A Characterized Ferricyanide Assay Leads to Improved Isolation of Intact Chloroplasts

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ABSTRACT

Plant-based photosynthesis occurs in an organelle called the chloroplast. Because of their widespread availability, spinach chloroplasts are a well characterized model used in photosynthetic studies. While the ferricyanide assay is a classic method used to measure photosynthetic activity, it remains relatively poorly characterized. In this work, we have redeveloped and characterized the classic ferricyanide assay and used it to improve current protocols in isolating intact spinach chloroplasts. Our assay was redeveloped for use with a JTS-10 pump probe spectrophotometer and optimized for chlorophyll and reactant concentrations, cuvette characteristics, actinic intensity, and detection wavelength, with 0.2 cm pathlength cuvettes and a detection wavelength of 420 nm being optimal. The final assay uses chloroplasts to an OD_{680} = 0.2 – 0.5, 2.5 mM ammonium chloride (de-couples electron and proton transfer), 1.5 mM of ferricyanide, and isotonic buffer (maintains osmolarity) in a 700 µL cuvette. This redeveloped assay became the metric for improving the protocol for isolation of intact chloroplasts. Our changes include releasing chloroplasts via grinding with a mortar and pestle instead of using a Warburg blender, adding a slow spin centrifugation step after the first filtration, and adding a second filtration step after the first centrifugation. This improved the percentage of intact chloroplasts as determined by our ferricyanide assay and decreased amounts of particulate residue in the final sample. Using the improved chloroplast isolation, we investigated the use of glycerol as a cryoprotectant and the effect of dibromothymoquinone (DBMIB) and 3-(3 4-dichlorophenyl)-1 1-dimethylurea (DCMU) on the rate of ferricyanide reduction. The results suggest that glycerol is not an effective cryoprotectant and that the primary site of reduction is in the cytochrome b_6f complex.

Methodology

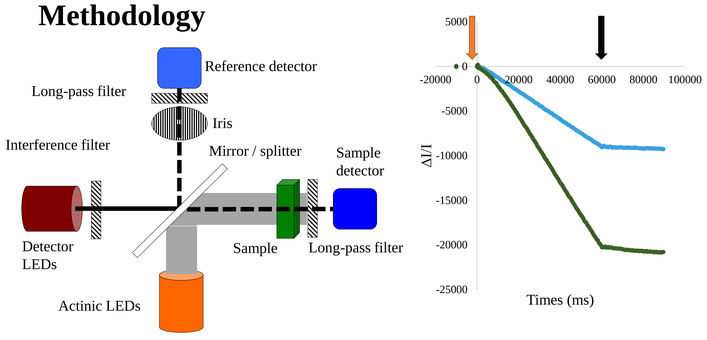

Figure 1. ***Left:*** Diagram of a JTS-10 dual-beam spectrophotometer with individual components labeled. The path of the detection pulses is shown as the bold line and hashes. The path of the actinic light is shown as the thick gray line. Note that the actinic light is prevented from striking the sample detector due to a long pass filter. ***Right:*** Sample trace from JTS-10 representing when light gets turned on (orange arrow) and when light gets turned off (black arrow). The blue trace represents assay with intact chloroplasts and the dark green trace represents assay with lysed chloroplasts.

Inhibition Studies

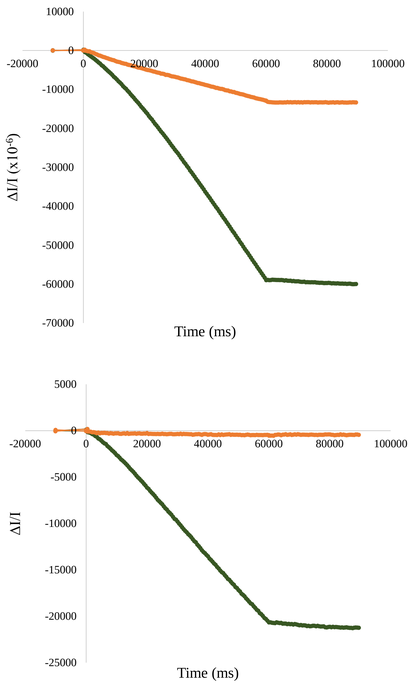

Figure 2. ***Top:*** Green trace represents ferricyanide assay with lysed chloroplasts. Orange trace represents addition of DBMIB in lysed ferricyanide assay displaying partial reduction within the Cytochrome b_6f complex. ***Bottom:*** Green trace represents ferricyanide assay with lysed chloroplasts. Orange trace represents addition of DCMU in lysed ferricyanide assay showing no reduction of ferricyanide within photosystem II.

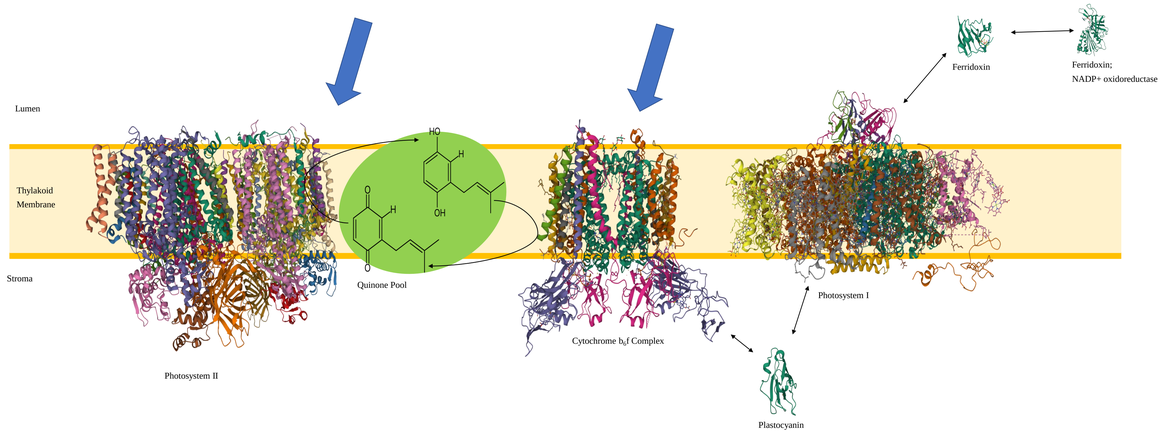

Figure 3. Diagram of electron transport in the thylakoid membrane within the chloroplast. The proteins are represented by their crystal structures taken from the RCSB PDB. Photosystem II (PDB ID 3WU2), Cytochrome b_6f Complex (PDB ID 2E74), Plastocyanin (PDB ID 1AG6), Photosystem I (PDB ID 4RKU), Ferridoxin (PDB ID 1A70), and Ferridoxin: NADP+ oxidoreductase (PDB ID 1QG0). Blue arrows represent sites of ferricyanide reduction sites Q_A in PSII and Q_o in Cytochrome b_6f Complex.

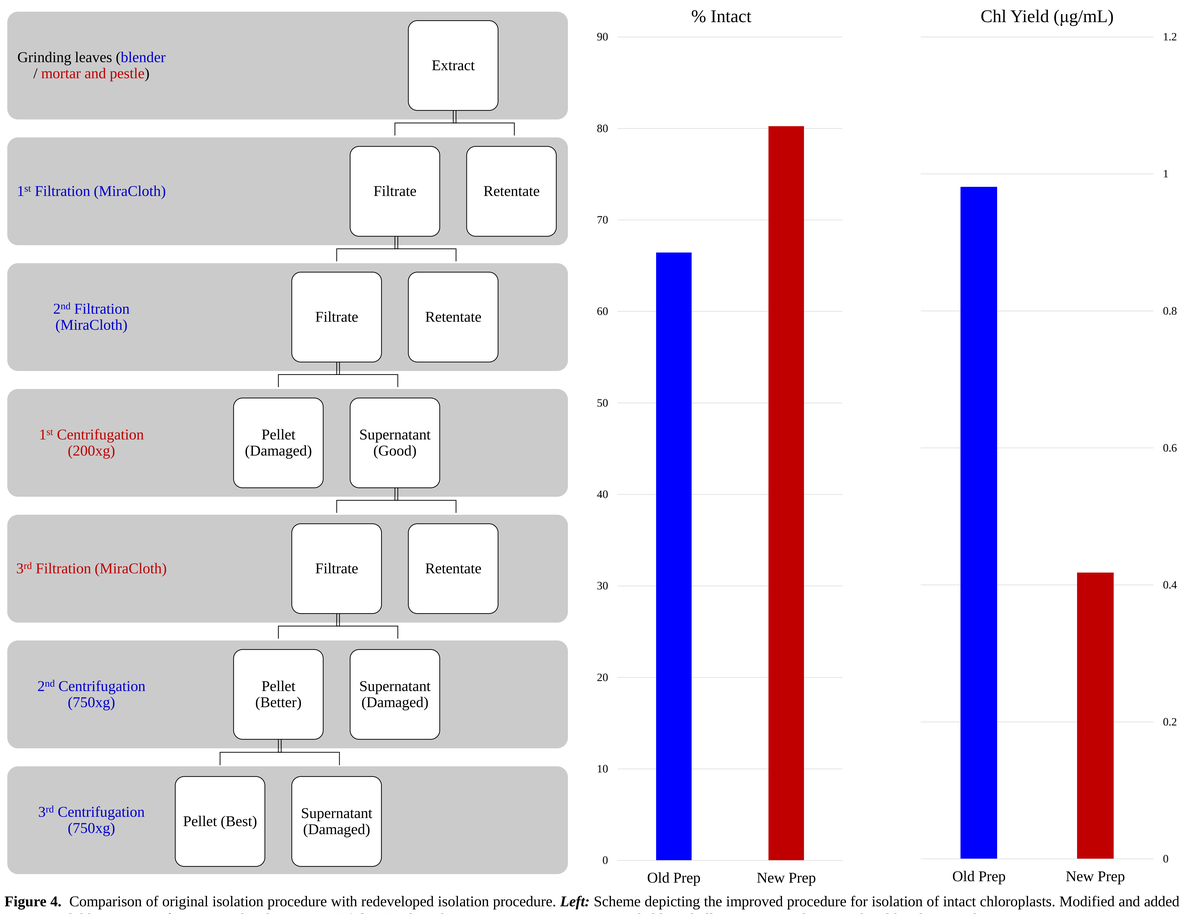

Figure 4. Comparison of original isolation procedure with redeveloped isolation procedure. ***Left:*** Scheme depicting the improved procedure for isolation of intact chloroplasts. Modified and added steps in red, blue steps are from original isolation prep. ***Right:*** Displays the average percent intactness and chlorophyll concentration between the old and new isolation prep. Percent intactness was determined with the redeveloped ferricyanide assay. Blue bars signify old isolation procedure and red bars represent new isolation procedure.

Assay Characterization

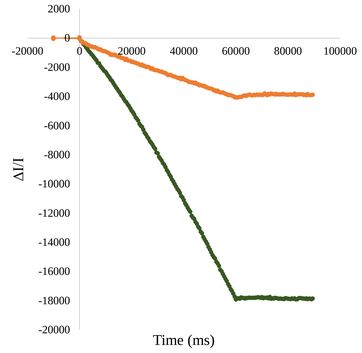

Figure 5. Removal of NH_4Cl from ferricyanide assay. Orange trace represents assay lysed with no NH_4Cl. Green represents assay lysed with NH_4Cl. between the concentration of chloroplasts and the rate of ferricyanide reduction.

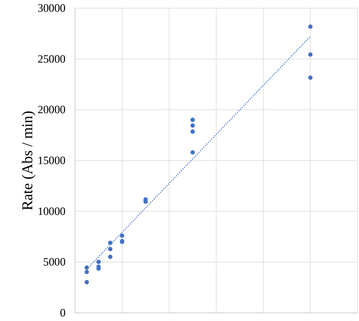

Figure 6. The relationship between the chloroplast concentration and the rate of ferricyanide reduction. The experimental conditions included increasing amounts of the chloroplast, 10 µL NH_4Cl, 8 µL ferricyanide, and Buffer B, assay volume 1 mL. The trendline on the graph shows the linear relationship between the concentration of chloroplasts and the rate of ferricyanide reduction.

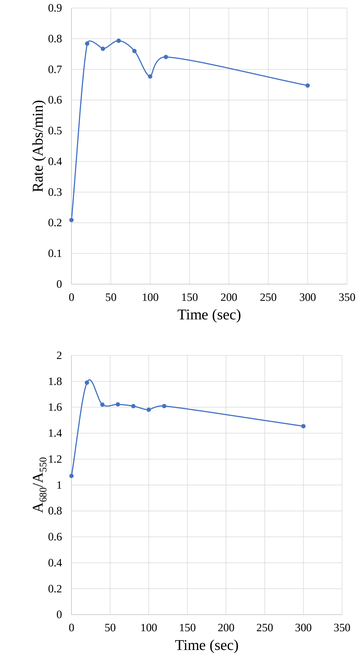

Figure 7. ***Top:*** Chloroplast lysing time course, where total lysing time was increased in 20 sec increments up to 120 sec. Trace was taken after a 5 min lysing time to determine if there was a significant decrease in rates. ***Bottom.*** The relationship depicting the time vs absorbance ratio. Absorbances were obtained from the same experiment as the time course.

References available upon request.