PLANT PROTEIN DETERMINATION

CONTENTS

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PLANT PROTEIN DETERMINATION

1.0 SCOPE AND APPLICATION

In order to compare the concentration of a specific component in a sample with the concentration of that same component in another sample, a common basis for the comparison must be provided. For instance, if the sample mass is the same for both samples a comparison on this basis might be valid in some situations. However, if the moisture of the two samples is vastly different, then a calculation would have to be made to account for this difference. The amount of the component in question is therefore often expressed per unit of the dry weight of the sample. This is called "normalizing" the data for the tested component.

For some types of analyses, however, determination of dry weight is not possible. The whole sample may be needed to assay the component in question, and drying the sample prior to analysis may damage that component. Chlorophyll and enzyme content are examples of sample components for which normalizing on the basis of dry weight may not be possible. It may also be impossible or impractical to normalize chlorophyll data on the basis of the area of leaf tissue analyzed. In these cases, the data may be normalized by directly relating it to the protein concentration in the same tissue sample on which the chlorophyll or enzyme analysis was made. Chlorophyll content is usually related to the total protein in the tissue, since this is most closely related to the dry matter mass. Enzymes are most commonly related to the soluble protein in the crude enzyme extract. The amount of substrate turned to product by the enzyme per unit of time per unit weight of protein is called the specific activity.

The procedure for determining the concentration of protein in plant tissues as a basis for normalizing chlorophyll or enzyme content is described in this Standard Operating Procedure (SOP). The procedures for chlorophyll and enzyme determinations are described in separate documents.

These are standard (i.e., typically applicable) operating procedures which may be varied or changed as required, dependent upon site conditions, equipment limitations or limitations imposed by the procedure. In all instances, the ultimate procedures employed should be documented and associated with the final report.

Mention of trade names or commercial products does not constitute U.S. Environmental Protection Agency (U.S. EPA) endorsement or recommendation for use.

2.0 METHOD SUMMARY

The pellet of leaf tissue remaining after extraction of chlorophyll (ERT/REAC SOP #2030, Chlorophyll Determination) is treated to remove lipids, then allowed to dissolve in 0.1 N Sodium Hydroxide (NaOH) for 15 minutes to generate a crude protein extract. The solution is centrifuged, and duplicate 0.1 milliliters (mL) aliquots are pipetted into separate test tubes. The protein content is determined on these duplicates using the Bicinchoninic Acid (BCA) method. This involves mixing the sample with reagents which react to form a product with a color intensity that is proportional to the amount of protein in the sample. The color is quantified as an absorbance reading in a spectrophotometer at a wavelength of 562 nanometers (nm).

To normalize enzyme activity, the determination is made directly on a portion of the same crude extract assayed for enzyme activity. Duplicate 0.1 mL aliquots are pipetted into separate test tubes and also assayed by the BCA method.

For any purpose, when protein is determined on unknown samples, solutions with known protein

concentrations must also be treated in the same manner as the samples in order to develop a standard curve. The standard curve matches the protein concentration to the absorbance reading from the spectrophotometer (see Appendix A). This is called a Beer's Law plot.

3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING AND STORAGE

After chlorophyll has been extracted, the pellet can be stored frozen (-10^{o}C) in a rubber-stoppered borosilicate glass centrifuge tube for no longer than one month; crude enzyme extracts can similarly be stored frozen. Screw-capped vials made for freezer storage can also be used.

4.0 INTERFERENCES AND POTENTIAL PROBLEMS

Inaccurate pipetting in diluting the samples will cause magnification of errors. A mechanical pipette capable of dispensing 0.1 mL accurately and proper training in its use will help to eliminate this problem. Additionally, personnel expected to perform this procedure will have experience in basic laboratory practices and will be trained in this protein determination procedure.

Certain buffers or other common reagents may interfere with the results. A list of common reagents that have been tested are included in the Pierce BCA Protein Assay Reagent Instructions (Appendix A).

5.0 EQUIPMENT

Equipment required in the laboratory will include:

- a tabletop centrifuge,
- 12-mL borosilicate glass centrifuge tubes,
- 5-mL and 10-mL graduated pipettes and bulb,
- 5-mL test tubes,
- a visible light spectrophotometer with cuvettes (1-cm cell),
- Pasteur pipettes and bulbs,
- squeeze bottle for distilled water,
- mechanical pipetters capable of dispensing 1 and 0.1 mL accurately,
- test tube racks,
- 100-mL graduated cylinders,
- an analytical balance,
- glassware to hold reagents,
- a constant temperature water bath,
- a logbook

6.0 REAGENTS

Reagents needed are:

1. Extraction reagents:

    - 1:1 Ethanol:ether (volume:volume [v:v)
    - 0.1N (0.4%) NaOH (0.4 grams (g)/100 mL distilled water)

2. Assay Reagent A:

- aqueous solution of 1% BCA, disodium salt (BCA-Na_2)
- 2% sodium carbonate, monohydrate (Na_2CO_3-H_2O)
- 0.16% sodium (Na_2) tartrate
- 0.4% NaOH
- 0.95% sodium bicarbonate ($NaHCO_3$) with pH adjusted to 11.25 using 50% aqueous NaOH

3. Assay Reagent B

- 4% copper sulfate, pentahydrate ($CuSO_4.5H_2O$).

Reagents A and B are stable indefinitely at room temperature and are commercially available (Pierce Chemical Co., Rockford, IL, Appendix A). Standard Working Reagent (S-WR) is prepared weekly or as needed by mixing 50 vol of Reagent A with 1 vol of Reagent B. S-WR is apple green in color. Further information on the stability of the reagents can be found in Appendix A.

4. Standard protein solution of Bovine Serum Albumin (BSA):

- 20 mg BSA per 10 mL of the same diluents for the samples (e.g., the buffer in which the enzyme was extracted or 0.1N NaOH for the leaf pellet tissue),
- Dilute to 200, 400, 600, 800, 1000 and 1200 micrograms (µg)/mL. Volumes of the stock to diluent for each concentration are given in Appendix A.

The prepared ampoules provided by Pierce (2 mg/mL BSA) may also be diluted in this manner.

7.0 PROCEDURES

The spectrophotometer will be turned on and allowed to stabilize. The water bath will be turned on and allowed to stabilize to 37°C. The instruments will be calibrated and operated according to the manufacturer's operating manual.

7.1 Protein Preparation after Chlorophyll Extraction

1. The pellet of leaf tissue remaining after chlorophyll extraction will be resuspended in 5 mL 1:1 ethanol:ether (v:v) to remove lipids.

2. Centrifuge for 2 minutes at high speed in a table-top centrifuge, then dispose of the supernatant in a waste container.

3. Resuspend the pellet in 5 mL 0.1N NaOH and allow it to dissolve for 15 minutes.

4. Repeat Step 2.

5. Assuming that all extractions were made from a similar amount of tissue, make dilutions

of 2 and 10 fold of one of the samples, using 0.1N NaOH. If the amount of tissue extracted varies, each of the samples will have to be tested to find the appropriate dilution for that sample. Pipette 0.1 mL aliquots of each dilution and the full-strength sample into separate 5 mL test tubes. Prepare a blank by pipetting 0.1 mL of 0.1N NaOH into a 5 mL test tube. Proceed with the steps in Section 7.3 below, then return to Step 6.

6. Select a dilution that is within the absorbance range of the BSA standards (this will usually be between about 0.3 and 1.3 absorbance units). Adjust all samples to this dilution factor with 0.1 N NaOH.

7. Pipette duplicate 0.1 mL aliquots of each appropriately diluted sample into separate 5 mL test tubes. Prepare duplicate blanks, as for Step 5. Proceed with the steps in Section 7.3 below.

7.2 Protein Preparation for Crude Enzyme Extracts

Follow Steps 5 through 7 above, except that blanks will consist of the same buffer used in the enzyme assay, and sample dilutions will also be made with this buffer rather than NaOH.

7.3 Protein Determination

The following steps are for the recommended 37^{o}C protocol. See Appendix A for a room temperature protocol and an enhanced protocol.

1. Pipette duplicate 0.1 mL aliquots of the BSA standard solution into 5 mL test tubes. Single measurements (rather than duplicates) can be made for the purpose of finding the appropriate sample dilution, after Step 5 (above, Sections 7.1 and 7.2).

2. Pipette 2 mL of S-WR into each test tube containing 0.1 mL of sample, BSA standard, or blank. Pipette forcefully and tap the bottom of each tube with the fingertips to make sure the solutions are well mixed.

3. Place the tubes in a test tube rack in the 37^{o}C water bath. Allow the reaction to proceed for 30 minutes in the water bath.

4. Adjust the spectrophotometer absorbance to zero with distilled water in the cuvette at a wavelength of 562 nm. Absorbances will be measured at this wavelength. The absorbance reading for the blank is subtracted from the reading of each individual unknown and standard.

5. After 30 minutes, the tubes will be removed from the bath and allowed to cool to room temperature, then read within 10 minutes. Each sample can be pipetted with a clean Pasteur pipette or poured into the spectrophotometer cuvette. After the reading, dispose of the sample to a waste container and shake the cuvette onto a paper towel. Do not rinse or dry the cuvette between samples. Begin with the blank and progress from the lighter to the darker samples.

6. The concentration of protein in the plant tissues is then determined from a plot of

concentration vs. absorbances obtained for the standard protein solutions.

8.0 CALCULATIONS

Average of all duplicates will be used. A regression equation will be calculated from the data obtained for the standard protein solutions. Computation of regression statistics can be performed manually. However, manual calculations are tedious, and the calculations can more easily be performed using computer statistical programs or calculators with statistical capabilities. The absorbance reading is the X variable and the BSA concentration is the Y variable. Absorbances of unknowns are then substituted into the equation and the protein concentration calculated. This concentration must then be divided by the dilution factor, if the extract was diluted. This is calculated as follows:

$$\frac{\text{Protein (mg/L) in sample assayed}}{\text{Dilution Factor}} = \text{Protein (mg/L) in Original Extract}$$

To find the total amount of protein in the original tissue sample, continue as follows:

$$\text{Protein (mg/L) in Original Extract} \times \text{Volume of Original Extract (L)} = \text{Total Protein in Tissue Sample (mg)}$$

Or, to find the amount of protein in a sample assayed for enzyme activity, the equation is as follows:

$$\frac{\text{Protein (}\mu\text{g/mL in Original Extract}}{\text{Dilution Factor before Assay (if applicable)}} \times \text{Volume used in enzyme reaction (mL)} = \text{Protein (}\mu\text{g) in enzyme reaction}$$

9.0 QUALITY ASSURANCE/QUALITY CONTROL

The following general QA procedures apply:

1. All field sampling data must be documented on field data sheets and logbooks.

2. All instrumentation must be operated in accordance with operating instructions as supplied by the manufacturer, unless otherwise specified in the work plan. Equipment checkout and calibration activities must occur prior to operation and they must be documented.

3. Samples collected from reference areas will be analyzed along with samples from the contaminated areas.

4. A sample plan, including numbers and sample size, will be diagrammed before sampling. A Quality Assurance Work Plan will be outlined before sampling.

5. Enough tissue will be collected so that two samples can be analyzed from each plant sampled in the field.

6. Results must be within ±20% variation between duplicates. Ten percent of all calculations will be checked by an additional person.

7. The order of analyses will be randomized between the impacted site samples.

8. All data and observations generated during the laboratory procedures will be documented in a laboratory notebook.

10.0 DATA VALIDATION

The data generated will be reviewed according to the QA/QC considerations listed in Section 9.0. In addition to duplicate analyses being carried out on each sample, random plots will be sampled and data will be statistically analyzed.

11.0 HEALTH AND SAFETY

Standard laboratory health and safety procedures will be followed. When working with potential hazardous materials, follow U.S. EPA, OSHA and corporate health and safety procedures.

12.0 REFERENCES

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