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Quantification of chrysolaminarin from the model diatom *Phaeodactylum tricornutum*

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ABSTRACT

We have established a protocol that allows for parallel quantification of three carbohydrate pools in the marine diatom *Phaeodactylum tricornutum*. This method utilizes a series of extraction and digestion steps followed by the employment of the 3-methyl-2-benzothiazolinone hydrazone (MBTH) reducing sugar assay. Comparing carbohydrate content between hydrolyzed and non-hydrolyzed soluble extracts enables quantification of soluble, non-reducing carbohydrate. The latter fraction contains chrysolaminarin as verified by ^{1}H NMR and monomer composition of the purified glucan. We applied this method to investigate carbon partitioning in two experiments. We observed the accumulation of chrysolaminarin during the day and near complete consumption in the dark, supporting its role for fueling heterotrophic metabolism at night. We then observed little change in chrysolaminarin accumulation or consumption during nitrogen starvation, a condition that is known to increase the cellular content of the biofuel precursor triacylglycerol. Overall, this method improves the resolution of major carbohydrate pools in diatoms, complementing physiological and systems biology studies interested in exploring diatom metabolism.

1. Introduction

Diatoms are important contributors to the biogeochemical cycling of carbon, contributing as much as one-fifth of annual net primary productivity [1]. Diatoms allocate organic carbon into two primary storage metabolites: the neutral lipid triacylglycerol (TAG) and the storage polysaccharide chrysolaminarin [2,3]. TAG is a major carbon reserve, especially during nutrient stress [4]. TAG productivity is of interest for biofuel production, as these neutral lipids are an important precursor for the production of biodiesel [5]. Diatoms have been identified as a promising platform for biodiesel production because of their relatively high biomass and lipid productivities relative to other algal species [6]. Simultaneously, diatoms synthesize chrysolaminarin as a reserve carbohydrate. Chrysolaminarin has been characterized as soluble polymer consisting of glucose monomers linked by a β-1,3 bond with limited β-1,6 branching [7]. The average molecular weight and number of branches of chrysolaminarin can vary significantly between species [8]. The structure and composition of chrysolaminarin is known; however, the enzymes responsible for its creation and consumption remain broadly uncharacterized [8]. Furthermore, the lack of an established quantification method limits our understanding of this reserve polysaccharide as a fraction of total cellular carbon.

Several studies have addressed the challenge of chrysolaminarin quantification by using a warm-water extraction that quantifies insoluble carbohydrates and soluble carbohydrates in parallel [9,10]. The diversity of insoluble and soluble carbohydrates produced by diatoms has recently been reviewed [11]. Briefly, insoluble carbohydrates contain structural carbohydrates associated with the frustule such as mannans [12], callose [13], and, in some species, chitin [14]. Soluble carbohydrates contain chrysolaminarin [7], exopolysaccharides [15], and free sugars in metabolism. These insoluble and soluble fractions are typically quantified with total carbohydrate assays such as phenol-sulfuric acid or anthrone methods [16,17]. These quantification methods do not differentiate between monosaccharides and polysaccharides. Several recent studies have opted to observe diatom carbohydrate biochemistry by measuring total carbohydrates with these assays [18–20]. However, quantifying soluble monosaccharides, soluble polysaccharides (chrysolaminarin), and insoluble polysaccharides in parallel with a reducing sugar assay [21] will provide more insight into carbon partitioning following genetic manipulation or physiological conditions.

Metabolic engineering strategies in many photoautotrophs seek to increase the flux of carbon and energy towards molecules of interest [5,22]. The proportions of carbon found in the two major carbon reserves of algae, neutral lipids and storage polysaccharides, can be

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manipulated through changing physiological status or by disruption of biosynthetic pathways. Nitrogen starvation is one of the best-characterized examples of manipulating metabolism: increased TAG accumulation has been observed in a broad range of organisms from diatoms [23–27] to green algae [27–30] when nitrogen is removed from the medium.

Carbon partitioning also can be altered by genetic engineering. It is known that some proportion of cellular organic carbon is allocated as carbon reserves (both TAG and storage polysaccharides). It was reasoned that disrupting polysaccharide metabolism might further increase the accumulation of TAG, as the displaced storage carbon from polysaccharides had to be partitioned somewhere in metabolism. For instance, mixotrophically grown starchless *Chlamydomonas* mutants accumulated even more TAG than wild type during nitrogen starvation [31,32]. Therefore, it is rational to suggest that manipulating chrysolaminarin metabolism may enhance TAG productivity in diatoms. Several groups have investigated this possibility in diatoms with a reverse genetics approach by knocking down or knocking out putative enzymes in chrysolaminarin biosynthesis [33,34], or by blocking β-1,3 glucanase activity [35]. All of these studies reported an increase in TAG content; however, they could only infer a reduction in chrysolaminarin accumulation. Conclusions about carbon repartitioning from chrysolaminarin to other carbon sinks have broadly been limited by the inability to specifically quantify chrysolaminarin from other major carbohydrate pools.

Our primary objective with this study was to establish a method to quantify chrysolaminarin and thereby improve the biochemical toolkit of diatoms to study central carbon metabolism. The method described here is based off of the sensitive and accurate 3-methyl-2-benzothiazolinone hydrazone (MBTH) assay [21,36]. The MBTH assay has already been applied to a range of carbohydrate quantification applications including field samples [37], glycolytic assays [38,39], and total carbohydrate content of algae [40]. It provides considerably greater accuracy and precision compared to the commonly used anthrone and phenol-sulfuric acid carbohydrate assays [40]. This assay is coupled to a workflow that quantifies three major carbohydrate pools from *Phaeodactylum tricornutum*, including a soluble, nonreducing fraction that contains chrysolaminarin. We then applied this workflow to quantify the changes in carbon partitioning to storage metabolites during nitrate starvation on a light/dark cycle. Our data indicate that, during nitrate starvation, chrysolaminarin per cell decreases and that TAG represents the dominant reserve carbon molecule.

2. Materials and methods

2.1. Extraction and analytical chemistry of carbohydrates

2.1.1. Culturing conditions

Batch cultures of axenic *Phaeodactylum tricornutum* CCAP 1055/1 (hereafter, *Phaeodactylum*) were grown in 1 L Roux flasks with 0.8 L of artificial seawater (Instant Ocean, 35‰ salinity). Nutrients were added per the stoichiometry described by Guillard [41], but at a 2.3-fold higher concentration to mitigate any nutrient limitation. Silicon was omitted from the medium, as it is not required for *Phaeodactylum* growth. Light was provided in a 12:12 day/night cycle, where daytime irradiance was set at 420 ± 20 μmol photons m^{-2} s^{-1} (LI-COR light meter, LI-250 A, 2 π sensor). Five replicate cultures were grown at 18 °C and were mixed by sparging with 1 L min^{-1} air. Cultures were maintained in exponential growth for at least two days prior to the start of the experiment with serial dilutions.

2.1.2. Extraction and purification of soluble carbohydrate

The 4 L volume was harvested at dusk during exponential growth and cells were pelleted by centrifugation (3220 ×*g*, 10 min, 18 °C) in 50 mL conical tubes. All harvested cells were combined into a single pellet, resuspended with 10 mL deionized water, and incubated at 50 °C for 30 min. The suspension was spun (3220 ×*g*, 10 min, 18 °C) and the 10 mL warm water extract was transferred to a fresh 50 mL conical tube. Then, 40 mL of 95% ethanol was added to the 10 mL warm water extract, inverted, and allowed to form a precipitate overnight at −20 °C [7]. Soluble, total carbohydrate content was monitored throughout the purification procedure (described below, 2.2.3.–2.2.4.). The precipitate was pelleted by centrifugation (3220 ×*g*, 10 min, 4 °C), supernatant discarded, and the pellet was thoroughly resuspended in 10 mL 95% ethanol by vigrous pipetting. This process was repeated once more to wash the pellet with 95% ethanol twice in total. After the second ethanol wash, the pellet was resolubilized with 5 mL deionized water. The resolubilized precipitate was enzymatically treated: a simultaneous DNase (Thermo Scientific, #EN0521) and RNase (Thermo Scientific, #EN0201) digest was performed for 1 h at 4 °C, followed by a 90 μg mL^{-1} Proteinase K treatment (Fisher Scientific, BP1700–100) for 2 h at 4 °C. Then, 4 volumes of 95% ethanol were added to this aqueous solution and allowed to precipitate overnight at −20 °C. Two 95% ethanol washes were performed the following day, and the pellet was resuspended with deionized water to a final volume of 3 mL. This 3 mL solution transferred into a 2 K MWCO Slide-A-Lyzer dialysis cassette (Thermo Scientific, #87718) and was dialzyed against deionized water for 24 h at 18 °C with continuous stir bar mixing. The dialysis volume was 3 × 0.6 L, where deionized water was replaced at 2 h and 4 h. The dialyzed retentate was passed through a 5 mL DEAE FF column (GE Healthcare, #17-515-01) on an ÄKTA start FPLC (GE Healthcare). Protein content was monitored during elution using an A280 detector on the FPLC. A 0–1 M NaCl gradient was applied using a 1 M NaCl buffer and 5 mL fractions were collected. Carbohydrate-containing fractions were pooled, frozen in liquid nitrogen, and lyophilized overnight. A polishing step took place by resuspending the lyophilized powder with deionized water to a final volume of 0.5 mL and applied to a Superdex 75 10/300 GL (GE Healthcare, #17-5174-01). One mL fractions were eluted using a flow rate of 0.5 mL min^{-1} and carbohydrate enriched fractions pooled, frozen, and lyophilized as described above, which yielded a purified polysaccharide sample used for compositional and structural analyses. Laminarin from *Laminaria digitata* was purchased from Sigma (#L-9634) and was directly used as a control for compositional and structural analyses.

2.1.3. Compositional analysis with alditol acetate derivatization and GC/MS

Compositional analysis of purified carbohydrate samples was performed using an established GC/MS approach [42]. Briefly, 1.0 μg of the internal standard, 3-O-methylglucose, was added to each sample: laminarin, the purified soluble glucan from *Phaeodactylum*, and the neutral sugar standard (rhamnose, arabinose, ribose, fucose, myo-inositol, mannose, galactose and glucose; 5 μg each). Dried samples were hydrolyzed with 250 μL 2 M trifluoroacetic acid (TFA) for 2 h at 120 °C. After cooling to room temperature (RT), samples were dried under nitrogen gas (N_2). The dried, hydrolyzed samples were reduced with 200 μL 10 mg mL^{-1} sodium borodeuteride in 1:1 of 1 M ammonium hydroxide and 95% ethanol for 2 h at RT, which was then terminated by adding 4–5 drops of glacial acetic acid. After drying the reduced samples under N_2, 200 μL 10% glacial acetic acid in methanol were added, and then were dried under N_2. This process was repeated 4–5 times. To the completely dried samples, 100 μL acetic anhydride was added, incubated at 100 °C for 1 h, and then were gently dried under N_2. Two mL chloroform and 1 mL deionized water (v/v, 2:1) were added to the acetylated samples to extract the derivatized products by centrifugation (1000 ×*g*, 1 min, RT). After removing the upper aqueous layers, 1 mL deionized water was added to the lower organic phase to repeat the extraction 4 more times. The organic phase was gently dried under N_2 and resuspended in 50 μL chloroform for gas chromatography/mass spectrometry (GC/MS) analysis. GC/MS analyses were carried out using a CP 3800 gas chromatograph (Varian) equipped with an MS320 mass spectrometer. Helium was used as the carrier gas with a flow rate of 1 mL min^{-1}. The samples were run on a DB 5 column (30 m × 0.20 mm i.d.). The oven temperature was