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Evaluating Thioredoxin-Mediated CF_oCF_1 Reduction Using an In Vitro Thylakoid Assay

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Abstract

The activity of chloroplast ATP synthase (CF_oCF_1) is precisely regulated through a thioredoxin (Trx)-mediated dithiol/disulfide reaction in response to varying light conditions. This regulatory mechanism is further controlled by ΔpH formation across the thylakoid membrane. To better understand this complicating regulatory function of CF_oCF_1, a method is required to evaluate the extent of CF_oCF_1 reduction by Trx under controlled ΔpH conditions and to directly evaluate the redox state of CF_oCF_1. In this study, we present a simple in vitro procedure to assess the CF_oCF_1 reduction system using spinach thylakoids. The method consists of three key steps: (A) simple preparation of intact thylakoids from spinach leaves; (B) reduction of CF_oCF_1 on the thylakoid membrane using recombinant Trx under light irradiation; and (C) in situ determination of the redox state of CF_oCF_1 by labeling thiol groups with a maleimide reagent followed by protein detection using western blotting. The redox state of CF_oCF_1 was determined by mobility shifts on non-reducing SDS-PAGE. This protocol provides a refined strategy for elucidating the regulatory mechanism controlling energy conversion by CF_oCF_1 under fluctuating photosynthetic conditions.

Key features

- A simple isolation method for intact thylakoids from *Spinacia oleracea* that enables the evaluation of the reduction of chloroplast ATP synthase by thioredoxin.
- The combination of LED light irradiation and electron mediators allows controlled adjustment of the proton electrochemical gradient across thylakoid membranes.
- The redox state of chloroplast ATP synthase can be distinguished by labeling free thiols with maleimide reagents and quantitative detection by western blotting.

Keywords: Chloroplast ATP synthase, Thioredoxin, Thylakoid membrane, Redox regulation, Thiol labeling, Western blotting, *Spinacia oleracea*

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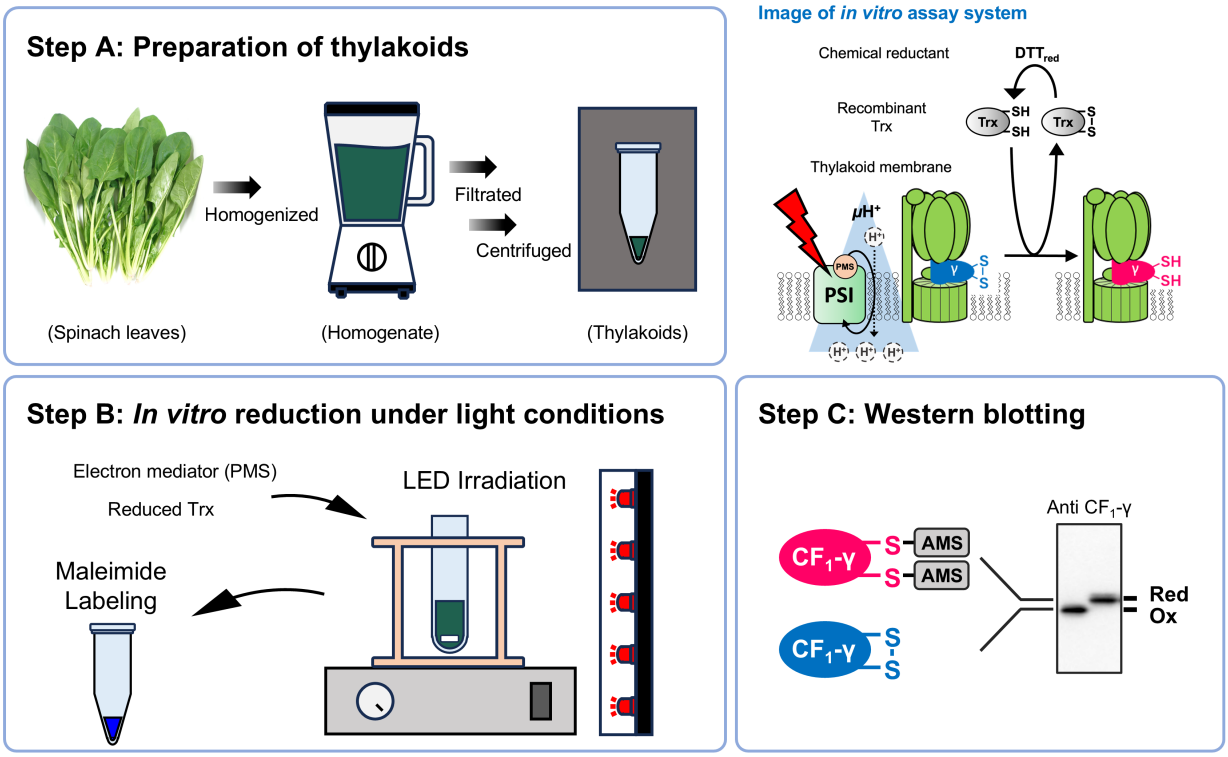
Graphical overview



Background

Chloroplast ATP synthase (CF_oCF_1) is a pivotal enzyme in photosynthetic energy conversion in green plants. When photosynthetic electron transport generates a proton electrochemical gradient ($\Delta\mu H^+$) across the thylakoid membrane under light conditions, this gradient is primarily consumed by CF_oCF_1 to drive ATP synthesis [1–3]. Therefore, CF_oCF_1 activity is strongly regulated to maintain efficient energy conversion under varying light conditions. One representative regulatory mechanism is redox control [4,5]. The γ subunit (CF_1-γ), which rotates within the enzyme complex during catalysis, harbors a redox-active cysteine pair [6,7]. These cysteines are targets of thioredoxin (Trx), which mediates the reducing power from the photosynthetic electron transport chain [8]. Furthermore, CF_1-γ is reduced by Trx only when ΔpH is formed across the thylakoid membrane under photosynthetic conditions [9–11]. Thus, CF_oCF_1 is regulated through a multistep control mechanism involving Trx-mediated redox reactions, closely linked to ΔpH formation, reflecting changes in light environment.

Over the past half-century of research on CF_1, including by our research group, methods for examining the redox state of CF_1-γ have been established. Jagendorf first demonstrated ATP synthesis driven by ΔpH across thylakoid membranes in isolated chloroplasts using the so-called acid–base transition experiment, in which chloroplasts were sequentially incubated in buffers of different pHs [12]. Subsequently, Gräber et al. applied the acid–base transition method to isolated thylakoids or liposomes containing isolated CF_oCF_1 in the presence of excess reducing agents, enabling kinetic analysis of the relationship between the redox state of CF_1-γ and CF_oCF_1 enzymatic activity [13,14]. In another approach, Schwarz et al. investigated the selectivity of Trx toward CF_1-γ by measuring ATP hydrolysis activity under defined pH gradients across thylakoid membranes in the presence of different Trx isoforms [15]. However, these studies focused primarily on the enzymatic activity of CF_oCF_1 rather than directly determining the exact redox state of CF_1-γ. Because the extent of ΔpH affects both ATP synthesis and hydrolysis activities of CF_oCF_1, a system capable of directly monitoring the redox state of CF_1-γ under ΔpH-forming conditions, independent of enzyme activity measurements, is required. In previous work, our laboratory successfully determined the redox state of CF_1-γ by directly applying a maleimide reagent to isolated spinach chloroplasts under light conditions [16]. Using this approach, we observed that CF_1-γ is rapidly reduced upon light irradiation, and that reduction is completely inhibited in the presence of an uncoupler in the chloroplast solutions.

In this study, we refine this conventional approach and provide a simple protocol for quantitative reduction analysis of CF_1-

γ using isolated spinach thylakoids combined with an artificial electron transfer mediator, which is required for membrane potential formation in the light [17], and recombinant Trx. Using this method, we successfully demonstrated the selectivity of major Trx family proteins toward CF_1-γ [18], oxidation of CF_1-γ by Trx-like proteins associated with ΔpH dissipation [19], and the distinct contributions of ΔpH and membrane potential to CF_1-γ reduction [20], supporting the sensitivity and accuracy of this method.

Materials and reagents

Biological materials

1. Fresh bunched spinach (*Spinacia oleracea*) (purchased from the market)

Reagents

1. Distilled water
2. Tricine (Wako, catalog number: 347-02844)
3. Magnesium chloride hexahydrate ($MgCl_2·6H_2O$) (Nacalai Tesque, catalog number: 20909-55)
4. Sodium chloride (NaCl) (Nacalai Tesque, catalog number: 31320-05)
5. Potassium chloride (KCl) (Nacalai Tesque, catalog number: 28513-85)
6. Sodium hydroxide (NaOH) (Nacalai Tesque, catalog number: 31511-05)
7. Sucrose (Nacalai Tesque, catalog number: 30404-45)
8. Dithiothreitol (DTT) (Wako, catalog number: 049-08972)
9. 1-methoxy-5-methylphenazinium methylsulfate (1-Methoxy PMS) (Sigma-Aldrich, catalog number: M8640)
10. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich, catalog number: C2920)
11. Ethanol (Nacalai Tesque, catalog number: 14713-24)
12. Sodium lauryl sulfate (SDS) (Nacalai Tesque, catalog number: 31607-65)
13. Glycerol (Nacalai Tesque, catalog number: 17018-83)
14. Bromophenol blue (BPB) (Wako, catalog number: 029-02912)
15. 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonate (AMS) (Invitrogen, catalog number: A485)
16. Tris(hydroxymethyl)aminomethane (Tris) (Nacalai Tesque, catalog number: 35434-21)
17. Glycine (Nacalai Tesque, catalog number: 17141-95)
18. Polyoxyethylene (20) sorbitan monolaurate (Tween 20) (Wako, catalog number: 167-11515)
19. Methanol (Nacalai Tesque, catalog number: 21914-74)
20. Disodium hydrogen phosphate (Nacalai Tesque, catalog number: 31801-05)
21. Potassium dihydrogen phosphate (Nacalai Tesque, catalog number: 28721-55)
22. SDS-PAGE protein marker (Bio-Rad, catalog number: 161-0373)
23. ECL Prime western blotting detection reagent (GE Healthcare, catalog number: RPN2232)
24. Primary antibody for the protein of interest
25. Secondary antibody for the protein of interest
26. Skim milk (Morinaga Nyugyo, catalog number: 0652842)
27. 100% (w/v) trichloroacetic acid (TCA) (Wako, catalog number: 200-08085)
28. Acetone (Wako, catalog number: 019-00353)

Solutions

1. 4× stock solution for grinding buffer (see Recipes)
2. Grinding buffer (see Recipes)
3. 25× DTT stock solution (see Recipes)
4. 50× PMS stock solution (see Recipes)
5. FCCP stock solution (see Recipes)
6. Thiol-labeling solution (see Recipes)
7. Electrophoresis buffer for SDS-PAGE (see Recipes)
8. Transfer buffer for western blotting (see Recipes)

9. PBS buffer (see Recipes)

Recipes

1. 4× stock solution for grinding buffer

Reagent	Final concentration	Quantity or volume
Tricine	200 mM	35.8 g
$MgCl_2·6H_2O$	20 mM	4.06 g
NaCl	40 mM	2.34 g
KCl	200 mM	14.9 g
Distilled water	N/A	Up to 1 L
Total	N/A	1 L

Adjust pH to 7.5 using NaOH. Prepare in a media bottle and store at 4 °C in a refrigerator; it is stable for several months.

2. Grinding buffer

Reagent	Final concentration	Quantity or volume
4× stock solution for grinding buffer	N/A	100 mL
Sucrose	0.4 M	54.8 g
Distilled water	N/A	Up to 400 mL
Total	N/A	400 mL

Prepare in a media bottle. Always use a freshly prepared grinding buffer for each experiment and maintain it at low temperature until just before use.

3. 25× DTT stock solution

Reagent	Final concentration	Quantity or volume
DTT	500 mM	77.1 mg
Distilled water	N/A	1 mL

Prepare in a microcentrifuge tube and store at -20 °C in a freezer; stable for several months. Dilute the 25× DTT stock solution to 1× (2 mM DTT) with grinding buffer before use.

4. 50× PMS stock solution

Reagent	Final concentration	Quantity or volume
1-Methoxy PMS	10 mM	3.36 mg
Distilled water	N/A	1 mL

Prepare in a microcentrifuge tube and store in a storage box away from light at room temperature; stable for several months. Dilute the 50× PMS stock solution to 1× (200 μM 1-Methoxy PMS) with grinding buffer before use.

5. FCCP solution

Reagent	Final concentration	Quantity or volume
FCCP	2 mM	1.02 mg
Ethanol	N/A	2 mL

Prepare in a microcentrifuge tube and store at -20 °C in a freezer; stable for several months.

6. Thiol-labeling solution

Reagent	Final concentration	Quantity or volume
Tris	62.5 mM	75.7 mg
SDS	2% (w/v)	200 mg
Glycerol	7.5% (w/v)	750 mg
BPB	0.01% (w/v)	1 mg
AMS	2 mM	10 mg
Distilled water	N/A	Up to 10 mL
Total	N/A	10 mL

Adjust pH to 6.8 using HCl. Prepare in a conical tube. Always use a freshly prepared thiol-labeling solution for each experiment. Store in a storage box away from light at room temperature until just before use.

7. Electrophoresis buffer for SDS-PAGE

Reagent	Final concentration	Quantity or volume
Tris	25 mM	30.25 g
Glycine	192 mM	144.1 g
SDS	0.1% (w/v)	10 g
Distilled water	N/A	Up to 10 L
Total	N/A	10 L

Prepare in a solution tank and store at room temperature; stable for several months.

8. Transfer buffer for western blotting

Reagent	Final concentration	Quantity or volume
Tris	40 mM	24.2 g
Glycine	242 mM	90.7 g
SDS	0.1% (w/v)	5 g
Ethanol	20% (v/v)	1 L
Distilled water	N/A	Up to 5 L
Total	N/A	5 L

Prepare in a solution tank and store at room temperature; stable for several months.

9. PBS buffer

Reagent	Final concentration	Quantity or volume
NaCl	137 mM	80 g
KCl	2.69 mM	2 g
Na_2HPO_4	7.75 mM	11 g
KH_2PO_4	1.47 mM	2 g
Tween 20	0.4% (v/v)	20 mL
Distilled water	N/A	Up to 10 L
Total	N/A	10 L

Prepare in a solution tank and store at room temperature; stable for several months.

Laboratory supplies

1. Newspaper
2. 1.5 mL microcentrifuge tube (WATSON, catalog number: 131-7155C)
3. 10 µL pipette tips (WATSON, catalog number: 110-201C)
4. 200 µL pipette tips (WATSON, catalog number: 110-705C)
5. 1,000 µL pipette tips (WATSON, catalog number: 110-804C)
6. Gauze (Iwatsuki, catalog number: 002-21540)
7. Absorbent paper (ATTO, catalog number: CB-09A)
8. Immun-Blot PVDF membrane (Bio-Rad, catalog number: 1620177)

Equipment

1. 10 µL pipette (Gilson, model: P10, catalog number: F144802)
2. 20 µL pipette (Gilson, model: P20, catalog number: F123600)
3. 200 µL pipette (Gilson, model: P200, catalog number: F123601)
4. 1,000 µL pipette (Gilson, model: P1000, catalog number: F123602)
5. Blender (Kuvings, model: KPB-351SP)
6. High-speed refrigerated centrifuge (Himac, model: CR20GIII)
7. Refrigerated microcentrifuge (TOMY, model: MX-307)
8. Equipment for SDS-PAGE (NIHON EIDO, model: NA-1012)
9. Equipment for western blotting (BIO CRAFT, model: BE-320)
10. Luminescence image analyzer (Fujifilm, model: LAS-3000 mini)

11. Spectrofluorometer (Jasco, model: FP-6500)
12. Quartz cell
13. Paintbrush
14. LED light (CCS Inc., model: ISLM-150150-HWHR)
15. Grass tube (AS ONE, catalog number: 14101005B)
16. Conical beaker (AS ONE, catalog number: 1-7117-03)
17. Funnel (AS ONE, catalog number: 6-319-06)
18. Magnetic stirrer (TOMY, model: FS-210)

Software and datasets

1. ImageJ, http://imagej.nih.gov/ij//

Procedure

A. Preparation of thylakoids from spinach leaves

1. Prepare grinding buffer (see Recipes) and keep it at 4 °C.
2. Obtain a bunch of spinach from a local supermarket one day before use.
3. Wash thoroughly with water, wrap in newspaper, and store overnight in the dark at 4 °C to deplete starch levels and improve the intactness of isolated thylakoids.
4. Excise approximately 10 g (fresh weight) of leaves (excluding petioles) in the dark.
5. Homogenize in 200 mL of grinding buffer using a blender (thrice for 3 s).
6. Filter through four layers of gauze using a funnel and collect the flowthrough.
7. Centrifuge at 3,000× *g* for 10 min at 4 °C.
8. Discard the supernatant and gently resuspend the pellet in 40 mL of grinding buffer on ice using a paintbrush.
9. Centrifuge at 300× *g* for 1 min at 4 °C, collect the supernatant, and centrifuge again at 3,000× *g* for 10 min at 4 °C.
10. Repeat steps A8–9 once more and resuspend the resulting pellet in 2 mL of grinding buffer using a paintbrush.
11. Dilute a 10 µL aliquot of the preparation solution 20-fold with the grinding buffer (e.g., 190 µL) to determine chlorophyll concentration.
12. Add 800 µL of 100% acetone to obtain 80% (v/v) aqueous acetone for extraction and vortex for 10 min.
13. Centrifuge at 20,400× *g* for 5 min at room temperature.
14. Transfer the supernatant to a quartz cell and measure absorbance at 646.6, 663.6, and 750 nm using a spectrophotometer.
15. Calculate the total concentration of chlorophyll (*a* + *b*) (mg/mL) using the following formula [21]:

$$[\text{Chlorophyll } a + b] = 17.76 \times (\text{Abs}_{646.6} - \text{Abs}_{750}) + 7.34 \times (\text{Abs}_{663.6} - \text{Abs}_{750})$$

Note: The chlorophyll concentration measured here is a 100-fold diluted value from the original extract.

16. Keep the preparation on ice in the dark for at least 1 h before the assay.
17. Before use, dilute the preparation to a chlorophyll concentration (Chl) of 200 µg/mL.

B. In vitro assay of Trx-mediated CF_oCF_1 reduction

1. Use grinding buffer as the reaction medium for the in vitro reduction assay; perform the assay at 25 °C.
2. Incubate 100 µL of 20 µM Trx-*f* with 100 µL of 2 mM DTT in a tube for 5 min at 25 °C.
3. Add 200 µL of the Trx-*f*/DTT mixture and 50 µL of 200 µg Chl/mL thylakoids to 1,730 µL of grinding buffer in a glass tube and stir gently for 1 min using a magnetic stirrer.
4. Add 20 µL of 200 µM 1-Methoxy PMS and stir for 1 min.
5. Add 1 µL of 2 mM FCCP as a negative control when required.
6. Final concentrations in the mixture are 5 µg Chl/mL thylakoids, 2 µM 1-Methoxy PMS, 100 µM DTT, and 1 µM Trx-*f*.
7. Irradiate this mixture horizontally with red light at 660 nm for 5 min using an LED to initiate $\Delta\mu H^+$ formation and Trx-*f*-

mediated reduction.
8. Continue stirring during the reaction and collect 100 µL aliquots at planned time points.
9. Immediately add 10% (w/v) TCA to stop the reduction by protein precipitation.

C. Determination of the protein redox state

This section is performed following step B of the procedure described in [22], with the following slight modifications:

1. Prepare a 10% polyacrylamide gel for SDS-PAGE, typically 85 × 60 × 1 mm.
2. Set the polyacrylamide gel in the electrophoresis tank and add the electrophoresis buffer.
3. Load 10 µL of each protein sample and run the electrophoresis.
4. Prepare the PVDF membrane and absorbent papers for western blotting. Hydrophilically treat the PVDF membrane with methanol (several seconds) and then equilibrate in the transfer buffer (for several minutes; see Recipes) prior to use.
5. Set the polyacrylamide gel after SDS-PAGE, the PVDF membrane, and the absorbent papers in the blotting equipment. Run the blotting.
6. After blotting, wash the PVDF membrane with distilled water and then PBS buffer (see Recipes) for several minutes.
7. Incubate the PVDF membrane with 1% (w/v) skim milk dissolved in PBS buffer overnight at 4 °C.
8. Incubate the PVDF membrane with the primary antibody diluted with PBS buffer at room temperature for 2 h.
9. Wash the PVDF membrane with PBS buffer thrice for 10 min.
10. Incubate the PVDF membrane with the secondary antibody diluted with PBS buffer at room temperature for 1 h.
11. Wash the PVDF membrane with PBS buffer thrice for 10 min.
12. Incubate the PVDF membrane with the ECL Prime solution and detect luminescence using a LAS-3000 Mini Imaging System.

Data analysis

1. The thiol-labeling maleimide reagent AMS (molecular mass 536.44) decreases protein mobility on SDS-PAGE (Figure 1A). The redox state of CF_1-γ is identified by an observable band shift. Protein reduction levels are calculated by quantifying signal intensities of reduced and oxidized forms using ImageJ software (Figure 1B).
2. Sequential sampling under changing environmental conditions (e.g., light) enables tracking of protein redox behaviors under environmental fluctuations.

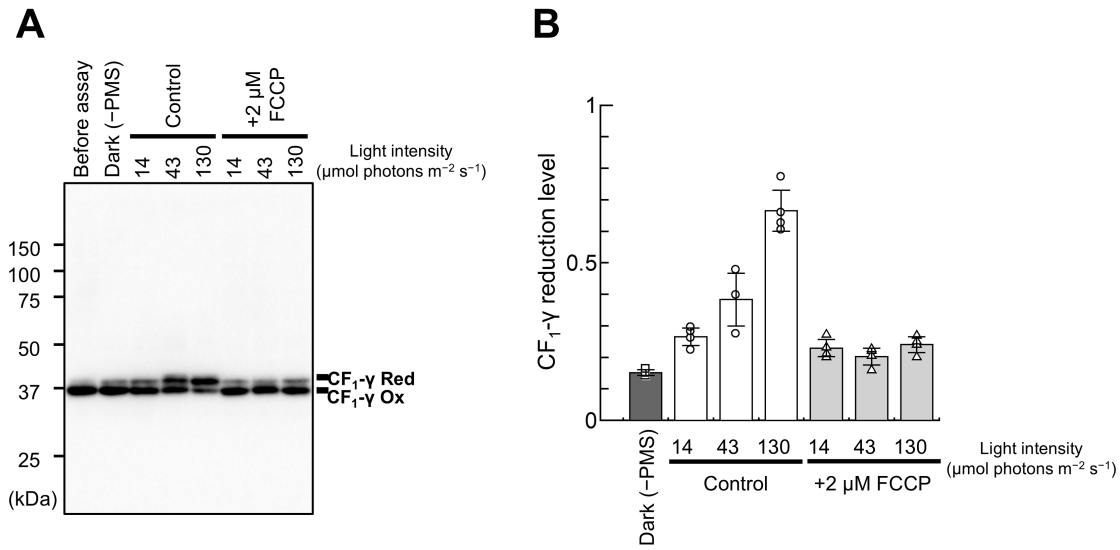


Figure 1. Example results of in vitro CF_1-γ reduction using this protocol. (A) Determination of the thylakoid CF_1-γ redox state. This protocol reduced CF_1-γ in the thylakoids (5 µg Chl/mL) for 5 min. The light intensity of LED irradiation for thylakoids was 14, 43, and 130 µmol photons $m^{-2} \cdot s^{-1}$. The results were also compared with the case with FCCP added.

Ox, oxidized form; Red, reduced form. (B) Quantification of CF_1-γ reduction levels for the data shown in (A). Data represent mean ± standard deviation (n = 3–4). Results were quoted with minor modifications from the original paper.

Validation of protocol

This protocol has been used and validated in the following research article:
• Sekiguchi et al. [20]. Proton gradient across the chloroplast thylakoid membrane governs the redox regulatory function of ATP synthase. *J. Biol. Chem.* 300(9): 107659 (Figure 2).

This protocol is an optimized version of our previous Trx-mediated CF_oCF_1 reduction protocol, which was used in the following research articles:
• Sekiguchi et al. [18]. Chloroplast ATP synthase is reduced by both *f*-type and *m*-type thioredoxins. *Biochim. Biophys. Acta Bioenerg.* 1861(11):148261 (Figures 1 and 2).
• Sekiguchi et al. [19]. Dissipation of the proton electrochemical gradient in chloroplasts promotes the oxidation of ATP synthase by thioredoxin-like proteins. *J. Biol. Chem.* 298: 102541 (Figures 2–4).

General notes and troubleshooting

General notes

This protocol provides a robust method for extracting thylakoids from spinach, enzymatic kinetic analysis, and western blotting. Although optimized with specific reagents and conditions, the underlying principles are broadly applicable to plant biochemistry research.
Plant type comparability: The protocol was developed and validated using spinach thylakoids, but its principles are expected to work across other plant types. However, the extraction efficiency of the thylakoids may vary; thus, a minor optimization of the components of the grinding buffer or the strength of homogenizing leaves in the blender may be required.
Detection of Trx target proteins: We successfully detected CF_1-γ on the thylakoid membrane using an in-house detection antibody via blotting analysis. If you wish to study a different Trx target protein on the thylakoid membrane, you could simply change the detection antibody.

Troubleshooting

Problem 1: Western blotting showed that CF_1-γ appeared to be completely reduced from the beginning of the reaction.
Possible causes: The spinach leaves may not have been kept in the dark long enough before use. Alternatively, the thylakoids may have been exposed to light for too long during isolation.
Solutions: Spinach leaves should always be kept in the dark overnight (or longer) before use, and thylakoid isolation should be performed under dark conditions as much as possible.

Problem 2: During the reaction, the temperature of the reaction solution increases.
Possible cause: The distance between the LED light source used for illumination and the reaction solution may be too short.
Solution: A water bath should be placed between the LED light source and the reaction solution to mitigate an increase in temperature.

Acknowledgments

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T.S. and T.H., conceptualization; T.S., investigation; K.Y., resources; T.S., writing—original draft; K.Y. and T.H., writing—

review and editing; T.H., supervision.

Competing interests

The authors declare that they have no conflicts of interest regarding the content of this article.

Ethical considerations

All experiments were performed using *S. oleracea*. No animal or human samples were used.

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