Variability of the Fatty Acid Composition during Development of the Green Microalga *Apatococcus constipatus*

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The green microalga *Apatococcus constipatus* was investigated for its fatty acid composition using GC and MS techniques. Considerable variations were found in individual fatty acid contents according to the stage of culture development. A set of saturated fatty acid homologues was distinguished as the main component regardless of the culture age. The occurrence of some uncommon fatty acids in minor amounts, such as hydroxylated ones, was found to be characteristic of the studied species. Depending on the development stage, those compounds were detected either only during initial growth phases or throughout the cultivation time.

Introduction

Apatococcus algae may be found in places, where moist conditions prevail. Indeed, the *A. constipatus* Printz species occurs in bright green patches on some Spanish monuments (Flores *et al.*, 1997), in the cover crusts on Norway spruce needles (Sochting, 1997) as well as attached to different insulating elements of African electric tractions (Gubański *et al.*, 2000). Although the occurrence of this alga is worldwide, it is not a subject of intense scientific studies. Thus, very little is known about the biochemistry of this species. Recently, the identification of the homologue series of exclusively saturated 5-n-alkylresorcinols in *Apatococcus* cells has been reported (Żarnowski *et al.*, 2000c). So far, any of another natural compounds included in *Apatococcus* cells have not been described as yet. In this study, we report the unusual fatty acid (FA) composition of total lipids of *A. constipatus* during the growth cycle. These compositions have not been reported in the literature previously.

Results and Discussion

The green unicellular microalga *A. constipatus* Printz (Gubański *et al.*, 2000) was examined for its FA composition during distinct phases of growth, respectively. Some extraordinarily interesting results were obtained for the tested organism. Total lipids were isolated from cells using continuous extraction with an appropriate chloroform-methanol-water mixture. Obtained lipids were hydrolysed, methylated and then analyzed by gas chromatography (GC) and mass spectrometry (MS) techniques. The identification of fatty acid methyl esters (FAME) was based upon their retention time (GC) and comparison either of their low resolution mass spectra with those of authentic FAs (EI-MS) or of their calculated molecule masses (FAB-MS). Table I lists all the identified compounds and indicates the presence of 14 main FAs during the *A. constipatus* development.

We have found significant, about 70-% differences in the FA patterns of algal cultures being at various cultivation stages. Application of two statistic comparative tests revealed the high variability of the FA composition (about 70%). Of total FA, saturated FAs comprised 24.7% in the log-phase up to 70.5% of the FAs in the stationary phase. Tetradecanoic (14:0) and hexadecanoic (16:0) acids were found as the predominant components. Their contents strongly increased within the growth of algal culture, from 8.2 and 12.5% to 28.5 and 27.5%, respectively. Furthermore, considerable amounts of octadecanoic acid (18:0) were found, comprising 2.2% during the log-phase and 9.1% of the total FAs in the stationary phase. Monounsaturated FAs were generally estimated in minor amounts. However, the content of octadecaenoic acid (18:1) significantly raised during the stationary phase, up to 9.3% of the total FAs. Polyunsaturated FAs (mainly octadecyldienoic, octadecyltrienoic and octadecyltetraenoic), were found during the initial period of algal growth, whereas their content drastically decreased along with the stationary phase's achievement. Also, a few unusual FAs substituted with a hydroxyl group were

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Table I. Relative compositions of total fatty acids [%] in *Apatococcus constipatus*.

Fatty acid	1-month-old culture (log-phase) [a,b]	3-month-old cultures (stationary phase) [a,c]
14:0	8.2	28.5
2-OH 14:0	2.9	*n.d.*
3-OH 14:0	5.6	3.8
15:0	*n.d.*	3.9
i-16:0	4.6	4.7
16:1	3.2	*n.d.*
16:0	12.5	27.5
2-OH 16:0	2.2	2.8
i-17:0	1.8	*n.d.*
17:0cyc	*n.d.*	1.6
18:0	2.2	9.1
18:1*cis*	2.4	9.3
18:2	4.7	*n.d.*
20:0	*n.d.*	1.5
Others (including PUFA)	49.7	7.3

[a] – Cultures were initiated by inoculation with 15 μg of the cells (dry weight) giving approximate cell concentration of 1 μg/cm^2.
[b] – Yielded 26 μg/cm^2.
[c] – Yielded 93 μg/cm^2.
n.d. – Not detected.

found in *A. constipatus* cells. 3-Hydroxyl-tetradecanoic (3-OH 14:0) and 2-hydroxyl-hexadecanoic (2-OH 16:0) were present in lesser amounts during the whole growth cycle, whereas 2-hydroxyl-tetradecanoic (2-OH 14:0) showed up only at the beginning stage. Moreover, relatively small amounts (1.6%) of methyleneoctadecanoic acid (17:0cyc), were detected only in algal cells of the stationary growth phase.

The presence of iso-branched FAs indicates bacterial contamination of the cultures. Similarly to algae, most bacteria have a specific FA profile, which is distinctive from that of algae. In all likelihood, these unidentified bacteria live in close symbiosis with algae. Undoubtedly, getting rid of those microbes from cultures using different doses of β-lactam antibiotics prevented the algae from further normal growth. However, the bacterial contribution to FAs have to be judged as insignificant, because the content of those branched FAs, found in studied samples, was relatively low (from 6.4 to 4.7%).

This work provides extensive and some useful information about chemotaxonomy of this species. The results odd indicate that this microalga is only partly of applied value. Polyunsaturated FAs are considered as essential FAs for human and animals, due to their high biological activity and participation in biosynthesis of eicosanoids (Mead *et al.*, 1984). From a nutritional point of view, the low contents of these compounds along with a high imbalance of FA composition during the whole growth cycle of *A. constipatus* bring about some inconveniences. After all, the use of such algae in i.e. food products may be expected to produce different biological effects. Namely, significantly long-lasting deficiencies of polyunsaturated FAs in consumed food may upset some steps of metabolic routes of such important compounds such as phospholipids. Thereby, functioning of certain internal organs consisting of those lipids may be also handicapped. Due to the low content of polyunsaturated FAs, this alga can not be recommended as a ingredient of safe food for both human and animals. However, our results also indicate the need of further work to understand the causes for differences in the FA composition during the growth stage of *A. constipatus*.

Experimental

The green unicellular microalga *A. constipatus* Printz (*Chlorophyta, Chaetophorales, Leptosiroideae*) strain B was isolated and identified previously (Gubański *et al.*, 2000). The alga was collected from silicone-rubber insulators in Tanzania in 1999. The agarized mineral medium of Slamer *et al.* (1971) was used for algae cultivation. The

cultures were grown on slants in glass tubes being incubated on a laboratory bench at approximately 25 °C under daylight conditions with approximately 12 hrs light : 12 hrs darkness cycles. Each of slant containing a sterile medium was inoculated with 15 µl of algae cells' suspension containing *ca.* 1 mg of the cells (dry weight) per 1 ml of deionized water. Such inoculum was prepared from 3-month old cultures. To compare FA patterns in the early (logarithmic) and late (stationary) phases of algal growth, the strain was cultured for 1 and 3 months, respectively. The growth of *Apatococcus* alga was monitored and individual growth stages were estimated basis of an assumption that the dry mass is in direct proportion to the number of algal cells, thereby being proportional to the culture growth stage. Briefly, the algae were scraped off the slants with a pen knife and placed into a small container with a lid. The whole was quickly shaken to remove agar slivers and the cell suspension was transferred to a small tube. The sample was frieze-dried and then weighted. Obtained data were expressed as dry weight mass of algae cells per area unit (µg/cm^2). The cultures grew on slants slowly, because the poor mineral medium was used for their cultivation. The generation time of algal cells for the early growth stage lasted on average 20 hrs. Unfortunately, the tested alga was able to grow only on agar-solidified media. Application of liquid one along with even intensive shaking (aeration) did not give satisfactory results.

Algal cells were rinsed out from slants with 0.1 M $MgSO_4$, and freeze-dried. Total lipids were extracted from the dry material using the modified method of Bligh and Dyer (1959). Briefly, the sample was dispensed into a separating funnel and was extracted three times. 5 ml of chloroform and methanol (1:2 by vol.) was added. The content was shaken for 1 min and allowed to stand for 1 hr. 5 ml of chloroform was added followed by 5 ml of distilled water. The lower layer that separated was filtered through a SP-1 filter (Whatman, UK) in order to remove any solid materials. The solution was transferred into a flask, the extracts were combined and the solvent was removed under nitrogen. Obtained fractions were converted into fatty acid methyl esters with small modifications as described previously (Żarnowski *et al.*, 2000b). Briefly, the hydrolysis of the lipids was achieved by resuspention of the material in 1 ml of solution 1 (45 g NaOH in 300 ml of 50% MeOH) and boiling for 30 min at 100 °C in a water-bath. Methylation of the lipids was done by adding of 2 ml of solution 2 (325 ml of 6 N HCl and 275 ml of MeOH) and incubation for 10 min at 80 °C, after that rapid cooling in an ice-bath. The extraction of the FAME was carried out by addition of 1.25 ml of solution 3 (200 ml of *n*-hexane and 200 ml of *tert*-methylbutylether) and the whole was shaken for 10 min. For the separation of the phases, the tube was incubated for 1 hr at −20 °C. Afterwards, the lower phase was discarded, and the upper phase transferred into a new tube and 3 ml of solution 4 (10.8 g of NaOH in 900 ml of H_2O) was added. The mixture was shaken for 5 min to remove contaminations and the upper phase was used directly for instrumental analyses.

FAME profiles of the studied alga were determined by gas chromatography. 1 µl of each sample was injected into a GC chromatograph Shimadzu GC-17A equipped with an autosampler AOC-1400, an autoinjector AOC-17 and a controller CBM101. A fused silica capillary column Permabond OV-1-DF (Macherey & Nagel, Germany) was used and column oven temperature was programmed as follows: 100 °C for 2 min, 100–210 °C for next 38 min. Flow rate of carrier gas (He) was 1 ml per min. Chromatograms were analysed using the Windows 3.11 PC-programme Class GC. FAs were identified by comparison of the relative retention times of their FAME with authentic standards, and their relative compositions were estimated from the area of the peaks in the chromatogram. Additional identification of particular FAs was achieved using FAB- and EI-MS. In this case, low-resolution spectra were recorded on an AMD 402 two sector mass spectrometer (AMD Intectra, Germany) of B/E geometry. Operating procedures were the same as described elsewhere (Żarnowski *et al.*, 2000a). The FA patterns were compared statistically using both the 1-r Pearson and joining discrepancy tests.

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