

Isolation of a Novel Oil Globule Protein from the Green Alga *Haematococcus pluvialis* (Chlorophyceae)

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Abstract Cytoplasmic oil globules of *Haematococcus pluvialis* (Chlorophyceae) were isolated and analyzed for pigments, lipids and proteins. Astaxanthin appeared to be the only pigment deposited in the globules. Triacylglycerols were the main lipids (more than 90% of total fatty acids) in both the cell-free extract and in the oil globules. Lipid profile analysis of the oil globules showed that relative to the cell-free extract, they were enriched with extraplastidial lipids. A fatty acids profile revealed that the major fatty acids in the isolated globules were oleic acid (18:1) and linoleic acid (18:2). Protein extracts from the globules revealed seven enriched protein bands, all of which were possible globule-associated proteins. A major 33-kDa globule protein was partially sequenced by MS/MS analysis, and degenerate DNA primers were prepared and utilized to clone its encoding gene from cDNA extracted from cells grown in a nitrogen depleted medium under high light. The sequence of this 275-amino acid protein, termed the *Haematococcus* Oil Globule Protein (HOGP), revealed partial homology with a *Chlamydomonas reinhardtii* oil globule protein and with undefined proteins from other green algae. The HOGP transcript was barely detectable in vegetative cells, but its level increased by more than 100 fold within 12 h of exposure to nitrogen depletion/high

light conditions, which induced oil accumulation. HOGP is the first oil-globule-associated protein to be identified in *H. pluvialis*, and it is a member of a novel gene family that may be unique to green microalgae.

Keywords Astaxanthin · *Haematococcus* · Oil globules · Oil globule protein · Triacylglycerols

Abbreviations

CFE	Cell-free extract
CGP	Carotene globule protein
DGDG	Digalactosyldiacylglycerol
DGTS	Diacylglyceroltrimethylhomoserine
EDA	Eicosadienoic acid
HOGP	<i>Haematococcus</i> oil globule protein
IEF	Isoelectric focusing
LHCP	Light-harvesting complex proteins
MGDG	Monogalactosyldiacylglycerol
ORF	Open reading frame
SQDG	Sulfoquinovosyldiacylglycerol
TFA	Total fatty acids

Introduction

Oil globules are discrete, sub-cellular organelles surrounded by a monolayer of polar phospholipids, glycolipids or sterols that encircle a hydrophobic core of neutral lipids [1]. These oil globules are ubiquitous in animals, plants, and microorganisms. In many microorganisms, such as yeasts, microalgae and bacteria, the accumulation of oil globules appears to be induced specifically in response to environmental stresses, such as nutrient limitation, high

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irradiance or osmotic stress [1, 2]. Although a variety of oil globule biogenesis models have been postulated, the actual process remains to be elucidated. Nonetheless, it is commonly accepted that globules are formed by vesiculation from the endoplasmic reticulum (ER) [3].

Two major groups of surface-associated proteins, oleosins and caleosins, are contained in the oil globules of plants. Oleosins comprise the most abundant family of oil-body-associated proteins identified in plants, but to date they have not been found in algae [4]. Caleosin, a calcium binding lipid-body protein that has also been found to be associated with ER membranes [5–7], is ubiquitous among higher plants, and a database search by the authors has revealed putative caleosin super-family proteins in green microalgae (unpublished data). A number of studies have attributed different roles to globule proteins, including globule formation, degradation, stabilization, and involvement in globule–globule or globule-other organelle interactions [1, 4, 7, 8]. *Drosophila*, yeast, and mammalian globules have also been shown to house refugee proteins that are not directly linked to lipid metabolism [9, 10].

It is known that some unicellular algae contain very large amounts of oil sequestered in oil globules. In some species, oil can amount to as much as 60% of the cell dry mass, with oil accumulation usually being accompanied by the cessation of cell growth [11–13]. However, little is known about the protein composition and functional roles of algal oil globules.

Of particular interest is the high-oil-producing unicellular green alga *Haematococcus pluvialis*. This alga is regarded as the best natural source of the high-value red pigment astaxanthin, a carotenoid that accumulates in algal cytoplasmic oil globules under various stress conditions [14]. The accumulation of astaxanthin in *H. pluvialis* is positively correlated with lipid accumulation, with the former depending on the latter but not vice versa [15]. Lipid accumulation, in turn, depends on de novo fatty acid synthesis [2, 15, 16]. Under nitrate deprivation, astaxanthin and fatty acids may comprise up to 4 and 40%, respectively, of cell dry mass [16]. As is the case for other organisms, oil globule formation in *Haematococcus* was suggested to be structurally related to ER membranes [17].

H. pluvialis is a unique organism for studying oil globule formation, since it accumulates both lipids and carotenoids in the same compartment. Analyses of lipid class distribution in isolated globules have previously been reported by Grunewald et al. [18], whose studies indicated the cytoplasmic origin of globule polar lipids. Zhekisheva et al. [2] found 16:0, 18:1 and 18:2 fatty acids to be the major fatty acids in TAG and astaxanthin esters. To deepen the current understanding of oil globule biogenesis and function, we isolated astaxanthin-rich oil globules from *H. pluvialis* to characterize and identify oil globule proteins.

Seven distinct protein bands associated with globules were identified. The mRNA for one 33-kDa protein was isolated and sequenced. The resulting sequence was found to encode a novel, hydrophobic protein that may have a specific function in green algae, since homologous sequences were found only in other species of green algae.

Materials and Methods

Haematococcus pluvialis Strain

H. pluvialis Flotow 1,844 em. Wille K-0084 (Chlorophyceae, order Volvocales) was obtained from the Scandinavia Culture Center for Algae and Protozoa (SCCAP) at the University of Copenhagen, Denmark.

Growth Conditions for Algal Cultures

H. pluvialis algal cultures were cultivated as previously described [2]. To induce the accumulation of astaxanthin-rich oil globules, cultures were inoculated into nitrate-free mBG-11 medium to a final cell concentration of 2×10^5 cell mL⁻¹ and subjected to 350 μmol photon m⁻² s⁻¹ (designated high light). After 7 days, red cells were harvested for globule isolation.

For RNA isolation, axenic cultures were grown up to a cell concentration of 2×10^5 cell mL⁻¹ in 250-mL flasks, each containing 100 mL of mBG-11; the flasks were held in a shaker (150 rpm) and exposed to light of 90 μmol photon m⁻² s⁻¹. Cells were then transferred to 250-mL flasks, each containing 50 mL of nitrogen-depleted mBG-11 medium and exposed to a light intensity of 200 μmol photon m⁻² s⁻¹ to induce astaxanthin accumulation. For each sample, a separate flask was harvested at 0, 12, 24, 48, and 72 h.

Growth Parameter Measurements and Pigment Extraction and Quantification

Culture cell concentration, chlorophyll quantification and total carotenoid quantification were evaluated as previously described [16].

Isolation of Globules

An *H. pluvialis* culture was harvested by centrifugation (1,500×g, 10 min), suspended in breakage buffer comprising 0.2 M sucrose (10 mM MOPS, pH 7.0; 10 mM KCl; 5 mM Na EDTA; 1 mM DTT; 1 mM PMSF; 1 mM benzamidine; and 0.5 mg/mL leupeptin), and ruptured with a Mini Beadbeater (BioSpec Products Inc., OK, USA) using 2.5-mm glass beads for 4 min. The resulting

homogenate was centrifuged (1,500×g, 10 min), and the cell-free extract obtained was fractionated by centrifugation (25,000×g, 60 min, Sorvall RC 5C plus) on a discontinuous sucrose flotation gradient (0.6, 0.4, 0.2, 0 M sucrose in breakage buffer). The upper layer of the sucrose gradient containing the oil globules was collected and centrifuged in an ultracentrifuge (100,000×g, 120 min, Sorvall Combi plus). The floating oily layer was collected in 0.3-mL aliquots and kept frozen (−20 °C) until analyzed. The microsomal fraction comprised the ultracentrifuge (100,000×g, 120 min) pellet of the sucrose gradient supernatant minus the oil globules.

High-Performance Liquid Chromatography Pigment Analysis

The pigment profile was determined by high-performance liquid chromatography (HPLC) as described in Zhekisheva et al. [2]. The chromatogram was recorded at 450 nm, but the quantification of pigments (in % based on pigment weights) was performed at 478 nm for xanthophylls, 655 nm for chlorophyll a and 645 nm for chlorophyll b. Astaxanthin and chlorophylls were identified by comparison to their standards, and astaxanthin isomers were identified from their UV–VIS absorbance spectra, according to Yuan and Chen [19]. Xanthophylls other than astaxanthin were identified by their absorbance spectra and calculated peak III/II ratios, according to Briton et al. [20].

Lipid Analysis

Lipids were extracted from the cell-free extract and oil globules by the method of Bligh and Dyer [21]. Total lipid extract was separated into neutral and polar lipids by silica Bond-Elute cartridges (Varian, CA) using 1.5% methanol in chloroform (v/v) and methanol to elute neutral and polar lipids, respectively.

Polar lipids were separated into individual lipids by two dimensional TLC on Silica Gel 60 glass plates (10 × 10 cm, 0.25 mm thickness [Merck, Darmstadt, Germany]) according to Khozin et al. [22]. Neutral lipids were resolved with petroleum ether:diethyl ether:acetic acid (70:30:1, v/v/v). Lipid spots on TLC plates were visualized with 0.05% 8-anilino-4-naphthosulphonic acid in methanol (w/v) and UV light exposure and then scraped from the plates and transmethylated for fatty acid analysis.

Fatty Acid Analysis

Fatty acid methyl esters (FAME) were obtained by transmethylation of the lipid extracts or individual lipids with dry methanol containing 2% H₂SO₄ (v/v) and heating at 80 °C for 1.5 h while stirring under an argon atmosphere.

Gas chromatographic analysis of FAME was performed on a Thermo Ultra Gas chromatograph (Thermo Scientific, Italy) equipped with PTV injector, FID detector, and a fused silica capillary column (30 m × 0.32 mm; ZB WAXplus, Phenomenex). FAME were identified by co-chromatography with authentic standards (Sigma Chemical Co., St. Louis, MO; Larodan Fine Chemicals, Malmö, Sweden) and FAME of fish oil (Larodan Fine Chemicals). Each sample was analyzed in duplicates of three independent experiments.

Protein Analysis

Protein samples were prepared as described in Wang et al. [23], and the BCA method was used for protein determination [24]. Separation of isolated protein fractions was performed by SDS PAGE (12%) according to Laemmli [25] with minor changes. Sample preparation entailed 1 h of incubation at room temperature in sample buffer containing 80 mM DTT. Bio Rad Precision Plus protein unstained standards were used as molecular weight markers.

Two-dimensional gels were prepared as described [26] with minor changes. Samples of 130 µl, each containing 50 µg of protein, were loaded on 7-cm isoelectric focusing (IEF) dry strips, pH 3–10 nonlinear (Amersham Biosciences AB, Uppsala). Protein spots of about 33 kDa were selected for analysis by mass spectrometry (MS).

Partial Amino Acid Sequencing

Protein spots were manually excised from 2D gels and digested in-gel with trypsin.

The peptide mixtures were subjected to solid phase extraction with a C18 resin filled tip (ZipTip Milipore, Billerica, MA USA) and nanosprayed into the Orbi-trap MS system in a 50% CH₃CN/1% CHOOH solution. MS was carried out with an Orbi-trap (Thermo Finnigen) using a nanospray attachment [27]. Data analysis was done using the Bioworks 3.3 package, and database searches were performed against the NCBI nr database with the Sequest and Mascot software packages (Matrix Science, England). De novo sequencing was performed using the Biolyx package (Micromass, England).

Isolation of Total RNA

Total RNA was isolated with an SV Total RNA Isolation Kit (Promega USA). From 40 ml of medium containing 2×10^5 cells mL⁻¹, cells were harvested, resuspended in the kit lysis buffer and broken in Mini Beadbeater (Bio-Spec Products Inc., OK, USA) using 2.5-mm glass beads for 4 min. RNA samples were quantified with a Nano Drop

(ND-1000, Thermo Scientific, USA) spectrophotometer and stored at -80°C .

cDNA Preparation, PCR and Sequencing

The Reverse iT 1st Strand Synthesis Kit (ABgene, UK) was used according to the manufacturer's instructions to synthesize cDNA from total RNA. The synthesized cDNA was used as the template in PCR amplification (with the degenerate primers listed in Table 3) using touch down PCR at 56 to 46 $^{\circ}\text{C}$. The PCR product was extracted and subcloned, and specific primers for 3' and 5' RACE were designed. Full-length cDNA was synthesized according to the protocol described in the manufacturer's instructions (BD SMART RACE, Clontech). Searches for homologues sequences were performed in the NCBI database using the BLAST program [28].

Results

Globule Isolation

Oil globule accumulation was induced by exposing exponentially growing cells (chlorophyll and astaxanthin were 5 and 1 $\mu\text{g mL}^{-1}$, respectively) to high light and to nitrogen deprivation. Chlorophyll and astaxanthin contents in red cells harvested after seven days were 5 and 168 $\mu\text{g mL}^{-1}$, respectively.

The isolation of pure oil globules from *H. pluvialis* was hampered by the need to first rupture the robust algal cell wall. In light of this problem, earlier studies have explored the rupture process, examining the methods available for cell breakage (including grinding, French press or freeze thaw cycles), algal strains with different cell wall morphologies, and the treatments for purifying the isolated globules, including high ionic strength washes, chaotropic agents and detergents [29]. Although it was found that the different procedures produced similar results in terms of the pigment, fatty acid, and protein compositions of the isolated globules, all preparations also contained some contaminating membranes and proteins that were probably introduced into the globules during cell breakage. We found that breaking the cells with a Mini Beadbeater was an efficient and reproducible method for producing a high oil globule yield. Light microscopy of our preparations showed a mixed-sized population of yellow–red globules ranging in diameter from about 200 nm to 4 μm (Fig. 1).

Pigment Analyses

Eight different astaxanthin esters, amounting to about 70% of the total pigments, were found in the cell-free extract

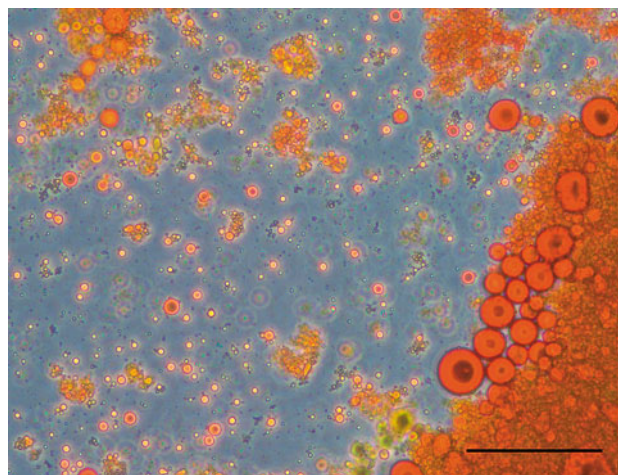


Fig. 1 Oil globules isolated from *H. pluvialis*. The oil globules were fractionated on a sucrose flotation gradient. Bar 20 μm

(CFE), while in the oil globules astaxanthin esters amounted to about 86% of the total pigments. The three major peaks on the HPLC chromatogram were attributed to astaxanthin esters 3, 5 and 6 (peaks 7, 8 and 9, respectively, in Fig. 2). UV–VIS absorbance spectra (not shown) indicated that peaks 8 and 9 were those of the *trans* isomer. In addition to the astaxanthin esters, minor amounts of chlorophyll and the chloroplast-derived xanthophylls—anthraxanthin, lutein and zeaxanthin—were also detected (Fig. 2). The retention time of zeaxanthin differed from the typical retention time previously reported by our group [2], and zeaxanthin may thus have been present in ester form. The relative chlorophyll content was higher in the CFE than in the oil globules—12.7% versus 4.5% (Table 1). The relative amounts of the other three xanthophylls were also higher in the CFE than in the oil globule fraction.

Fatty Acid and Lipid Analyses

Fatty acid composition of the total homogenate was similar to that of the oil globules and was characterized by the four major fatty acids: 16:0, 18:1, 18:2 and 18:3n-3 (not shown). To further analyze fatty acid composition and estimate the distributions of polar and neutral lipids in the oil globule enriched fraction, we performed a detailed lipid analysis of the representative sample of oil globules fractionation by two-dimensional TLC followed by GC analysis of fatty acid composition and content. About 70% of the total fatty acids (TFA) in CFE were recovered in the oil globule fraction, but polar membrane glycerolipids (PL), which constituted only 3.5% of the TFA in CFE, were reduced to 1.5% in the oil globules. Accordingly, neutral lipid TAG was a major component of both fractions (Table 2), accounting for more than 70% of the TFA in neutral lipids (NL). Diacylglycerol (DAG) and free fatty acids (FFA)

Fig. 2 HPLC chromatogram (recorded at 450 nm) of the pigment extract from *H. pluvialis* oil globules. Peaks: 1, antheraxanthin; 2, lutein; 3, zeaxanthin; 4, astaxanthin ester 1; 5, chlorophyll b; 6, astaxanthin ester 2; 7, chlorophyll a + astaxanthin ester 3; 8–11, astaxanthin esters 5, 6, 7 and 8

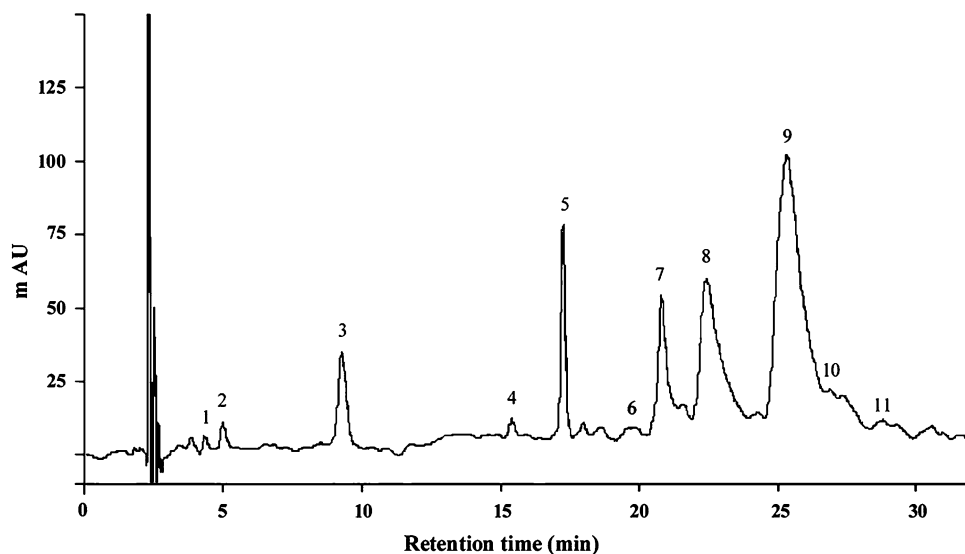


Table 1 Pigment composition^a of cell free extract and of isolated oil globules

Rt (min)	Ant 4.10	Lut 4.70	Zea 8.50	Ast 1 14.90	Chl b 16.90	Ast 2 19.20	Chl a 20.40	Ast 3 20.50	Ast 4 20.90	Ast 5 22.00	Ast 6 24.60	Ast 7 26.40	Ast 8 27.80
Cell free extract	2.6	4.0	13.9	0.0	6.4	0.0	6.3	7.6	1.5	17.4	36.7	2.0	1.7
Oil globules	1.5	1.7	5.8	1.9	1.9	1.1	2.7	10.5	3.0	23.5	42.9	3.7	0.0

Samples containing 10 µg of total pigments were injected into the HPLC. Bold values indicate three major peaks

Ant antheraxanthin, *Lut* lutein, *Zea* zeaxanthin, *Ast* astaxanthin ester, *Chl* chlorophyll, *Rt* retention time

^a Values are expressed as the percentages of total pigment weight

constituted 7% each of the TFA in NL. FFA were also included in the calculation due both to their relatively high proportion and to their potential involvement in NL turnover. A relatively large percentage of the fatty acids in the NL fraction were esterified to astaxanthin in its mono- and diester forms, with the majority existing as monoesters.

The most salient difference between the oil globules and the CFE was the decreased proportion of the major thylakoid and inner chloroplast membrane lipid [30] monogalactosyldiacylglycerol (MGDG) in the oil globules. The ratio of two galactolipids, MGDG to digalactosyldiacylglycerol (DGDG), decreased from 1.3 to 0.4 during oil globule purification. Consequently, the relative proportions of the polar lipids—DGDG, sulfoquinovosyldiacylglycerol (SQDG) and diacylglyceroltrimethylhomoserine (DGTS)—increased, while that of phosphatidylcholine (PtdCho) remained unchanged.

There were no substantial differences between the fatty acid compositions of individual lipids in the CFE (not shown) and in the oil globule fraction. Despite tiny amounts of PL, a characteristic fatty acid signature was evident for each lipid class (Table 2). The galactolipids MGDG and DGDG were typically enriched with chloroplast PUFA, specifically linoleic acid (LNA, 18:2n-6) and

α -linolenic acid (ALA, 18:3n-3), and with C16 PUFA 16:3n-3 and 16:4n-3. Another chloroplast-associated lipid, SQDG, contained less PUFA but was rich in saturated palmitic acid, 16:0. Phosphatidylglycerol (PtdGro) contained about 3% 16:1 Δ 3-*trans*—a distinctive feature of this class of chloroplast lipids (not shown). It appeared that *H. pluvialis* oil globules contained the phospholipid PtdCho and the betaine lipid DGTS as major extraplastidial polar lipids. PtdCho and DGTS featured similar fatty acid compositions, namely, the highest percentages of γ -linolenic acid (GLA; 18:3 Δ 6,9,12, n-6) and stearidonic acid (SDA; 18:4 Δ 6,9,12,15, n-3), indicating their possible involvement in the Δ 6 lipid-linked desaturation of LNA (18:2 Δ 9,12, n-6) and of ALA (18:3 Δ 9,12,15, n-3), respectively. Phosphatidylethanolamine (PtdEth), the minor component of the PL fraction, contained the highest proportions of C20 long-chain PUFA, particularly, of arachidonic acid (AA; 20:4 Δ 5,8,11,14, n-6) at 16%. In addition eicosadienoic acid (EDA; 20:2 Δ 11,14, n-6) and eicosapentaenoic acid (EPA; 20:5 Δ 5,8,11,14,17, n-3) comprised for about 4% each of TFA in this lipid class. This characteristic fatty acid composition allows speculation on the involvement of PtdEth in the Δ 8 desaturation of 20:2n-6 and Δ 17 (n-3) desaturation of AA. In this context, although it is worth

Table 2 Fatty acid profiles^a of individual lipids from cell free extract (CFE) and isolated oil globules (OG)

	16:0	16:2 n-6	16:3 n-3	16:4 n-3	18:0	18:1 n-9	18:1 n-7	18:2 n-6	18:3 n-6	18:3 n-3	18:4 n-3	20:2 n-6	20:3 n-6	20:4 n-6	20:5 n-3	Others ^b	OG	CFE
Polar lipids																	% PL	% PL
MGDG	18.3	1.9	9.2	12.7	1.9	4.8	0.7	12.4	0.7	30.4	0.7	0.3	0.3	0.8	0.3	4.5	9	24
DGDG	29.5	1.7	12.1	1.6	0.9	2.9	0.2	13.9	0.2	34.2	0.3	0.1	0.0	0.1	0.0	2.2	23	19
SQDG	54.8	0.0	0.3	0.1	0.8	4.3	0.4	21.8	0.6	13.5	0.6	0.1	0.4	0.3	0.1	1.8	14	10
PtdGro	42.6	1.8	1.2	0.3	2.2	6.0	6.8	19.5	0.2	8.4	0.9	1.8	0.3	1.6	0.4	6.2	6	3
DGTS	26.9	0.3	0.9	0.6	1.3	1.5	1.0	30.1	7.1	19.0	7.8	0.3	0.1	1.3	0.6	1.3	25	21
PtdCho	34.6	0.3	0.9	0.5	1.4	1.4	1.1	23.2	8.3	15.7	9.1	0.2	0.2	0.8	0.8	1.5	16	16
PtdEtn	26.4	0.3	1.6	0.5	1.9	2.7	9.5	14.3	1.9	6.8	2.1	4.3	0.2	16.2	3.6	7.7	1	2
PtdIns	43.5	0.0	0.4	0.4	2.5	1.8	6.4	31.6	0.5	3.9	0.5	0.4	1.4	1.1	0.3	5.1	6	4
Neutral lipids																	% NL	% NL
TAG	19.8	0.9	3.1	4.3	1.2	26.9	0.7	26.4	1.0	9.9	0.9	0.8	0.5	0.6	0.5	2.4	76	73
FFA	3.7	0.3	0.3	0.4	1.4	33.0	0.6	26.3	0.2	7.2	0.3	24.8	0.2	0.3	0.0	1.0	6	7
DAG	4.2	0.0	0.3	0.2	1.8	44.1	0.8	35.7	0.2	8.3	0.2	0.7	0.5	0.5	0.3	2.1	7	7
Astaxanthin monoester	13.5	0.4	1.9	2.4	2.3	23.3	1.4	25.9	1.3	18.7	2.0	1.0	0.5	1.2	0.5	3.7	9	10
Astaxanthin diester	12.4	0.3	0.9	0.7	3.0	37.3	1.5	23.5	1.1	10.8	1.6	2.1	0.5	0.8	0.2	3.5	2	3

Data are presented as the means of replicated representative samples of oil globules and CFE

^a Fatty acid composition (% of total fatty acids)

^b Includes 14:0, isomers of 16:1, 20:0, 20:1, and 22:0

noting the abundance of EDA in the FFA pool, we realize that more thorough studies of PUFA biosynthesis in *H. pluvialis* are obviously needed to confirm the predictions made in the present work.

The neutral acyl lipids TAG and DAG and astaxanthin esters were enriched with oleic acid (OA, 18:1n-9) and LNA. The astaxanthin monoesters contained the highest relative proportions of ALA, and the percentage of palmitic acid was lowest in DAG and FFA.

Protein Analysis

Four major protein bands with estimated molecular weights of 20–30 kDa (Fig. 3 lane 8) that appeared in the isolated globule preparation were also evident in the total homogenate fraction of both green (astaxanthin and oil free) and red (astaxanthin and oil rich) cells (Fig. 3, lanes 1 and 7). These proteins were identified as chloroplast light-harvesting proteins on the basis of their positive cross-reactivity with anti light-harvesting complex protein (LHCP) antibodies (not shown). To reveal the native proteins of the globules, we followed the changes in protein profile during 14 days of exposure to nitrate depletion and high light. During this period, astaxanthin accumulated and chlorophyll levels decreased (Fig. 4).

As the globules accumulated, the relative abundances of only a few protein bands seemed to increase while the total

protein content decreased. Of the proteins that did increase, we could distinguish seven different protein bands that appeared in the globule fraction but not in the microsomal fraction. These proteins were thus all considered to be globule-associated proteins (Fig. 3).

Globule proteins were also analyzed by 2D gel electrophoresis. Two major protein spots, both of about 33 kDa but with different PI values (Fig. 5), were cleaved with trypsin and analyzed by MS/MS. Identical partial sequences comprising 12 peptides were obtained for the two 2D protein spots, and these were designated *Haematococcus* oil globule protein (HOGP). Based on the sequences of five peptides, forward and reverse degenerate nucleotide primers were designed and used in different combinations for PCR amplification on cDNA from induced cells. The three peptides whose primers produced PCR amplification products are shown in Table 3, and the five peptides identified in the final amino acid sequence are indicated in bold letters in Fig. 6. A PCR product was initially cloned, and the sequencing was completed by 3'-RACE and 5'-RACE extensions (see Table 3 for the primers). A DNA product of 910 bp was obtained with an open reading frame (ORF) of 825 bp (accession number HQ213938) that encoded for a protein of 275 amino acids. An NCBI search with the protein query produced significant hits only from green microalgae. Multiple sequence alignment of HOGP with putative green algal orthologs revealed identities of 40, 38, and 36% with

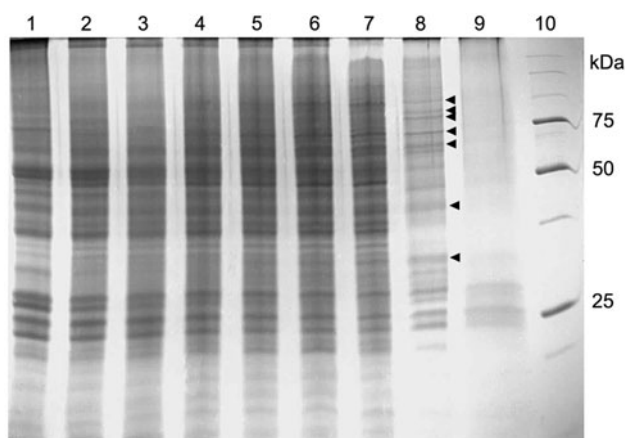


Fig. 3 Protein analysis of *H. pluvialis* total cells and oil globules by SDS–PAGE: (1–7) protein extracts of total cell homogenates during stress induction on days 0, 2, 4, 6, 8, 10, and 12; (8), oil globules; (9), microsomes; (10), molecular weight markers. Arrows between lanes 8 and 9 indicate the proteins thought to be globule-associated

Volvox carteri f. nagariensis, *Polytomella parva*, and *Chlamydomonas reinhardtii*, respectively (Fig. 6). A significant but smaller homology of 27% identity was also found with *Coccomyxa* sp., accession number GW230985 (not shown).

A comparison of the secondary structure of HOGP with its *C. reinhardtii* ortholog, which is the only full-length published sequence, was performed by hydrophobicity plot analyses [31]. The two proteins showed very similar patterns, with the majority of their hydrophobic regions situated between amino acids 55–65 and 170–190 of HOGP (Fig. 7). A short hydrophilic region that is evident in HOGP at amino acids 105–113 also appeared in the *P. parva* sequence but is absent in the *C. reinhardtii* and *V. carteri f. nagariensis* sequences (Figs. 6, 7).

To characterize the induction of the *Hogp* gene, cultures were induced to accumulate astaxanthin (see above) for a period of 72 h, during which RNA was isolated at different time intervals. Specific primers were designed from our cDNA sequence, while actin control primers were adopted from Huang et al. [32]. Astaxanthin accumulation could be detected spectrophotometrically in a total pigment extract after as short a time as 12 h, and after three days astaxanthin increased by more than tenfold (Fig. 8). Transcript levels of the *Hogp* gene, which were almost undetected in green non-stressed cells, increased by >100-fold after 12 h of induction. They remained almost constant for 48 h and started to decrease after 72 h (Fig. 9).

Discussion

The ability of plant or algal cells to accumulate secondary carotenoids depends on the availability of a sink for carotenoid deposition [33, 34]. It is likely that deposition of

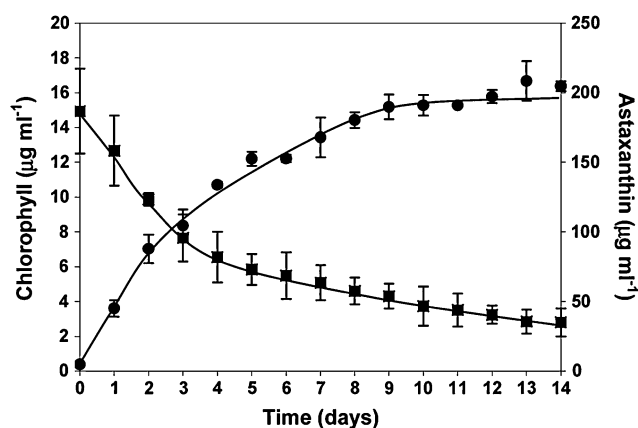


Fig. 4 Changes in chlorophyll (filled square) and astaxanthin (filled circle) contents during 14 days of cultivation in a column under high light and nitrogen starvation to induce the accumulation of oil globules for protein extraction

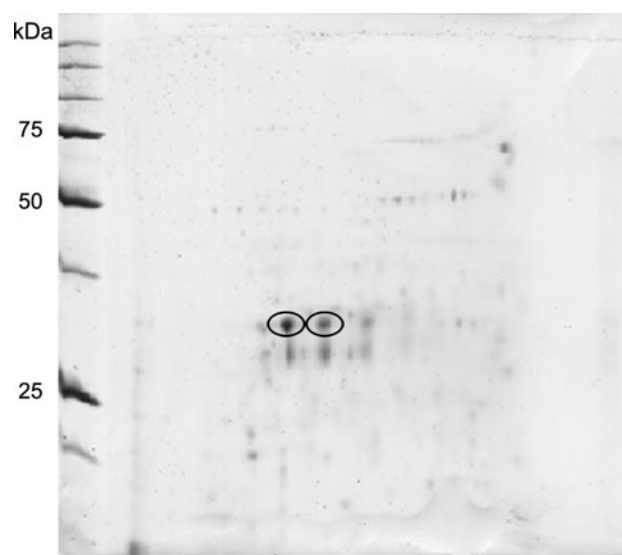


Fig. 5 2D gel electrophoresis of *H. pluvialis* oil globule proteins. The marked bands were excised and subjected to MS analysis

carotenoids in a lipid sink will prevent end product inhibition of the biosynthetic pathway [35]. In yeasts, for example, the expression of carotenogenic genes leads only to minor astaxanthin accumulation [36–38], supposedly as a consequence of the lack of sinks for the carotenoid end product. This “sink hypothesis” is supported by our earlier studies with the carotene globule protein (Cgp), a protein that is associated with plastidic β -carotene oil globules in *Dunaliella bardawil* and that functions in globule stabilization [8]. The “sink hypothesis” may also be applicable to TAG biosynthesis, where the availability of oil globules or the enhanced ability to produce them should facilitate the parallel synthesis and accumulation of storage lipids. Identifying the main globule-associated enzymes and

Table 3 Primers used in this work

Degenerative	
Peptide no. 1: NDAWNMCAS	
1FOR:	5' GAY GCN TGG AAY ATG TG 3'
1REV:	5' CA CAT RTTCCA NGC RTC 3'
Peptide no. 2: TADPVTQTGDDGF	
2FOR:	5' ACN GCN GAY CCN GTN AC 3'
2REV:	5' TC NCC NGT YTG NGT NAC 3'
Peptide no. 3: TAPVVAQAQDL	
3FOR:	5' CCN GTN GTN GCN CAR GC 3'
3REV:	5' GCY TGN GCN CAN CAN GG 3'
Differential expression of <i>Hogp</i>	
For	5' AGCGGGAGATAGTGCGGGACA 3'
Rev	5' ATGCCACCGCCTCCATGC 3' (actin control primers)
For	5' CAGCACGCCCTGGACACCCTGAAC 3'
Rev	5' GGTTTGGGTGACTGGGTCGGCTGT 3' (specific primers)
RACE	
GSP3	5' ACAAGGCGGTGGCAGACGGGAAG 3'
NGSP3	5' CAGCACGCCCTGGACACCCTGAAC 3'
5 ukII	5' ATCAACTACGCCCTTCTTCCCGTCTG 3'
5 ukIII	5' AAAGTAGCTGTTGCTCGTGGCGATG 3'

proteins taking part in the assembly of oil globules can therefore be a key to future biotechnological manipulation of microalgae for the high yield production of oil and carotenoids. Indeed, carotenoid biosynthetic enzymes have already been shown to be localized in oil globules [18, 39].

The lack of sufficient molecular information and sequence data for *H. pluvialis* limits the use of bioinformatics to identify specific protein genes. We therefore applied proteomics approaches to identify possible globule-associated proteins, an approach requiring enriched and contaminant-free oil globule preparations. This approach poses a major challenge when organelles have to be isolated from cells with a rigid cell wall, as is the case for *Haematococcus*, which necessitates aggressive cell breakage. This is particularly true for the isolation of hydrophobic organelles such as oil globules, which can easily adsorb hydrophobic proteins or pigments released from the chloroplast during aggressive lysis in apparatuses such as a French press or a bead beater [40]. Indeed, the chlorophylls, xanthophylls and some of the plastid lipids in our preparations undoubtedly originated in chloroplast membranes, and the consistent detection of LHCPs as the major proteins in isolated globules (also verified by MS analysis), even after purification, may be an artifact of isolation. A possible solution may lie in the production of spheroplasts by enzymatic treatment, followed by gentle lysis via osmotic shock. Although previous studies have reported the successful application of this approach in *H. pluvialis* [41, 42], our group has never succeeded in reproducing

their results, even after successful removal of the cellulose layer from the cell wall [43]. As an alternative, we tried to use flagellated (motile) cells with reduced cell walls. Our attempts to isolate oil globules using different breakage methods and/or motile cells, however, resulted in almost the same globule composition as that presented in Fig. 1 and Tables 1 and 2.

The globule preparations obtained were rich in astaxanthin and in the fatty acids that are characteristic of TAG in microalgae as compared to the CFE. Moreover, some chloroplast lipids (MGDG) were substantially reduced in globule fraction (Table 2). We are therefore confident that our preparations are largely representative of oil globules composition in vivo, with the exception of some contaminating pigments, lipids and proteins, as mentioned above.

Based on the pigment content of the globules, we concluded that astaxanthin is the only carotenoid accumulating in the globule fraction. This conclusion is supported by earlier findings of Grunewald and Hagen [44]. Three astaxanthin esters comprise the majority of astaxanthin contributing to the absorbance maximum at 476.6–480 nm. None of the intermediates of the astaxanthin biosynthesis pathway or free astaxanthin or β -carotene was detected in globules, although minor amounts of these pigments were reported in the total homogenate of red *H. lacustris* cells [19]. The fatty acids content of oil globule resembled that of the CFE, which is to be expected, since oil globule TAG comprise the vast majority of lipids [16] (Table 2). These results are in agreement with the findings of Zhekisheva et al. [2], who induced astaxanthin accumulation in *H. pluvialis* only by high light exposure, and those of Wang et al. [45] for *C. reinhardtii* cytosolic isolated oil globules.

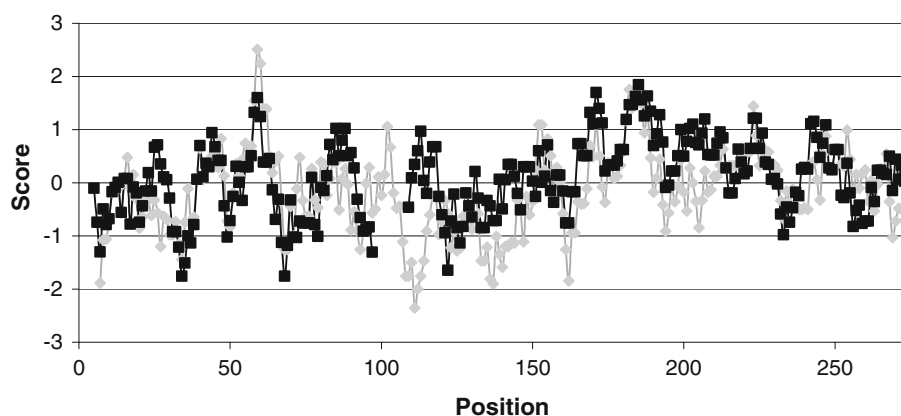
The results of oil globule individual lipid analysis are largely in agreement with the data of Grunewald et al. [18], who estimated the proportions of different lipid classes in oil globules of vegetative cells of *H. pluvialis* by densitometry of charred lipid spots on TLC plates. In the course of their preparations, the authors also detected lipids of plastid origin—DGDG, SQDG, and PG—but they reported the complete absence of MGDG. We were able to detect a relatively low proportion of MGDG in our oil globule preparations; however, this lipid class was substantially reduced in oil globules compared to in CFE, indicating enrichment of oil globules with other oil globule-specific lipid classes. The betaine lipid DGTS and the phospholipid PtdCho constituted the major portion of extraplastidial lipids in oil globules (about 40% of PL). It appears that DGTS contributed significantly to the formation of an oil globule polar lipid monolayer in green algae [18, 46] in contrast to higher plants whose oil globule phospholipid monolayer is enriched in PtdCho and PtdEtn. DGTS shares some properties with PtdCho, and is abundant in some algal groups and low plants,

Polytomella	-----	
Haematococcus	MSE---KQLKRLGFVHQGASYAYSITGTAEKLYKTARSFAPTFVEPTLAQVEDRVVAIT TA	57
Chlamydomonas	MAESAGKPLKHLEFVHTYAHKFASGAAYVEGGYQKAKTYVPAVAQPYIAKAEETCLAYAA	60
Volvox	MAD---DRKLRKLGFDAYTHKLANGAAYVEGAYKKVKPLVPQQVQFPFLAKVEDAVLAYTA	58
	*::: : **:* ** . : . : . * *::: . * . : * : * : * : * : * : *	
Polytomella	----NAQDKAEKILKKADDQVDKVVSNAHNIYQAGRSTVDTAVNNIMEIHQNNIETYQQT	56
Haematococcus	PVVAQAQDL SEKALHIADDQVEICVNTTDAKAVADGKKGVIDCMNGVKEMHEKNMQTYIAT	117
Chlamydomonas	PLATKATDHAEKILRSTDAQLDALYAASASWLSSSQK-----LADSNIAAFRGA	109
Volvox	PVVAKASDQAEKFLRITDEQVDYLYVETAAAYLTQTRK-----LTQSNIDTFRSA	107
	*::: * * : * * * : * * : : : : : . * : * : * : * : * : * : :	
Polytomella	THKYFQLVSSSTAEEVVAARVAPTQAFQKAHEILRLSLNKAQECADDPKAVKIVYDSWVVS	116
Haematococcus	SNRYFEYIKGMSEWAKDKMNP IKGQHALDNLNAAIAKAEATDPDVAAKMAL DWN SFA	177
Chlamydomonas	ADKYIDLKSTAQHVTSKLPDLSVAKARELLSASLEQAKALADPDAVAALDAWTKFA	169
Volvox	ADKYQMVKSTADYLASKLSYDISVQAARDFISKSVEKAKELSDPDAAVRIVYDSWQQFA	167
	::: * : : . : : : . * : : : : * : : * * * . . * * * :	
Polytomella	SVPVVASVLPYLEPAAARAFQNFERSIHDSLVSVPHYKQGYDMASATLQWATTTSPFRLGA	176
Haematococcus	SV PVVAKVLE TADPVTQTGL SSFYKLHDTLVSWPLYSK VVSTGVSTLSWATT MPYKLG	237
Chlamydomonas	AIPAVAKVLSAASPLTGKGVAAFTAAHDLVHSALYRYGVSVGASTLGWATSTTPYKLSA	229
Volvox	AIPAVAKTLEKTAPVTRKGFETFAAHDALVSSLVYKRSVSLGATTLGWATTTTPYKLG	227
	::: * * * . * : . . * * * * * . . : * * * * * * : * * * :	
Polytomella	NVM-----	179
Haematococcus	QMYPLVQPVADPALAKITNSKVINGLTSLYWKPTASAA-	275
Chlamydomonas	AYLYPLVQPVADPALDKVSKSTYVNAAIKYWAPAVAAA	268
Volvox	QYLYPMVQSVADPAL-----	242
	: * * : * * . * * * * * * : * : : : * : * * * * * : * * * * * :	

Fig. 6 Multiple sequence alignment of *H. pluvialis* HOGP protein with putative green algal orthologs from *C. reinhardtii* (XP_001697668), *Volvox carteri f. nagariensis* (FD812477), and *Polytomella parva* (EC748417). The peptides identified in MS/MS are marked in bold. The

insert is shaded. The region of misalignment in the hydropathy plot is underlined. Symbols: identical (asterisk), conserved (colon), semi-conserved (dot)

Fig. 7 Hydropathy plots for *H. pluvialis* HOGP (filled diamond) and for *C. reinhardtii* protein (filled square). Word = 9. The *C. reinhardtii* sequence was introduced with a gap from aa 98–108



but it is absent in higher plants. In *C. reinhardtii*, PtdCho is missing, and DGTS is present as a major polar lipid in the oil globule-enriched fraction [46].

Protein analysis of the isolated globule fraction by SDS PAGE identified at least seven protein bands that were not present in the microsomal fraction. The same proteins accumulated during astaxanthin accumulation, as can be seen in the protein pattern of total cell homogenates (Fig. 3). These findings support the notion that the bands are indeed authentic oil-globule-associated proteins

(Fig. 3). One of the protein bands showed two major 33 kDa spots on 2D gel electrophoresis and was termed HOGP. The different PIs of the two HOGP spots could be due to protein phosphorylation. HOGP was analyzed by MS/MS, and the partial amino acid sequences were utilized to prepare degenerate DNA primers that were used to clone the gene encoding HOGP. A database search revealed that HOGP has orthologs only among green algae. One such ortholog was recently identified in *C. reinhardtii* oil globules and has been shown to affect globule size [46].

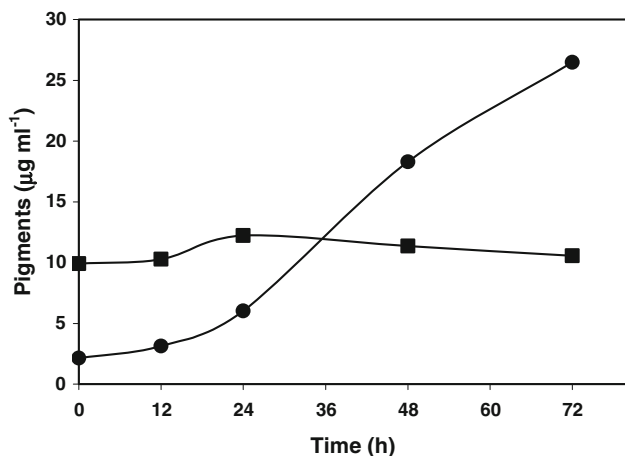


Fig. 8 Changes in chlorophyll (filled squares) and astaxanthin (filled circles) contents during 72 h of cultivation in an Erlenmeyer flask under nitrogen starvation and $200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ to induce the accumulation of oil globules for RNA extraction

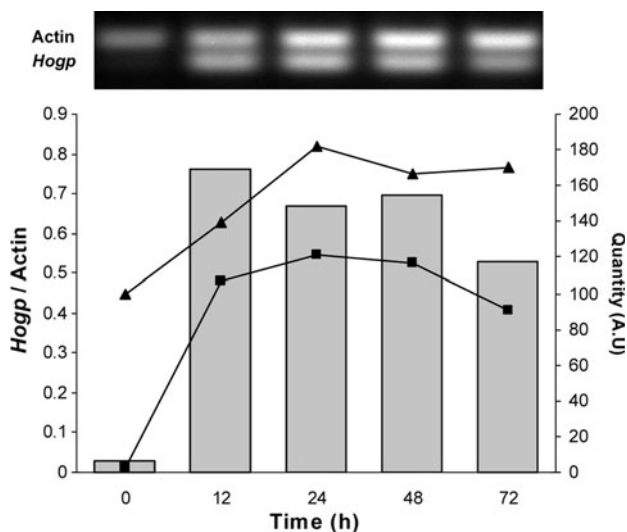


Fig. 9 Transcript levels of actin (filled triangles) and *Hoggp* gene (filled squares) during 72 h of cultivation under nitrogen starvation and $200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ to induce the accumulation of oil globules for RNA extraction. PCR product on agarose gel and densitometric representation. Bars refer to left axis

The novel family of globule-associated proteins differs completely in sequence and in secondary structure from plant oleosins and caleosins, suggesting that green algae evolved these unique globule-associated proteins, which may or may not share similar functions with plant oleosins. A comparison of the orthologous amino acid sequences from four microalgal species showed that the *H. pluvialis* and *P. parva* sequences each have an insert of 11 amino acids (Fig. 6) that is not found in *C. reinhardtii* or *V. carteri f. nagariensis*. The hydropathy plot comparison between the *H. pluvialis* and *C. reinhardtii* proteins revealed another significant difference between the two

proteins, i.e., a highly hydrophilic 9-amino-acid domain following the 11-amino-acid insertion in *H. pluvialis* (and *Polytomella*) that is hydrophobic in *C. reinhardtii* and *Volvox*. Both differences may account for the divergent functions of the two proteins. The induction pattern of HOGP is consistent with its predicted localization in oil globules: the appearance of the 33-kDa protein in nitrate depletion/high light inductive conditions is roughly correlated with astaxanthin induction (Figs. 3, 4). The transcript of the *Hoggp* gene is hardly detectable in green cells but is highly induced upon exposure of the cells to astaxanthin-inducing conditions, preceding the appearance of the protein as expected. The findings presented in this paper thus indicate that the HOGP protein is a promising candidate for involvement in globule biogenesis and the stress response in *Haematococcus* and related species of green algae.

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