Quantification of Rubisco by CE

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Warren CR, Adams MA, Chen Z (2000) Effect of N-source on concentration of Rubisco in *Eucalyptus diversicolor*, as measured by capillary electrophoresis. *Physiologia Plantarum* 110: 52-58 [PDF 236 KB]

Warren CR, Adams MA, Chen Z (2000) Is photosynthesis related to concentrations of nitrogen and Rubisco in leaves of Australian native plants? *Australian Journal of Plant Physiology* 27: 407-416 [PDF 344 KB]

DISCLAIMER
Before using this or any other analytical method it is imperative that you check that it works with your samples. The bare minimum is to test accuracy and precision.

- Test accuracy by creating a standard curve by serial dilution of a sample and/or via spike and recovery tests. Both tests will show if the analysis is affected by the sample matrix.
- Test precision by repeated analysis of the same sample. It's best to do separate precision tests for the analytical method (replicate analyses of the same extract) and for the entire extraction and analysis procedure (extract the same sample several times and carry each extract through the analysis procedure). These tests will show you where poor precision is creeping into your analysis.

Remember that your results are qualitative if you rely on a standard curve with a purified analyte.

1 Introduction

Proteins are analysed as complexes with sodium dodecyl sulfate (SDS). SDS binds to water-soluble proteins approximately in proportion to the length of the polypeptide chain, with one SDS molecule bound per two amino acid residues. Therefore, SDS-protein complexes will possess the same charge-to-mass ratio independent of polypeptide chain length and has similar denatured shape for each complex. In free solution the mobilities of the complexes is similar. In a sieving medium the electrophoretic mobility is proportional to the logarithm of the molecular weight. Hence we can separate proteins on the basis of molecular weight in any seiving medium whether it is an acrylamide gel (e.g. SDS-PAGE) or a polymer-filled capillary (e.g. CE).

Rubisco is a large protein (c. 550 kDa) comprised of 8 small subunits (SSU) and 8 large subunits (LSU). In the presence of SDS (and heat), Rubisco is denatured to its two subunits with molecular masses 12-18 kDa (SSU) and 45-55 kDa (LSU).

2.Preparation of samples

For most herbaceous species, and many non-sclerophyllous woody species, extraction and analysis of proteins is easy - proteins can be analysed in crude extracts with no clean-up required.

Sclerophyllous species such as *Eucalyptus* are the most difficult to work with since they contain high concentrations of waxes, oils, phenols and tannins that may precipitate those proteins extracted or interfere with their analysis. The basic extraction protocol may not work with sclerophyllous species. The tell-tale signs of failure are a low yield of extracted proteins and the inability to resolve any proteins by CE – the CE electropherogram will have an unstable baseline with no identifiable peaks, or exceptionally broad peaks. Extraction with large amounts of insoluble PVPP can overcome these problems in some cases (Loomis and Battaile 1966; Warren et al. 2000a). A combination of extraction

with PVPP and precipitation allows extraction and analysis of Rubisco from the most difficult species (Warren et al. 2000b).

N.B. there is no universal way of successfully extracting proteins from leaf tissue – the methods outlined here may or may not work.

2 Safety hazards

2.1 2-mercaptoethanol is toxic, see chemical risk assessment and read MSDS. Wear suitable PPE (gloves, labcoat, eye protection) and work in a fume hood at all times. Solid wastes (used gloves, eppendorfs, pipette tips) should be stored in a sealed container (e.g. 2 x ziplok bags) in the fume hood until they can be disposed of.

3 Reagents

Beckman-Coulter SDS 14-200 gel buffer (one bottle will do ~200 samples)

The same extraction buffer is used throughout:

- 50 mM Tris-HCl (pH 8.0)
- 1% (w/v) SDS
- 15% (v/v) glycerol
- 0.1 M 2-mercaptoethanol (toxic!)*

* 2-mercaptoethanol is toxic, wear appropriate PPE and work in a fume hood at all times

A stock solution of extraction buffer can be made without 2-mercaptoethanol and stored at room temperature.

To make a 500-mL stock solution add:

- 3.0285 g of Tris;
- 75 mL of glycerol
- 5 g of SDS
- dissolve and make up to 500 mL with DI water

on the day required, add 2-mercaptoethanol to a small working volume (e.g. 50-100 mL) of extraction buffer.
To make a 100-mL working solution add 0.7 mL of 2-mercaptoethanol

4 Standards

The normal range for protein standards is 0.1 to 1 mg/L. We have partially purified Rubisco (Sigma) which is OK for determining the migration time of small and large subunits and for spiking samples to verify peak identification. However, this is not suitable for quantification because it is only partially purified (besides, it is expensive). Purified Rubisco is not commercially available. Use purified (99% or greater) BSA for generating a standard curve. Seeing as "all" proteins have similar absorbance at 220 nm we can use BSA for quantification of Rubisco.

5 Method

5.1 Collection and storage of plant material

The way in which samples are collected and stored affects subsequent analysis of proteins. Generally, fresh or frozen material works well. Do not microwave tissues to dry/kill them, this results in seemingly irreversible cross-linking of polyphenols with proteins. Freeze drying may result in similar problems.

5.2 Basic extraction method

1. Place 4 frozen leaf discs (no. 3 cork borer is OK) in a 2-mL eppendorf, together with 2 3-mm ball bearings and a small volume of PVPP (use enough PVPP to fill the eppi to a height of ~5 mm. Too much PVPP will make grinding difficult; too little and you may have problems with polyphenols)
2. In batches of 8 eppendorfs, freeze in liquid N. (trying to freeze and grind more than 8 at a time doesn't work – they thaw out and wont grind)
3. Immediately transfer the 8 frozen eppendorfs to mixer mill and grind for 1.5 min at 20 hz. Check to see if material is ground adequately. If not re-freeze and repeat the grinding step
4. Add 1.0 mL of extraction buffer
5. Using mixer mill, mix for 1.5 min at 20 hz
6. Centrifuge for 2mins at max speed
7. Transfer supernatant to a labelled 2-mL eppendorf
8. Repeat steps 4, 5, 6, 7
9. Centrifuge pooled supernatant
10. Transfer 400 µL of supernatant to a 600- µL Axygen microtube
11. Cap and heat at 95-100ºC in a water bath for 10 minutes
12. Cool to room temperature
13. Cut off lid and replace with a blue Beckman rubber cap
14. Centrifuge and analyse by CE on the same day
15. If using fresh or fresh-frozen material, calculate dry weight fraction of a separate subsample of foliage, i.e. measure fresh and dry (80ºC for 72 h) weight

5.3 Extraction methods for sclerophylls- MCW precipitation

This precipitation procedure selectively precipitates proteins. It can also be used to clean-up samples for analysis of total protein by the Lowry method, in which case samples should be re-dissolved in NaOH, not extraction buffer (2-mercaptoethanol interferes with the Lowry assay). If proteins are dilute, they can be concentrated during the precipitation procedure by either increasing the volume of sample added and/or decreasing the volume of extraction buffer that precipitated proteins are re-dissolved in

Reference: Wessel, D. Fluegge , U.I. (1984), Anal. Biochem. 138, 141-143

-it might be difficult to redissolve pellets of more than 0.4mg protein
-do the precipitation at RT, otherwise the solutions become turbid

1. Place 4 frozen leaf discs (no. 3 cork borer is OK) in a 2-mL eppendorf, together with 2 3-mm ball bearings and a small volume of PVPP (use enough PVPP to fill the eppi to a height of ~5 mm. Too much PVPP will make grinding difficult; too little and you may have problems with polyphenols)
2. In batches of 8 eppendorfs, freeze in liquid N. (trying to freeze and grind more than 8 at a time doesn't work – they thaw out and wont grind)
3. Immediately transfer the 8 frozen eppendorfs to mixer mill and grind for 1.5 min at 30 hz. Check to see if material is ground adequately. If not re-freeze and repeat the grinding step
4. Add 1.0 mL of extraction buffer
5. Using mixer mill, mix for 1.5 min at 30 hz
6. Centrifuge for 2mins at max speed
7. Transfer supernatant to a labelled 2-mL eppendorf
8. Repeat steps 4, 5, 6, 7
9. Centrifuge the pooled supernatant for 2 mins at max speed
10. Pipette 150ul of supernatant into an empty 2-mL eppendorf

11. -add 4 volumes of MeOH (600µL) and 1 volume of Chloroform (150 l)
12. -vortex and then check that there is only one phase
13. -add 3 volumes of DI water (450 µL)
14. -vortex thoroughly, centrifuge 2 mins at max speed
15. -remove upper organic phase without disturbing interface (contains the proteins !) and collect for disposal
16. -add at least 3 volumes of MeOH (450 µL)
17. -vortex thoroughly, centrifuge for 2 mins at max speed
18. -remove supernatant; be careful not to lose the pellet (you could mark tube side if especially anal)
19. -air dry pellet, but not completely (easier to dissolve afterwards)
20. Take up in 400 µL of extraction buffer
21. Re-dissolve/denature by heating in a water bath for 10 min at 95-100ºC (vortex once to ensure pellet is re-dissolved
22. Transfer the re-dissolved and denatured proteins into a 600- µL Axygen microtube
23. Remove the cap, replace with a blue Beckman rubber cap
24. Centrifuge and analyse by CE on the same day

5.4 CE setup

NB. The set-up and analysis of Rubisco depends on which analysis kit is being used. The best thing to do is follow the instructions in the kit.

The ends should always be immersed in vials of SDS gel buffer or H2O so as to prevent them drying ouyt. Therefore, you must work quickly when loading/unloading samples from the CE.

The method described here is for a reverse injection, i.e. sample being injected from the outlet side. This decreases the analysis time two-fold (but decreases resolution) because proteins are being separated over a shorter distance compared to a normal inlet-side injection

Always program a shutdown method that rinses the capillary with SDS gel buffer. To ensure the capillary is left immersed in gel buffer vials you must make the last rinse step between two vials of gel buffer (i.e. not from gel buffer to waste). The shutdown should also turn off the lamp

Analysis Method. Protein_reverse.met
Cartridge and carousel temp: 20°C
Polarity: normal
Run voltage:9 kV
Run time: 7 minutes
Detector: 220 nm, 10 nm bandwidth, 8 Hz
Sample injection: 15 sec * 0.5 psi (reverse)
Rinse cycles (2): 1 M HCl (1 min x 20 PSI, reverse), run buffer (3 min x 20 PSI, reverse)
Wait cycles (1): H2O

6 References

Loomis WD, Battaile J (1966) Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5: 423-438
Warren CR, Adams MA, Chen ZL (2000a) Is photosynthesis related to concentrations of nitrogen and Rubisco in leaves of Australian native plants? Aust. J Plant Physiol. 27: 407-416.

Warren CR, Chen ZL, Adams MA (2000b) Effect of N source on concentration of Rubisco in Eucalyptus diversicolor, as measured by capillary electrophoresis. Physiol. Plant. 110: 52-58
Wessel, D. Fluegge , U.I. (1984), Anal. Biochem. 138, 141-143
Yokata S, Mae T, Makino A, Ojima K (1990) Effect of sodium dodecylsulfate on the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in the extract of rice (Oryza sativa L.) leaves. Soil Sci. Plant Nutr. 36: 91-96