Composition and Biosynthesis of Fatty Acids in *Pyramimonas grossii*

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ABSTRACT

The green alga *Pyramimonas grossii* orginating in the coastal waters of the Atlantic Ocean Argentina was subcultured until a monoalgal culture was obtained. The fatty acid composition of the alga grown in a mineral medium at 12 C was determined by gas liquid chromatography (GLC) on 2 columns. The major fatty acids were oleic, linoleic, palmitic and α-linolenic acids, but the 20-carbon polyunsaturated acids, 20:4ω6 and 20:5ω3, respectively, belonging to the linoleic and α-linolenic series, were also found. Incubation with [^{14}C] oleate, [^{14}C] acetate, [^{14}C] linoleate and [^{14}C] α-linolenate suggests that linoleate is not directly converted to α-linolenate. [^{14}C] Acetate was easily converted to palmitic, palmitoleic and oleic acids. However, after 48 hr of incubation, only traces of radioactivity were detected in linoleic acid and no label was found in α-linolenic acid.

INTRODUCTION

It has been established that the main fatty acids present in marine planktonic green algae belonging to the classes Chlorophyceae and Prasinophyceae are 16:4ω3, 18:3ω3 and 16:0, with smaller quantities of the acids 18:2ω6, 18:3ω6, 16:3ω6 and 16:3ω3 (1). In addition, it is reported that some species belonging to the genera *Dunaliella* and *Heteromastix* also contain significant quantities of 20:5ω3, 18:4ω3 and 22:5ω3 acids (2). The presence of smaller quantities of odd carbon number saturated acids and the corresponding anteiso acids were also reported in a freshwater Chlorophyte (3).

Two different routes have been proposed for the synthesis of α-linolenic acid in plants. In one pathway, it would be produced by the oxidative desaturation of linoleic acid in the ω3-bond position of the chain (4). The other route was reported by Kannangara et al. for the green alga *Chlorella pyrenoidosa* (5). These authors suggest the presence of an enzymatic system capable of desaturating dodecanoic acid to dodecatrienoic acid which is then elongated to α-linolenic acid.

We report the fatty acid composition of the unicellular Chlorophyte *Pyramimonas grossii* isolated from coastal waters of Argentina and the results of a preliminary study on fatty acid biosynthesis in this species.

EXPERIMENTAL PROCEDURES

Microorganisms

P. Grossii Parke (6) was isolated from a coastal sea water sample (San Antonio Oeste Bay, Río Negro, 42°44′ S-64° 54′W, Argentina). The sample volume of 300 ml was enriched with a mixture of potassium phosphate and sodium nitrate according to Castellvi's method (7). After 1 week, *P. grossii* became dominant and a monoalgal culture was obtained by successive dilutions.

Cultures were maintained in a lipid-free liquid medium, which consisted of (mmol/ℓ): $NaNO_3$ (1), NaH_2PO_4 (1×10^{-1}), $NaSiO_2$ (2×10^{-1}), Na_2 EDTA (3×10^{-2}), Tris (5), NaCl (400, $MgSO_4$ (20), $MgCl_2$ (10), KCl (10), $NaCO_3H$ (2), $FeCl_3$ (2×10^{-3}), H_3BO_3 (2×10^{-1}), $MnCl_2$ (7×10^{-3}), $ZnCl_2$ (8×10^{-4}), $COCl_2$ (2×10^{-5}), $CuCl_2$ (2×10^{-7}), Vit. B_{12} (10 μg/ℓ), and distilled water (1,000 ml). The cultures were continuously illuminated with a 15-watt tungsten lamp at 30 cm from the culture surface.

Prior to incubation with labeled sustances, the cells were grown in 1-ℓ flasks with 400 ml of the medium, maintained in an incubator at 12 C ± 0.5 with continuous bubbling of sterile air at ca. 400 ml/min. When cultures reached half of the logarithmic growth phase (between 1.5 and 2.0×10^6 cells/ml), cells were either separated for fatty acid analysis or incubated with radioactive substances.

No contamination of cell cultures with bacteria could be detected by microscopic examination and only a few colonies appeared after culturing in the same medium with 3% agar for 9 days. Therefore, as bacterial contamination was negligible, the labeling products were ascribed specifically to the alga.

Incubation with Labeled Substrates

[1-^{14}C] Sodium acetate (61 mCi/mmol), [1-^{14}C] oleic acid (62 mCi/mmol), [1-^{14}C]-

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linoleic acid (61 mCi/mmol) and [1-^{14}C]α-linolenic acid (58 mCi/mmol) of 98% purity were purchased from Radiochemical Centre, Amersham, England for use as radioactive tracers.

Algal cultures were incubated with 2 μmol sodium acetate (2 μCi) for 48 hr. One μmol (1 μCi) of each of the other precursors was added to cultures as ammonium salt and incubated for 24 hr under the conditions already described.

Some incubations with [1-^{14}C]sodium acetate and [1-^{14}C]ammonium oleate were carried out in the presence of 20 μmol of nonradioactive linoleic acid. After incubation, the cells were separated by centrifugation at 2,500 x g for 10 min and washed with small amounts of fresh culture medium.

Fatty Acid Separation

Lipids and pigments were extracted from whole cells by the Folch et al. procedure (8). However, the yield of fatty acids was less than the amount obtained by a direct saponification of cells. Therefore, cells were saponified with 10% KOH in ethanol. Nonsaponifiable lipids were extracted with petroleum ether, radioactivity counted and then discarded. The solution was acidified with HCl and free fatty acids were extracted with petroleum ether. They were immediately converted to the methyl esters by heating with HCl-methanol.

The radioactivity of the different fractions was measured by dissolving the samples in Bray's scintillation liquid (9) and counting them in a Packard instrument.

Fatty Acid Analysis

Total lipid fatty acid composition of the alga was determined by gas liquid chromatography (GLC) in a Packard 420 apparatus with hydrogen flame detector. Glass columns of 180 cm x 0.4 cm id packed with 15% EGSS-X on Chromosorb WHP (80-100 mesh) at 180 C and 15% EGSS-Y on Chromosorb W aw (80-100 mesh) at 190 C were used. A flow rate of 60 ml/min of nitrogen as gas carrier was used. Quantitation was performed by triangulation.

Chromatographic peaks obtained from both columns were tentatively identified by comparison of their relative retention times (rrt 18:0) with standards and checked by determination of their equivalent chain length values (ECL) (10). The number of carbons in the fatty acids was determined by hydrogenation (11) and GLC of the methyl esters of the saturated acids thus formed.

The number of double bonds was determined in some cases by separating the methyl esters by TLC-$AgNO_3$ (10%) and developing twice in hexane/diethyl ether/acetic acid (94:4:2) (12). Each fraction was reanalyzed by GLC.

Radioactive fatty acid analyses were carried out by gas liquid radiochromatography in a Model 893 Packard apparatus equipped with a proportional counter using 15% DEGS on Chromosorb W AW (80-100 mesh). The relative radioactivity in the different fatty acid peaks was determined by measuring the area of the counter output peaks by triangulation (13).

RESULTS AND DISCUSSION

The total fatty acid composition of *P. grossii* is shown in Table I. Two GLC columns of different liquid phase polarities were used to help in the identification of some peaks. Only saturated fatty acids of 12, 14, 16, 18 and 20 carbons were detected when the total fatty acid methyl esters were hydrogenated and rechromatographed.

Fractionation of the fatty acid methyl esters by TLC-$AgNO_3$ revealed the presence of saturated esters and unsaturated compounds of 1, 2, 3, 4 and 5 double bonds. Each fraction was analyzed again by GLC to confirm the

TABLE I

Fatty Acid Composition of *P. grossii*

Fatty acids	ECL[a]	Composition[b] (wt %)	
12:0	12.00	2.0	(0.2)
14:0	14.00	0.9	(0.0)
16:0	16.00	14.3	(0.6)
16:1ω7 (?)	16.63	4.7	(0.3)
16:2	17.45	4.8	(0.2)
16:3ω6	18.00	10.0[c]	(0.4)
18:0	18.00		
16:3ω3	18.52	21.4[d]	(0.9)
18:1	18.52		
16:4ω3	19.05	7.4	(0.2)
18:2ω6	19.42	17.1	(0.4)
18:3ω6	20.00	1.0	(0.1)
18:3ω3	20.42	10.6[e]	(0.5)
18:4ω3	21.06	2.2	(0.1)
20:4ω6	22.39	1.2	(0.1)
20:5ω3	23.47	1.4	(0.1)

[a]Equivalent chain length calculated from retention times measured on EGSS-X at 180 C.

[b]Results are the mean of 3 determinations ± extreme deviations of the mean, in brackets.

[c]The 16:3ω6 acid amounts to ca. 6% of total fatty acids.

[d]The 16:3ω3 acid amounts to ca. 1% of total fatty acids.

[e]May include some 20:1 acid.

TABLE II

Radioactivity Distribution in the Fatty Acids of *P. grossii* after Incubation with Labeled Substrates

Labeled fatty acids	Substrates					
	[1-^{14}C] Na Ac	[1-^{14}C] Na Ac + 18:2ω6	[1-^{14}C]18:1ω9	[1-^{14}C]18:1ω9 + 18:2ω6	[1-^{14}C]18:2ω6	[1-^{14}C]18:3ω3
16:0	29.7 (2.2)[a]	19.9 (1.1)[a]	8.7	2.2	9.8	5.2
16:1	34.5 (3.6)	15.6 (1.2)			10.4	3.4
16:3ω6 (?)	–	–	1.2	1.7	–	1.2
18:0	trace	1.6 (1.0)	trace	trace	–	18.8
18:1	35.8 (2.4)	62.9 (3.3)	84.7	83.4	11.3	10.6
18:2ω6	*trace*	trace	trace	trace	68.5	3.2
18:3ω3	–	–	2.8	5.9	–	57.6
20:1	–	–	2.6	6.8	–	–

[a]Results are the mean of 2 determinations ± extreme deviations of the mean, in brackets.

previously assigned identities of fatty acids.

Results show that the most abundant fatty acids are: octadecenoic, linoleic, palmitic and α-linolenic with values similar to those of *C. pyrenoidosa* as shown by Matucha et al. (3). Although *P. grossii* is a marine alga, it contains a high proportion of linoleic acid. However, a significant amount of linoleic acid was also reported in the marine unicellular green algae *Chlamydomonas sp.* (2) and *Dunaliella salina* (14). Nevertheless, the quantitative fatty acid composition of *P. grossii* is significantly different from the one described for unicellular green algae, including other members of the class *Prasinophyceae* (2). Other saturated acids of 12, 14 and 18 carbons accompanied palmitic acid. Mono-, di-, tri-, tetra- and pentaethylenic unsaturated acids belonging to the ω3 and ω6 families also were found (Table I). The abundance of 18:1 and 18:2ω6 acids in *P. grossii* and the presence of 18:4ω3, 20:4ω6 and 20:5ω3 show a similarity with the fatty acid composition of some nonplanktonic members of the Chlorophyceae group (15,16). However, we must take into account that the nitrogen content in the medium, light intensity, or growth cycle phase may affect the fatty acid composition in algae (17).

Fatty Acid Biosynthesis

The results obtained by gas liquid radiochromatography after incubating the alga with labeled substrates are shown in Table II. When cells were incubated for 48 hr with [1-^{14}C] sodium acetate, saponified and lipids extracted with petroleum ether, a part of the radioactivity (76%) remained in the aqueous phase. A significant amount of the label was found in the unsaponifiable material (19%) and only a small proportion in the fatty acids. Although significant decarboxylation of sodium acetate is possible, this suggests that sodium acetate dissolved in the medium is incorporated by the cell, but that it is not used preferentially for fatty acid synthesis but for other syntheses, such as for sterols. At any rate, incorporation in 16:0, 16:1, 18:0 and 18:1 acids and traces in 18:2 acid was demonstrated. The lack of incorporation into the other acids, especially 18:3, could be explained by a slower biosynthesis, compartmentalization of acetate usage, or alternative substrates.

When cells were incubated with [1-^{14}C] 18:2ω6 and [1-^{14}C] 18:3ω3 salts, labeling was only detected in the incubated precursors and the shorter chain acids, but not in the higher homologs. This suggests that these fatty acids were incorporated but were possibly broken down to acetyl-CoA which was then

used by the cell in the synthesis of new short chain fatty acids. This is the most likely interpretation since it is very difficult to accept either a biohydrogenation of 18:2 to 18:1, or 18:3 to 18:2, 18:1 and 18:0 or a chain shortening of 18:3 to 16:3. In addition, the results of Table II suggest that in *P. grossii*, $\Delta 6$ as well as $\omega 3$ desaturases and elongases are inactive in our experimental conditions or that the added substrates do not reach the corresponding enzymes. $\Delta 6$ Desaturase is considered a typical enzyme of animals and $\omega 3$ desaturase is typical of plants. However, activity of both enzymes has been reported in some unicellular photosynthetic, as well as heterotrophic organisms (18-21).

Therefore, these results suggest the existence of other mechanisms different from those of direct desaturation of 18:2$\omega 6$ and 18:3$\omega 3$ to 18:3$\omega 3$ and 18:4$\omega 3$ acids, respectively. A possible route for the synthesis of α-linolenic acid in *P. grossii* could be the one proposed by Stumpf et al. for spinach chloroplasts, *C. pyrenoidosa* and *Candida bogoriensis* (5,22,23) starting from acids of low molecular weight.

After incubating the alga with [1-^{14}C] 18:1$\omega 9$, labeling was observed in some acids of lower molecular weight as well as in 18:2$\omega 6$, 18:3$\omega 3$ and 20:1. It seems likely that 20:1 is synthesized by chain elongation of the 18:1 precursor. With regard to the polyunsaturates, however, 2 possible synthetic pathways must be considered. One involves degradation of 18:1$\omega 9$ to acetate and de novo synthesis of products; the second is the conversion of 18:1$\omega 9$ to 18:2$\omega 6$ by a $\omega 6$ desaturase and thence to 18:3$\omega 3$ by a $\omega 3$ desaturase. However, as already discussed, [1-^{14}C] 18:2$\omega 6$, when directly supplied to the alga, was not converted to 18:3$\omega 3$. Therefore, the evidence points strongly toward degradation of 18:1$\omega 9$ to acetate and de novo synthesis of 16:3$\omega 3$, which is then elongated to 18:3$\omega 3$, by Stumpf's pathway. This de novo pathway is supported by the observation of labeled short chain fatty acids as additional reaction products.

The conversion of [^{14}C]18:1 to 18:3 acid is facilitated by adding unlabeled linoleic acid to the medium (Table II, column 4), whereas the conversion to palmitic acid is decreased. In addition, when unlabeled linoleic acid is added to the culture medium, the incorporation of [^{14}C]acetate into fatty acids is also affected markedly (Table II, column 2). Linoleic acid facilitates the incorporation of acetate into 18:1 but incorporation into palmitic and 16:1 acids is reduced. Thus, *P. grossii* is able to synthesize saturated and unsaturated fatty acids, resulting in a composition rich in palmitic, stearic, oleic, linoleic and α-linolenic acids, but the type and amount of acids synthesized would be controlled by the concentration of linoleic acid and also probably by other fatty acids in the medium.

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