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Extraction of Intracellular and Cell Wall Proteins from Leaves and Roots of Harsh Hakea

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[Abstract] Plant proteins can be targeted to intracellular (*i.e.,* cytosol, vacuole, organelles *etc.*) or extracellular (*i.e.*, cell walls, apoplast) compartments. Dual targeting is a key mechanism with important implications for plant metabolism, growth, development and defense *etc*. Harsh Hakea (*Hakea prostrata* R.Br.) is a perennial species and member of the Proteaceae family that thrives on extremely phosphate impoverished soils of southwestern Australia. Harsh Hakea is not a common model organism, but has been widely developed for physiological and molecular/biochemical studies of the endogenous adaptations of an 'extremophile' plant species to abiotic stress, including low phosphorus tolerance. Tissues of Harsh Hakea contain large amounts of compounds (*e.g.*, phenolics) that interfere with the extraction of soluble proteins. We previously optimised extraction of intracellular proteins from Harsh Hakea proteoid roots to improve soluble protein yield by at least 10-fold (Shane *et al.*, 2013). Here, we describe the protocol for extraction and separation of intracellular from 'loosely bound' cell-wall proteins in Harsh Hakea.

Materials and Reagents

1. 2 ml microcentrifuge tubes (Thermo Fisher Scientific, catalog number: 05-408-138)
2. Spectrum™ Spectra/Por™ 4 RC Dialysis Membrane Tubing 12,000 to 14,000 Dalton MWCO (Thermo Fisher Scientific, catalog number: 08-667B)
3. 25 mm syringe filters (0.45 µm) (Sarstedt AG & Co, catalog number: 83.1826)
4. Zeba™ spin desalting columns 7K MWCO (0.5 ml) (Thermo Fisher Scientific, catalog number: 89882)
5. Plastic pellet pestle (Kimble-Chase Kontes) (Thermo Fisher Scientific, catalog number: K749521-0590)
6. Harsh Hakea leaves or roots
7. Dewar (MVE 20/15*)* with liquid N_2, and ice bucket
8. Imidazole (Bioshop, catalog number: IMID508)
9. Triton X-100 (Bioshop, catalog number: TRX777)
10. Glycerol (Bioshop, catalog number: GLY002)
11. Magnesium chloride (Bioshop, catalog number: MAG508)
12. Polyethylene glycol 8000 (Bioshop, catalog number: PEG800)

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13. Phenylmethylsulfonyl fluoride (PMSF) (Geno Technology Inc., G-Biosciences, catalog number: 786-0555)
14. Thiourea (Sigma-Aldrich, catalog number: T-7875)
15. Poly (vinylpolypyrrolidone) (PVPP) (Sigma-Aldrich, catalog number: P-6755)
16. Poly (vinylpyrrolidone) (Bioshop, catalog number: PVP504)
17. Intracellular extraction buffer (see Recipes)
18. Resuspension buffer (RB) (see Recipes)
19. Cell wall extraction buffer (CWEB) (see Recipes)
20. Dialysis buffer (DB) (see Recipes)

Equipment

1. Porcelain mortar (70 mm inner diameter) and pestle
2. Macrosep® Advance Centrifugal Concentrators (10 kDa MWCO) (Pall Corporation, catalog number: MAP010C37)
3. Microcentrifuge (Beckman Coulter, model: microfuge 22R)
4. PT-3100 Polytron Homogenizer (Kinematica AG, model: PT-3100)
5. Magnetic stirrer
6. 4 °C cold room

Procedure

This protocol applies to extraction of intracellular and cell-wall proteins from Harsh Hakea leaves, but can also be applied to roots (*e.g.*, Shane *et al.*, 2014; Shane *et al.*, 2013). For protocols optimized for extracting intracellular and cell wall proteins from leaves, stems etc. of the model plant *Arabidopsis thaliana* see Shane *et al.* (2014); Shane *et al.* (2013) and Robinson *et al.* (2012).

A. Extraction of soluble intracellular proteins
   1. Quickly freeze freshly harvested tissues in liquid N_2. Store at -80 °C until use.
   2. Grind frozen tissues (*e.g.*, ~1 g, comprising a biological replicate) to a powder under liquid N_2 using a pre-chilled mortar and pestle.
   3. Transfer an aliquot (*e.g.*, ~200 mg) of frozen tissue powder to a pre-cooled, 2 ml microcentrifuge tube. Do not allow tissue to thaw during this step. Maintain samples in liquid N_2 until all samples are weighed out.
   4. Add ice-cold Intracellular extraction buffer (IEB) at 1:4 (w/v) to each sample and vortex until tissue thaws into extraction buffer. Briefly homogenize sample using the Polytron (2 to 4 pulses at high speed for 10 to 15 sec per pulse) while maintaining the microcentrifuge tube on ice. Retain homogenates on ice until all extractions are

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completed (it is recommended not to exceed 8 to 10 samples per batch to reduce probability of protein proteolysis *etc.*).

5. Centrifuge homogenates at 17,500 *x g* for 15 min at 4 °C.
6. Carefully pipette off supernatant (clarified extract) and desalt using a Zeba™ spin desalting column previously equilibrated in IEB lacking PVPP and PVP. The desalted extract is designated the intracellular fraction of soluble proteins (*e.g.*, ~2-10 mg protein/ml depending upon the tissue type and stage of development).

B. Washing the insoluble pellets

1. The pellet containing insoluble cell debris is washed twice by resuspending in 1.5-2.0 ml of re-suspension buffer (RB) using a pellet pestle, and centrifugation as above (supernatants are discarded).
2. The pellet is washed as above an additional four times in RB lacking Triton X-100. When completely washed, the pellets will be nearly colourless.

C. Extraction of cell-wall bound proteins

1. The extraction of cell wall proteins from the washed insoluble pellets is done using a cell wall extraction buffer (CWEB) (see Recipes) (~ 2 ml) while stirring for 30 min at 4 °C.
2. Samples are centrifuged as above and concentrated using the Macrosep® centrifugal concentrator to ~0.2 ml yielding a protein concentration of ~2 mg/ml [for spectrophotometric determination of protein concentration see Knowles and Plaxton (2013)].
3. Clarify extracts by passing through the 0.45 syringe filter, and then dialyse overnight at 4 °C against 4 L of the dialysis buffer (DB) using Spectra/Por dialysis tubing (12-14 kDa MWCO, 25 mm flat width).
4. The resultant sample contains ‘loosely-bound’ cell-wall proteins.
5. SDS-PAGE of the cell wall versus intracellular fractions followed by immunoblotting using anti-aldolase and anti-phosphoenolpyruvate carboxylase antibodies is performed to confirm absence of immunoreactive -40 kDa aldolase and -100 kDa phosphoenolpyruvate carboxylase polypeptides (*i.e.*, cytoplasmic marker proteins) in the cell wall extracts [for detailed procedure see Shane *et al.* (2014)].

Recipes

All buffers are made up fresh the day before and stored at 4 °C until used, but no sterilization was done.

1. Intracellular extraction buffer (IEB, keep on ice)
   50 mM imidazole-HCl (pH 7.0)
   0.1% (v/v) Triton X-100

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10% (v/v) glycerol
10 mM thiourea
2 mM $MgCl_2$
2% (w/v) polyethylene glycol 8,000
1% (w/v) polyvinyl (polypyrrolidone) (PVPP)
Note: Add fresh to IEB immediately prior to tissue extraction.
1% (w/v) polyvinylpyrrolidone (PVP)
Note: Add fresh to IEB immediately prior to tissue extraction.
1 mM phenylmethylsulphonyl fluoride (PMSF)
Notes:

a. *Add fresh to IEB immediately prior to tissue extraction.*
b. *Prepare a 100 mM stock of PMSF in absolute ethanol and store at -20 °C; PMSF is unstable in aqueous solution. Handle PMSF with care as this protease inhibitor is quite toxic.*

2. Resuspension buffer (RB, keep in ice)
   50 mM Tris-HCl (pH 7.4)
   10 mM $MgCl_2$
   1% (v/v) Triton X-100
3. Cell wall extraction buffer (CWEB, keep in ice)
   1 M NaCl in 40 mM Tris-HCl (pH 7.4)
   10 mM $MgCl_2$
4. Dialysis buffer (DB, keep in 4 °C cold room)
   40 mM Tris-HCl (pH 7.4)
   10 mM $MgCl_2$

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