Video Article

Determination of the Glycogen Content in Cyanobacteria

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Abstract

Cyanobacteria accumulate glycogen as a major intracellular carbon and energy storage during photosynthesis. Recent developments in research have highlighted complex mechanisms of glycogen metabolism, including the diel cycle of biosynthesis and catabolism, redox regulation, and the involvement of non-coding RNA. At the same time, efforts are being made to redirect carbon from glycogen to desirable products in genetically engineered cyanobacteria to enhance product yields. Several methods are used to determine the glycogen contents in cyanobacteria, with variable accuracies and technical complexities. Here, we provide a detailed protocol for the reliable determination of the glycogen content in cyanobacteria that can be performed in a standard life science laboratory. The protocol entails the selective precipitation of glycogen from the cell lysate and the enzymatic depolymerization of glycogen to generate glucose monomers, which are detected by a glucose oxidase-peroxidase (GOD-POD) enzyme coupled assay. The method has been applied to *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, two model cyanobacterial species that are widely used in metabolic engineering. Moreover, the method successfully showed differences in the glycogen contents between the wildtype and mutants defective in regulatory elements or glycogen biosynthetic genes.

Video Link

The video component of this article can be found at https://www.jove.com/video/56068/

Introduction

Cyanobacteria accumulate glycogen as the major carbohydrate store of carbon from CO_2 fixed in light through photosynthesis. Glycogen is a glycan consisting of linear α-1,4-linked glucan with branches created by α-1,6-linked glucosyl linkages. Glycogen biosynthesis in cyanobacteria starts with the conversion of glucose-6-phosphate into ADP-glucose through the sequential action of phosphoglucomutase and ADP-glucose pyrophosphorylase. The glucose moiety in ADP-glucose is transferred to the non-reducing end of the α-1,4-glucan backbone of glycogen by one or more glycogen synthases (GlgA). Subsequently, a branching enzymes introduce the α-1,6-linked glucosyl linkage, which is further extended to generate the glycogen particle. In the dark, glycogen is broken down by glycogen phosphorylase, glycogen debranching enzymes, α-glucanotransferase, and malto-dextrin phosphorylase into phosphorylated glucose and free glucose. These feed into catabolic pathways, including the oxidative pentose phosphate pathway, the Embden-Meyerhof-Parnas pathway (glycolysis), and the Entner-Doudoroff pathway[1,2,3,4].

Glycogen metabolism in cyanobacteria has garnered increasing interest in recent years because of the potential for cyanobacteria to develop into microbial cell factories driven by sunlight to produce chemicals and fuels. Glycogen metabolism could be modified to increase the yield of the products, because glycogen is the largest flexible carbon pool in these bacteria. An example is the cyanobacterium *Synechococcus* sp. PCC 7002, which has been genetically engineered to produce mannitol; the genetic disruption of glycogen synthesis increases the mannitol yield 3-fold[5]. Another example is the production of bioethanol from glycogen-loaded wildtype *Synechococcus* sp. PCC 7002[6]. The wildtype cell glycogen content may be up to 60% of the dry weight of the cell during nitrogen starvation[6].

Our understanding of glycogen metabolism and regulation has also expanded in recent years. While glycogen is known to accumulate in the light and to be catabolized in the dark, detailed kinetics of glycogen metabolism during the diel cycle was only recently revealed in *Synechocystis* sp. PCC 6803[7]. Moreover, several genes affecting the accumulation of glycogen have been identified. A notable example is the discovery that the putative histidine kinase PmgA and the non-coding RNA PmgR1 form a regulatory cascade and control the accumulation of glycogen. Interestingly, the *pmgA* and *pmgR1* deletion mutants accumulate twice as much glycogen as the wildtype strain of *Synechocystis* sp. PCC 6803[8,9]. Other regulatory elements are also known to affect the accumulation of glycogen, including the alternative sigma factor E and the transcriptional factor CyAbrB2[10,11].

As interest in glycogen regulation and metabolism grow, a detailed protocol describing the determination of the glycogen content is warranted. Several methods are used in the literature. Acid hydrolysis followed by the determination of the monosaccharide content through high-pressure anion exchange liquid chromatography coupled with a pulsed amperometric detector or spectrometric determination following treatments with acid and phenol are widely used methods to approximate the glycogen content[9,10,12,13]. However, a high-pressure anion exchange liquid

chromatographic instrument is very expensive and does not discriminate glucose derived from glycogen from that derived from other glucose-containing glycoconjugates, such as sucrose[14], glucosylglycerol[15], and cellulose[16,17,18], which are known to accumulate in some cyanobacterial species. The acid-phenol method can be performed using standard laboratory equipment. However, it uses highly toxic reagents and does not distinguish glucose derived from different glycoconjugates, nor does it distinguish glucose from other monosaccharides that constitute cellular materials, such as glycolipids, lipopolysaccharides, and extracellular matrices[12]. Notably, the hot acid-phenol assay is often used for the determination of total carbohydrate content rather than for the specific determination of glucose content[12]. Enzymatic hydrolysis of glycogen to glucose by α-amyloglucosidase followed by the detection of glucose through an enzyme-coupled assay generates a colorimetric readout that is highly sensitive and specific to glucose derived from glycogen. The specificity can be enhanced further with the preferential precipitation of glycogen from cell lysates by ethanol[5,8,19].

Here, we describe a detailed protocol for an enzyme-based assay of the glycogen content in two of the most widely studied cyanobacterial species, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, in the wildtype and mutant strains. In order to ensure efficient hydrolysis, a cocktail of α-amylase and α-amyloglucosidase is used[8]. The endo-acting α-amylase hydrolyzes the α-1,4-linkages in various glucans into dextrins, which are further hydrolyzed to glucose by exo-acting α-amyloglucosidase[20]. The synergistic effects of these enzymes are well known, and these enzymes are routinely used for the selective hydrolysis of starch, which is an α-linked glucan like glycogen, without affecting other glycoconjugants, such as cellulose, in the plant biomass[21]. The released glucose is quantitatively detected following an enzyme-coupled assay consisting of glucose oxidase-which catalyzes the reduction of oxygen to hydrogen peroxide and the oxidation of glucose to a lactone-and peroxidase-which produces a pink-colored quinoneimine dye from hydrogen peroxide, a phenolic compound, and 4-aminoantipyrine[22].

Protocol

1. Preparation

1. **Cyanobacterial cultures**
    1. Grow *Synechocystis* sp. PCC 6803 at 30 °C in liquid BG11 medium[8], with a constant supply of air supplemented with 1% (v/v) CO_2. Illuminate the cultures continuously with light at a photosynthetic photon flux density of 50 µmol photon/m^2/s.
    2. Grow *Synechococcus* sp. PCC 7002 in liquid A+ medium[23] (BG11 medium can also be used), with a constant supply of air supplemented with 1% (v/v) CO_2. The temperature should be 37 °C. Illuminate the cultures continuously with light at a photosynthetic photon flux density of 150 µmol photon/m^2/s.
    3. Measure the optical density (OD) of the culture at 730 nm using a cuvette with a light path of 1 cm. If the OD value is above 0.8, make appropriate dilutions to obtain OD measurements that are proportional to the cell concentration.
    NOTE: The protocols presented below are suitable for liquid cultures, with a cell density corresponding to an OD_{730nm} value of 2 or higher. When cultures in the exponential growth phase are desired, which typically have an OD_{730nm} value below 1, concentrate the cell density by centrifugation and resuspension in a buffer or medium to achieve an OD_{730nm} value of 2 or higher.

2. **Buffers and reagents**
    1. Make 50 mM Tris-HCl buffer at pH 8.
    2. Make 50 mM sodium acetate buffer at pH 5.
    3. Make a stock solution of 8 U/mL amyloglucosidase in 50 mM sodium acetate buffer, pH 5.
    4. Make a stock solution of 2 U/mL α-amylase in 50 mM sodium acetate buffer, pH 5.
    5. Using distilled water, makeglucose standard solutions at concentrations ranging between 0 and 100 µg/mL,.
    6. Prepare GOD-POD reagent from the D-Glucose Assay Kit (GODPOD Format), following the manufacturer´s instruction.

2. Determination of the Cell Dry Weight (Optional)

1. Transfer 2 mL of a culture or a cell resuspension (see step 1.1) to a 2.0 mL tube and centrifuge at 6,000 x g and 4 °C for 5 min. Discard the supernatant.
2. Resuspend the pellet in 1 mL of water and centrifuge at 6,000 x g and 4 °C for 5 min. Discard the supernatant and resuspend the cell pellet in 0.5 mL of water.
3. Transfer the suspension to a pre-weighed aluminum tray. Transfer the tray to a drying oven at 105 °C for overnight drying (approximately 18 h).
CRITICAL: It is important that the tray is handled with forceps to avoid the transfer of material from the fingers. Dry an empty, pre-weighed aluminum tray under the same conditions to determine any weight loss from the trays during drying.
4. After drying, remove the tray from the oven and allow it to equilibrate at ambient conditions for 5 min before weighing it with an accuracy of 0.0001 g.
NOTE: The value can be used to normalize the glycogen content on a cell-dry-weight basis.

3. Lysis of Cyanobacterial Cells

1. Transfer 1 mL of a culture or a cell resuspension (see step 1.1) to a 1.5 mL tube and centrifuge at 6,000 x g and 4 °C for 10 min. Discard the supernatant.
2. Resuspend the pellet in 1 mL of 50 mM Tris-HCl, pH 8, and centrifuge at 6,000 x g and 4 °C for 10 min. Discard the supernatant and resuspend the cell pellet in 1 mL of the Tris-HCl buffer. Repeat the process.
3. Thoroughly resuspend the pellet in 500 µL of 50 mM Tris-HCl buffer, pH 8.
CRITICAL: Resuspend the pellet well for an efficient cell lysis. Keep the resuspension in ice.

4. **Lyse the resuspended cells at 4 °C by performing 30 cycles of ultrasonication, each cycle consisting of 30 s at a frequency of 20 kHz with the maximum amplitude, followed by 90 s without.**
   NOTE: This method can efficiently lyse *Synechococcus* sp. PCC 7002.
   1. Alternatively, transfer the cell resuspension to a screw-cap tube and add zirconium oxide beads to the tube, following manufacturer's instructions. Set the tube in a tissue homogenizer and lyse the cells at 4 °C by performing 2 cycles of beating, each cycle consisting of 5 min at the frequency setting of 5.
      NOTE: This method can efficiently lyse *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002.
5. Centrifuge the tube containing the lysate for 10 min at 6,000 x g and 4 °C.
   NOTE: The pellet should mainly consist of large cell debris. If there is a significant number of unbroken cells, repeat step 3.4. Keep the supernatant on ice.
6. Determine the protein concentration using a commercial BCA Protein assay kit.
   NOTE: The value can be used to normalize the glycogen content on a protein content basis.

4. Glycogen Precipitation

1. Remove chlorophyll *a* from the cell lysate by mixing 900 µL of 96% (v/v) ethanol and 100 µL of the supernatant from step 3.5 in a 1.5 mL screw-cap tube. After closing the cap, heat the tube at 90 °C for 10 min using a standard laboratory heating block.
2. Incubate the tube on ice for 30 min.
3. Centrifuge the tube at 20,000 x g and 4 °C for 30 min and carefully remove the supernatant; the pellet contains glycogen. Lightly dry the pellet in air to remove excess ethanol.
   CRITICAL:Excessive drying of the pellet must be avoided, otherwise it becomes difficult to solubilize in step 5.1.
4. OPTIONAL: Measure the absorbance of the supernatant obtained in step 4.3 at 664 nm to determine the chlorophyll *a* content. Use an absorption coefficient of 84.6 L/g/cm[24].
   NOTE: The value can be used to normalize the glycogen content.

5. Enzymatic Hydrolysis and Glycogen Determination

1. Solubilize the pellet obtained in step 4.3 in 100 µL of 50 mM sodium acetate, pH 5, and add 50 µL of 8 U/mL amyloglucosidase and 50 µL of 2 U/mL α-amylase. Mix these materials well using a vortex.
   NOTE: Mixing by pipetting is not recommended because the glycogen pellet is viscous.
2. Incubate the mixture at 60 °C on a heating block for 2 h to enable the digestion of glycogen into glucose molecules.
3. Centrifuge the sample at 10,000 x g for 5 min and transfer the supernatant to a new 1.5 mL tube.

6. Determination of the Total Glucose Content Using the GOD-POD Reagent

1. Measure the concentration of glucose in the supernatant obtained in step 5.3 using the GOD-POD reagent. Transfer 100 µL of the supernatant from step 5.3 to a well in a 96-well plate. As a negative control, use 100 µL of 50 mM sodium acetate, pH 5. For generating a calibration curve, also measure the glucose standard solutions.
2. Add 150 µL of GOD-POD reagent to each sample and quickly mix by pipetting.
3. Incubate the plate statically at 25 °C for 30 min. Record the absorbance value at 510 nm using a plate reader.
4. Calculate the concentration of glucose using a calibration curve obtained from the glucose standards.
   NOTE: The glycogen concentration in the cell lysate is expressed as the concentration of glucose (µg/mL).

Representative Results

10 mL of wildtype *Synechocystis* sp. PCC 6803 were grown under photoautotrophic conditions until the OD_{730nm} value reached approximately 0.8. The cells were harvested and resuspended in 50 mM Tris-HCl, pH 8. The OD_{730nm} value was adjusted to 2-3. The glycogen content was analyzed following the protocol described above. The glycogen content per the OD_{730nm} was 13 ± 1.8 µg/mL/OD_{730nm} (N = 12). The glycogen content relative to the protein content was 0.24 ± 0.03 µg/µg (N = 12), and the glycogen content relative to the chlorophyll *a* content was 5.7 ± 0.6 µg/µg (N = 12). Due to the small amount of material available, the measurement of the cell dry weight was omitted. Given that the protein content in cyanobacteria cultivated under comparable conditions is about 50% of the cell dry weight[25], the glycogen content is estimated to be 12% of the cell dry weight, which is consistent with previous studies[26].

The detection limits of the GOD-POD assay showed a linear correlation between the glucose concentration and absorbance at 510 nm in the glucose concentration range between 10 and 100 µg/mL, corresponding to absorbance values of 0.08 and 0.7 at 510 nm. The minimum limit is likely due to the instrumental detection limit. When glucose concentrations higher than 150 µg/mL were used, dark green precipitates formed, causing a large variation in the absorbance readout. Concerning the amount of cell materials used, we routinely obtained reproducible glycogen contents when the OD_{730nm} value of cell resuspension prior to cell lysis was between 2 and 10. Cell resuspensions with an OD_{730nm} value of 1 or below gave rise to signals close to the minimum detection limit, leading to highly variable results. Cell resuspension with an OD_{730nm} value higher than 20 was not suitable because cell lysis was incomplete, and either extended lysis or dilutions were required.

Figure 1 shows representative results of the glycogen contents in *Synechocystis* sp. PCC 6803 wildtype and two mutant strains (*ΔpmgA* and *ΔpmgR1*). The cells grown at the exponential growth phase were used. The glycogen contents were first normalized by the total protein contents and were subsequently expressed relative to the value for the wildtype. The results show that the mutant strains have glycogen contents that are at least two-fold higher than the wildtype strain.

Next, the glycogen content was analyzed in strains of *Synechococcus* sp. PCC 7002 engineered to produce mannitol[5]. The first strain (Glg^+) contains the wildtype *glgA1* and *glgA2* genes encoding two functional glycogen synthases, whereas the second strain (Glg^-) lacks functional copies of these genes[5]. The glycogen and mannitol contents were then measured in both strains (**Figure 2**). The results show that the Glg^- lacked a detectable level of glycogen, while it produced more mannitol than the Glg^+ strain. This suggests that the carbohydrate synthesized by photosynthesis is redirected to mannitol in the mutant strain that lacks the ability to synthesize glycogen. The OD_{730nm} values of the cultures were approximately 10, providing sufficient cell materials for cell dry weight analysis. The glycogen contents were normalized using the cell dry weight.

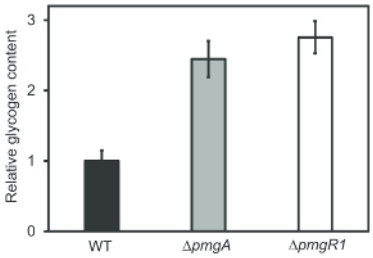

Figure 1: The glycogen contents measured in different lines of *Synechocystis* sp. PCC 6803. Relative glycogen contents in the WT and in two mutants (*ΔpmgA*[9] and *ΔpmgR1*[8]) are shown. The glycogen levels were normalized by the total protein contents. Means of three biological replicates are shown, with error bars representing standard deviations. Please click here to view a larger version of this figure.

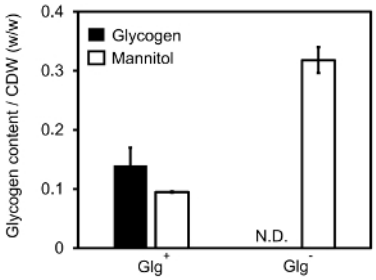

Figure 2: Glycogen and mannitol production in genetically manipulated *Synechococcus* sp. PCC 7002[5]. Glg^+, the strain synthesizing mannitol and glycogen. Glg^-, the strain synthesizing mannitol but no glycogen. The values depicted are the average of three biological replicates, with error bars representing the standard deviations. CDW: cell dry weight, N.D.: not detected. Please click here to view a larger version of this figure.

Discussion

Critical steps within the protocol are glycogen precipitation and resuspension. After centrifugation following ethanol precipitation, glycogen forms a translucent pellet that loosely adheres to the walls of the microcentrifuge tubes. Therefore, when removing the supernatant, special attention needs to be given so as not to remove the pellet. The glycogen pellet is sticky, and solubilization can be difficult if it dries out. Note that the complete solubilization of the glycogen pellet is important because incomplete solubilization will lead to inefficient enzymatic digestion and will therefore give rise to large variations between technical replicates. The application of sonication prior to solubilization by vortexing may facilitate the process.

Concerning the choice of normalization method, the cell dry weight is a widely-used reference. It is more laborious and requires more cell biomass than determinations of protein and chlorophyll *a*. The total protein content provides an alternative reference to the cell biomass and can be determined on a small scale, as described in the present protocol. The total protein content per dry cell biomass is typically in the range between 40% and 50%, although the exact value depends upon growth conditions[25,28]. The chlorophyll *a* content can be determined readily and can be used as a proxy to the cell amount present in the sample. However, it is well known that the amount of chlorophyll *a* per cell varies significantly depending upon the growth conditions, particularly in response to changing nutrient concentrations and light intensities[29].

One of the significant advantages of the presented technique with respect to other methods is that it is highly selective. The hot acid and phenol protocol and the monosaccharide composition analysis following acid hydrolysis are relatively simple methods and have been used in previous studies[9,10,12,13]. However, these methods can overestimate the glycogen content because non-glycogen glucose and additional sugars can contribute to the measurement, depending on the carbohydrate detection technique[12]. The described technique selectively detects glucose in glycogen. It can also discriminate glucose from cellulose, because α-amylase and α-amyloglucosidase do not hydrolyze the β-linked glucosyl linkages present in cellulose. In previous studies, the enzymatic hydrolysis of glycogen was performed solely by α-amyloglucosidase[7,27]. Inclusion of the endo-acting α-amylase together with the exo-acting α-amyloglucosidase, as presented in this protocol, can ensure the efficient hydrolysis of glycogen. A similar selective hydrolysis is routinely applied to treatments of plant biomass, wherein the combination of α-amylase α-amyloglucosidase is used to hydrolyze starch without affecting other glucose-containing polysaccharides, such as cellulose[21].

The main limitation of the technique is that the procedure is low-throughput because individual samples are processed separately using microcentrifuge tubes. The glycogen precipitation step is the primary factor preventing higher throughput. Implementation as a high-throughput procedure would require the use of deep-well plates and the ability to centrifuge these at a high speed (20,000 x *g*). While centrifuges that can

centrifuge 96-well plates at the necessary speed are available, most deep-well plates cannot tolerate force larger than 6,000 x *g*. Hence, the careful choice of materials is required to adapt the protocol for high-throughput analysis.

Disclosures

The authors have nothing to disclose.

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