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BCA analysis of hydrolyzable proteins in pollen using the Pierce BCA assay kit

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ABSTRACT

This protocol quantifies total protein contents of small amounts of pollen (10 mg) using the bicinchoninic acid (BCA) assay of the Pierce BCA protein assay kit (Thermo Scientific product number 23225). This protocol adapts the kit protocol for analysis of anther pollen, corbicular pollen, and stored pollen from bee colonies. Pollen samples are subjected to acid hydrolysis to digest refractory proteins and to degrade sugars and other pollen substances that interfere with total protein assays. The acid hydrolysis releases substantially more protein from pollens than occurs with soluble proteins but may result in some protein losses due to the harsh acidic conditions. Similar BCA analysis methods without hydrolysis may be used to quantify soluble proteins (proteins obtained without acid hydrolysis or other digest methods) which may exclude insoluble pollen proteins from full quantification. The total protein contents obtained using BCA analysis of acid hydrosylates represents the maximum protein available to consumers and likely includes some refractory proteins that may not be digested by the consumer.

The assay is modified from the original standard protocol to work for pollen analysis: Document Connect (thermofisher.com). Please note that our steps differ from the commercial source protocol.

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Grand Challenge - Assessing the Nutrient Contents of Pollen for Bees

ATTACHMENTS

Pierce BCA protein assay kit 23225.pdf

DeGrandi Hoffman et al 2018 JIP 109 114 Connecting the nutrient composition of seasonal pollens.pdf

Protocol status: Working
We use this protocol and it's working

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GUIDELINES

- Sample contents are quantified by comparison against known amounts of BSA external standards.
- The external standards are not processed through acid hydrolysis (as the samples are) but are subjected to the BCA reaction.
- The working detection range of the BCA reaction is approximately 20 mg to 2000 mg per sample.
- The 10 mg sample mass is based on dried protein estimates within this range.
- Both sample and standard reaction solutions are run in triplicate on the 96 well plate.
- The BCA reaction is time sensitive and continuous (no true end point) so maintain similar time intervals with each plate preparation.
- External standards should be included on every BCA reaction plate.
- If the sample is too concentrated, dilute spare acid hydrosylate and rerun the reaction.

MATERIALS

This protocol uses reagents from the Pierce BCA protein assay kit (Thermo Scientific 23225).

Consumables and benchtop equipment

crimp cap vials, 12 mm x 32 mm (Thermo Scientific 200 000)
crimp caps, 11 mm red rubber crimp cap (Wheaton W224211-01SP)
P200 and P1000 pipettors and pipette tips
aluminum foil
aluminum blocks (capable of fitting 12 mm crimp cap vials)
crimper and decrimper, for 12 mm vials
96 well flat bottom plate (Costar 9018)

Instruments

cooler and lid (capable of holding liquid nitrogen)
digital hot plate (HP 30A digital hot plate, Torrey Pines Scientific)
microplate reader (BioTek microplate reader Synergy HT and Gen5 program, Bio Tek)
vacuum centrifuge (Savant SpeedVac SPD 2010, ThermoScientific)

Chemicals

bovine serum albumin (2000 µg/mL stock ampule, in BCA kit, Thermo Scientific 23209)
concentrated hydrochloric acid
liquid nitrogen
phosphate buffer saline (PBS) tabs (Sigma P4417-50TABS)
Solutions A & B (BCA working reagent (WR) components, in BCA kit)

SAFETY WARNINGS

Read the SDS safety sheets for all chemicals used in this assay, especially hydrochloric acid and liquid nitrogen. Wear full eye protection, lab coats and gloves, and work in a ventilated hood, particularly while handling strong acids. Store and dispose of hazardous wastes and chemicals in accordance with national, state, and local laws.

Liquid nitrogen is used in a potentially hazardous manner in this protocol. Contact with liquid nitrogen can cause severe cryogenic burns and breathing in a nitrogen atmosphere can cause rapid blackout and asphyxiation without warning (possibly into the liquid nitrogen container). Always work in a well ventilated area. Wear protective gloves and sleeves that provide resistance to liquid nitrogen cryogenic burns. Avoid gloves or sleeves that absorb liquid nitrogen (trap liquid nitrogen against body tissues). **Do not lean into the cooler and breathe in the nitrogen atmosphere.** Work with another person present at your side.

Selection of the red rubber crimp caps used to seal the acid hydrolysis is critical. Only certain red rubber crimp caps are sufficiently resistant to strong acids such as refluxing hydrochloric acid. The susceptibility of a given commercial source of caps to corrosion may change without warning from the manufacturer. Test out new batches of red rubber crimp caps before general use.

BEFORE START INSTRUCTIONS

Read the section on the handling of the liquid nitrogen-nitrogen atmosphere cooler. The method described here is both difficult and potentially hazardous given the need for pipetting near open liquid nitrogen. Check the resistance of the red rubber crimp caps to acid hydrolysis before attempting large scale processing of samples.

Acid Hydrolysis of Pollen

1 Dry down the pollen sample in a freeze dryer.

2 Weigh out 10.0 mg of pollen into a crimp cap vial. Place the sample vials into a 24 well plate (acts as a vial holder).

3 *Warning – read the precautions and hazards regarding handling of liquid nitrogen for creation of a nitrogen atmosphere.This step is potentially hazardous in several ways.*

Set up a nitrogen atmosphere in a tall thin styrofoam cooler (at least 30 cm) by adding enough liquid nitrogen to cover the bottom about 1.5 cm deep. Cover the cooler with a lid when not in use. Place a small piece of styrofoam in the liquid nitrogen to act as a bench inside the cooler. The bench should be well grounded and not float or wiggle around in the liquid nitrogen. The goal is to create a cold nitrogen atmosphere around the vials yet avoid contact of the researcher or vials with liquid nitrogen.

4 Place the open vials in their vial holder on the cooler bench and cover the nitrogen atmosphere cooler with the lid for 10 minutes. This sealing will allow air within the vial to be largely displaced by cold nitrogen gas.

5 Carefully add 1000 µL concentrated (12M) hydrochloric acid to each vial in the cooler. Avoid spilling acid droplets on the vial lip where the crimp cap will be sealed. Cover the vials in the cooler again for 10 more minutes to allow the nitrogen atmosphere to enter the vials again.

6 Carefully reach into the cooler and use a vial crimper to seal each vial securely with a red rubber crimp cap.

7 Remove the vials from the cooler.Inspect the vials to ensure that the caps are well sealed. Double crimp the cap at two different angles to ensure a tight seal.

8 Incubate the vials in a heating block on a hot plate at 110°C for 16 hours. Cover the heating blocks with aluminum foil to limit convention heat loss. Visually check the vial caps for leaks (corrosion will be notably visible) after the plate reaches temperature.

Preparing Sample Protein Hydrosylates For the BCA Assay

9 Allow the vials and their heating blocks to cool to room temperature. Examine the vial caps for leakage and corrosion. Spin the vials down for a few seconds in a vacuum centrifuge (**without vacuum**) to draw down condensation.

10 Pipette 300 µL of the 1000 µL acid digest into a new crimp vial. Re-seal and save the remainder of the vial hydrosylate as backup material.

11 Completely remove all of the acid and water from the vials by evaporation in a vacuum centrifuge. Hydrochloric acid is volatile but requires several hours to evaporate.

12 Reconstitute the dried material in 1000 µL PBS buffer. Vortex for 15 seconds, sonicate for 30 seconds, and vortex again for 15 seconds. Examine representative vial contents under a microscope to ensure that the material went into solution.

13 Make a step dilution series of BSA (bovine serum albumin) standards from 0 to 2000 µg/mL using the BSA stock ampule (2000 µg/mL). Work up 500 µL standards for each concentration since the standards will be run in triplicate. Use the same exact batch of diluent solution (PBS buffer) as your samples. Add the following materials to a crimp cap vial, cap with a red rubber crimp cap, and briefly vortex.

target conc. (µg/mL)	**BSA solution (µL)**	**diluent solution (µL)**
2000	500 of 2000 µg/mL	0
1500	373 of 2000 µg/mL	127
1000	250 of 2000 µg/mL	250
750	187.5 of 2000 µg/mL	312.5
500	250 of 2000 µg/mL	750 (1000 µL total - use excess in later dilutions)
250	250 of 500 µg/mL	250
125	125 of 500 µg/mL	375
25	25 of 500 µg/mL	475
0	0	500

BCA Total Protein Assay Reaction

14 Turn on the plate reader for sufficient time (30 minutes for a BioTek Synergy HT microplate reader) for the optics to reach operating temperature and stabilize. Set the plate reader temperature to 37°C if your plate reader has thermal control.

Each plate has sufficient wells to run 9 standard concentrations (27 wells total on the left side) and 23 samples (69 wells total) in triplicate.

15 Prepare working reagent (WR in the kit manual) from kit Solutions A and B. For a full 96 well plate (200 µL per well, 19.2 mL total), add 392 µL Solution B to 20.000 mL Solution A to make 20.392 mL WR. This reagent lasts up to one week at room temperature.

16 Add 200 µL working reagent (WR) to each well in a 96 well plate.

17 Add 25 µL of standard or sample solution to each well to start the reaction.

18 Incubate the plate(s) at 37°C for 30 minutes.

If multiple plates are run, record the time and maintain a uniform stagger for the incubation time and subsequent plate reading. The reaction is continuous and absorbance will increase over time.

19 Place the plate into a plate reader and read the 562 nm absorbance for each well. Set the plate reader at 37°C if the reader has thermal control.

Calculation of Total Protein from Absorbance

20 Export the 562 nm absorbance data. Calculate the average 562 nm absorbance for each sample and standard triplicate.

21 Generate a standard curve from the standards. Calculate the net 562 nm absorbance for each standard concentration as:

562 nm absorbance of the standard concentration – 562 nm absorbance of the 0 µg/mL standard

22 Plot the net 562 nm absorbance for each standard concentration against the standard protein concentration (ug protein/mL). Apply a linear best fit line with a zero intercept.

23 Calculate the average net 562 nm absorbance for each sample triplicate as:

562 nm absorbance of the sample concentration – 562 nm absorbance of the 0 µg/mL standard.

If the sample 562 nm net absorbance exceeds the 2000 µ*g/mL BSA protein standard net absorbance maximum, repeat the BCA reaction with a 1:4 diluted sample. Do not dilute the standards and use the same diluent solution (PBS solution) as the standards and other samples.*

24 Calculate the amount of total protein present in each sample. Use the best fit line equation to calculate the amount of total protein present in each sample well. Steps that were performed differently between standards and samples (i.e. dilutions, taking only a fraction of the total sample) need corrections in calculations. Apply a correction factor to adjust for the reduced proportion of sample protein hydrosylate material used in the BCA analysis (300 µL of 1000 µL total, so 3.33x). Apply a dilution correction factor if samples were further diluted (i.e. a dilution of 1:4 (to 1/5x original concentration) requires a 5x dilution factor).

25 Divide the amount of total protein present by the pollen mass to obtain a total protein concentration for the pollen sample.