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Enrichment of γ-linolenic acid from fungal oil by lipase-catalysed reactions

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Summary. Various lipases have been evaluated as biocatalysts for the enrichment of γ-linolenic acid from a commercial fungal oil derived from *Mucor* sp. by selective esterification of the fungal oil fatty acids with *n*-butanol or by selective hydrolysis of the oil. Lipase from *M. miehei* (Lipozyme), as compared to lipases from *Candida cylindracea, Penicillium cyclopium,* and *Rhizopus arrhizus,* was found to be most effective in the enrichment of γ-linolenic acid in unesterified fatty acids upon esterification of the fungal oil fatty acids with *n*-butanol. Thus, the γ-linolenic acid content could be raised from 10.4% in the starting material to 68.8% in the unesterified fatty acids. Selective hydrolysis of the fungal oil triacylglycerols using Lipozyme resulted in about 1.5-fold enrichment of γ-linolenic acid in the unhydrolysed acylglycerols. Other lipases tested, such as those from *P. cyclopium, C. cylindracea, R. arrhizus, Penicillium* sp. (Lipase G), porcine pancreas and *Chromobacterium viscosum*, were also rather ineffective in the enrichment of γ-linolenic acid by selective hydrolysis of the fungal oil triacylglycerols.

Introduction

γ-Linolenic acid (all *cis*-6,9,12-octadecatrienoic acid) is of considerable commercial interest due to its biomedical properties (Horrobin 1982). Common sources of γ-linolenic acid are the seed oils of evening primrose, *Oenothera biennis* L. (Hudson 1984; Mukherjee and Kiewitt 1987), borage, *Borago officinalis* L. (Whipkey et al. 1988) and *Ribes* spp. (Traitler et al. 1984). Lately, microorganisms, such as *Mortierella* spp. (Hansson and Dostálek 1988; Kamisaka et al. 1990) and *Mucor ambiguus* (Fukuda and Morikawa 1987) have been considered for the production of oils containing γ-linolenic acid, and such oils are now commercially available.

The methods used so far for the enrichment of γ-linolenic acid from natural resources include urea adduct formation (Traitler et al. 1988), separation on Y-zeolite (Arai et al. 1987), and solvent winterization (Yokochi et al. 1990). Work from this laboratory has shown recently that γ-linolenic acid can be efficiently enriched by lipase-catalysed selective esterification with *n*-butanol of the fatty acids from evening primrose oil with the exception of γ-linolenic acid, which is thus obtained as a concentrate in the unesterified fatty acid fraction (Hills et al. 1989, 1990b). Selective hydrolysis of the triacylglycerols of evening primrose oil catalysed by lipases also led to enrichment of γ-linolenoyl moieties in the unhydrolysed acylglycerols (Hills et al. 1989). Here we report the application of these enzymatic fractionation techniques for the enrichment of γ-linolenic acid from a commercially available fungal oil.

Materials and methods

Materials. Fungal oil from *Mucor* sp. was purchased from Sigma (Deisenhofen, FRG). Lipase from *M. miehei* (Lipozyme), provided by Novo Industrie (Mainz, FRG), had an activity of 25 batch interesterification units (BIU)/g; 1 BIU corresponds to 1 μmol palmitic acid incorporated into trioleoylglycerol/min from an equimolar mixture at 40° C. A lipase preparation from *Penicillium* sp. (Lipase G) was a product of Amano Pharmaceutical (Nagoya, Japan), with an activity of 55000 units/g at pH 5.6 using vinyl laurate as substrate. The lipases supplied by Biocatalysts (Pontypridd, Mid-Glamorgan, UK) had the following activity, determined with olive oil as substrate: *P. cyclopium*, 1250 units/g; *Candida cylindracea*, 85000 units/g; porcine pancreas, 4500 units/g; *Chromobacterium viscosum,* 144000 units/g. Activity of the lipase suspension from *Rhizopus arrhizus* (Boehringer, Mannheim, FRG), determined using olive oil as substrate, was 50000 units/ml. All other chemicals, reagents, and adsorbents were of analytical grade purchased from E. Merck (Darmstadt, FRG).

Preparation of fatty acids. Fungal oil was saponified with KOH at room temperature and the resulting soaps treated with HCl in order to liberate the fatty acids that were extracted with hexane (Kates 1986).

Esterification. The reactions were carried out at 30° C for various periods in sealed vials with magnetic stirring using 250 mM of the fungal oil fatty acids with 500 mM *n*-butanol in 1 ml hexane in the

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presence of lipase powder (10% w/w of the substrate). The reactions were terminated by separating the lipase preparation from the reaction products by centrifugation.

Hydrolysis. The reactions were carried out at 40° C for various periods in sealed vials with magnetic stirring using 174 mg fungal oil with 18 mg lipase powder (or 18 µl lipase suspension) in the presence of 12 µl added water. The reactions were stopped by separating the lipase preparation from the reaction products by centrifugation.

Analysis of reaction products. The reaction products resulting from esterification reactions were fractionated into butyl esters and unesterified fatty acids by TLC on Silica Gel H with hexane:diethyl ether:acetic acid (80:20:1, v/v) as developing solvent. The fractions of unesterified fatty acids and butyl esters were eluted from the adsorbent with water-saturated diethyl ether. Known amounts of methyl heptadecanoate was added to each fraction as an internal standard and the unesterified fatty acids were converted to methyl esters by treatment with diazomethane. Subsequently, each of the fractions of butyl esters and methyl esters (derived from the unesterified fatty acids) were analysed by gas chromatography in a Perkin Elmer (Überlingen, FRG) F-22 instrument equipped with flame ionization detectors. The separations were carried out on a 30 m × 0.25 mm capillary column coated with a 0.25 µm layer of the stationary phase, DB-23-50% cyanopropyl (Carlo Erba Instruments, Hofheim/Ts, FRG) using a temperature programme of 170–230° C, 2° C/min; nitrogen, 2 ml/min, was used as carrier gas. The composition of the butyl esters and methyl esters was determined and the relative proportion of butyl esters formed and unesterified fungal oil fatty acids remaining in the product were calculated from the ratios of the respective peak areas of the butyl esters and methyl esters to the peak area of the internal standard, methyl heptadecanoate.

The reaction products resulting from hydrolysis of the fungal oil were fractionated by TLC on Silica Gel H containing 5% (w/w) sodium carbonate using diethyl ether:methanol (97:3, v/v) as the developing solvent (Hills and Mukherjee 1988). The fraction of unesterified fatty acids, located at the origin of the chromatogram, and that of acylglycerols (i.e. mono-, di- and triacylglycerols) migrating close to the solvent front, were removed. The fractions containing unesterified fatty acids or acylglycerols were converted to methyl esters according to Chalvardjian (1964). A known amount of the internal standard, methyl heptadecanoate, was added to each methyl ester fraction, which was subsequently purified by TLC on Silica Gel H with hexane:diethyl ether (70:30, v/v). Finally, the methyl esters were analysed by gas chromatography as described above, and, the relative proportions and composition of the fatty acids, liberated from the fungal oil, and those of the unhydrolysed acylglycerols were determined as described above.

Results

The fatty acid mixture obtained from the fungal oil was reacted with *n*-butanol in the presence of hexane as solvent using four different lipases as esterification catalysts. Tables 1–4 record for lipases from *P. cyclopium*, *C. cylindracea*, *R. arrhizus* and *M. miehei* the relative proportions and compositions of unesterified fatty acids remaining in the reaction product and the corresponding figures for butyl esters formed by lipase-catalysed esterification for various periods of time. It is quite evident from these data that although all the four lipases tested led to extensive esterification (85–95%) after 6 h reaction, their substrate selectivities were markedly different.

Thus, with the lipase from *P. cyclopium* the level of γ-linolenic acid was raised from about 10% in the starting material to about 25% in the unesterified fatty acids. Despite extensive esterification of all the major fatty acids of the fungal oil with *n*-butanol, only very little γ-linolenic acid was converted to butyl esters (Table 1). Enrichment of γ-linolenic acid paralleled a decrease in the levels of oleic and linoleic acids in the unesterified fatty acids and a concomitant increase in the proportion of these two acids in the butyl esters (Table 1). It is also seen from the data given in Table 1 that the increase in the enrichment of γ-linolenic acid during the course of the reaction was paralleled by a decrease in the yield of γ-linolenic acid in the unesterified fatty acids.

The data given in Table 2 show that the lipase from *C. cylindracea*, compared to that from *P. cyclopium*, was much more effective in the enrichment of γ-linolenic acid. Thus after 1 h reaction, when as much as about

Table 1. Enrichment of γ-linolenic acid in unesterified fatty acids by selective esterification of fatty acids from fungal oil with *n*-butanol catalysed by lipase from *Penicillium cyclopium*

Reaction time (h)	Component	Amount in total products (%)	Composition of acyl constituents[a] (%)						Enrichment of γ-18:3 in fatty acids (%)	Yield of γ-18:3 in fatty acids (%)
			16:0	18:0	18:1	18:2	γ-18:3	Others[b]		
0	Fatty acids	100.0	21.4	4.1	36.6	21.6	10.4	5.9	1.0	100
1	Fatty acids	49.9	25.1	5.5	32.0	17.4	12.9	7.1	1.2	62
1	Butyl esters	50.1	18.0	3.3	47.3	25.3	1.0	5.1		
2	Fatty acids	36.0	24.0	5.6	27.5	16.2	18.8	7.9	1.8	65
2	Butyl esters	64.0	18.8	3.5	45.8	25.3	1.5	5.1		
4	Fatty acids	15.5	26.7	6.5	23.6	13.0	20.8	9.4	2.0	31
4	Butyl esters	84.5	20.5	3.9	43.8	24.3	2.1	5.4		
6	Fatty acids	15.3	26.2	6.2	19.3	11.0	25.6	11.7	2.5	38
6	Butyl esters	84.7	22.0	4.2	42.2	23.1	2.5	6.0		

[a] Acyl constituents are designated by number of carbon atoms:number of *cis*-double bonds
[b] Including 14:0, 16:1, α-18:3, 20:0, 20:1, 22:0, 22:1, 24:0 and 24:1

Table 2. Enrichment of γ-linolenic acid in unesterified fatty acids by selective esterification of fatty acids from fungal oil with *n*-butanol catalysed by lipase from *Candida cylindracea*

Reaction time (h)	Component	Amount in total products (%)	Composition of acyl constituents[a] (%)						Enrichment of γ-18:3 in fatty acids (%)	Yield of γ-18:3 in fatty acids (%)
			16:0	18:0	18:1	18:2	γ-18:3	Others[b]		
0	Fatty acids	100.0	21.4	4.1	36.6	21.6	10.4	5.9	1.0	100
1	Fatty acids	8.4	20.5	5.3	14.0	3.8	46.3	10.1	4.5	37
1	Butyl esters	91.6	23.8	4.4	41.6	23.9	0.0	6.3		
4	Fatty acids	0.8	9.8	3.1	6.4	2.2	57.6	20.9	5.5	4
4	Butyl esters	99.2	26.6	5.1	40.2	21.3	1.4	5.4		
6	Fatty acids	2.4	6.0	1.7	10.1	6.0	63.0	13.2	6.1	15
6	Butyl esters	97.6	24.5	4.8	39.3	22.4	2.6	6.4		

[a] Acyl constituents are designated by number of carbon atoms:number of *cis*-double bonds
[b] Including 14:0, 16:1, α-18:3, 20:0, 20:1, 22:0, 22:1, 24:0 and 24:1

Table 3. Enrichment of γ-linolenic acid in unesterified fatty acids by selective esterification of fatty acids from fungal oil with *n*-butanol catalysed by lipase from *Rhizopus arrhizus*

Reaction time (h)	Component	Amount in total product (%)	Composition of acyl constituents[a] (%)						Enrichment of γ-18:3 in fatty acids (%)	Yield of γ-18:3 in fatty acids (%)
			16:0	18:0	18:1	18:2	γ-18:3	Others[b]		
0	Fatty acids	100.0	21.4	4.1	36.6	21.6	10.4	5.9	1.0	100
1	Fatty acids	88.7	22.4	4.4	36.8	20.6	9.7	6.1	0.9	83
1	Butyl esters	11.3	18.9	3.0	42.3	25.5	0.4	9.9		
2	Fatty acids	85.0	24.0	4.9	35.6	19.1	10.6	5.8	1.0	87
2	Butyl esters	15.0	27.0	4.4	44.0	19.1	0.2	5.3		
4	Fatty acids	46.2	22.7	5.2	33.2	17.5	14.1	7.3	1.4	63
4	Butyl esters	53.8	22.8	3.7	42.9	24.4	0.5	5.7		
6	Fatty acids	5.8	27.6	7.0	25.8	10.5	17.0	12.1	1.6	9
6	Butyl esters	94.2	24.2	4.2	42.3	23.1	0.7	5.5		

[a] Acyl constituents are designated by number of carbon atoms:number of *cis*-double bonds
[b] Including 14:0, 16:1, α-18:3, 20:0, 20:1, 22:0, 22:1, 24:0 and 24:1

Table 4. Enrichment of γ-linolenic acid in unesterified fatty acids by selective esterification of fatty acids from fungal oil with *n*-butanol catalysed by lipase from *Mucor miehei*

Reaction time (h)	Component	Amount in total products (%)	Composition of acyl constituent[a] (%)						Enrichment of γ-18:3 in fatty acids (%)	Yield of γ-18:3 in fatty acids (%)
			16:0	18:0	18:1	18:2	γ-18:3	Others[b]		
0	Fatty acids	100.0	21.4	4.1	36.6	21.6	10.4	5.9	1.0	100
1	Fatty acids	38.8	19.3	4.3	33.6	18.3	17.4	7.1	1.7	65
1	Butyl esters	61.2	24.6	4.6	40.5	23.2	0.7	6.4		
2	Fatty acids	19.6	14.2	3.5	27.1	14.2	33.5	7.5	3.2	63
2	Butyl esters	80.4	24.9	4.7	40.6	22.9	1.1	5.8		
4	Fatty acids	8.9	5.6	1.6	10.9	5.0	68.8	8.1	6.6	59
4	Butyl esters	91.1	25.1	4.8	39.7	22.3	2.2	5.9		
6	Fatty acids	4.2	3.4	0.8	10.0	3.0	64.8	18.0	6.2	26
6	Butyl esters	95.8	24.5	4.6	38.3	22.4	3.9	6.3		

[a] Acyl constituents are designated by number of carbon atoms:number of *cis*-double bonds
[b] Including 14:0, 16:1, α-18:3, 20:0, 20:1, 22:0, 22:1, 24:0 and 24:1

92% of the fungal oil fatty acids were esterified, the level of γ-linolenic acid in the unesterified fatty acids was raised to about 47% whereas virtually no γ-linolenic acid was converted to butyl esters. Enrichment of γ-linolenic acid paralleled a decrease in the levels of palmitic, oleic and linoleic acid in the unesterified fatty acids and a moderate increase in the proportion of these acids in the butyl esters. Extension of the reaction time to 6 h led to an increase in the level of γ-linolenic acid to 63% in the unesterified fatty acids; however, the yield of γ-linolenic acid was considerably reduced.

As compared to lipases from *P. cyclopium* and *C. cylindracea*, the lipase from *R. arrhizus* was found to be much less effective for the enrichment of γ-linolenic acid by selective esterification with *n*-butanol (Table 3). Thus, the level of γ-linolenic acid in the unesterified fatty acids could be raised only up to 14 and 17%, respectively, after 4 and 6 h of reaction, which resulted in about 54% and 94% esterification of the fungal oil fatty acids.

Of all the lipase preparations tested, the lipase from *M. miehei* was found to be most effective for enrichment of γ-linolenic acid by selective esterification of the fungal oil fatty acids with *n*-butanol (Table 4). Thus, the level of γ-linolenic acid in the unesterified fatty acids could be raised to about 34% and 69%, respectively, after 2 and 4 h of reaction, which resulted in about 80% and 91% esterification of the fungal oil fatty acids; the corresponding yields of γ-linolenic acid were 63% and 59%. Increasing the reaction time to 6 h led to some decrease in the level of γ-linolenic acid to about 65% and to a substantial reduction in yield to 26%. It is also evident from the data presented in Table 4 that the time course of enrichment of γ-linolenic acid was accompanied by a decrease in the levels of palmitic, oleic and linoleic acid in the unesterified fatty acids and a moderate increase in the proportion of these acids in the butyl esters.

Another approach that was followed involved selective hydrolysis of fungal oil triacylglycerols aimed at cleavage of most of the fatty acids other than γ-linolenic acid, and enrichment of this acid in unhydrolysed acylglycerols, i.e. tri-, di- and monoacylglycerols. The data presented in Table 5 show the relative proportions and composition of unhydrolysed acylglycerols and fatty acids in the reaction products obtained upon partial hydrolysis of the fungal oil triacylglycerols using the lipase from *M. miehei* for various periods. The results show that the proportions of fatty acids cleaved from the triacylglycerols progressively increased to about 22% after 12 h of reaction. All the fatty acids were readily cleaved with the exception of γ-linolenic acid, which was found to be enriched in the unhydrolysed acylglycerols to the extent of about 15%. Extending the reaction time to 24 h raised the level of γ-linolenic acid in the acylglycerols to about 17%, although the total amount of fatty acids formed was reduced to about 15%. This is likely to be due to re-esterification of the fatty acids to acylglycerols. The extent of enrichment of γ-linolenic acid in the unhydrolysed acylglycerols by selective hydrolysis of the fungal oil triacylglycerols using the lipase from *M. miehei* (Table 5) was, however, far less impressive than the enrichment of this acid in the unesterified fatty acids by selective esterification of the fungal oil fatty acids with *n*-butanol using lipases from *P. cyclopium* (Table 1), *C. cylindracea* (Table 2) and *M. miehei* (Table 4).

Several other lipases, i.e. those from *P. cyclopium*, *C. cylindracea*, *R. arrhizus*, *Penicillium* sp. (Lipase G), porcine pancreas and *Chromobacterium viscosum*, were also examined for their ability to catalyse selective hydrolysis of the fungal oil triacylglycerols for the enrichment of γ-linolenic acid in the unhydrolysed acylglycerols. The relative proportions and composition of the fatty acids and unhydrolysed acylglycerols formed upon hydrolysis of the fungal oil for 8 h by each of the

Table 5. Enrichment of γ-linolenic acid in unhydrolysed acylglycerols by selective hydrolysis of fungal oil using lipase from *M. miehei*

Reaction time (h)	Component	Amount in total products (%)	Composition of acyl constituents[a] (%)						Enrichment of γ-18:3 in acylglycerols (%)
			16:0	18:0	18:1	18:2	γ-18:3	Others[b]	
0	Acylglycerols	~100.0	21.4	4.1	36.6	21.6	10.4	5.9	1.0
1	Acylglycerols	92.0	14.5	3.1	35.7	19.9	14.2	12.6	1.4
1	Fatty acids	8.0	30.9	5.2	29.7	22.6	3.7	7.9	
4	Acylglycerols	81.9	14.5	2.0	28.5	24.0	13.9	17.1	1.3
4	Fatty acids	18.1	31.7	5.8	30.3	20.4	3.9	7.9	
8	Acylglycerols	80.1	12.5	2.5	26.0	27.3	15.7	16.0	1.5
8	Fatty acids	19.9	31.9	5.4	30.1	20.0	4.6	8.0	
12	Acylglycerols	77.7	12.9	2.7	27.5	27.1	15.4	14.4	1.5
12	Fatty acids	22.3	27.8	5.8	32.0	20.4	5.5	8.5	
24	Acylglycerols	85.1	9.5	2.4	21.4	29.1	16.8	20.8	1.6
24	Fatty acids	14.9	29.5	6.3	34.0	17.2	4.1	8.9	

[a] Acyl constituents are designated by number of carbon atoms:number of *cis*-double bonds
[b] Including 14:0, 16:1, α-18:3, 20:0, 20:1, 22:0, 22:1, 24:0 and 24:1

Table 6. Enrichment of γ-linolenic acid in unhydrolysed acylglycerols by selective hydrolysis of fungal oil for 8 h using various lipases

Lipase	Component	Amount in total products (%)	Composition of acyl constituents[a] (%)						Enrichment of γ-18:3 in acylglycerols (%)
			16:0	18:0	18:1	18:2	γ-18:3	Others[b]	
None	Acylglycerols	~100.0	21.4	4.1	36.6	21.6	10.4	5.9	1.0
P. cyclopium	Acylglycerols	82.4	11.7	2.3	20.4	31.0	13.1	21.5	1.3
	Fatty acids	15.8	23.5	3.7	37.8	25.1	5.2	4.7	
C. cylindracea	Acylglycerols	46.4	16.1	2.9	26.0	24.4	15.3	15.3	1.5
	Fatty acids	53.6	23.0	4.4	37.7	22.5	7.2	5.2	
R. arrhizus	Acylglycerols	67.3	12.6	2.3	26.8	27.7	16.2	14.4	1.6
	Fatty acids	32.7	34.6	6.8	29.0	19.0	2.4	8.2	
Penicillium	Acylglycerols	87.3	8.2	1.6	15.9	33.5	15.4	25.4	1.5
sp. (Lipase G)	Fatty acids	12.7	23.4	3.6	41.6	23.1	4.1	4.2	
Porcine	Acylglycerols	89.4	12.2	2.1	23.2	27.7	15.5	19.3	1.5
pancreatic	Fatty acids	10.6	32.7	6.7	29.4	19.0	2.6	9.6	
Chromobacterium	Acylglycerols	65.2	17.2	3.0	37.5	23.9	11.6	6.8	1.1
viscosum	Fatty acids	34.8	27.8	7.0	27.6	21.1	9.6	6.9	

[a] Acyl constituents are designated by number of carbon atoms:number of *cis*-double bonds
[b] Including 14:0, 16:1, α-18:3, 20:0, 20:1, 22:0, 22:1, 24:0 and 24:1

above lipases are given in Table 6. These data show that the degree of hydrolysis attained with the various lipases were in the order: *Candida cylindracea* > *Chromobacterium viscosum* > *R. arrhizus* > *P. cyclopium* > Lipase G > porcine pancreas. The extent of enrichment of γ-linolenic acid attained by the lipases from *Candida cylindracea*, *R. arrhizus*, Lipase G and porcine pancreas were of a similar order of magnitude as that attained with the lipase from *M. miehei*; the lipases from *P. cyclopium* and *Chromobacterium viscosum* were even less effective in this regard (Tables 5 and 6).

Discussion

Lipases from oilseed rape (Hills et al. 1989, 1990a) and those from *M. miehei* (Hills et al. 1989, 1990b; Rangheard et al. 1989), *Candida rugosa*, porcine pancreas, as well as *Geotrichum candidum* (Rangheard et al. 1989) have been shown to discriminate against γ-linolenic acid in lipase-catalysed esterification of fatty acids with primary alcohols, such as *n*-butanol or *n*-propanol. Similarly, lipase from *R. delemar* has been found to discriminate against γ-linolenoyl moieties and γ-linolenic acid in the lipase-catalysed transesterification of various triacylglycerols with stearic acid and of trioleoylglycerol with various fatty acids, respectively (Osterberg et al. 1989). Moreover, lipase from oilseed rape was shown to catalyse the hydrolysis of tri-γ-linolenoylglycerol at a much lower rate than trioleoylglycerol (Hills et al. 1990a). These substrate selectivities of the lipases from oilseed rape and *M. miehei* have been utilized for the enrichment of γ-linolenic acid from evening primrose oil by selective esterification of the fatty acids of this oil with *n*-butanol or by selective hydrolysis of the oil to yield, respectively, fatty acids or acylglycerols with elevated levels of γ-linolenic acid (Hills et al. 1989, 1990b). Here we report similar approaches of enzymatic fractionation by kinetic resolution for the enrichment of γ-linolenic acid from fungal oil using several commercially available lipases.

The results of the lipase-catalysed reactions reported here show that selective esterification of fungal oil fatty acids with *n*-butanol, rather than selective hydrolysis of fungal oil triacylglycerols, should be the method of choice for enrichment of γ-linolenic acid by enzymatic fractionation.

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