Enzymatic Fractionation of Fatty Acids: Enrichment of γ-Linolenic Acid and Docosahexaenoic Acid by Selective Esterification Catalyzed by Lipases

Matthew J. Hills, Irmgard Kiewitt and Kumar D. Mukherjee*
Federal Center for Lipid Research, Institute for Biochemistry and Technology, H.P. Kaufmann-Institute, Piusallee 68, D-4400 Münster, Federal Republic of Germany

Immobilized lipase preparations from seedlings of rape (*Brassica napus* L.) and *Mucor miehei* (Lipozyme) used as biocatalysts in esterification and hydrolysis reactions discriminate strongly against γ-linolenic and docosahexaenoic acids/acyl moieties. Utilizing this property, γ-linolenic acid contained in fatty acids of evening primrose oil has been enriched seven to nine-fold, from 9.5 to almost 85% by selective esterification of the other fatty acids with butanol. Similarly, docosahexaenoic acid of cod liver oil has been enriched four to five-fold, from 9.4 to 45% by selective esterification of fatty acids (other than docosahexaenoic acid) with butanol. As long as the reaction is stopped before reaching equilibrium, very little of either γ-linolenic acid or docosahexaenoic acid are converted to butyl esters, which results in high yields of these acids in the unesterified fatty acid fraction.

KEY WORDS: Cod liver oil, docosahexaenoic acid, enzymatic fractionation of fatty acids, evening primrose oil, γ-linolenic acid, lipase-catalyzed esterification, *Mucor miehei* lipase, rape lipase.

Several polyunsaturated fatty acids belonging to the (n-6) series, such as γ-linolenic acid (γ-18:3) and dihomo-γ-linolenic acid (γ-20:3), as well as those of the (n-3) series, such as docosahexaenoic acid (22:6) and eicosapentaenoic acid (20:5), are of considerable pharmaceutical interest due to their biomedical properties (1–4). Common sources of γ-linolenic acid are the seed oils of evening primrose (*Oenothera biennis L.*) (5–7), borage (*Borago officinalis* L.) (8), and *Ribes* spp. (9). Lately, microorganisms such as *Mortierella* spp. (10,11) and *Mucor ambiguus* (12) have been explored for the production of γ-linolenic acid and dihomo-γ-linolenic acid. Marine lipids, such as fish oils, are a common source of the (n-3) polyunsaturated fatty acids (4), although recently microorganisms, such as *Mortierella* spp., are also being considered for the production of eicosapentaenoic acid (13,14). Methods used so far for the enrichment of these polyunsaturated fatty acids from their natural sources include urea adduct formation (15), separation on Y-Zeolite (16) and supercritical fluid extraction (17). Lipase-catalyzed transesterification of cod liver oil has been described for the enrichment of triacylglycerols with n-3 polyunsaturated fatty acids (18).

We recently found that lipase, isolated from rape seedlings and immobilized on celite, catalyzes under nonaqueous conditions in the presence of hexane the esterification of fatty acids with primary alcohols in a similar manner as many microbial lipases do (19). In both esterification and hydrolysis reactions, however, the rape lipase was found to strongly discriminate against unsaturated fatty acids/acyl moieties having a *cis*-6 or a *cis*-4 double bond, e.g., γ-linolenic acid and docosahexaenoic acid, respectively (20). In this context it is of interest to note that porcine pancreatic lipase also discriminates against fatty acids/acyl moieties containing a double bond or methyl branching close to the carboxyl group, such as *trans*-3 fatty acids (21) or 2,2-dimethyl stearic acid (22), respectively. The above property of the rape lipase was utilized in "enzymatic fractionation" of fatty acids of evening primrose oil for the enrichment of γ-linolenic acid by selective esterification of fatty acids, other than γ-linolenic acid, with butanol (23). A similar approach was used recently for the enrichment of docosahexaenoic acid via selective esterification of fatty acids with methanol, interesterification of triacylglycerols or methyl esters with fatty acids, or hydrolysis of methyl esters (24).

Here we report the application of the enzymatic fractionation of fatty acids using lipase from rape as well as microbial lipase (Lipozyme) for the enrichment of γ-linolenic acid and docosahexaenoic acid by selective esterification of fatty acids of evening primrose oil and cod liver oil with butanol.

EXPERIMENTAL PROCEDURES

Evening primrose oil and the pure fatty acids used as reference substances were obtained from Sigma Chemie GmbH, Deisenhofen, Federal Republic of Germany. Cod liver oil was purchased from a local pharmacy. The fatty acids were prepared from evening primrose oil and cod liver oil by saponification at room temperature, followed by hydrolysis with hydrocholric acid (25). All reagents and adsorbents were from E. Merck AG, Darmstadt, Federal Republic of Germany.

Lipase from seedlings of rape (*Brassica napus* cv. Ceres) was isolated and immobilized onto celite as described elsewhere (19,26). Immobilized lipase (Lipozyme) from *Mucor miehei* was kindly provided by Novo Industrie GmbH, Mainz, Federal Republic of Germany.

Esterification reactions were carried out at 30°C for various periods in sealed vials with magnetic stirring using 125 mM, each, of the fatty acids from either evening primrose oil or cod liver oil together with 250 mM butanol in 1.5 mL hexane solution. Catalyst used in one set of reactions was 10 mg immobilized rape lipase containing 5 μL Bis-Tris-Propane/HCl (20 mM, pH 7.5). Alternatively, Lipozyme (10% w/w of the reaction partners) was used as the catalyst. Aliquots of the reaction mixture were withdrawn at definite intervals and centrifuged to remove the catalyst.

*To whom correspondence should be addressed.

The products formed by reaction of the fatty acids from evening primrose oil with butanol were treated with diazomethane in order to convert the unesterified fatty acids to methyl esters. The resulting mixture of methyl esters and butyl esters were analyzed by gas chromatography at 210°C, using Silar 5CP (Applied Science Laboratories, State College, PA) as described earlier (23,27). The relative proportion and composition of unesterified fatty acids in the reaction product was calculated from composition of the mixture of methyl and butyl esters (23).

The products formed by reaction of the fatty acids from cod liver oil were fractionated by thin-layer chromatography (TLC) on Silica Gel H with hexane:diethyl ether:acetic acid (70:30:1, v/v). The fractions corresponding to 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 internal standard, and the fatty acids converted to methyl esters by treatment with diazomethane. Subsequently, each of the methyl ester and butyl ester fractions were analyzed as described earlier by GC on Silar 5CP (23,27), using a temperature program of 160–190°C, 1°C/min, followed by 190–230°C, 2°C/min. Relative proportion of unesterified fatty acids in the reaction product was calculated from the peak areas of the internal standard in the fractions of methyl esters and butyl esters.

RESULTS AND DISCUSSION

The ability of the lipase from rape to discriminate against γ-linolenic acid in esterification reactions (20) was utilized for the enrichment of this acid in the fatty acids derived from evening primrose oil by selective esterification with butanol. The data presented in Table 1 show, in agreement with our recent findings (23), that with lipase from rape as biocatalyst, all the fatty acids of evening primrose oil, with the exception of γ-linolenic acid, were extensively esterified with butanol. This resulted in enrichment of γ-linolenic acid in the unesterified fatty acids. Thus, after 48 hr of reaction, 17.8% of the fatty acids remained unesterified, but they contained as much as 54.5% γ-linolenic acid as compared to 9.5% in the starting material. Increasing the reaction time to 72 hr resulted in a further decrease in the proportion of unesterified fatty acids to 14.4%, and an increase in the γ-linolenic acid content

TABLE 1

Fractionation of Fatty Acids from Evening Primrose Oil by Selective Esterification with Butanol Using Rape Lipase

Reaction time (hr)	Component	% of Total product	Composition (%) of acyl constituents					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		
0	fatty acids	100	8.6	1.7	6.0	72.9	9.5	1.0	100
48	fatty acids	17.8	6.7	2.2	3.4	33.1	54.5	5.7	100
48	butyl esters	82.2	9.0	1.6	6.3	83.2	tr		
72	fatty acids	14.4	5.6	1.1	1.0	27.8	64.6	6.8	98
72	butyl esters	85.6	9.0	1.6	6.4	82.7	0.2		

TABLE 2

Fractionation of Fatty Acids from Evening Primrose Oil by Selective Esterification with Butanol Using Lipozyme

Reaction time (hr)	Component	% of Total product	Composition (%) of acyl constituents					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		
0	fatty acids	100	8.6	1.7	6.0	72.9	9.5	1.0	100
1	fatty acids	38.7	7.8	1.3	3.4	62.5	26.1	2.7	100
1	butyl esters	61.2	9.2	1.6	6.5	82.7	tr		
2	fatty acids	23.4	9.4	tr	3.8	43.2	43.6	4.6	100
2	butyl esters	76.6	8.3	1.4	6.0	84.2	tr		
4	fatty acids	9.3	3.2	tr	tr	13.9	82.8	8.7	81
4	butyl esters	90.8	8.7	1.1	6.7	81.5	2.0		
6	fatty acids	7.2	1.4	tr	tr	13.9	84.7	8.9	64
6	butyl esters	92.8	7.9	1.3	6.9	80.1	3.7		
8	fatty acids	6.5	1.5	tr	tr	16.9	81.5	8.6	56
8	butyl esters	93.6	8.2	1.3	6.7	79.3	4.5		
20	fatty acids	2.5	8.0	tr	tr	36.0	56.0	5.9	15
20	butyl esters	97.6	7.7	1.6	6.5	75.9	8.3		

TABLE 3

Fractionation of Fatty Acids from Cod Liver Oil by Selective Esterification with Butanol Using Rape Lipase or Lipozyme

Reaction time (hr)	Component	% of Total product	Composition (%) of major acyl constituents								Enrichment of 22:6 in fatty acids	Yield (%) of 22:6 in fatty acids
			14:0	16:0	16:1	18:1	20:1	20:5	22:1	22:6		
0	fatty acids	100	6.5	13.7	10.9	19.7	9.6	8.4	9.3	9.4		
Rape lipase												
24	fatty acids	50.0	3.0	12.0	5.7	19.5	11.5	9.3	12.0	12.1	1.3	64
24	butyl esters	50.0	11.4	17.7	14.2	21.6	9.2	5.2	7.0	1.1		
48	fatty acids	15.2	1.6	9.5	3.3	16.8	11.8	8.0	13.7	16.6	1.8	27
48	butyl esters	84.8	9.0	16.3	12.7	22.2	9.4	8.6	8.2	1.9		
Lipozyme												
1	fatty acids	43.0	12.2	5.0	7.7	17.8	9.4	10.3	9.7	12.7	1.4	58
1	butyl esters	57.0	7.0	15.5	10.4	22.4	12.3	5.8	10.2	1.3		
4	fatty acids	10.3	3.3	4.6	2.9	6.3	2.4	7.8	3.2	39.5	4.2	43
4	butyl esters	89.7	6.7	15.1	10.0	22.0	12.5	9.5	10.2	2.5		
6	fatty acids	7.4	2.6	2.2	1.8	3.5	1.4	4.8	1.9	45.9	4.9	36
6	butyl esters	92.6	6.5	14.7	9.7	21.4	11.8	9.7	11.7	2.7		
16	fatty acids	2.0	3.2	2.7	1.5	5.7	1.3	1.3	1.5	54.3	5.8	11
16	butyl esters	98.0	5.9	13.6	9.0	20.1	11.2	10.0	11.2	3.8		

to 64.6%. Concomitantly, the linoleic acid content was increased from 72.9% in the starting material to 82.7% in the butyl esters after 72 hr of reaction. Very little γ-linolenic acid is converted to butyl esters, which resulted in its almost quantitative yield in the unesterified fatty acid fraction.

Studies on substrate specificity of several lipases revealed that the microbial lipase preparation, Lipozyme, shows similar discrimination against γ-linolenic acid, as does the rape lipase (28). Therefore, Lipozyme also was explored for selective esterification aimed at enrichment of γ-linolenic acid. The results given in Table 2 show that the reaction of fatty acids of evening primrose oil with butanol, catalyzed by Lipozyme, for a period of 6 hr led to progressive esterificaiton of all the fatty acids except γ-linolenic acid, and concomitant enrichment of this acid in the unesterified fatty acids. After 6 hr of reaction the unesterified fatty acids contained as much as 84.7% γ-linolenic acid. Further increase in reaction time led to a decrease in the enrichment of γ-linolenic acid and a substantial lowering of its yield in the unesterified fatty acids.

The ability of lipase from rape to discriminate against docosahexaenoic acid (20) was used for enrichment of this acid in the fatty acids derived from cod liver oil by selective esterification with butanol. Lipozyme was also examined in this regard. The data given in Table 3 show that esterification of fatty acids of cod liver oil with butanol using rape lipase led to an almost two-fold enrichment of docosahexaenoic acid, from 9.4 to 16.6% in 48 hr. Further increase in enrichment was not observed with prolonged reaction period. With Lipozyme as catalyst, 4–6 hr reaction led to about 90% conversion of the fatty acids to butyl esters and concomitant enrichment of docosahexaenoic acid to 40–46%. Further increase in reaction time to 16 hr increased the enrichment of docosahexaenoic acid to 54%, but the yield of this acid in unesterified fatty acids was greatly reduced. The data for Lipozyme in Tables 2 and 3 illustrate a feature of the kinetic resolution of different substrates by an enzyme in which there is a trade-off between increasing the concentration of a poorly reacting substrate and its yield when the reaction is terminated. If the reaction is allowed to proceed too long, the poor substrate will also be converted and reach a similar equilibrium to the other substrates. Thus, the progress of reactions for kinetic resolution of compounds must be monitored and the reaction stopped when the optimal balance between enrichment and yield is reached. A method for modeling kinetic resolution reactions has been described by Chen *et al.* (29).

The data presented here, as well as those reported earlier (23,29,30), show that the enzymatic fractionation of lipids based on substrate selectivity of lipases in synthetic or hydrolytic reaction should be eminently suitable for large scale preparation of fatty acids and other lipids of biomedical interest.

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