Enzymatic Enrichment of C_{20} *cis*-5 Polyunsaturated Fatty Acids from *Biota orientalis* Seed Oil

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ABSTRACT: Enrichment of *cis*-5 polyunsaturated fatty acids [20:3(5*c*,11*c*,14*c*), 4.3% and 20:4(5*c*,11*c*,14*c*,17*c*), 11.3%] from *Biota orientalis* seed oil was carried out by lipase-catalyzed selective esterification and hydrolysis reactions. Lipases from *Rhizomucor miehei* (Lipozyme), *Candida cylindracea* and porcine pancreas were used. Lipozyme-catalyzed esterification of *Biota* fatty acids with *n*- butanol in *n*-hexane allowed 20:3 and 20:4 (as fatty acids) to be enriched to a maximum level of 52.9%, and in the presence of *C. cylindracea* lipase 61.5% enrichment was achieved. Esterification with pancreatic lipase was poor with low levels of enrichment of 20:3 and 20:4 (22%). A multigram scale esterification of the free fatty acids from *Biota* seed oil by repeated treatment of the isolated fatty acid fraction with *n*-butanol in *n*-hexane in the presence of *C. cylindracea* lipase furnished an enrichment yield of 72.5% of a mixture of 20:3 and 20:4 fatty acids. Urea fractionation of the free fatty acids of *Biota* oil gave an initial enriched fraction of 20:3 (9.5%) and 20:4 (25.2%) which, upon treatment with *C. cylindracea* lipase in *n*-butanol and *n*-hexane, gave an enriched fraction of 85.3% of 20:3 and 20:4 fatty acids. Partial hydrolysis of the triglycerides of *Biota* oil by *C. cylindracea* lipase in potassium phosphate buffer at 25°C resulted in a 2.8-fold enrichment of *cis*-5 polyunsaturated fatty acids (40.8% of 20:3 and 20:4) as contained in the unhydrolyzed acylglycerol fractions.
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KEY WORDS: Enrichment, enzymatic, fatty acid selectivity, lipase, polyunsaturated fatty acids.

The fatty acid composition of the seed oil of *Biota orientalis* (also known as *Platycladus orientalis* or *Thuja orientalis* or, more commonly, *Arbor vitae*) was reported earlier (1) to contain 16:0, 18:0, 18:1(9*c*), 18:2(9*c*,12*c*), 20:0, 20:1(11*c*), 20:2(5*c*,11*c*), 20:2(11*c*,14*c*), 20:3(5*c*,11*c*,14*c*) (4.3%) and 20:4(5*c*,11*c*,14*c*,17*c*) (11.3%). The *cis*-5 polyunsaturated fatty acids (20:3 and 20:4) isolated from *Biota* seed oil have recently been shown to exhibit essential fatty acid effects on the lipid metabolism in the rat (2). In view of the biological importance, there is a need to develop procedures for the enrichment of the *cis*-5 polyunsaturated fatty acids (20:3, 20:4) in *B. orientalis* seed oil for biomedical applications. Enzymatic methods involving lipases have been reported for the enrichment of various biologically active fatty acids, including γ-linolenic acid from evening primrose oil (3), docosahexaenoic acid and eicosapentaenoic acid from fish oil (4,5). In this paper we report the results of the enzymatic enrichment of *cis*-5 polyunsaturated fatty acids (20:3 and 20:4) found in *Biota* oil.

EXPERIMENTAL PROCEDURES

Biota orientalis seeds were purchased from herbal shops in Guangzhou (China). Lipozyme (*Rhizomucor miehei* lipase immobilized on cationic ion exchange resin) with an activity of 6 BAUN/g (1 BAUN corresponds to 1 μmol of decanoic acid incorporated into trioleoylglycerol/min from an equimolar mixture at 70°C) was a gift from Novo Industrie A/S (Bagsvaerd, Denmark). Lipases from *Candida cylindracea* (type VII, activity 905 U/mg and type VIIA immobilized on acrylic beads, activity 179 U/mg) and porcine pancreatic lipase (type II, activity 179 U/mg, 1 U will hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in 1 h at pH 7.2 at 37°C using olive oil) were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were reagent grade and redistilled before use. All other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Gas–liquid chromatography (GLC) was performed with a Hewlett–Packard (Palo Alto, CA) model 5890 instrument. The GLC column used was a capillary column coated with a polar SP-2380 stationary phase (30 m × 0.53 mm, 1 μm film thickness) and fitted with a flame-ionization detector. Nitrogen was the carrier gas (2 mL/min). The column oven temperature was programmed from 100 to 240°C at the rate of 5°C/min. The temperature of the injection port and detector was maintained at 280°C. The GLC peak areas were recorded on a Hewlett–Packard model HP3394A electronic integrator. Methyl fatty esters were prepared by the method described by Schulte and Weber (6) with minor modification as follows: a mixture of fatty acid (2 mg), 1,2-dichloroethane (100 μL) and trimethylsulfonium hydroxide (0.2 M in methanol, 50 μL) was shaken for 30 s. The aliquot was injected directly onto the GLC column. Trimethylsulfonium hydroxide (0.2 M in methanol) was purchased from Macherey-Nagel (Duren, Germany).

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General procedure for enzymatic esterification of fatty acids on a micro-scale. A mixture of the free fatty acids (200 mg) of the seed oil of *B. orientalis*, *n*-butanol (0.12 mL), *n*-hexane (1.7 mL) and lipase (30 mg) (Lipozyme or porcine pancreatic lipase or *C. cylindracea* lipase) was stirred in a glass culture tube at 25°C for the indicated period (see Tables 1 and 2). The reaction mixture was centrifuged, and the *n*-hexane solution was loaded onto a short silica gel (2 g) column (250 mm × 10 mm i.d.) and eluted with a mixture of *n*-hexane and diethyl ether (95:5, vol/vol, 30 mL) to remove the butyl esters. This was followed by a mixture of *n*-hexane and diethyl ether (4:1, vol/vol, 30 mL) to isolate the free fatty acids. The solvent of the collected fractions was evaporated under a stream of nitrogen. Yields and fatty acid composition of the fractions were analyzed.

Enzymatic enrichment of cis-5 *unsaturated fatty acids from* B. orientalis *on a multi-gram scale.* A mixture of *B. orientalis* fatty acids (10.0 g), *n*-butanol (1.7 mL) and *C. cylindracea* lipase (1.2 g) was stirred for 6 h under reduced pressure (20 mm Hg) by connecting the reaction flask to a water aspirator. *n*-Hexane (20 mL) was then added and the mixture was filtered. The residue on the filter was washed with *n*-hexane (2 × 5 mL). The filtrate was loaded onto a silica gel (100 g) column. The column was eluted with a mixture of *n*-hexane and diethyl ether (9:1, vol/vol, 150 mL), which furnished the butyl esters (6.0 g, 60% w/w). Further elution with a mixture of *n*-hexane, diethyl ether and acetic acid (80:20:1, vol/vol/vol, 200 mL) gave, on evaporation of the solvent, the unreacted free fatty acids (3.2 g, 32% w/w). GLC analysis of the methyl ester derivatives of the isolated free fatty acid fraction showed a total amount of 40% of 20:3 and 20:4. The enrichment process was repeated by stirring the isolated free fatty acid fraction (3.2 g) with 0.3 g of *C. cylindracea* for 6 h. The isolated free fatty acid fraction (2.3 g) showed a total of 60% of 20:3 and 20:4. The process was repeated once more to yield a free fatty acid fraction (2.2 g) with a total of 72.5% of 20:3 and 20:4.

Urea fractionation of fatty acids from B. orientalis. Urea (57.0 g) was dissolved in 90% aqueous ethanol (150 mL) at 70°C. Fatty acids from *B. orientalis* (19.0 g) in 90% ethanol (20 mL) was added, and the mixture was allowed to cool to room temperature and kept for 15 h at 4°C. The crystalline urea adduct was filtered. The filtrate was diluted with water (180 mL) and acidified with dilute HCl (3 M, 25 mL). The solution mixture was successively extracted with light petroleum (b.p. 40–60°C, 75 mL), diethyl ether (75 mL) and light petroleum (75 mL). The combined organic extract was washed with water (50 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and silica (20 g) column chromatography using a mixture of light petroleum and diethyl ether (200 mL, 3:2, vol/vol) as the eluent furnished a mixture of fatty acids (3.1 g). The fatty acid composition of the latter was: 18:1 (0.8%), 18:2 (22.6%), 18:3 (41.9%), 20:3 (9.5%) and 20:4 (25.2%). This mixture of fatty acids was esterified with *n*-butanol at 25°C in the presence of *C. cylindracea* for 12 h. The enrichment process was repeated twice by stirring the isolated free fatty acid with *C. cylindracea* for 12 h each time.

The urea adduct was dissolved in water (80 mL), and the mixture was extracted with diethyl ether (3 × 75 mL). The organic extract was washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent under reduced pressure furnished fatty acids (13.5 g, 71%) composed of 16:0 (6.5%), 18:0 (4.9%), 18:1 (16.7%), 18:2 (26.1%), 20:1 (0.4%), 20:2 (1.0%), 20:2 (1.1%), 20:3 (5.1%) and 20:4 (7.6%).

Hydrolysis of triacylglycerols of B. orientalis *using* C. cylindracea *lipase.* A mixture of triacylglycerols from *B. orientalis* (200 mg), potassium phosphate buffer (2 mL, pH 7.0) and *C. cylindracea* lipase (20 mg) was stirred in a culture tube

TABLE 1
Enrichment of *cis*-5 Polyunsaturated Fatty Acids from *Biota orientalis* Seed Oil by Lipozyme-Catalyzed Esterification of *Biota* Fatty Acids with *n*-Butanol in *n*-Hexane

Reaction time (min)	Reaction temperature (°C)	Component[a]	Amount (% w/w)	Fatty acid composition (wt%)					20:3 + 20:4	Enrichment 20:3 + 20:4 (-fold)	Yield of 20:3 + 20:4 (%)
				18:1	18:2	18:3	20:3	20:4			
0	20	FA	100	13.8	25.7	34.3	4.8	9.8	14.6	1.0	100
20	20	FA	34	12.9	22.6	24.4	9.7	17.0	26.7	1.8	62
		BE	66	13.6	28.5	39.7	2.8	5.5	8.3	—	—
30	20	FA	24	9.7	18.3	21.7	12.8	26.6	39.4	2.7	65
		BE	76	11.1	28.0	39.7	4.4	8.2	12.6	—	—
60	20	FA	9	14.3	22.9	22.6	10.2	20.8	31	2.1	19
		BE	91	11.9	29.3	37.5	4.5	8.8	13.3	—	—
30	10	FA	29	12.9	21.6	25.3	10.7	21.0	31.7	2.2	63
		BE	71	9.9	28.7	43.7	3.2	5.0	8.2	—	—
30	30	FA	14	7.9	15.3	17.7	16.8	36.1	52.9	3.6	51
		BE	86	13.5	26.8	37.5	5.3	7.3	12.6	—	—
30	40	FA	12	13.9	19.5	17.5	14.3	28.3	42.6	2.9	35
		BE	88	34.5	19.6	23.0	5.7	8.1	13.8	—	—

[a]FA, fatty acids; BE, butyl esters. The average amount (wt%) of FA and BE of 16:0, 18:0, 20:1 and 20:2 are 4.8, 2.6, 0.5 and 1.3 and 6.0, 1.9, 0.4 and 0.8, respectively.

TABLE 2
Enrichment of *cis*-5 Polyunsaturated Fatty Acids from *Biota orientalis* Seed Oil by *Candida cylindracea* Lipase-Catalyzed Esterification with *n*-Butanol in *n*-Hexane at 25°C

Reaction time (h)	Component[a]	Amount (% w/w)	Fatty acid composition (wt%) 18:1	18:2	18:3	20:3	20:4	20:3 + 20:4	Enrichment 20:3 + 20:4 (-fold)	Yield of 20:3 + 20:4 (%)
0	FA	100	13.8	25.7	34.3	4.8	9.8	14.6	1.0	100
4	FA	67	13.5	26.1	30.0	6.8	14.7	21.5	1.5	99
	BE	33	6.6	24.4	62.9	0.3	0	0.3	—	—
6	FA	52	15.6	23.9	15.8	9.7	17.8	27.5	1.9	98
	BE	48	11.0	27.6	54.7	0.2	0	0.2	—	—
12	FA	43	16.2	28.8	10.5	8.5	22.0	30.5	2.1	90
	BE	57	25.5	19.2	49.5	0.1	0	0.1	—	—
20	FA	22	7.3	6.9	12.9	21.0	40.5	61.5	4.2	93
	BE	78	13.4	27.9	51.2	0.1	0	0.1	—	—

[a]FA, fatty acids; BE, butyl esters. The average amount (wt%) of FA and BE of 16:0, 18:0, 20:1 and 20:2 are 6.2, 2.7, 0.5 and 3.2, and 3.5, 1.8, 0.4 and 0.7, respectively.

at 25°C for 2 h. Diethyl ether (2 mL) was added and the mixture was mixed using a vortex mixer for 15 s, and it was then centrifuged. The diethyl ether phase was isolated and the aqueous layer was extracted with diethyl ether (2 × 2 mL). The ethereal extract was evaporated under a stream of nitrogen. The residue was separated by silica thin-layer chromatography using a mixture of *n*-hexane, diethyl ether and acetic acid (70:30:1, vol/vol/vol) as the developing solvent. By this technique the free fatty acid fraction was separated from the mixture of mono-, di- and triacylglycerols. The acylglycerols were converted to methyl esters by transesterification with trimethylsulfonium hydroxide in methanol as described for the methylation of free fatty acids above.

RESULTS AND DISCUSSION

Lipozyme (*R. miehei*) is known as an efficient biocatalyst for esterification and interesterification purposes (7). The enzyme from *C. cylindracea* has the ability to selectively esterify unsaturated fatty acids containing a *cis*-9 double bond in the alkyl chain (8).

When a mixture of the free fatty acids from *B. orientalis* [containing a total of 15% w/w of 20:3(5*c*,11*c*,14*c*) and 20:4(5*c*,11*c*,14*c*,17*c*)] was stirred with *n*-butanol in *n*-hexane in the presence of Lipozyme at 20°C for 30 min, the unesterified free fatty acid fraction showed a 3.6-fold (52.9%) enrichment of 20:3 and 20:4 with a 51% recovery yield (Table 1). However, the butyl ester fraction also showed a substantial amount of the 20:3 and 20:4 (total of 12.6% w/w) fatty acids. When the same reaction was carried out at a higher temperature (60°C) for 30 min, 96% of the total amount of fatty acids was converted to the butyl esters. Lipozyme displayed no selectivity at 60°C toward positional isomers of unsaturated fatty acids, as fatty acids containing the *cis*-5 or *cis*-9 double bond were equally and readily esterified. It was also found that the same reaction conducted at 4°C or even at -20°C for 72 h resulted in the total esterification of the fatty acids to the corresponding butyl esters. From these results it can be concluded that Lipozyme remains an efficient biocatalyst for esterification of long-chain saturated and unsaturated fatty acids, but constitutes a nonselective enzyme for esterifying *cis*-5 and *cis*-9 positional isomers at 60°C or under the specified conditions.

When a similar reaction was conducted in the presence of porcine pancreatic lipase at 30°C for 12 h, esterification was very slow yielding only 10% w/w of butyl esters. By raising the reaction temperature to 60°C and prolonging the reaction to 24 h, the esterification process was only slightly improved, leaving 60% w/w of the fatty acids unesterified. The composition of the free fatty acid fraction in this experiment showed a low enrichment (22%) of the 20:3 and 20:4 fatty acids. In view of the low rate and nonspecific esterification behavior of pancreatic lipase, this biocatalyst was not regarded a suitable choice for enriching the *cis*-5 unsaturated fatty acids in *Biota* seed oil.

Lipase from *C. cylindracea*, on the contrary, has been reported to hydrolyze fatty esters and to esterify free fatty acids containing a *cis*-9 double bond with a high degree of selectivity (9). When a mixture of *B. orientalis* fatty acids, *n*-butanol and *n*-hexane was stirred in the presence of *C. cylindracea* lipase at 25°C for 20 h, most of the unsaturated fatty acids containing a *cis*-9 double bond (oleic acid, linoleic and linolenic acids) were converted to the butyl esters. None of the *cis*-5 unsaturated 20:3 and 20:4 fatty acids was esterified. The unesterified fatty acid fraction contained a total of 61.5% w/w of 20:3 and 20:4 (Table 2). Palmitic acid and stearic acid were found in about equal amounts in both the free fatty acid and butyl ester fractions, whereas 60% of the amount of oleic acid was converted to the butyl ester derivative. When the reaction period was extended to 48 h, the level of enrichment of the *cis*-5 unsaturated fatty acids was not further improved.

Applying this procedure to a gram scale operation (10 g) of *B. orientalis* fatty acids, the isolated free fatty acid fraction was found to contain 40% w/w of the *cis*-5 unsaturated 20:3 and 20:4 fatty acids after 24 h of reaction. No solvent was used in this reaction. The reaction time could be reduced to

TABLE 3
Multigram Esterification of Fatty Acids from *Biota orientalis* Seed Oil by Repeated Treatment with *n*-BuOH in the Presence of *Candida cylindracea* in *n*-Hexane at 20°C

Reaction time (h)	Component[a]	Amount (% w/w)	Fatty acid composition (wt%) 18:1	18:2	18:3	20:3	20:4	20:3 + 20:4	Enrichment 20:3 + 20:4 (-fold)	Yield of 20:3 + 20:4 (%)
0	FA	100	13.8	25.7	34.3	4.8	9.8	14.6	1.0	100
6	FA	35	15.6	18.0	15.6	13.4	26.4	39.8	2.7	95
	BE	65	27.7	20.1	41.3	0.6	0	0.6	—	—
6	FA	43	8.5	7.5	6.1	27.5	32.4	59.9	4.1	65
	BE	57	60.6	10.0	9.5	2.2	3.4	5.6	—	—
6	FA	61	6.9	3.0	1.1	38.6	33.9	72.5	5.0	74
	BE	39	20.4	13.5	19.2	11.2	12.5	23.7	—	—

[a]FA, fatty acids; BE, butyl esters. The average amount (wt%) of FA and BE of 16:0, 18:0, 20:1 and 20:2 are 4.4, 2.1, 1.5 and 6.7, and 8.1, 2.4, 1.5 and 3.9, respectively.

six hours with an enrichment yield of 40% w/w of 20:3 and 20:4, when the process was carried out under reduced pressure (20 mm Hg) by connecting the reaction vessel to a water aspirator. Prolonging the reaction time for this experiment by a further 24 h caused a substantial amount of 20:3 and 20:4 fatty acids to be esterified. To circumvent this problem, the isolated free fatty acid fraction was instead subjected twice to a fresh treatment with *C. cylindracea* lipase for six hours under reduced pressure. The result showed an overall enrichment of the *cis*-5 unsaturated 20:3 and 20:4 fatty acids to a level of 72.5% with a yield of 20% w/w of fatty acids. This concentrate also contained a 12.1% w/w of 20:2 as determined by gas chromatography. The composition of this fraction is given in Table 3.

In order to improve further on the result of the enrichment process involving *C. cylindracea* lipase, removal of the saturated fatty acids prior to enzymatic treatment was considered beneficial to the enrichment process. Urea fractionation of the free fatty acids from *B. orientalis* seed oil allowed all saturated fatty acids (16:0, 18:0 and 20:0) including the trace amounts of 20:1 and 20:2 fatty acids to be separated. The uncomplexed urea fraction (filtrate) consisted of four fatty acids, *viz.* linoleic acid (26%), linolenic acid (41.8%), 20:3 (9.5%) and 20:4 (25.2%). Esterification of this mixture of polyunsaturated fatty acids with *C. cylindracea* lipase afforded an 85% enrichment of 20:3 and 20:4 fatty acids after repeating the process three times with fresh lipase on the isolated fatty acid fraction. It can therefore be concluded from these results (Table 4) that an optimum (85.3%) enrichment condition for 20:3 and 20:4 fatty acids from *B. orientalis* seed oil can be achieved by first performing a urea fractionation to remove the saturated fatty acids followed by repeated esterification with *C. cylindracea* lipase.

Attempts to esterify fatty acids from *Biota* seed oil with *n*-butanol in *n*-hexane in the presence of *C. cylindracea* lipase immobilized on acrylic beads have failed even after stirring the mixture for 24 h at 25°C. The starting materials were recovered instead.

The lipase of *C. cylindracea* is also known to preferentially hydrolyze triacylglycerols, where the acyl chain contains a *cis*-9 double bond. When a mixture of triacylglycerols (100 mg) from *Biota* oil was stirred in potassium phosphate buffer (pH 7.0) in the presence of *C. cylindracea* lipase at 25°C for 30 min, the fatty acid composition of the combined isolated mono-, di- and triacylglycerols showed 37.2% w/w of 20:3 and 20:4 fatty acids. Extending the reaction time to 2 h, the enrichment of 20:3 and 20:4 fatty acids by this procedure reached a maximum level of 40.8%. However, when the

TABLE 4
Enrichment of *cis*-5 Polyunsaturated Fatty Acids in Urea Fractionated *Biota orientalis* Fatty Acids by Repeated *Candida cylindracea* Lipase-Catalyzed Esterification with *n*-Butanol in *n*-Hexane at 25°C

Time (h)	Component[a]	Amount (% w/w)	Fatty acid composition 18:1	18:2	18:3	20:3	20:4	20:3 + 20:4	Enrichment 20:3 + 20:4 (-fold)	Yield of 20:3 + 20:4 in FA
0	FA[b]	100	0.8	22.6	41.8	9.5	25.2	34.7	1.0	100
12	FA	43	0.7	17.4	24.7	13.6	43.6	57.2	1.6	71
	BE	57	0.7	28.5	65.1	1.6	4.1	5.7	—	—
12	FA	77	0.8	16.2	14.2	19.3	49.5	68.8	2.0	93
	BE	23	0.8	27.0	63.2	2.1	6.9	9.0	—	—
12	FA	53	0.5	9.7	4.5	24.6	60.7	85.3	2.5	66
	BE	47	1.3	36.7	45.0	4.3	12.7	17.0	—	—

[a]FA, fatty acids, BE, butyl esters.
[b]Nonurea complexed fraction obtained after urea fractionation.

TABLE 5
Partial Hydrolysis of Triglycerides of *Biota orientalis* Seed Oil by *Candida cylindracea* Lipase in Potassium Phosphate Buffer (pH 7.0, 0.1 M) at 25°C

Reaction time (h)	Component[a]	Amount (% w/w)	Fatty acid composition (wt%)					20:3 + 20:4	Enrichment 20:3 + 20:4 (-fold)	Yield of 20:3 + 20:4 (%)
			18:1	18:2	18:3	20:3	20:4			
0	AG	100	13.8	25.7	34.3	4.8	9.8	14.6	1.0	100
0.5	AG	36	14.7	15.8	11.9	13.7	23.5	37.2	2.5	92
	FA	64	16.4	32.7	36.9	1.9	2.8	4.7	—	—
1.0	AG	32	18.4	22.9	15.7	11.4	15.6	27.0	1.8	59
	FA	68	11.0	30.0	46.7	1.7	4.0	5.7	—	—
2.0	AG	23	12.4	14.5	11.6	14.5	26.3	40.8	2.8	64
	FA	77	15.5	29.7	35.8	3.4	5.5	8.9	—	—

[a]AG, acylglycerols; FA, fatty acids. The average amount (wt%) of AG and FA of 16:0, 18:0, 20:1 and 20:2 are 6.2, 6.4, 1.2 and 3.4, and 5.1, 2.7, 0.3 and 0.6, respectively.

reaction time was prolonged, hydrolysis of the acyl chains became less controlled, causing the *cis*-5 unsaturated fatty acids to be also hydrolyzed. From this result (Table 5) it appears that acylglycerols enriched in *cis*-5 20:3 and 20:4 up to 41% can be achieved by the use of the lipase from *C. cylindracea* on hydrolysis of triacylglycerols of the oil of *B. orientalis.*

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