Preparation of Eicosapentaenoic Acid (EPA) Concentrate from *Porphyridium cruentum*

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The polyunsaturated fatty acid eicosapentaenoic acid (EPA) has attracted increased attention due to its pharmaceutical properties. The main source is marine fish oil which contains approximately 15% EPA. However, pharmaceutical applications of EPA will probably require higher concentrations, perhaps as high as 90%. The red microalga *Porphyridium cruentum* is a potential source, because its EPA content approaches 44.1% of the total fatty acids. Three methods were attempted for EPA concentration and arachidonic acid (AA) removal from the oil of this alga. Separation of the glycolipids, formation of a urea inclusion complex and reverse phase chromatography on C-18 Sep-Pak filters resulted in an EPA concentrate of 97% purity. Similar methods resulted in an AA concentrate of 80% purity.

KEY WORDS: AA, algal oil, EPA, *Porphyridium*, PUFA purification.

The polyunsaturated fatty acids eicosapentaenoic acid (EPA) and arachidonic acid (AA) have attracted increased attention in recent years. EPA was shown to reduce blood pressure in rats (1,2) as well as in humans (3,4). It has been suggested that EPA competitively inhibits the conversion of n-6 fatty acid precursors to AA, suppresses the production of eicosanoids of the 2 series (5) and increases the formation of eicosanoids of the 3 series, some of which are hypotensive (6). EPA was also found to reduce blood cholesterol levels (7). AA serves as a starting material for a biosynthetic production of prostaglandin PGE_2. Also, being a component of human mother's milk, it is of potential value as an ingredient in various formulations of artificial baby food.

The main source of EPA is marine fish oil which contains approximately 15% EPA. However, it contains also significant amounts of cholesterol. Daily consumption of marine fish oil eventually results in an increased cholesterol uptake which could be counterproductive for reducing blood lipid level. Pharmaceutical applications of EPA will probably require preparations of considerably higher EPA concentrations. An n-3 fatty acid concentrate was made from cod liver oil by urea inclusion (8). Although the product contained 85% n-3 fatty acids, only 28% consisted of EPA.

The red microalga *Porphyridium cruentum* is a potential source for the pharmaceutically valuable fatty acids EPA and AA (9). In previous studies we elucidated the environmental conditions leading to high contents of either EPA or AA (9-11). In this paper, methods are described for the fractionation of *P. cruentum* oil resulting in EPA and AA concentrates.

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MATERIALS AND METHODS

Growth of cells: P. cruentum strain 1380.1d was obtained from the Göettingen Algal Culture collection (Göettingen, Germany). Cultures were grown on Jones' medium (12) in glass tubes that were incubated in a temperature-regulated water bath and illuminated with four cool-white fluorescent lamps providing 170 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the side of the water bath. The cultures were mixed by bubbling an air — CO_2 mixture (99:1) through a sintered glass tube placed at the bottom of each tube.

Lipids fractionation. Freeze-dried samples of biomass were extracted with chloroform-methanol-water (2:1:0.8) according to Bligh-Dyer (13). Separation into neutral lipid, glycolipid (GL) and phospholipid classes was achieved on a silica gel column. The individual classes were successively eluted with chloroform, acetone and methanol, respectively.

Lipid transmethylation. The GL fraction (0.65 g) was treated with 13 mL of methanol-acetyl chloride (95:5) at 0°C. The mixture was sealed in a light-protected Teflon-lined vial under an Argon atmosphere and heated to 80°C for 1 hr. The vial contents were then cooled, diluted with 5 mL water and extracted with 5 mL of hexane containing 0.01% butylated hydroxytoluene. The hexane layer was dried over Na_2SO_4, evaporated to dryness and redissolved in hexane.

Fatty acid analysis. Gas chromatographic analysis was performed with a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm) at 200°C (injector and flame ionization detector temperatures 230°C, split ratio 1:100). The results were formulated with an HP 3390A integrator. For quantitative analyses, 17:0 methyl ester was added as an internal standard. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma, St. Louis, MO) and by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed with a Finnigan 500 mass spectrometer equipped with a carbowax capillary column (30 m × 0.25 mm). Chemical ionization spectra were obtained at 250 ev with isobutane as the reactant gas. The data shown are mean values of at least two independent samples, each analyzed in duplicate.

Fractionation with urea. One gram of fatty acid methyl esters (FAME) was dissolved in 10 mL methanol containing 4 gram urea (E. Merck, Darmstadt, Germany) by heating the mixture to 65°C until clear. The urea and the urea inclusion complexes were allowed to crystallize at room temperature and then were refrigerated overnight at 4°C and then at −15°C. The mother liquor was separated by vacuum filtration and was extracted with 3 mL methylene chloride after addition of 6 mL water and 3 mL concentrated hydrochloric acid. The lower phase was separated and the aqueous

layer was reextracted with methylene chloride. The combined extracts were evaporated to dryness and dissolved in methanol.

Reverse phase chromatography. C-18 Sep pak filters (Waters Associates, Milford, MA) were preconditioned with 5 mL each of acetonitrile, water and 25% acetonitrile-water. A solution of 1 mg of the FAME mixture in 100 μL methanol was loaded onto the filter and eluted by washing the column with 10 mL vol of acetonitrile-water mixtures of increasing acetonitrile concentrations (58%–100%), at a flow rate of approximately 5 mL/min. Heptadecanoic acid methyl ester was added as an internal standard. Subsequently, each fraction was extracted twice with methylene chloride and water. The combined organic phases were evaporated to dryness, reconstituted in hexane and analyzed by GC.

RESULTS AND DISCUSSION

A preliminary evaluation of several strains of *P. cruentum* indicated that strain 1380.1d was the most promising EPA producer (11). It had the highest content of EPA, i.e., 2.4% of ash-free dry weight. This strain had also a relatively low AA content. The AA to EPA ratio (R) reflects the degree of difficulty in the purification of EPA. The lower the R value, the easier the separation from AA.

In previous studies we have demonstrated that by environmental and nutritional control it is possible to either increase or decrease the EPA and AA content and thus the R value (9,10). Under optimal growth conditions EPA was found to be the major fatty acid, while under reduced growth rate AA becomes the main fatty acid.

P. cruentum strain 1380.1d was cultivated under conditions leading to a high EPA content, i.e. a temperature of 25°C, high light intensity and low cell concentration. Under these conditions, the extracted oil contained 40.7% EPA (% of total fatty acids) and 14.6% AA. Moreover, approximately 90% of the EPA was restricted to the GL while the AA was primarily contained in the neutral lipid and the phospholipid fractions. Therefore, in order to purify EPA from other fatty acids and especially from AA, a preliminary fractionation of the GL from the oil was undertaken. In the acetone fraction which included the GL, the proportion of EPA increased to 47.5% while that of AA decreased from 14.6% to 5.4% (Table 1).

EPA enrichment by urea fractionation. The GL fraction was transmethylated and, by forming urea inclusion complexes, almost all of the saturated and monounsaturated fatty acids were effectively removed. The EPA and AA contents increased to 81.9% and 6.6% respectively. It is interesting to note that the removal was more efficient for C-18 FAME than for C-16 FAME (Table 2). Also, while the urea inclusion complexes are primarily formed with saturated and monounsaturated fatty acids, the inclusion complexes contained some linoleic acid and AA, while EPA was completely excluded. Thus, not only was EPA purified from saturated and monounsaturated FAME, but a significant purification from AA was also achieved, as is evidenced in the R values which decreased from 0.11 to 0.08 (Table 2). Similarly, Traitler *et al.* (14) have shown that by urea fractionation of black currant seed oil, it was possible to selectively enrich γ-linolenic acid over α-linolenic acid. It appears therefore that the urea fractionation method could be utilized for more than just the removal of saturated and monounsaturated

TABLE 1

EPA Enrichment by Silica Gel Fractionation of *P. cruentum* Lipids

	Fatty acid composition (% of total fatty acids)									
	16:0	16:1	18:0	18:1	18:2	20:2	20:3	20:4	20:5	R
Total lipids	30.6	5.3	0.3	0.9	4.8	0.8	2.0	14.6	40.7	0.36
Glycolipids fraction	34.8	0.4	0.8	1.3	9.6	0.1	0.2	5.4	47.5	0.11

TABLE 2

EPA and AA Concentration by Urea Treatment

	Fatty acid composition (% of total fatty acids)									
	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	R
FAME[a]	34.8	0.4	0.8	1.3	9.6	0.1	0.2	5.4	47.5	0.11
FAME after urea treatment	3.1	0.3	—	0.6	7.3	0.1	0.2	6.6	81.9	0.08
FAME after chromatography	0.3	—	—	—	0.9	—	0.2	1.4	97.3	0.01

[a]FAME of glycolipid fraction.

TABLE 3

EPA Purification by Reverse Phase Chromatography[a]

	Fatty acid composition (% of total fatty acids)									
% CH_3CH[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	% of total EPA[c]
58	14.2	2.0	1.2	1.6	3.2	2.9	1.4	6.1	67.3	2.7
59	1.6	0.1	0.2	0.0	1.7	0.4	0.5	2.3	93.2	29.1
59	3.2	0.2	0.3	0.0	2.8	0.8	0.9	3.0	88.9	27.2
59	2.4	0.3	0.4	0.2	4.0	1.0	1.5	4.8	85.4	25.2
59	19.0	1.0	0.9	0.8	16.8	1.1	0.5	15.2	44.8	7.1
60	32.4	1.1	0.8	1.3	21.3	0.7	1.9	17.1	23.4	4.2
61	46.7	1.2	0.2	1.3	25.1	1.2	1.8	15.1	7.4	1.5
62	60.8	1.2	0.7	1.7	15.9	0.0	3.9	10.1	5.6	0.9
63	63.7	1.3	1.2	2.3	13.1	0.6	3.8	6.7	7.2	0.6
65	68.8	1.5	0.7	3.1	7.6	2.0	1.9	5.3	9.1	0.5
100	74.3	1.7	4.5	2.7	4.6	0.4	3.9	2.4	5.5	0.4
Pre-separation mixture[d]	33.8	0.4	0.8	1.2	9.3	0.1	0.2	8.2	46.1	

[a]Representative example.
[b]Percent of acetonitrile in acetonitrile-water.
[c]Percent of total EPA — the ratio of EPA content in fraction to total EPA eluted.
[d]Composition of FAME mixture loaded on the column.

fatty acids. The inclusion complex formation reported here was not optimized, and it is quite possible that optimization could result in an even more efficient removal of AA.

The same procedure was utilized for the preparation of an AA concentrate. *P. cruentum* strain 1380.1b was previously identified as a rich source of AA (11). At the stationary phase, the AA content increased to 41.7% of total fatty acids. The oil was transmethylated and treated with urea as above resulting in the AA content increasing to 80.0% (data not shown).

EPA purification by reverse phase chromatography. Reverse phase chromatography is known to separate fatty acids according to chain length and by degree of unsaturation (15). However, separation is generally achieved by high-performance liquid chromatography (HPLC) which is quite expensive. In this work, we attempted to concentrate EPA by open-bed reverse phase chromatography. C-18 Sep pak filters were utilized as a model. Acetonitrile-water mixtures proved to be more selective than methanol-water in the separation of EPA from AA. Equal volumes of increasing concentrations of acetonitrile in water were utilized to elute the FAME mixtures (Table 3). The first two fractions (40% and 55% acetonitrile) consisted of unretained FAME. However, the percent of total EPA found in these fractions combined was less than 0.7%. EPA started to elute in the third fraction (58% acetonitrile) which still contained some unretained material. No attempts were made to improve the separation at this point, as this fraction contained only 2.7% of total EPA. The bulk of the EPA was eluted in the next 3 fractions (59% acetonitrile) which contained 81.5% of total EPA. The EPA content in each fraction ranged from 85.4% to 93.2%, while AA did not exceed 4.8%. The AA and 18:2 contents peaked at 60% and 61% acetonitrile respectively. While this procedure is effective for removal of saturated and monounsaturated FAME, it is envisaged that on a large scale it would be advantageous to pretreat the mixture with urea. Indeed, when reverse phase chromatography was preceded by urea treatment, 85% of the EPA was recovered at a 97.3% purity.

ACKNOWLEDGMENTS

The competent technical assistance of S. Didi is gratefully acknowledged. We are indebted to B. Kessler for helpful discussion. This study was supported by grants from the U.S. Agency for International Development (DPE-5544-G-SS8017-00) and from the Israeli National Council for Research and Development. Contribution No. 44 from the Laboratory for Microalgal Biotechnology, Jacob Blaustein Institute for Desert Research.

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[Received April 27, 1990; accepted September 13, 1990]