Preparation of Arabinogalactan Glycoproteins from Plant Tissue

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[Abstract] This supplements an earlier protocol (Popper, 2011) for the extraction and assay of cell surface arabinogalactan proteins (AGPs). These highly glycosylated glycoproteins (~95% carbohydrate) contain numerous glycomodules with paired glucuronic acid residues that bind Ca^{2+} in a pH dependent manner (Lamport and Varnai, 2013). Classical AGPs comprise the bulk of cell surface glycoproteins and are thus integral components of a Ca^{2+} oscillator involved in a signalling pathway where calcium is a "universal signalling currency" analogous to ATP as the universal energy currency. The central role of these peripheral glycoproteins is thus reason enough for their further study. However, problems arise due to the extensive glycosylation and its apparent microheterogeneity generally assumed to preclude a simple reductionist approach.

Here I describe a simple partial purification of classical AGPs based on their specific interaction with the β-D-glucosyl or galactosyl Yariv reagent, a synthetic diazo dye that precipitates AGPs as an insoluble complex in salt solutions at neutral pH. (The solubility of this complex in dilute alkali provides a rapid sensitive quantitative assay for AGPs.) Reduction of the Yariv diazo linkage releases soluble AGPs for further analysis. For example deglycosylation of AGPs in anhydrous hydrogen fluoride followed by column chromatography yields just a few major AGP polypeptides purified to homogeneity (Zhao *et al.*, 2002). However, purification of individual AGP glycoproteins to homogeneity is rarely achieved (Darjania *et al.*, 2002); not only do the closely related AGP glycosylation profiles vastly outweigh any contribution from the amino acid composition but the glycan polydispersity made isolation of a single molecular entity well-nigh impossible until AGPs genetically engineered with a hydrophobic green fluorescent protein tag allowed chromatographic purification (Zhao *et al.*, 2002). New approaches to AGP fractionation into discrete classes is now also a distinct possibility based on their calcium content hitherto ignored!

[Principle] Disrupted plant tissues release soluble AGPs that can be precipitated as their Yariv complex. This procedure yields mainly classical AGPs; these comprise the bulk of cell surface AGPs. Extraction with $CaCl_2$ rather than the more usual NaCl has two advantages:

1. It results in Ca^{2+} tightly bound by the glucuronic acid residues (Lamport and Varnai, 2013) at > pH 4.5 thus enhancing AGP solubility after its release from the insoluble Yariv complex.
2. It removes pectin as insoluble calcium pectate crosslinked by *inter*molecular Ca^{2+} bridges while AGPs with *intra*molecular Ca^{2+} remain soluble.

Materials and Reagents

1. Tobacco BY-2 cells or other plant tissue
2. Liquid nitrogen
3. $CaCl_2$ (2% w/v)
4. Distilled water
5. Na metabisulphite (Sigma-Aldrich, catalog number: S-1256)
6. Dialysis tubing 12 kDa MW cutoff (3.2 cm flat width) (Sigma-Aldrich, catalog number: D-0530)
7. Superose-6, 10/300 GL (GE Healthcare, catalog number: 17-5172-01)
8. Hydroxyproline
9. Glucuronic acid
10. Gum arabic (Sigma-Aldrich, catalog number: G-9752)
11. NaCl
12. NaOH
13. Yariv reagent (Biosupplies Australia Pty, catalog number: 100-2) (see Recipes)

Equipment

1. Blender/coffee mill
2. Minifuge centrifuge
3. 2 ml Sarstedt tube (with screw cap)
4. Spectrophotometer or microplate reader
5. Mass spectroscope
6. Microfuge
7. Fine-tip pipette
8. Fine tip sonic probe
9. Block heater

Procedure

1. Freeze ~10 to 100 g plant tissue in liquid nitrogen.
2. Pulverise frozen fresh tissue to a fine powder in a cold blender/coffee mill.
3. Stir tissue in 2% w/v $CaCl_2$ for 2-3 h at RT (2 ml for each gram of tissue).
4. Centrifuge 30 min at ~10,000 *x g* (*e.g.* minifuge at RT).
5. Assay 10, 50 and 100 µl aliquots to estimate total AGP in extract (see Notes).
6. Add a slight excess (see Notes) of Yariv reagent to the remaining extract.

7. Allow to precipitate at least 1 hour or overnight at RT.
8. Collect precipitate by low speed centrifugation (10 min at 2,000 *x g*).
9. Resuspend precipitate in 1.5 ml distilled water.
10. Transfer to 2 ml Sarstedt tube (with screw cap).
11. Add ~25 mg Na metabisulphite (final conc.70 mM) to reduce the diazo linkage (top up with H_2O to exlude oxygen which otherwise results in the formation of elemental sulfur).
12. Cap tube tightly and heat at ~50 °C until decolourised (5-20 min).
13. Transfer to small (~5 ml) dialysis bag; stir overnight at RT in 500 ml distilled H_2O and change H_2O three times.
14. Freeze dialysate in liquid nitrogen, lyophilise, then and weigh the product (AGP yields vary from 30-300 µg AGP/g fresh weight depending on tissue source) (see Notes).
15. Validate classical AGPs by size and composition:
    a. Gel filtration on Superose-6 (Lamport, *et al*., 2006).
    b. Hydroxyproline content (Kivirikko and Liesmaa, 1959).
    c. Uronic acid content (Blumenkrantz and Asboe-Hansen, 1973).
    d. Bound calcium via colourimetry (Gindler and King, 1972) or ICPMS (inductively coupled plasma mass spectroscopy) (Lamport and Varnai, 2013).
    e. Amino acid and sugar analyses.

Notes

1. Arabinogalactan protein assay via Yariv reagent.
   All steps at RT.
   a. Add test samples to Eppendorf microfuge tubes.
   b. Make up to ~500 µl in 1% $CaCl_2$.
   c. Use 10 and 20 µg gum arabic as AGP "standards" as follows:
      Add 10 µl gum arabic (1 mg/ml) to 500 µl 1% $CaCl_2$
      Add 20 µl gum arabic (1 mg/ml) to 500 µl 1% $CaCl_2$
   d. Use 500 µl 1% $CaCl_2$ as a reagent blank.
   e. Then to each tube:
      Add 200 µl β-D-Galactosyl-Yariv reagent (1 mg/ml in 2% $CaCl_2$)
      Or use β-D-Glucosyl-Yariv reagent – choice depends on availability.
   f. Mix well and leave for at least 30 min at room temperature.
   g. Spin 10 min at ~15,000 *x g* in microfuge.
   h. With CARE use fine-tip pipette to remove & discard supernate.
   i. Wash pellet twice with 1 ml 2% $CaCl_2$.
   j. Add 1 ml 20 mM NaOH.

k. Shake vigorously to dissolve pellet or sonicate 10-20 sec with fine tip probe or in a sonic bath. If solution is turbid, spin at ~15,000 *x g* to clarify.

l. Read A_{457} nm against reagent blank within an hour or so. A_{457} avoids phenolic interference when eluting Yariv from intact BY-2 cells; for general assays read at 440 nm.

m. Plot "standard curve" and calculate unknowns as µg AGP/tube.

Note: Gum arabic quantification is only an approximation as each AGP binds different amounts of Yariv.

As a general guide, however, a given weight of Yariv reagent will precipitate the same weight of AGP. So for AGP isolation a 10% excess of Yariv reagent generally suffices to precipitate all the AGP.

2. AGP cellular distribution (background information):

Classical AGPs are essential glycoproteins distributed in three cell surface compartments: bound to the outer surface of the plasma membrane by a GPI anchor; soluble in the periplasm; and "bound" or trapped in the wall matrix.

Thus AGP cellular distribution T = M + S + W (Lamport *et al*., 2006)

M = AGPs bound to plasma membrane

S = Soluble AGPs released by cell disruption

W = AGPs bound to cell wall

Table 1. Total of AGPs in tobacco BY-2 cells. In tobacco BY-2 cells, T = 600 µg AGPs g fresh weight.

BY-2 cells (data in [6])	T Total	M Membrane bound	S Soluble periplasmic	W Wall bound
Salt-adapted*	600	60	354	186
Control	600	210	282	108

* AGPs upregulated by high salt appear in the growth medium.

Recipes

1. Preparation of Yariv reagent (1,3,5-tri-(p-β-D-galactosyloxyphenylazo)-2,4,6-trihydroxybenzene) or the β-D-glucosyl derivative

Dissolve 100 mg β-D-galactosyl Yariv in 100 ml 2% w/v $CaCl_2$

Acknowledgments

This protocol is adapted from Popper (2011), Lamport and Varnai (2013) and Lamport *et al*. (2006).

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