

Measurement of Ascorbic Acid and Glutathione Content in Cyanobacterium *Synechocystis* sp. PCC 6803

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[Abstract] Ascorbic acid (AsA) and glutathione (GSH) are two key components of the antioxidant machinery of eukaryotic and prokaryotic cells. The cyanobacterium *Synechocystis* sp. PCC 6803 presents both compounds in different concentrations (AsA, 20-100 μ M and GSH, 2-5 mM). Therefore, it is important to have precise and sensitive methods to determine the redox status in the cell and to detect variations in this antioxidants. In this protocol, we describe an improved method to estimate the content of both antioxidants (in their reduced and oxidized forms) from the same sample obtained from liquid cultures of *Synechocystis* sp. PCC 6803.

Keywords: Cyanobacteria, Antioxidants, Glutathione, Ascorbic acid, Oxidative stress

[Background] The redox status in the cell can be altered by multiple factors generating oxidative stress. We used this protocol to quantify GSH and AsA contents in *Synechocystis* sp. PCC 6803 exposed to heat (50 °C). As described for *Arabidopsis thaliana*, heat stress resulted in a decline in the content of both antioxidants and triggered cell death by ferroptosis (Distéfano *et al.*, 2017; Aguilera *et al.*, 2019 preprint). Although this protocol was set for *Synechocystis* sp. PCC 6803, it can be applied to determine GSH and AsA contents in other cyanobacteria. In cyanobacteria AsA contents are very low, μ M range in normal conditions and pM under some treatments. For that reason we propose this sensitive method with an improved cell lysate procedure.

Materials and Reagents

1. Pipette tips: 0.1-10 μ l, 1-200 μ l and 100-1,000 μ l
2. 1.5 ml microcentrifuge tubes
3. 50 ml conical centrifuge tubes
4. Sterile syringes: 1 ml and 10 ml
5. Sterile acid-washed glass beads (150-212 μ m) (Sigma-Aldrich, catalog number: G1145)
6. 0.22 μ m nylon membrane filters (e.g., GVS, catalog number: FJ13BNPNY002AD01)
7. *Synechocystis* PCC 6803 liquid cultures
8. Ice
9. Liquid nitrogen
10. Sterile Mili-Q water
11. Water for HPLC (Sigma-Aldrich, catalog number 900682)
12. Bond Elut C18 cartridges (Agilent Technologies, catalog number: 12102028)
13. TFA (Trifluoroacetic acid) (Carlo Erba, catalog number: 411564)
14. DTT (Dithiothreitol) (Sigma-Aldrich, catalog number: D0632)
15. Methanol HPLC-grade (Sigma-Aldrich, catalog number: 34860)
16. Phosphoric acid for HPLC (85%) (Sigma-Aldrich, catalog number: 49685)
17. Potassium phosphate dibasic (Sigma-Aldrich, catalog number: P8281)
18. Potassium dihydrogen phosphate anhydrous (Sigma-Aldrich, catalog number: 543841)
19. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: 431788)
20. NADPH (β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate \geq 93% (HPLC) (Sigma-Aldrich, catalog number: N1630)
21. DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)) (Sigma-Aldrich, catalog number: D8130)
22. GSSG (L-Glutathione oxidized) (Sigma-Aldrich, catalog number: G4376)
23. Glutathione Reductase from baker's yeast (*S. cerevisiae*) (Sigma-Aldrich, catalog number: G3664)
24. 4-Vinylpyridine (Sigma-Aldrich, catalog number: V3204)
25. L-Glutathione reduced (Sigma-Aldrich, catalog number: G4251)
26. L-Ascorbic acid (Sigma-Aldrich, catalog number: A5960)
27. Neubauer counting chamber (Brand™ Blaubrand™, catalog number: 10350141)
28. Phosphate buffer 100 mM pH7 (see Recipes)
29. Phosphate buffer 100 mM pH 7.5 + EDTA 5 mM (see Recipes)

Equipment

1. Pipettes
2. Neubauer counting chamber

3. Refrigerated centrifuge (50 ml conical tubes: Thermo Scientific™, model: IEC™ CL31; 1.5 ml tubes: Thermo Scientific™, model: Sorvall™ Legend™ Micro 17R)
4. Vortex mixer (Scientific Industries, model: VORTEX-GENIE 2)
5. Spectrophotometer (Shimadzu, model: UV-160A-Vis)
6. pH-meter (Milwaukee, model: MI-150, catalogue number: 11861)
7. HPLC system (Shimadzu Scientific Instruments) equipped with:
 - a. LC pump (Shimadzu Scientific Instruments, model: LC-10 AT)
 - b. UV-VIS detector (Shimadzu Scientific Instruments, model: SPD-10AV)
 - c. Microsphere C-18 column (Agilent technologies, SS 100 x 4.6 mm, 3 µm; catalog number: CP28076)

Software

1. Microsoft Excel (Microsoft Corporation, Available from: <https://office.microsoft.com/excel>)

Procedure

A. Cell harvest and antioxidants extraction

1. Place fifty milliliters of exponentially-growing culture ($\sim 2.5 \times 10^7$ cells ml⁻¹) in 50 ml conical centrifuge tubes.
2. Harvest cells by centrifugation (9,300 × g for 10 min, 4 °C).
3. Discard the supernatant and place the pellet with a sterile spatula in a new and sterile 1.5 ml microcentrifuge tube.
4. Place the tube on ice. Resuspend cells in 1 ml 3% TFA and add approximately 100 µl of sterile acid-washed glass beads (150-212 µm).
5. Lyse the cells by mixing vigorously (vortex, 1 min), freeze with liquid nitrogen and thaw in ice.
Note: Broken cells can be observed under an optical microscope, in order to ensure a correct rupture of the cells.
6. Repeat Step A5 at least 3 times.
7. Centrifuge the homogenate (16,000 × g 15 min, 4 °C).
8. Collect the supernatant in a new 1.5 ml microcentrifuge tube and keep it on ice before use.
9. Activate Bond Elut C18 columns by applying in the following order:
 - a. 1.5 ml methanol.
 - b. 1.0 ml water for HPLC.
 - c. 1.0 ml phosphate buffer 100 mM pH 7.00.
 Discard the solution from the washing steps.
10. Pass 500 µl of the supernatant (Step A8) through a previously activated Bond Elut C18 cartridge using a syringe.
11. Discard flow-through (500 µl) and pass 1.5 ml phosphate buffer 100 mM pH 7.00 to elute (pH

of the elution around 2). Collect the filtrate in a new 1.5 ml microcentrifuge tube and mix thoroughly by vortexing for 10-20 s.

This filtrate is used for ascorbic acid and glutathione determination.

Note: It is highly recommended to perform the measurements immediately after obtaining the filtrate. In particular, ascorbic acid determination needs to be done after the preparation. Freezing the samples is not recommended because after thawing a drastic reduction in the antioxidant content is observed. For glutathione measurements, the supernatant can be stored at -80 °C.

B. Reduced ascorbic acid and total ascorbate measurements

- To determine reduced ascorbic acid mix in a new 1.5 ml microcentrifuge tube:
 - 240 µl of the filtrate obtained in Step A10
 - 240 µl of K₂HPO₄ 100 mM pH 8.5
 - 50 µl of TFA 10%
 - 15 µl of water for HPLC
- To determine total ascorbate mix in a new 1.5 ml microcentrifuge tube:
 - 240 µl of the filtrate obtained in Step A10
 - 240 µl of K₂HPO₄ 100 mM pH 8.5
 - 15 µl of DTT 100 mM (final concentration: 3 mM)
 Incubate for 5 min at room temperature and stop the reaction by adding 50 µl of TFA 10%.

C. Reduced ascorbic acid and total ascorbate quantification

- To quantify the reduced ascorbic acid concentration, filter the solution obtained in Step B1 through a 0.22 µm nylon membrane filter and inject 10 µl in a HPLC equipped with a C-18 column (100 × 4.6 mm, 3 µM). As mobile phase use a solution of KH₂PO₄ 100 mM (pH 3.00) in water for HPLC (adjust pH with phosphoric acid 85%) at 0.35 and 0.5 ml min⁻¹ flow.
- Quantify the area under the peak that displays a maximum at 265 nm (Figure 1).
- To quantify total ascorbate concentration filter and inject 10 µl of the second solution obtained in step B2 using the same equipment and conditions described in Steps C1 and C2.
- Prepare a calibration curve using a series of standard L-ascorbic acid dilutions (e.g., final concentrations of 0.1, 0.5 and 1 µM) and measure the peak area following the same procedure as described in Step C2 (Figure 1).
- Adjust the linear function of ascorbic acid concentration vs. peak area and use the slope to calculate the concentration of the unknown samples as described in the formula:

Total ascorbate or reduced ascorbic acid (µmol/mg fresh weight)

$$= \frac{\text{curve slope} \times \text{peak area} \left(\frac{\text{picomol}}{\text{ml}} \right) \times 1000000 \times \text{final volume of the eluate (ml)}}{\text{sample weight (mg)}} \times 2 \times 3$$

The x2 is included in the formula because cells are resuspended in 1 ml TFA (Step A4) but only half of the supernatant is passed through the column (Step A9). The x3 is included because in Step A10 one volume of sample is passed through the column (500 µl) and three volumes are collected (1.5 ml).

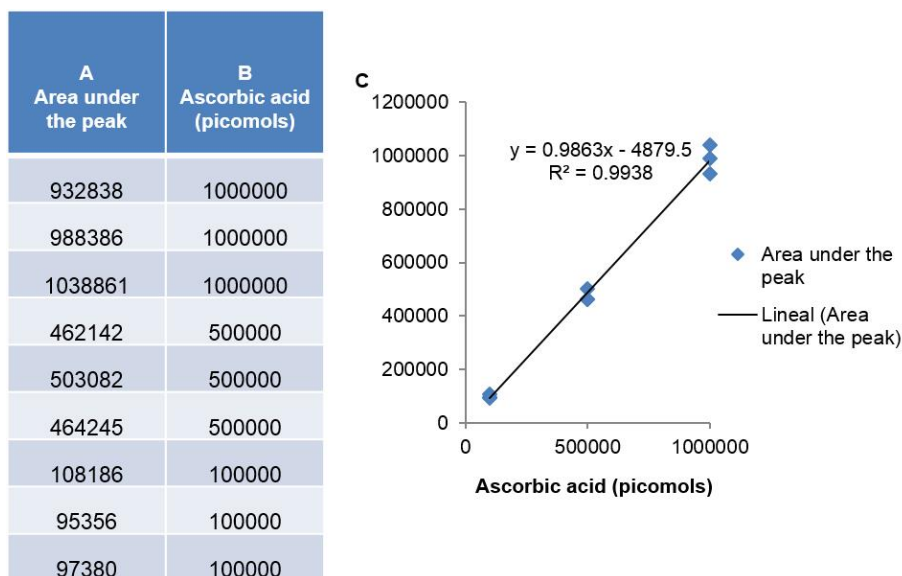


Figure 1. Area under the peak and standard curve to Asa quantification

D. Measurement of glutathione content

The quantitative determination of the total amount of glutathione (GSH + GSSG) employs an enzymatic method. In this, the reduction of DTNB to 5-thio-2-Nitrobenzoate (TNB) by NADPH is quantified spectrophotometrically. This reaction is used to measure the reduction of GSSG to GSH. The rate of the reaction is proportional to the GSH and GSSG concentration.

1. To determine the total glutathione, in a new 1.5 ml microcentrifuge tube add:

650 µl phosphate buffer 100 mM pH 7.5 + EDTA 5 mM

25 µl DNTB (5 mg/ml) (final concentration 0.2 mM)

5 µl NADPH (1 mg/100 µl)

5 µl l glutathione reductase 1:30 (0.5 U)

300 µl of filtrate obtained in Step A10

Note: The blank solution contained all reagents except the filtrate obtained in Step A10.

2. Mix gently and read the absorbance at 412 nm using a spectrophotometer.
3. To extract the oxidized glutathion (GSSG), add 3 µl of Vinylpyridine (95%) to 300 µl of the filtrate obtained in Step A10, gently mix by vortexing and incubate at room temperature for 25 min.
4. Centrifuge (16,000 × g 10 min, 4 °C) and place the tube on ice
5. In a new 1.5 ml microcentrifuge tube add
365 µl phosphate buffer 100 mM pH 7.5 + EDTA 5 mM.

- 25 μ l DNTB (5 mg/ml).
- 5 μ l NADPH (1 mg/100 μ l)
- 5 μ l l glutathione reductase 1:30 (0.5 U)
- 100 μ l of the supernatant obtained in Step D4
- 6. Mix gently and read the absorbance at 412 nm.
- 7. Quantification of glutathione levels is based on the oxidized form of glutathione (GSSG). To quantify the glutathione levels, prepare a calibration curve with 0-100 μ M standard L-Glutathione oxidized solution.

Note: The content of the antioxidant can be expressed either as per mg of fresh or dry weight or as pmol per cell (in the case of ascorbic acid) and nmol per cell (in the case of glutathione). The content of antioxidants can be correlated with cell number determined with a Neubauer counting chamber.

Recipes

1. Phosphate buffer 100 mM pH 7
Add 1.071g K_2HPO_4 and 0.524 g KH_2PO_4 in 100 ml Sterile Mili-Q water
2. Phosphate buffer 100 mM pH 7.5 + EDTA 5 mM
Solution 1: mix 0.681 g of KH_2PO_4 and 0.093 g of EDTA in 50 ml sterile Mili-Q water
Solution 2: mix 0.871 g of K_2HPO_4 and 0.093 g of EDTA in 50ml sterile Mili-Q water
Adjuts the pH of solution 2 by adding solution 1 till reach pH 7.5

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This protocol was adapted from previously published studies (Griffith, 1980; Bartoli *et al.*, 2006; Narainsamy *et al.*, 2016).

Competing interests

The authors declare no competing financial interests.

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