0273-2289/99/80/0141/$12.50

Eicosapentaenoic Acid Release from the Red Alga *Pachymeniopsis lanceolata* by Enzymatic Degradation

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Received September 10, 1998; Accepted February 9, 1999

Abstract

Forty-eight species of seaweeds from Japanese waters were screened for the valuable polyunsaturated fatty acids eicosapentaenoic acid (EPA). The eight species that contained the highest levels of these compounds were analyzed in detail. Of all species tested the red alga *Pachymeniopsis lanceolata* contained the highest EPA concentration, and it was present as both the free and bound forms. EPA constituted 38.7% of total fatty acids, and polar lipids were the main constituent of the total lipids in *P. lanceolata*. EPA was obtained from the marine algae *P. lanceolata* by enzymatic hydrolysis of the total lipids extract using phospholipase A_2 (PLA_2). The release of EPA reached a plateau after 10 min of enzymatic treatment. These results suggest that *P. lanceolata* is a useful natural source of EPA and that PLA_2 treatment is a convenient method for obtaining EPA from the red alga.

Index Entries: Eicosapentaenoic acid; phospholipase A_2; enzymatic hydrolysis; seaweeds; *Pachymeniopsis lanceolata*.

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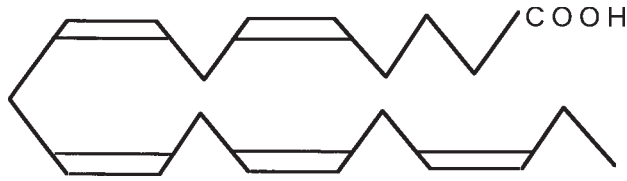

Fig. 1. Structure of *cis*-5,8,11,14,17-eicosapentaenoic acid.

Introduction

Eicosapentaenoic acid (EPA), a polyunsaturated fatty acid comprising 20 carbon atoms with 5 double bonds (Fig. 1), is known to play an important role in mammals as an agent for the prevention of blood platelet aggregation *(1,2)* and to be useful for the reduction of blood cholesterol *(3,4).*

It has been demonstrated that EPA is effective in the prevention and cure of thrombosis *(5,6)*, arteriosclerosis, and other blood-circulatory diseases *(7–9).* Greenland Eskimos have few of these diseases, and this is thought to be because they eat fishes that are rich in EPA. Fish oils contain EPA, which originates from marine micro- and macroalgae, and is concentrated in fish via the food chain. Commercial sources of EPA are almost solely limited to fish oils. However, in addition to the difficulty in separating it from other fatty acids, EPA derived from fish oils has considerable disadvantages such as its unpleasant odor. Therefore, there is considerable medical, nutritional, and commercial interest in finding alternative natural sources of easily isolated EPA.

Microalgae have been promoted as a potential source of EPA use in health foods and pharmaceuticals. EPA has been found in microalgae such as *Isochrysis galbana (10)*, *Phaeodactylum tricornutum (11)* and *Porphyridium cruentum (12)*, and its production from such sources has been reported *(13–15).* However, very little is known about macroalgae that contain high concentrations of EPA *(16,17).* In a previous paper, Hatakeda et al. *(18)* characterized the fatty acid components in the lipids of *Pachymeniopsis lanceolata*, a red marine seaweed. To obtain EPA from marine algae in its free, usable form, it is necessary to find EPA-rich algae and to develop a convenient way to release it from such algae.

In this study, we report the screening of marine algae for EPA by gas chromatographic analysis of their fatty acids. The seasonal variation in the EPA content of harvested *P. lanceolata*, the species found to have the highest EPA content, was also studied. The efficient release of EPA from that marine algae on treatment with phospholipase A_2 (PLA_2) is demonstrated.

Materials and Methods

Materials

Forty-eight species of marine algae, including *P. lanceolata*, were harvested at the coast of Onagawa, Miyagi prefecture and Kamakura,

Table 1
Seaweeds of High-Level EPA[a]

Division	Species
Rhodophyta	*Chondria crassicaulis*
	Pachymeniopsis lanceolata
	Porphyra tenera
Phaeophyta	*Analipus japonicus*
	Colpomenia sinuosa
	Desmarestia ligulata
	Dictyopteris divaricata
	Spatoglossum pacificum

[a]Seaweeds were collected on the Pacific Ocean coast (Onagawa, Miyagi prefecture) in Japan.

Kanagawa prefecture, Japan from March 1988 to July 1995. There were 5 species of green algae, 23 species of red algae, and 20 species of brown algae. The eight species given in Table 1 were found to have the highest concentrations of EPA and were examined in detail.

The algae were carefully cleaned, rinsed in freshwater, and lyophilized. The dried algae were used in the lipid extractions after crushing.

PLA_2 (EC 3.1.1.4), fatty acid methyl esters (FAMEs), linoleic acid, *cis*-5,8,11,14,17-EPA, and reference phospholipids (phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Extraction and Lipid Analysis

To 250 g of each of the dried 48 algal species 1 L of chloroform:methanol (2:1, v/v) was added, which was then kept in a cold room (4°C) for 2 wk. Lipids were extracted by the method of Folch et al. *(19).* Each algal suspension was filtered and the filtrate allowed to separate into chloroform and aqueous layers. The organic layer was then evaporated under vacuum, and the total lipids were dried in a desiccator and weighed.

Lipids were fractionated into hydrocarbons, triglycerides, free fatty acids, and polar lipids on a silicic acid column (100–200 mesh). To 215 g of dried *P. lanceolata* 2 L of acetone were added and mixed in a blender at room temperature. The algal suspension was filtered and divided into acetone soluble and insoluble fractions. Lipids were eluted using *n*-hexane, 3% ether/*n*-hexane, 15% ether/*n*-hexane, ether, and methanol *(20).* Fractions were evaporated to dryness and the lipid content was determined gravimetrically. The purity of the lipids in each fraction was assessed using thin-layer chromatography (TLC).

For the analysis of phospholipids, the extracts of *P. lanceolata* in chloroform:methanol (2:1) containing 0.05% butylated hydroxytoluene were spotted on silica plates and developed in chloroform:methanol:water (65:35:5).

After development, phospholipids on the plates were stained by the method of Ryu and MacCoss *(21).* Lipids containing phosphate groups were detected using the reaction of phosphate and molybdate. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine were used as reference standards.

Gas chromatography (GC) analysis was carried out to determine the fatty acid composition and content of the different algae. The extracted lipids were added to 0.5 *N* KOH-95% ethanol and saponified for 2 h under reflux and esterified using sodium methoxide/methanol solution at 100°C for 30 min. FAMEs were analyzed on a Shimadzu-15A or a Hitachi G-5000 GC equipped with a flame ionization detector (FID) using a Silar 10C (5%) column (GL Sciences Inc., Tokyo, Japan, 3 mm i.d. × 4 m). The FID and injector port were set at 280°C, while the oven temperature was programmed to rise from 160 to 260°C at 4°C min^{-1}, and held at the maximum temperature for 10 min. Individual peaks of FAMEs were identified by comparison with the retention time of known standards on GC and by GC-mass spectrometry (JEOL DX300) using the same column as for the fatty acid analysis. Fatty acid contents were determined from the integrated peak areas. The data shown are mean values of at least three independent samples, each one analyzed in duplicate.

Enzymatic Hydrolysis and High-Performance Liquid Chromatography Analysis for EPA

One milliliter of 50 m*M* Tris-HCl buffer (pH 8.0), 50 µL of 0.2 *M* $CaCl_2$, 50 µL of PLA_2 (50 U), and 2 mL of diethyl ether saturated with water were added to the dried lipid extract. The enzymatic digestion was carried out for 10–30 min at room temperature, and then the mixture was centrifuged at 3000 rpm (800*g*) for 5 min. The ether layer of the supernatant was analyzed for EPA content using high-performance liquid chromatography (HPLC) with a C18 column (TSKgel ODS-80Ts, Tosoh Corporation, Tokyo, Japan).

EPA was separated by elution with a mixture of acetonitrile: 10 m*M* phosphate buffer (pH 2.2) (80:20, v/v). EPA was identified by cochromatography with authentic samples and its concentration calculated from the peak areas. EPA released by the enzymatic hydrolysis was calculated as the difference between the PLA_2-treated samples and untreated controls.

Results and Discussion

Screening and Fatty Acid Composition

Table 2 shows the types, percentages, and amount of the various fatty acids (mg/100 g biomass) in the eight seaweeds with the highest EPA levels. The highest level of EPA, 347 mg /100 g of biomass, was found in *P. lanceolata.* Concentrations of more than 100 mg of EPA/100 g of biomass were observed in two red algae (*Chondria crassicaulis* and *P. lanceolata*) and two brown algae (*Colpomenia sinuosa* and *Dictyopteris divaricata*). *Analipus japonicus* and *Desmarestia ligulata* contained 98 mg and 99 mg of EPA/100 g of biomass, respectively.

Table 2
Fatty Acid Composition and Content of Seaweeds

Fatty acid	*Analipus japonicus*	*Chondria crassicaulis*	*Colpomenia sinuosa*	*Desmarestia ligulata*	*Dictyopteris divaricata*	*Pachymeniopsis lanceolata*	*Porphyra tenera*	*Spatoglossum pacificum*
Fatty acid composition (wt % of total fatty acids)								
C14:0	6.3	9.2	8.2	5.1	2.4	2.9	0.0	6.5
C16:0	21.1	44.8	23.5	19.7	22.5	33.9	31.8	20.0
C16:1	1.5	3.2	1.2	2.8	6.8	2.1	2.4	4.2
C16:2	0.9	0.0	0.0	0.5	1.3	0.0	0.0	1.5
C18:0	0.5	1.5	1.9	1.9	1.3	0.0	1.0	1.1
C18:1 (n-9)	20.7	17.8	21.8	10.9	12.8	7.3	3.4	17.9
C18:2 (n-6)	12.0	1.8	8.5	10.8	8.8	0.9	2.5	7.8
C18:3 (n-3)	7.5	0.0	7.2	13.2	13.1	0.0	0.0	12.0
C18:4 (n-3)	11.9	1.5	8.8	14.4	12.9	0.0	4.1	18.2
C20:3 (n-6)	0.6	0.0	1.2	0.0	0.0	0.5	2.2	0.7
C20:4 (n-6)	7.8	3.4	5.7	11.0	9.8	13.2	4.4	7.4
C20:5 (n-3)	8.0	16.7	12.0	7.4	7.3	38.7	48.1	0.9
Fatty acid content (mg/100 g biomass)								
C14:0	77	67	96	67	41	26	0	163
C16:0	257	323	276	262	383	303	21	505
C16:1	18	23	14	38	116	19	2	105
C16:2	11	0	0	7	23	0	0	38
C18:0	6	11	23	25	23	0	1	28
C18:1 (n-9)	253	129	256	145	218	64	2	450
C18:2 (n-6)	146	13	100	144	150	9	2	197
C18:3 (n-3)	91	0	84	176	224	0	0	301
C18:4 (n-3)	146	11	103	191	219	0	3	459
C20:3 (n-6)	8	0	14	0	0	5	1	17
C20:4 (n-6)	95	24	67	146	168	134	3	186
C20:5 (n-3)	98	120	140	99	125	347	31	23

Table 3
Fatty Acid Composition (wt %) of Lipid Classes of *P. lanceolata*[a]

Fatty acid	Free fatty acids	Polar lipids	Triglycerides
C14:0	3.9	2.2	2.0
C15:0	1.0	0.0	0.0
C16:0	46.7	38.0	21.4
C16:1	2.2	1.6	2.0
C18:0	1.3	0.0	0.0
C18:1 (n-9)	11.3	7.7	8.3
C18:2 (n-6)	0.9	0.0	1.0
C20:3 (n-6)	0.6	0.0	0.9
C20:4 (n-6)	19.3	10.7	31.3
C20:5 (n-3)	12.7	39.8	33.0

[a]See ref. *18*.

P. lanceolata was found to contain the fewest types of fatty acid. Only 8 of the 12 fatty acids found in the different seaweeds were present in *P. lanceolata*—C14:0, C16:0, C16:1, C18:1, C18:2, C20:3, C20:4, and C20:5. The main fatty acids were C16:0 and C20:5, comprising 33.9 and 38.7% of the total fatty acids, respectively. These results suggest that *P. lanceolata* is a good source of EPA, which may be easily separated from other fatty acids.

Fatty Acid Composition of Lipid Classes

The lipids of *P. lanceolata*, which had the highest EPA content, comprised free fatty acids (5.9%), hydrocarbons (12.2%), polar lipids (72.4%), and triglycerides (9.5%). Polar lipids are the main constituents of the lipids in *P. lanceolata.* EPA is distributed among the free fatty acids, polar lipids, and triglycerides (Table 3). The main fatty acids of the polar lipid fraction were C16:0 (38.0%) and C20:5 (39.8%). The polar lipid fraction contained more EPA than the triglyceride and free fatty acid fractions. Polar lipids such as phospholipids are known to occur as membrane lipids in seaweeds.

Phospholipids such as phosphatidylethanolamine, phosphatidylchlorine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine were identified in the lipid extracts of *P. lanceolata* using TLC (data not shown).

PLA_2 Treatment

PLA_2 is known to hydrolyze selectively the ester linkage at the C-2 position of phospholipids (Fig. 2), where, in the lipids of *P. lanceolata*, X may be choline, ethanolamine, inositol, or serine, and R_2 is EPA.

Figure 3 shows the effect of PLA_2 treatment on the release of EPA from phospholipids in extracts of *P. lanceolata.* The enzymatic hydrolysis dramatically increased the amount of EPA in the chloroform:methanol extracts. The amounts of EPA obtained with and without PLA_2 treatment were

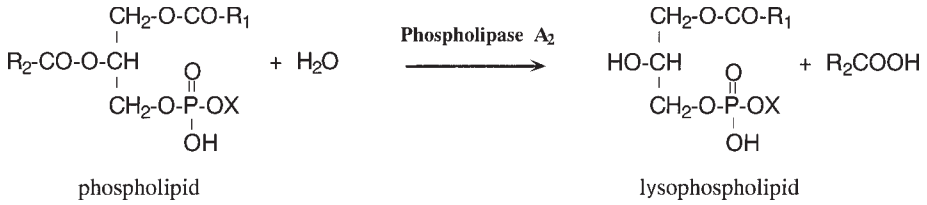

Fig. 2. Enzymatic hydrolysis of phospholipids by PLA_2.

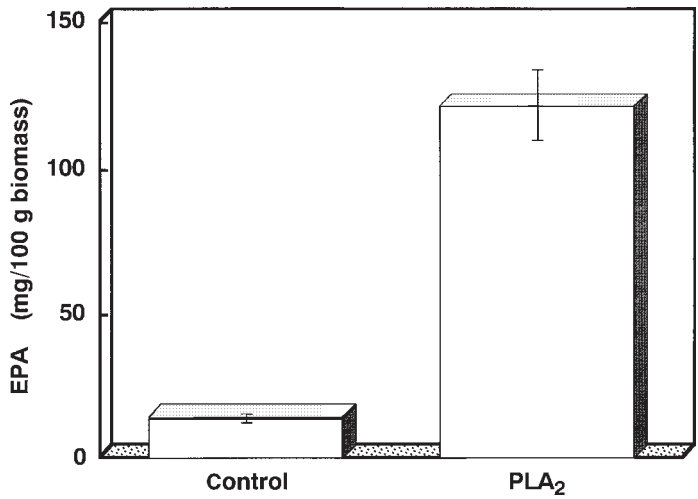

Fig. 3. Effect of PLA_2 treatment on EPA release from phospholipids in extracts of *P. lanceolata.* Assay methods are described in Materials and Methods.

121.62 and 13.53 mg/100 g of biomass, respectively. The amount of bound EPA in the phospholipids was 108.09 mg/100 g of biomass. Thus, both free and bound EPA exist in extracts of *P. lanceolata.* Since EPA and palmitic acid were found to amount to 38.7 and 33.9%, respectively, of the total fatty acids in *P. lanceolata,* the high proportion of EPA in the total lipids of *P. lanceolata* and the low levels of unsaturated fatty acids should simplify methods for the recovery of EPA. The enzymatic degradation of *P. lanceolata* lipids by PLA_2 is an effective method for the release of EPA from phospholipids.

Time Course of EPA Release

Lipid extracts of *P. lanceolata* were incubated with PLA_2 in the presence of $CaCl_2$. After incubation with PLA_2 as shown in Fig. 4, the amounts of EPA released were determined using HPLC. The amount of EPA released increased for 10 min (Fig. 4), then remained constant until 120 min (data not shown). Thus, a reaction time of 10 min is sufficient to release practically all the bound EPA by enzymatic hydrolysis. The hydrolysis of EPA by PLA_2 is quite efficient and rapid.

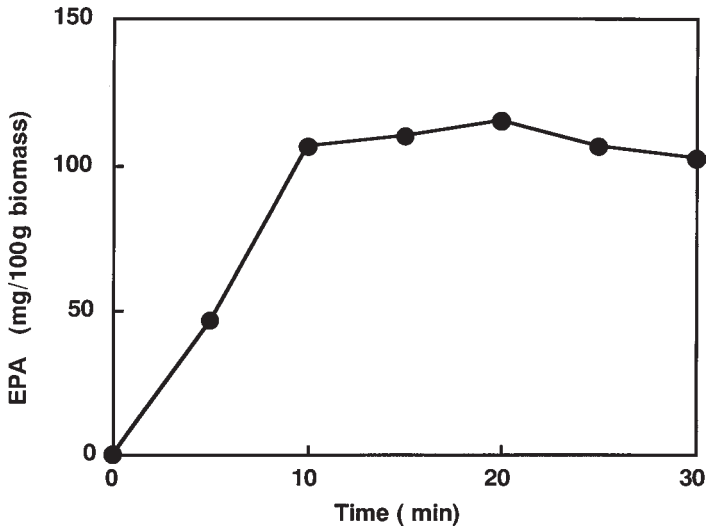

Fig. 4. Time course of EPA release on PLA_2 treatment. Assay methods are described in Materials and Methods.

Seasonal Variation in EPA Content

The seasonal variation in EPA content and seawater temperature from December to July are shown in Fig. 5. The EPA content of *P. lanceolata* collected in January was higher than other samples while the average seawater temperature in January was 10°C, the lowest of all examined. These results suggest that the proportion of EPA increases at low temperatures. It has been reported that the fatty acid composition of marine algae is influenced by environmental conditions, in particular, temperature *(22,23).*

It is thought that the lower freezing point of unsaturated fatty acids and of compounds that contain them (e.g., triglycerides and phospholipids) is advantageous in cold conditions, by preserving membrane fluidity *(24)*. It has been shown that desaturases are activated at low temperatures *(25)*, and that mutants defective in such desaturases are less tolerant to cold *(26)*. The converse is true in that the more heat-tolerant species and mutants contain low levels of unsaturated membrane lipids. The observed seasonal variation in EPA suggests that *P. lanceolata* is harvested during the cold season, at the time when EPA levels peak.

It is known that brown and red algae generally contain higher proportions of C20:4 and C20:5 fatty acids than other taxa. EPA accounted for 41% of the total fatty acids of *Porphyra tenera (27)*, and 48% of the total fatty acids of *Porphyra yezoensis (28)* and *Porphyra umbilicalis (17)*. EPA accounted for 38.7% of the total fatty acids of *P. lanceolata*, which contained 347 mg of EPA/100 g of biomass. This is nearly 10 times higher than the EPA content of *P. tenera* (Table 2). Thus, *P. lanceolata* is an important potential source of EPA. Although there have been many reports of EPA production from microalgae *(13–15,29–32)*, the disadvantages of microalgae are that the

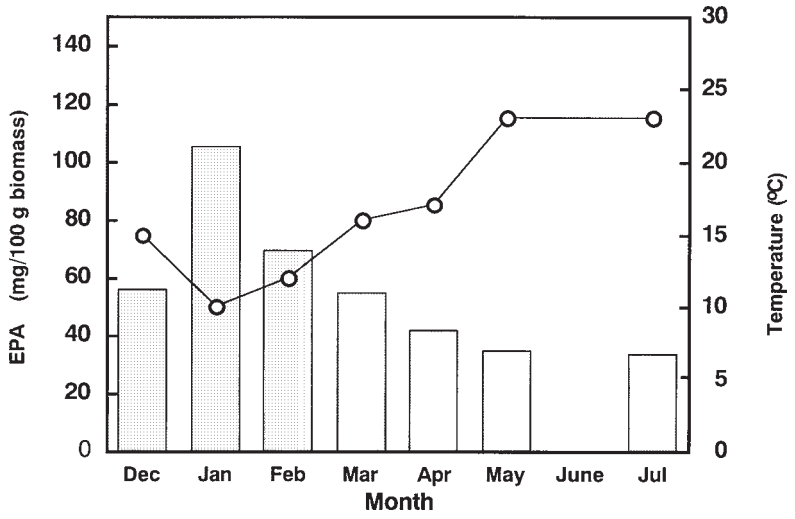

Fig. 5. Seasonal variation of EPA content of *P. lanceolata* and seawater temperature. EPA content was determined by HPLC analysis after enzymatic treatment. —○—, Seawater temperature.

amount of biomass is small and that the production costs are higher. In light of our study, the use of macroalgae such as *P. lanceolata* as a source for the production of EPA seems to be a commercially attractive option. Further studies focusing on the screening for high-EPA-producing cultivars, optimization of culture conditions, application of molecular biology to increase yields further, and the development of industrial scale digestion by PLA_2 are all necessary to develop an EPA-producing industry based on *P. lanceolata.*

Conclusion

We screened 48 types of marine algae for EPA content. The highest level of EPA was found in the red alga *P. lanceolata*. Polar lipids are the main constituents of the lipids of *P. lanceolata,* and EPA is one of the main fatty acids of the polar lipid fraction. EPA from *P. lanceolata* was effectively released by 10 min of digestion with PLA_2. The amount of EPA obtained after PLA_2 treatment was 121.62 mg/100 g of biomass. The level of EPA in lipid extracts of *P. lanceolata* harvested in January was highest when the temperature of the sea was lowest at 10°C. Our results suggest that *P. lanceolata* is a promising natural source of EPA, and that PLA_2 treatment is a convenient method of releasing this compound.

Acknowledgments

The authors wish to thank Professor Koga, Department of Fisheries in Kitasato University for identification of seaweeds. They also would like to thank Dr. Scott McNiven for linguistic assistance.

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