Isolation of chloroplasts from marine microalgae *Isochrysis galbana* Parke suitable for organelle lipid composition analysis

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Abstract

Background: Marine microalgae, *Isochrysis galbana* Parke, is an important diet microalgal species with high nutritional value. Different from other unicellular microalgae, its cell contains two chloroplasts which are the major sites for lipid synthesis.

Results: Here, we optimized a chloroplast isolation approach suitable for *I. galbana* Parke, and evaluated the purity and integrity of the isolated chloroplasts by microscopic observations and biochemical assays. The chloroplast lipids were sequenced by ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Glycolipids were the main chloroplast lipids, and DGDG, MGDG, and SQDG were the most abundant glyceroglycolipids. DGMG and SQMG were not observed among the chloroplast lipids. In addition, DG was the most abundant neutral lipid. A part of fatty acyl R1/R2 with MGMGs, DGDGs, MGDGs, SQDGs, PEs, and PCs were not found in chloroplasts. The fatty acid proportion of chloroplast lipids were increased, decreased, or remained unchanged compared with the whole-cell.

Conclusions: This newly developed isolation approach was a simple and reliable method to isolate chloroplasts with high integrity and purity. Collectively, our findings show that such an isolation approach may be used in studies on many different aspects of chloroplast biology, and offers a useful reference for isolation of chloroplast from other marine microalgae.

Background

Chloroplasts are the feature components of the microalgae, macroalgae, and plants. As the sites for photosynthesis, they are important to organismal survival and are responsible for carbohydrate metabolism, amino acid biosynthesis, chlorophyll biosynthesis, and lipid biosynthesis [1]. The structure of the chloroplast can be divided into three sub-compartments, and each performs its own functions. The envelope, a double-membrane system surrounding the organelle, contains enzymes involved in carbon metabolism and oxidative stress response, which are associated with lipid metabolism and communication between plastids [2]. The stroma between the inner envelope and the thylakoid membrane contains soluble proteins involved in the Calvin cycle and protein synthesis [3]. The thylakoid membrane, composed of a number of thylakoids stacked on top of each other to form grana and lamellae, is the site for oxygenic photosynthesis. Therefore, chloroplasts are the main sites of organismal biosynthesis and metabolic pathways and have a substantial control on cell survival.

Chloroplast lipids are indispensable for oxygenic photosynthesis. They are the integral components of photosynthetic protein complexes and constituents of mechanisms to protect the photosynthetic machinery [4,5]. Besides, lipids constitute the membranes for compartmentalization of the chloroplast and can contribute to the flexibility of protein complexes and stabilize its bilayers [6]. In general, chloroplast lipids play an important role in photosynthesis, which can modify their energetic metabolism and affect organism life activities. A comprehensive understanding of the chloroplast lipid composition

facilitates the clarification of lipid metabolic machinery, which plays a fundamental role in rational engineering of oils and fatty acids for fuel, industrial feedstocks, and nutritional improvement.

Until recently, only a limited number of studies have focused on chloroplast lipid composition and metabolism in land plants. Chloroplasts are particularly rich in monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosylmonoacylglycerol (SQDG). In higher plant *Arabidopsis*, galactolipids are essential to support the growth and photosynthesis [7,8]. MGDG, DGDG, SQDG, and phosphatidylglycerol (PG) constitute the thylakoid membranes, which are the integral ingredients of the photosynthetic complexes [5,9]. Phosphatidylcholine (PC), lyso-phosphatidylcholine (lyso-PC), or phosphatidic acid (PA) for chloroplast lipids can be produced in the endoplasmic reticulum (ER) and the chloroplast [10]. In unicellular green alga *Chlamydomonas reinhardtii*, a model species, the building blocks for triglyceride (TG), and membrane lipids are biosynthesized in the chloroplast. PA and its dephosphorylated product diacylglycerol (DG), are produced in the chloroplast, and they primarily function as precursors for structural lipids of the photosynthetic membrane system [11]. In starved cells, galactolipids are maintained to preserve sufficient chloroplast integrity [12]. In general, neutral lipid metabolism of chloroplast has been extensively studied in *C. reinhardtii*. However, there are no comprehensive studies on the microalgal chloroplast lipid composition.

Marine microalgal biomass plays a critical role in the animal food chain. As a golden-brown flagellate marine microalga, *Isochrysis galbana* Parke is widely adopted as an aquaculture feed for young fish and in bivalve hatcheries due to its high content of polyunsaturated fatty acids (PUFAs) [13]. Previous investigations have shown that the nutritional value of *I. galbana* to Mollusca larvae is greatly attributed to its unique lipid composition [14-16] and that different cultivation temperatures or times can alter the lipid composition of *I. galbana*. Therefore, many studies have focused on identifying growth conditions that can promote lipid production [17,18]. While the whole-cell lipidomics of *I. galbana* is widely studied, the lipid synthesis and biosynthesis of chloroplast remain largely unknown.

A major deterrent in studying the lipid composition of the chloroplast is the technical challenge associated with the isolation and purification of intact chloroplasts. The currently used techniques employ physical or chemical means to break the cell wall and the plasmalemma [19,20]. The isolation techniques of chloroplast have been confined to several higher plants and a model alga. For *Arabidopsis*, a method for chloroplast isolation relies on protoplastation [21]. However, this method is time-consuming and expensive. To resolve this problem, a simple and cost-effective approach has been developed to isolate a large number of intact chloroplasts from *Arabidopsis* seedlings [22,23]. For marine plant *Posidonia oceanica*, an optimized protocol has been adapted from terrestrial plants and can be used to isolate chloroplasts from as minimal tissue as possible [24]. For *C. reinhardtii* mutant, cells are broken by passing them through a 27-gauge syringe needle [15,25]. However, thus far, no universal method for isolation and purification of the chloroplast is available due to the differences in the number, size, gravity, thylakoid membrane structure, and lipid composition of the chloroplast of different plant cells. Thus, developing a suitable chloroplast isolation protocol that can be applied to different plant species is essential. The marine microalgae *I. galbana* Parke, in which one cell contains two chloroplasts, is

different from others [26]. The possibility of obtaining intact chloroplasts, which are active in lipid synthesis, from a no cell wall microalga prompted us to attempt the isolation of chloroplasts from *I. galbana*. There are no established techniques for the isolation of intact chloroplasts from *I. galbana*, and there are no studies on its chloroplast lipid composition. Therefore, a chloroplast isolation technique for the marine microalgae *I. galbana* that is suitable for studying the organelle lipid composition is an emerging need.

In the present study, we developed a rapid approach for cellular fractionation of *I. galbana*, by which intact chloroplasts could be isolated. The chloroplasts purified using this approach were mostly intact and contained only minimal contamination. The isolated chloroplasts and their integrity were analyzed by phase-contrast fluorescence microscopy (Leica) and transmission electron microscopy (TEM), and their purity was analyzed by enzyme-linked immunosorbent assay (ELISA). The lipid composition of the chloroplasts isolated from *I. galbana* was identified by ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

Methods

Microalgal culture and sample collection

I. galbana Parke was provided by the Marine Biotechnology Laboratory of Ningbo University, China. Seawater (pH 8.2, salinity 22‰) was subjected to filtration using 0.45-µm cellulose acetate membranes, followed by heat sterilization. The culture medium was supplemented with the following nutrients: 100 mg/L KNO₃, 10 mg/L KH₂PO₄, 2.5 mg/L MnSO₄·H₂O, 2.5 mg/L FeSO₄·7H₂O, 10 mg/L EDTA-Na₂, 6 µg/L vitamin B₁, and 0.05 µg/L vitamin B₁₂. Microalgae were maintained in 5,000 mL conical flasks at 20 ± 2 °C, with a light intensity of 4,000 Lux (LED) under a 12:12-h light-dark (L:D) photoperiod. Cells were incubated at 4 °C overnight to metabolize the pyrenoid and starch granules in the chloroplasts, followed by sample collection at the late stationary phase. The cells were centrifuged at 6,000×g for 10 min. A blood counting chamber was used to determine the cell density at each time point. All experiments were performed in triplicates, and the results were expressed as the mean ± standard deviation.

Isolation and purification of intact chloroplast

Chloroplasts from *I. galbana* were isolated and purified at 4 °C or on ice to protect the integrity of chloroplasts, and the pellet was washed and resuspended gently. First, 100 mg of wet weight microalgae was washed twice with 2 mL of 50 mM HEPES-KOH (pH 7.8) and centrifuged at 800×g for 10 min to remove salts. The chlorophyll concentration of cells was measured according to the study by Arnon [27]. The key step was to gently separate the cell membrane from the plastids, and to this end, the cells were provided with a hypotonic environment by two steps. First, the cells were resuspended in 2 mL of solution A (300 mM sucrose, 50 mM HEPES-Tris, 2 mM EDTA-Tris, 1 mM MgCl₂, 1% PMSF as a protease inhibitor, pH 7.8) and centrifuged. Second, the cells were subjected to a lower osmotic shock by resuspending the cell pellet in 2 mL of solution B (100 mM sucrose, 50 mM HEPES-Tris, 2 mM EDTA-Tris, 1 mM MgCl₂, 1%

PMSF, pH 7.8) and allowing to stand on ice for 20 min. Subsequently, the cells were successively homogenized at 2,500 r/min for 10 s and 6,800 r/min for 15 s, and this process was repeated 3-4 times, followed by centrifugation at 800×g for 10 min. For effective separation, the supernatant was set aside, and solution B was added to the cell pellet and subjected to homogenization for 3-4 turns.

The supernatant was centrifuged at 1,000×g for 10 min to remove nuclei and intact cells, and the crude chloroplasts were collected by centrifugation at 4,500×g for 10 min. The crude chloroplast pellet was gently resuspended in 1 mL of solution C (300 mM D-sorbitol, 50 mM HEPES-Tris, 10 mM EDTA-Tris, 5 mM MgCl₂, 1% PMSF, pH 7.8) using a pipette to avoid disrupting the chloroplasts. The resuspended sample was gently loaded onto several Percoll density gradients (top to bottom: 2 mL 10% Percoll, 3 mL 30% Percoll, 3 mL 50% Percoll). All gradient media were prepared in solution C. The Percoll gradients were subjected to centrifugation at 8,000×g for 30 min. The intact chloroplast constituted a band at the 30–50% interface and was collected, diluted with three volumes of solution D (300 mM D-sorbitol, 50 mM HEPES-Tris, 10 mM EDTA-Tris, 5 mM MgCl₂, pH 7.8), centrifuged at 3,500×g for 10 min twice, and the chlorophyll concentration of the chloroplast was determined. Pellets were stored at -80 °C.

Electron microscopy

The modified Spurr method [28] was used to prepare the samples for TEM. Briefly, samples were incubated in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.8) at 4 °C for 48 h, washed with phosphate buffer, and then post-fixed with 1% osmium tetroxide (OsO₄) for an additional 3 h. Subsequently, samples were dehydrated in a graded series of ethanol, followed by acetone. The specimens were incubated in a mixture of acetone and final Spurr resin mixture overnight, followed by incubation at 70 °C for more than 9 h. The samples were sectioned using a LEICA EM UC7 ultratome, and the sections were stained by uranyl acetate and alkaline lead citrate, followed by observation using a Hitachi TEM (Model H-7650) at an acceleration voltage of 80 kV.

ELISA assays

Proteins were extracted according to a modified method [29]. Briefly, the samples were ground with liquid nitrogen in a pre-cooled mortar and subsequently sonicated in lysis buffer (8 M urea, 1% PMSF) on ice three times. The remaining debris was removed by centrifugation at 12,000×g at 4 °C for 10 min. The supernatant was collected, diluted with four volumes of cold acetone, and incubated at -20 °C for 24 h, followed by centrifugation at 12,000×g at 4 °C for 10 min. Finally, the obtained pellet was dissolved in phosphate buffer saline (PBS).

According to literature, cytoplasm and mitochondria are common contaminants in chloroplast isolation. The purity of isolated chloroplasts was biochemically assessed using ELISAs to estimate the activities of Phosphoenolpyruvate carboxylase (PEPC) and Cytochrome C oxidase (COX) [24,25,30]. The chloroplast-specific enzyme, Ribulose biphosphate carboxylase oxygenase (Rubisco), was used as a positive control. The enzyme activities were estimated using ELISA kits (Meimian Biotechnology, Jiangsu, China),

according to the manufacturer's instructions, and the color change was measured spectrophotometrically at 450 nm. Each experiment was conducted with three biological replicates.

Total lipid extraction

The total lipid was obtained from samples using a modified Bligh and Dyer method [31]. Briefly, freeze-dried samples were extracted with chloroform/methanol/water (2:2:0.8, v/v/v) and dried under a gentle stream of N₂, and the residue was preserved at -20 °C. The final lipid residue was resuspended in CH₃OH before further analysis. The above-mentioned procedures were carried out in triplicates. The results were expressed as mean ± standard deviation.

UHPLC conditions

A Thermo Fisher U3000 High-Performance LC system (UHPLC) with an ACQUITY UPLC BEH C8 analytical column (i.d. 2.1 mm × 100 mm, particle size 1.7 μm, pore size 130 Å) was used to perform reversed-phase analysis. Mobile phase A consisted of acetonitrile/water (6:4, v/v), mobile phase B consisted of isopropanol/acetonitrile (9:1, v/v), and both phases contained 0.1 % formic acid and 10 mM ammonium acetate. The flow rate was set at 0.2 mL/min. The gradient elution was programmed as follows: 0-15 min, 60-45% A; 15.0-18.0 min, 45-35% A; 18.0-26.0 min, 35% A; 26.0-28.0 min, 35-0% A; 28.0-30.0 min, 0% A; 30.0-30.5 min, returning to an initial 60% A; 30.5-40 min, 60% A. The temperature of the sample chamber was maintained at 8 °C, and the column temperature was maintained at 45 °C. Samples were filtered using a 0.22-μm ultrafiltration membrane (Millipore, Bedford, MA, USA) before injection.

Mass spectrometric conditions

Mass spectrometry was carried out on a Thermo Scientific™ Q Exactive hybrid quadrupole-Orbitrap mass spectrometer equipped with a HESI-II probe. The instrument was operated according to a data-dependent LC-MS/MS method in positive mode and negative mode, respectively. Data were obtained in a centroid mode from 200 to 2,000 m/z at a resolution of 70 K, and in a high energy collisional dissociation (HCD) MS/MS mode at a resolution of 17.5 K. The automatic gain control (AGC) target was set at 1e⁶ for MS and 2e⁵ for MS². The capillary voltage was maintained at 3.5 kV, and the capillary temperature was set at 350 °C. The sheath gas was 45 arb, and the aux gas was 10 arb. MS² analysis was carried out on the mass spectrometer with different collision energy and ramp of 25, 30 V in a positive ion mode and 20, 24, 28 V in a negative ion mode based on the type of lipids. The instrument was previously calibrated in positive mode and negative mode, respectively.

Data processing

The raw data acquired from LC/MS runs were analyzed on the Thermo Xcalibur™ system. Lipidsearch software version 4.1 (Thermo Scientific™) was used to perform peak identification, lipid identification, peak extraction, peak alignment, peak area, and intensity. According to characteristic fragment ions and fragmentation pathways, glycolipids, phospholipids (PLs), betaine lipids, and non-polar lipids could be

determined [32-39]. Identification of each lipid class and the acyl chains were further confirmed by MS/MS in positive or negative ion mode, as previously described. The semi-quantitative determination of each lipid was based on the lipid standards (Avanti Polar Lipids Inc., Alabaster, AL) area [32,40].

Results

Chloroplast yield

The chlorophyll concentration of the whole-cell and chloroplast were determined. The average yield of intact chloroplasts was $10 \pm 1\%$ based on the chlorophyll recovery (Additional file 1: Table S1).

Chloroplast characterization and integrity

Chloroplast debris was accumulated at the 10-30% percoll gradient interface, the intact chloroplasts constituted a band at the 30-50% interface, and intact cells were found at the bottom of the tube (Fig. 1). The microscopic pictures for each fraction are shown in Fig. 2 and Fig. 3.

Fig. 3a and Fig. 3b show that there were no intact cells in the purified chloroplasts. Under a phase-contrast microscope, the intact chloroplasts were green and surrounded by a bright halo due to the presence of the chloroplast envelope, and had an integrity of $>85\%$ (Fig. 3d). When observing the intact cells and isolated chloroplasts using fluorescence microscopy, the isolated chloroplasts emitted red light and were smaller than intact cells (Fig. 3e and Fig. 3f). Examination of cell ultrastructure showed that purified chloroplasts retained their shape, and were intact (Fig. 3g and Fig. 3h).

Chloroplast purity

PEPC and COX activities in the cell homogenates, crude chloroplasts, and purified chloroplasts were determined, and the data is shown in Table 1. The differential centrifugation could reduce 54% cytoplasm and 45% mitochondrial contamination, and a discontinuous density gradient centrifugation could further reduce 41% cytoplasm and 51% mitochondrial contamination. This method resulted in only low activities of PEPC and COX, confirming that there was no significant organelle contamination in the isolated chloroplasts.

Lipid composition of the whole-cell and isolated chloroplast

In the present study, we analyzed the contents of glycolipids, PLs, sphingolipids, betaine lipids, and acylglycerols in the whole-cell and chloroplast of *I. galbana* Parke. Figure 4 lists the relative amounts of lipid classes in total lipid and the relative amounts of major lipid subclasses in total lipid.

Glycolipids were the main lipid components in the chloroplast and accounted for 55.7% of the total lipid. As the most abundant glyceroglycolipid in the chloroplast, DGDG, MGDG, and SQDG made up approximately 27.0%, 18.4%, and 8.2% of the total lipid, respectively, which were much higher than those in the whole-cell. The fatty acyl R1/R2 were mostly occupied by 16:1/18:4, 16:1/18:3, and 18:4/18:5 in

DGDGs, 18:4/18:5, 18:4/18:4, 18:4/18:3, and 14:0/18:4 in MGDGs and 18:4/18:3, 16:0/18:3, and 18:1/18:1 in SQDGs (Additional file 2: Table S2). The percentage of trigalactosyldiacylglycerol (TGDG) in the chloroplast was 1.2%, which was nearly the same as that in the whole-cell. Remarkably, monogalactosylmonoglyceride (MGMG) constituted 9.5% of glycolipids in the whole-cell, while it only accounted for 0.7% in the chloroplast. Besides, the fatty acid chains of MGMGs with 16:2 were present in the whole-cell but absent in the chloroplast. Compared to the whole-cell, digalactosylmonoglyceride (DGMG) and sulfoquinovosylmonoacylglycerol (SQMG) were nearly undetectable in the chloroplast. Therefore, our data suggested that these two types of lipids were not components of the chloroplast lipids.

Based on our experimental results, the proportion of acylglycerols was second only to glycolipids, which were abundant in both the whole-cell (25.1%) and the chloroplast (28.9%) of *I. galbana*. Microscopic observations confirmed that chloroplast contained lipid droplets (LD), which were conserved neutral lipid storage organelles (Fig. 5). The proportion of DG in total chloroplast lipid was 20.4%, which was more than that in the whole-cell. Meanwhile, the proportions of monoglyceride (MG) and TG in chloroplast were less than those in the whole-cell.

The proportion of PLs in the total lipid of chloroplast was only slightly more than that in the whole-cell. The proportion of PG in PL was greater compared with the other four PLs in the whole-cell and chloroplast, and the second most abundant PL was PC. The chloroplast exhibited a slight increase in PG and a slight decrease in PC compared with the whole-cell. Other PLs in the chloroplast were detected at lower amounts. PA content of the chloroplast was slightly higher than that of the whole-cell, while phosphatidylinositol (PI) and phosphatidylethanolamine (PE) showed the opposite trends compared with PA. PEs containing fatty acyl R1/R2 with 16:0/20:4, 16:0/22:6, 18:1/20:4, 18:0/20:4, and 18:1/22:6 were not found in chloroplast compared with the whole-cell, and PCs with 18:0/20:4 were also not found in the chloroplast.

We also found sphingolipids, which made up 8.3% in the whole-cell and 10.4% in the chloroplast. There was a lot of cerebroside and a small amount of ceramide in sphingolipids. The proportion of betaine lipids was low, which was nearly the same between the whole-cell and chloroplast. Moreover, 14:0 lyso-DGCC (lyso-diacylglycerylcarboxyhydroxymethylcholine) and 16:0 lyso-DGCC were not found in chloroplast compared with the whole-cell.

For *I. galbana* Parke, the fatty acid composition of major lipid classes in whole-cell and chloroplast was given in Figure 6. Compared with the whole-cell, in DGs of the chloroplast, the proportion of 18:1 fatty acids was sharply increased, the proportions of 16:0, 16:2, 18:0, 19:1, and 20:5 fatty acids were slightly increased, and the proportions of 14:0, 18:4, and 22:6 fatty acids were slightly decreased. The proportions of 16:1, 18:2, and 18:3 fatty acids in DGs remained unchanged (Fig. 6a). Moreover, in TGs of chloroplast, the proportion of 18:1 fatty acid was sharply decreased, the proportions of 16:1, 18:1, and 18:2 fatty acids were slightly decreased, and the proportions of other fatty acids were slightly increased (Fig. 6b). The PC composition was deeply modified in the chloroplast, with a dramatic elevation in 18:1 and 19:1

fatty acids and a remarkable reduction in 16:0, 18:2, and 20:4 fatty acids (Fig. 6g). In addition, in PGs of the chloroplast, the proportion of 18:1 fatty acid was substantially increased, and the proportions of other fatty acids were slightly reduced (Fig. 6h). In contrast, the proportions of DGDG, MGDG, SQDG, and glycosphingolipid (GerGI) in the chloroplast were essentially similar to those in the whole-cell.

Discussion

The marine algae of *I. galbana* differ from other marine unicellular microalgae in their lack of the cell wall. Due to this difference in structure, chloroplast isolation techniques need to be specifically adapted for *I. galbana*, and here, we report a new isolation approach. SDS, frequently presented in isolation buffers to break up the cell wall, was not added to the isolation buffer in this study. Referred to the disruption method for animal cells [41], our improved method used two-step hypotonic buffers, which was better compared with simplex lysis buffers. Buffer A contained 300 mM sucrose, and buffer B contained only 100 mM sucrose. The two-step hypotonic buffers allow the microalgae cells to gradually detach from the plasma membrane, and the amount of buffers added needs to be strictly controlled. However, as the plasma membrane of *I. galbana* was extremely hard, separation could further be achieved by homogenization to save time. Meanwhile, the two-step hypotonic buffers provided conditions for the next homogenization. A rapid isolation method of chloroplast consisting of the two-step hypotonic buffers and homogenization could save time and increase the yield.

In particular, the multiple homogenization steps were a key feature of the isolation procedure and ensured a high yield of intact chloroplasts. During homogenization, quartz sand mixture (0.1mm and 0.4mm) was added into the tube to break the cells of *I. galbana*. The homogenization needs to be thorough, and several short-time homogenizations were used until very little sediment was observed after centrifugation at $800 \times g$ for 10 min. The supernatant was examined using phase-contrast fluorescence microscopy to ensure the absence of intact cells. The speed of homogenization is also vital for intact chloroplast isolation, and we adopted the intermittent low-speed and high-speed method. With this modification, we could not only isolate the intact chloroplasts but could also avoid thylakoid membrane fragmentation that is usually caused by mechanical heat generated during continuous homogenization. Additionally, continuous low-speed homogenization increases the isolation time substantially, which is not conducive to the separation and protection of intact chloroplasts. Therefore, our method of successive low and high-speed homogenization in this experiment mitigates these issues. The homogenization condition presented here are optimal for *I. galbana* Parke, and we demonstrated that the osmotic shock and homogenization play a fundamental role for effective cell lysis, organelle separation, and the final isolation of intact chloroplasts.

After homogenization, intact chloroplasts were purified from damaged chloroplasts and intact cells by a Percoll density gradient centrifugation, and two-step gradients were employed for most experiments. Before gradients were added, the tube was rinsed with 1% BSA. Then the gradients were added slowly along the tube wall to form distinct interfaces. The gradient centrifugation step allowed for the purification of intact, photosynthetically active chloroplasts from *I. galbana* Parke.

Microscopy and ELISAs were used to estimate the integrity and purity of the isolated chloroplast. ELISAs is a powerful method for measuring the enzymatic activity and is often applied in biochemical assays not only for animal cells but also for plants and algae [42,43]. ELISAs are rapid, sensitive, specific, and precise [44], therefore, we used it to quantify enzymatic activity. To avoid the inactivation of the enzymes, the samples should go through the entire experimental process, and the sampling process should be synchronous. Cell homogenate, rather than cell fractionation, was selected to determine 100% of individual enzymatic activity. PEPC and COX are enzymes unique to the cytoplasm and mitochondria, respectively, and chloroplasts do not contain these enzymes [25]. Microscopic images and the Rubisco activity data confirmed the integrity of the isolated chloroplast. The results of the PEPC and COX activities in the cell homogenates, crude chloroplasts, and purified chloroplasts ensured the purity of the isolated chloroplast, indicating that they were suitable for chloroplast lipid composition analysis.

To fully understand the chloroplast lipid composition in *I. galbana* Parke, we chose the late stationary phase of microalgae for analysis because it is the optimal period with the most lipid accumulation [45]. The chloroplast lipid composition of *I. galbana* Parke was qualitatively similar to that of other photosynthetic plants. The results of the cellular and chloroplast lipid composition in *I. galbana* Parke indicated that chloroplasts were the major sites for fatty acid synthesis and lipid biosynthesis. The results of glycolipids were consistent with those reported for chloroplast from land plants like *Arabidopsis* [46,47]. One important exception was that the content of DGDG was higher compared with the MGDG in this alga. This was probably a consequence of different plant species or cultivation conditions as depending on the plant species, changes in growth conditions can affect the amounts of DGDG and MGMG [12,48].

According to previous reports, acylglycerols are present in minor amounts in *Vicia faba* [49] and in significant amounts in *Mimosa* [50]. Our results indicate similar acylglycerols levels in *I. galbana* Parke and *Mimosa*. In eukaryotes, lipid droplets serve as the primary depot for energy and neutral lipid storage [51,52], and consistent with other literature, we also observed lipid droplets in the chloroplasts of *I. galbana* Parke (Fig. 5) [53,54]. *I. galbana* chloroplast lipid droplets contained substantial quantities of storage lipids, and DGs were used for the synthesis of glycolipids and PGs.

In plants and algae, galactolipid, sulfolipid, and PGs are synthesized by DAG, which is derived from the eukaryotic pathway or eukaryotic and prokaryotic pathways [55,56]. *I. galbana* chloroplast lipids had high amounts of unsaturated fatty acids, and were mostly occupied with 14:0, 16:1, 18:3, 18:4, and 18:5 in DGDGs, 14:0, 16:1, 18:1, 18:3, 18:4, and 18:5 in MGMGs, and 14:0, 16:0, 18:1, 18:3, and 18:4 in SQDGs. In PGs, 16:0 and 18:1 were the main fatty acids. The position of these fatty acids in the glycerol backbone was different. Fatty acyl residues of DGDGs and SQDGs were mainly with 18 carbon atoms at the *sn*-2 position of the glycerol backbone. In *I. galbana*, DGDG and SQDG were mostly derived from the eukaryotic pathway. The other two photosynthetic membrane lipids, fatty acyl residues with 16 and 18 carbon atoms, were both distributed at the *sn*-2 position. Thus, in *I. galbana* Parke, the MGMG and PG were produced by both the prokaryotic and eukaryotic pathways.

Conclusions

To our knowledge, this is the first method that enabled the isolation of intact chloroplasts and delivered lipid yield that was sufficient for sub-cellular lipid composition studies in *I. galbana* Parke. Our study compares the difference between cellular and chloroplast lipid composition of the marine microalgae *I. galbana*, and analyze the fatty acids of chloroplast lipids. Further work should focus on the changes in chloroplast lipids during the entire growth stage.

Abbreviations

AGC: automatic gain control; BSA: bovine serum albumin; Chlp: chloroplast; COX: Cytochrome C oxidase; DG: diacylglycerol; DGDG: digalactosyldiacylglycerol; DGMG: digalactosylmonoglyceride; ER: endoplasmic reticulum; GerGI: glycosphingolipid; HCD: high energy collisional dissociation; LD: lipid droplet; lyso-DGCC: lyso-diacylglycerylcarboxyhydroxymethylcholine; lyso-PC: lyso-phosphatidylcholine; M: mitochondrion; MG: monoglyceride; MGDG: monogalactosyldiacylglycerol; MGMG: monogalactosylmonoglyceride; OsO₄: osmium tetroxide; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PEPC: Phosphoenolpyruvate carboxylase; PG: phosphatidylglycerol; PI: phosphatidylinositol; PLs: phospholipids; PUFAs: polyunsaturated fatty acids; Py: pyrenoid; Rubisco: Ribulose biphosphate carboxylase oxygenase; SDS: sodium dodecyl sulfate; SQDG: sulfoquinovosyldiacylglycerols; SQMG: sulfoquinovosylmonoacylglycerol; TEM: transmission electron microscopy; TG: triglyceride; TGDG: trigalactosyldiacylglycerol; UHPLC–MS/MS: ultrahigh-performance liquid chromatography tandem mass spectrometry; WC: whole-cell.

Declarations

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Authors' contributions

The project was coordinated by Ye Zhong and Yanrong Li, and Jilin Xu conducted the experiments. Jiaye Cao and Chengxu Zhou contributed to data collection and analysis. Jilin Xu and Xiaojun Yan designed the experiments and reviewed the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

All data supporting the conclusions of this article are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors are consent for publications.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1 Comparison of enzyme activities in cell homogenate, crude, and purified chloroplasts from *I. galbana* Parke

Enzyme name	cell homogenate	crude chloroplasts	purified chloroplasts
Rubisco	11.85±0.8	10.38±0.1	11.09±0.6
COX	0.24±0.0	0.11±0.0	0.06±0.0
PEPC	9.64±0.4	5.64±0.5	2.72±0.2

Activity is expressed as U·per L of chlorophyll in three independent replicates. Cell homogenate was obtained by homogenization. Crude chloroplasts were obtained by differential centrifugation. Purified chloroplasts were obtained by differential centrifugation followed by purification using discontinuous density gradient centrifugation.

Figures

sample

a 10%

b 30%

c 50%

Figure 1

Typical Percoll gradient after centrifugation, and the percentages of Percoll in buffer for the various layers. a: broken chloroplasts, b: intact chloroplasts, c: intact cells.

Figure 2

Microscopic images of the broken chloroplasts at 50,000× magnification (a) and the intact cell at 40,000× magnification (b).

Figure 3

Micrographs of whole-cell and isolated chloroplasts from *I. galbana* Parke. a, c, e, g: Light and electron micrographs of *I. galbana* Parke. Light micrograph of *I. galbana* at 400× magnification (a), Phase-contrast micrograph of *I. galbana* at 400× magnification (c), Fluorescence micrograph of *I. galbana* at 400× magnification (e), and TEM image of *I. galbana* at 30,000× magnification (g). b, d, f, h: Light and electron micrographs of purified chloroplasts. Chloroplasts purified from *I. galbana* Parke were analyzed

by phase-contrast fluorescence microscopy at 400× magnification, light (b), phase contrast (d), and fluorescence (f), microscopy, and majority of the isolated chloroplasts were intact. The integrity of the chloroplasts was validated using TEM at 40,000× magnification (h). Chlp: chloroplasts; Py: pyrenoid.

Figure 4

Total lipid composition of whole-cell (black bars) and chloroplast (gray bars) in *I. galbana* Parke. Lipid content is expressed as nmol lipid mg⁻¹ of dry weight and then converted to the percentage of total lipid ±

standard error ($n = 3$).

Figure 5

TEM micrographs of lipid droplets present in the chloroplast. Chlp: chloroplasts; LD: lipid droplet; M: mitochondrion.

Figure 6

Fatty acid profiles of major lipid subclasses in the whole-cell and chloroplast of *I. galbana* Parke. Results are shown for some of the major lipid subclasses, DG (a), TG (b), DGDG (c), MGDG (d), SQDG (e), GerGI(f), PC (g), and PG (h), whole-cell (black bars) and chloroplasts (gray bars). Lipid content is expressed as nmol fatty acids mg⁻¹ of dry weight and then converted to the percentage of total lipid $\pm$ standard error (n = 3).

Supplementary Files

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