO (i.e., when the ratio <1; cf. Table II).

The other modification of the lipase-mediated hydrolysis (Method II) (Ref.[20]) was performed by employing the immobilized lipases of the studied series of enzymes (Table III). The reaction was carried out in a two-phase system consisting of a buffer and isooctane. Free lipases (those from *M. miehei* and *P. fluorescens*) were found inconvenient for performing the enzymic hydrolysis in this particular two-phase system. The free lipase was always present in aqueous phase, and any stirring or shaking of the mixture in order to enhance a contact between the enzyme and the substrate was ineffective. The rate of such a hydrolysis of BCO was substantially nil. Further effort in this study was stopped, and attention was focused on screening of immobilized lipases from *C. cylindracea*, *M. miehei* (immobilized on Sol-Gel-AK), *P. cepacia*, and Lipozyme® (the lipase from *M. miehei* immobilized on macroporous ion exchange resin). These enzymic reactions were performed at 30 °C for 4 h. Increasing of the temperature up to 40 °C resulted in a decrease in chemical yield of the products of the enzymic transformations.

As shown in Table III, no considerable enrichment with either γ-linolenic acid or α-linolenic acid in any fraction of the evaluated experiments was observed. However, when comparing the ratio values calculated for the collective fractions of diacylglycerols and monoacylglycerols with those calculated for the fractions of free fatty acids in the reactions mediated by either immobilized form of the lipase from *M. miehei*, discrimination of either α-linolenic acid (ratio values 1.74 and 1.31) or γ-linolenic acid (ratio values 0.24 and 0.12) is obvious (Table III). The same principle as described above (Method I) was observed concerning the linoleic acid and oleic acid contents in the respective fractions, and concerning even a decrease in the linoleic acid content accompanying the discrimination of γ-linolenic acid (ratio value <1).

Table II
Fatty acid composition of glycerol esters and free fatty acids obtained by enzymic hydrolysis of blackcurrant with the tested lipases – Method I

Source of Lipase	Fraction[a]	18:1*n*-9 [%][b]	18:2*n*-6 [%][b]	18:3*n*-6 [%][b]	18:3*n*-3 [%][b]	18:3*n*-6/18:3*n*-3 ratio
Candida cylindracea	TG	14.2	45.4	12.3	10.7	1.15
	FFA+DG+MG	20.1	37.6	4.6	5.5	0.84
Mucor miehei	TG	13.7	46.0	12.5	11.2	1.12
(immobilized on Sol-Gel-AK)	FFA	22.5	32.5	1.2	3.2	0.37
	DG+MG	14.4	47.6	16.8	9.6	1.75
Lipozyme®	TG	13.4	45.0	13.3	11.2	1.19
	FFA	22.4	32.9	1.5	3.6	0.42
	DG+MG	25.3	14.3	1.8	0.9	2.00
Pseudomonas cepacia	TG	19.5	40.6	7.6	6.9	1.10
	FFA	14.5	39.4	5.5	6.1	0.90
	DG+MG	24.8	40.6	6.6	4.6	1.43
Mucor miehei	TG	36.0	13.3	0.0	0.0	0.00
(non-immobilized)	FFA	20.3	28.8	5.9	2.8	2.11
	DG+MG	16.1	10.7	0.8	0.8	1.00
Pseudomonas fluorescens	TG	14.8	46.1	6.8	6.6	1.03
	FFA	18.0	44.3	11.8	10.4	1.14
	DG+MG	14.5	44.1	15.7	13.4	1.17

[a] Triacylglycerols (TG), free fatty acids (FFA), diacylglycerols (DG), monoacylglycerols (MG), [b] mole percents

Table III
Fatty acid composition of glycerol esters and free fatty acids obtained by enzymic hydrolysis of blackcurrant with selected lipases – Method II

Source of Lipase	Fraction[a]	18:1*n*-9 [%][b]	18:2*n*-6 [%][b]	18:3*n*-6 [%][b]	18:3*n*-3 [%][b]	18:3*n*-6/18:3*n*-3 ratio
Candida cylindracea	TG	13.5	46.1	12.7	11.6	1.09
	FFA+DG+MG		not determined			–
Mucor miehei	TG	14.7	45.8	11.7	10.8	1.08
(immobilized on Sol-Gel-AK)	FFA	19.7	31.1	1.0	4.2	0.24
	DG+MG	18.0	45.7	11.8	6.8	1.74
Lipozyme®	TG	14.5	46.5	12.7	10.6	1.20
	FFA	17.0	44.3	1.0	8.0	0.12
	DG+MG	23.4	35.1	5.0	3.8	1.31
Pseudomonas cepacia	TG	17.8	44.8	9.5	9.1	1.04
	FFA	17.2	11.1	0.5	0.5	1.00
	DG+MG	28.5	1.4	0.0	0.0	0.00

[a] Triacylglycerols (TG), free fatty acids (FFA), diacylglycerols (DG), monoacylglycerols (MG), [b] mole percents

3.2. Enzymic esterification

The enzymic esterification (Method III) (Ref.[25]) of free fatty acids, obtained by chemical hydrolysis of BCO, was performed for 2 h because the selectivity of the lipases to fatty acids decreased with increasing time due to their deactivation (Table IV). It was observed that after 3 h of enzymic transformation, no residual free fatty acids were present and, therefore, the screened enzymes, immobilized lipases from *C. cylindracea*, *M. miehei* (immobilized on Sol-Gel-AK), *P. cepacia*, and Lipozyme® (the lipase from *M. miehei* immobilized on macroporous ion exchange resin) showed no fatty acid specificity. Using Lipozyme® as biocatalyst, a content of 2.3 % of butyl γ-linolenate was found in the fatty acid butyl ester fraction under conventional heating, while 16.9 % of γ-linolenic acid was identified in the residual fatty acids. When using the lipase from *P. cepacia* as biocatalyst under conventional heating, the quantity of butyl γ-linolenate rose to 20 % in the fatty acid butyl ester fraction, while 12.2 % of γ-linolenic acid remained in the residual free fatty acids. When evaluating

Table IV
Composition of fatty acid butyl esters and residual free fatty acids after enzymic esterification performed under conventional heating – Method III

Source of Lipase	Fraction[a]	18:1*n*-9 [%][b]	18:2*n*-6 [%][b]	18:3*n*-6 [%][b]	18:3*n*-3 [%][b]	18:3*n*-6/18:3*n*-3 ratio
Candida cylindracea	FABE			not detectable		–
	RFFA	13.7	46.1	12.3	11.4	1.08
Mucor miehei	FABE	16.0	54.5	2.2	15.2	0.14
(immobilized on Sol-Gel-AK)	RFFA	14.0	47.6	13.3	12.4	1.07
Lipozyme®	FABE	15.8	56.2	2.3	16.0	0.14
	RFFA	13.8	45.2	16.9	10.8	1.56
Pseudomonas cepacia	FABE	6.8	46.1	20.1	12.9	1.56
	RFFA	15.1	47.9	12.2	11.9	1.02

[a] Fatty acid butyl esters (FABE), residual free fatty acids (RFFA), [b] mole percents

Table V
Composition of fatty acid butyl esters and residual free fatty acids after enzymic esterification performed under microwave irradiation – Method IV

Source of Lipase	Fraction[a]	18:1*n*-9 [%][b]	18:2*n*-6 [%][b]	18:3*n*-6 [%][b]	18:3*n*-3 [%][b]	18:3*n*-6/18:3*n*-3 ratio
Candida cylindracea	FABE	11.0	7.0	8.0	8.7	0.92
	RFFA	14.4	41.4	9.6	8.9	1.08
Mucor miehei	FABE	13.3	44.3	5.9	13.9	0.42
(immobilized on Sol-Gel-AK)	RFFA	15.6	47.8	9.9	11.0	0.90
Lipozyme®	FABE	15.0	52.7	5.5	14.8	0.37
	RFFA	11.4	35.4	29.9	8.2	3.65
Pseudomonas cepacia	FABE	7.6	47.0	19.6	13.8	1.42
	RFFA	16.2	50.0	9.2	11.4	0.81

[a] Fatty acid butyl ester (FABE), residual free fatty acids (RFFA), [b] mole percents

the γ-linolenic acid/α-linolenic acid ratios in the individual fractions after the enzymic esterification, both forms of the immobilized lipase from *M. miehei* were found to display discrimination to this ratio given in the original BCO. A remarkable discrimination of γ-linolenic acid during this esterification is well documented by the ratio values (0.14; Table IV). The γ-linolenic acid/α-linolenic acid ratio in residual free fatty acid fractions (1.07 and 1.56) shows, in turn, enrichment with γ-linolenic acid in this fraction only when Lipozyme® was used as enzyme mediator of esterification. The linoleic acid and oleic acid contents seemed to be even in accordance with increasing or decreasing contents of α-linolenic acid in the product fractions.

Under microwave irradiation (Method IV) (Ref.[25]), 5.5 % of butyl γ-linolenate was found in the fatty acid butyl ester fraction, and 29.9 % of γ-linolenic acid in the residual free fatty acids as products of esterification mediated by Lipozyme® (Table V). Using the lipase from *P. cepacia* as biocatalyst under microwave irradiation, the ratio of butyl γ-linolenate and the residual γ-linolenic acid changed as well (19.6 % *versus* 9.2 %; Table V).

Comparing the ratio values (Table V), again both immobilized lipases from *M. miehei* showed discrimination of γ-linolenic acid in this esterification reaction. A remarkable result was achieved with Lipozyme® as biocatalyst. The fraction of residual free fatty acids (29.9 %) was enriched with γ-linolenic acid, and a high γ-linolenic acid/α-linolenic acid ratio was obtained (3.65). The action of other enzymes seemed to be different from that found in the esterification under conventional conditions. This finding may contribute to the general idea that microwave-irradiated reactions should be studied more intensively to understand the processes, which seem to be more often used as a "black box". The dependence of linoleic acid and oleic acid contents in the mixtures showed again accordance with the content of α-linolenic acid, but some differences were observed (Table V) compared to the alternative modification of this enzymic transformation of fatty acids (Table IV).

The results obtained indicate clearly that differences do exist if the effect of microwave irradiation is compared with that of conventional heating. This finding supports the original hypothesis, in which such results had been expected even if many differences in results obtained by conventional heating and microwave irradiation have still been considered with

certain skepticism. The methods of heating the reaction mixtures are different. The focused microwave irradiation supplies energy in a more concentrated and controlled way to the system than conventional heating is able to do[27–28]. This difference results in more effective and more rapid getting over the transition state energy barrier under microwave irradiation even if these energies are substantially decreased by the catalytic action of the enzyme.

4. Conclusion

Several important findings were found during this investigation:

(*a*) Enzymic hydrolysis, Method I: Highest enrichment with γ-linolenic acid was achieved in the fraction of mono- and diacylglycerols using the lipase from *M. miehei* immobilized on Sol-Gel-AK (16.8 %) and that from *P. fluorescens* (15.7 %). However, only the lipase from *M. miehei* immobilized on Sol-Gel-AK showed simultaneously discrimination of α-linolenic acid during this enzymic transformation (γ-linolenic acid/α-linolenic acid ratio = 1.75).

(*b*) Enzymic hydrolysis, Method II: Almost no enrichment with γ-linolenic acid was observed (12.7 %) in the triacylglycerol fractions resulting from the enzymic transformations performed by the lipase from *C. cylindracea* and by Lipozyme®. However, important discrimination of α-linolenic acid was found in experiments with *M. miehei* immobilized on Sol-Gel-AK (γ-linolenic acid/α-linolenic acid ratio = 1.74).

(*c*) Enzymic esterification, Method III: Enrichment with γ-linolenic acid was achieved either by the lipase from *P. cepacia* (20.1 %) in the fraction of butyl esters of fatty acids or by Lipozyme® in the fraction of residual free fatty acids (16.9 %). Maximum γ-linolenic acid/α-linolenic acid ratio was found identical together with the above-mentioned results (1.56).

(*d*) Enzymic esterification, Method IV: Enrichment with γ-linolenic acid was achieved either by the lipase from *P. cepacia* (19.6 %) in the fraction of butyl esters of fatty acids or by Lipozyme® in the fraction of residual free fatty acids (29.9 %), which was the best enrichment found in this series of experiments for γ-linolenic acid. The absolute maximum of the γ-linolenic acid/α-linolenic acid ratio was found with Lipozyme® (3.65).

(*e*) Enzymic esterification, in general: While Lipozyme® discriminates γ-linolenic acid (16.9 % and 29.9 % found in the fraction of residual free fatty acids), the lipase from *P. cepacia* works in the opposite way, because it causes enrichment with γ-linolenic acid in the fraction of fatty acids butyl esters (20.1 % and 19.6 %).

(*f*) Enzymic transformations, in general: The lipase from *M. miehei* (*sn*-1,3-regiospecific lipase) was the best lipase for performing the evaluated enzymic processes in the way they had been designed.

(*g*) Enzymic transformations, in general: Lipozyme® was the best biocatalyst among all three forms of the lipase from *M. miehei*, which were subjected to this screening. This form of immobilization of the lipase from *M. miehei* seems to meet all basic requirements of the immobilized lipase to be considered for potential industrial application in the enrichment with γ-linolenic acid and α-linolenic acid from natural plant oils as shown in this study with BCO.

Financial support of this research through the COST network project D13/0014/01 (D13.10; Ministry of Education of the Czech Republic) and through the internal grant No. 2/8 of the Institute of Organic Chemistry and Biochemistry is gratefully acknowledged. We thank the University of La Rochelle, France (Professor M.-D. Legoy), for enabling us to perform experiments with a Synthewave S 402 microwave reactor in the frame of the COST exchange program for scientists. The authors appreciate skilful technical assistance of Mrs. M. Wimmerová and Mrs. H. Ernyeiová.

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The ability of enzymes to mediate some transformations of blackcurrant oil was described and evaluated. Four commercially available lipases, both in their free and immobilized forms were selected for the investigation. The selected enzymes were the lipases from *Candida cylindracea*, *Mucor miehei*, *Pseudomonas cepacia* and *Pseudomonas fluorescens*. Two target enzymic processes were investigated: (*a*) Enzymic hydrolysis of blackcurrant oil was studied and potential selectivity was evaluated of several commercially available lipases to discriminate polyunsaturated fatty acids, namely α-linolenic acid and γ-linolenic acid in products under mild conditions. Two modifications of the process were used, of which employing aqueous media gave a higher enrichment in γ-linolenic acid in the obtained mono- and diacylglycerols (up to 16.8 % of γ-linolenic acid). (*b*) Enzymic esterification of fatty acids obtained by chemical hydrolysis of blackcurrant oil was studied and evaluated to find commercially available enzyme(s) capable of mediating similar discrimination to that under (*a*). Two modifications of the process were again used: Enrichment with γ-linolenic acid up to 20 % was achieved under conventional heating, and up to 30 % under microwave irradiation. The methods employed were compared.