Pressure Cycling Technology (PCT) mediated sample preparation schemes enabling high quality two-dimensional gel electrophoresis of *Fagus grandifolia* tree bark proteins

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ABSTRACT

Pressure Cycling Technology (PCT) was used to extract proteins from *Fagus grandifolia* tree bark samples. PCT in combination with newly developed extraction reagents yielded 5-10 times more protein than homogenization with conventional buffers. However, tannins from the tree bark interfered with two-dimensional gel electrophoresis (2DGE) of proteins. The inclusion of polyvinylpyrrolidone (PVP) in the extraction media resulted in the removal of tannins, but with concomitant loss of proteins. A scheme using ultrafiltration to concentrate the protein samples, followed by the use of polyvinylpolypyrrolidone (PVPP) spin columns to remove interfering polyphenols, enabled high quality 2DGE. Alternatively, high molecular weight tannins were effectively removed by ultrafiltration using 100,000 Da nominal molecular weight limit (NMWL) membranes where proteins were partitioned to the filtrate rather than the retentate. Likewise, such filtrates produced high quality 2D gels with minimal tannin interference.

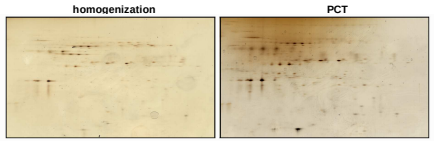


Figure 1. *2D gels comparing proteins isolated from* F. grandifolia *phloem by homogenization in standard buffer (left) and by PCT using the ProteoSOLVE CE Lysis Reagent (right). The homogenized sample was boiled for 10 minutes in 100 mM Tris, 5% SDS, 1% BME. The PCT sample was not boiled. Protein yields from duplicate phloem samples were 1.13 ± 0.10 mg/mL by PCT compared to 0.24 ± 0.02 mg/mL by homogenization. Gel loads were normalized to initial tissue mass. IPGs were pH 3-10.*

1. INTRODUCTION

The American beech tree *Fagus grandifolia* is the only species of this genus in North America. Beech bark disease is initiated by scale insect manifestation, such as *Cryptococcus fagisuga*, which renders the tree bark susceptible to secondary infection by *Neonectria* species of fungi. Genetic diversity in *F. grandifolia* has lead to apparent scale insect resistance in some subpopulations of this deciduous tree. An understanding of the biochemical factors contributing to susceptibility or resistance to parasitic infection can be contrived through genetic and proteomic analyses. However, the preparation of suitable samples from tree bark is challenging for several reasons including (*i*) the cellulosic nature of wood, which makes it difficult to quantitatively extract analytes, (*ii*) the relatively low yields of protein, and (*iii*) the overabundance of lignans, tannins, and other polyphenols which interfere with protein analyses.

A pressure cycling technology (PCT) in which hydrostatic pressure rapidly oscillates between 1 and 2,380 atmospheres was investigated for the extraction of proteins from *F. grandifolia* bark. Pressure-mediated extraction schemes were developed which employed different chemistries in an effort to optimize the disruption of cell walls and protein solubilization and recovery. Compared to conventional homogenization methods, PCT yielded 5-10 times more total protein, depending on which extraction reagent was used. The inclusion of PVP in the lysis reagent as previously described generally resulted in lower recovery of proteins, regardless of whether tree bark was processed by PCT or homogenization and regardless of which lysis reagent was used. However, interference from tannins and other polyphenols results in inaccurate protein assay determinations and greatly diminishes the number of proteins spots resolvable by 2DGE. Hence, removal of tannins downstream of the extraction process was mandated. Tannins were effectively removed by two processes: (*i*) by passage of extracts over centrifugal or gravitational PVPP columns, or (*ii*) by ultrafiltration using 100,000 Da NMWL membranes where proteins were partitioned to the filtrate rather than the retentate.

2. MATERIAL AND METHODS

2.1 Diseased and non-diseased phloem samples

Beech bark disease was first reported in Ludington State Park, Michigan by O'Brien (*Plant Disease* 2001, 85, 921) and considerable levels of beech scale infestation are currently observed. Diseased and non-diseased trees were initially selected based on visual assessment of the presence or absence of the beech scale insect. Subsequently, the visual assessment was confirmed by placing 200 scale eggs on a foam pad and affixing it to the bark of the selected trees. After one year, the pads were removed and the number of scale insects that had colonized the tree was determined. This challenge was repeated for two more years. In all three years, diseased trees supported large scale infestations, ranging from 33 to 200 healthy adult scale insects establishing under the foam test pads. On non-diseased trees, the maximum number of adult scale insects that infested the bark underneath the foam pad was four, and in two of the replicates no scale insects were found. Tissue for protein extractions was collected by removing branches from the canopy. The outer bark was removed and the phloem/cambium layer just beneath the outer bark was collected by peeling this layer off with a stainless steel vegetable peeler.

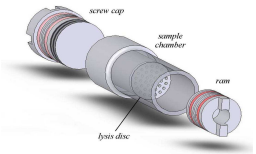


Figure 2. *Exploded view showing the components of the PULSE Tube FT-500. Under high pressure, the ram forces tissue and fluid through the perforated lysis disc. Upon return to ambient pressure, the ram retracts pulling in solvent from the other chamber.*

2.2 Pressure Cycling Technology (PCT)

The NEP-3229 and NEP-2320 Barocycler PCT instruments and PULSE Tubes (Figure 2) were from Pressure BioSciences (West Bridgewater, MA, USA). Bark peels were ground in a stainless steel blade coffee grinder (KitchenAid. Mississauga, Canada). Approximately 450 mg of ground phloem was placed into duplicate PULSE Tubes with 1 mL of each reagent listed in Table 1. All samples were supplemented with protease inhibitors and dithiothreitol (DTT), beta-mercaptoethanol (BME), or tributylphosphine (TBP) was used as a reducing agent (Sigma-Aldrich Chemicals, St. Louis, MO, USA). Samples were processed for 60 pressure cycles, each cycle consisting of 20 seconds at 2,380 times atmospheric pressure followed by rapid depressurization and hold for 5 seconds at atmospheric pressure. Alternatively, 300 mg of ground phloem was processed in 3 mL in an Ultra-Turrax Homogenizer (IKA Labortechnik, Staufen, Germany) for 2X 20 seconds at maximum speed, followed by boiling for 10 minutes.

2.3 Protein assays

Since the carryover of tannins inflates protein assay values, all samples were concentrated by ultrafiltration or by overnight precipitation in 87% acetone and exchanged into 7M urea, 2M thiourea, 4% CHAPS prior to modified Bradford assay.

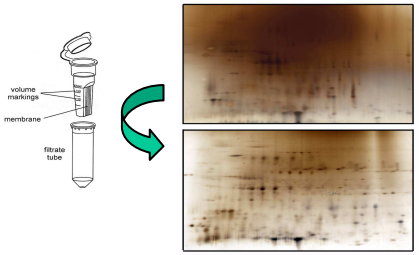
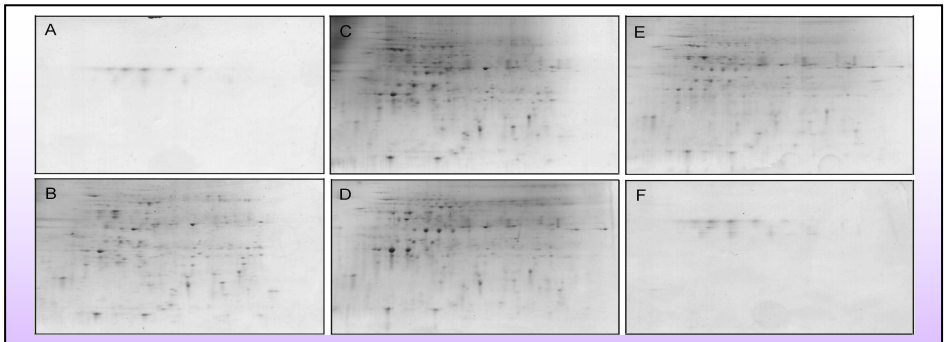


Figure 3. *Tannins and other polyphenols that interfere with 2DGE (top gel) are effectively removed by ultrafiltration (bottom gel). High molecular weight tannins are retained by a 100,000 Da NMWL membrane while smaller proteins are collected in the filtrate. (In converse to usual ultrafiltration, which uses a 10,000 Da NMWL membrane to concentrate proteins in the retentate.)*



gel	method	reagent	mg/mL	CBB	silver
A	PCT	60 mM Tris, 125 mM BME, 5 mM EDTA, 10% PVP	0.02	24	47
B	PCT	100 mM Tris, 125 BME, 5% SDS	0.18	377	547
C	PCT	7M urea, 2M thiourea, 4% CHAPS, 100 mM DTT	1.66	303	591
D	PCT	9M urea, 70 mM SDS, 40 mM dodecylmaltoside	0.70	274	-
E	PCT	ProteoSOLVE CE, 10 mM TBP	1.13	294	611
F	homogenizer	60 mM Tris, 125 mM BME, 5 mM EDTA, 10% PVP	0.04	25	81

Figure 4. *Image analysis of Coomassie stained 2D gels (A-F) of proteins extracted from phloem using PCT and various solvents. IPGs were pH 4-7. Gel loads were normalized to initial tissue mass. Total protein concentration in each extract is indicated (mg/mL). Protein spots were counted in Coomassie stained gels (CBB), then recounted after the gels were restained with silver stain.*

2.4 PVPP Spin Columns

Insoluble PVPP was from Martin-Vialatte Cenologie (Cedex, France). Four milliliters of a 10% slurry was packed by centrifugation in an Ultrafree CL microfiltration device (Millipore Corporation, Danvers, MA, USA). The column was equilibrated twice with 7M urea, 2M thiourea, 4% CHAPS. The samples were passed through PVPP columns by gravity flow for 10 minutes, followed by centrifugation at 1,000 RCF for one minute to evacuate remaining fluid in the column bed.

2.5 IEF, 2DGE, and Image Analysis

First dimension IEF was performed on IPGs pH 3-10 and 4-7 (BioRad, Hercules, CA, USA). 2DGE was performed as previously described by Smejkal *et al.* (*J. Proteomic Research* 5, 983- 987). Gels were stained with ProteomiQ Blue colloidal Coomassie stain (Proteome Systems, Woburn, MA, USA) or SilverQuest Silver Stain Kit (Invitrogen, Carlsbad, CA, USA) and analyzed using PDQuest Version 8.0 Image Analysis Software.

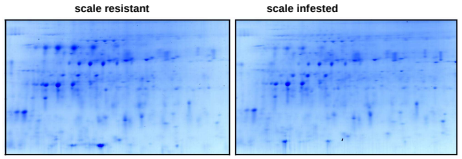


Figure 5. *Enlarged regions of 2D gels comparing phloem proteins isolated from uninfested resistant (left) and highly scale-infested F. grandifolia trees (right). Samples were extracted using PCT and the ProteoSOLVE CE Lysis Reagent, concentrated by ultrafiltration, and passed over PVPP columns. Sample loads were normalized to total protein. IPGs were pH 4-7.*

3. RESULTS AND DISCUSSION

Homogenization in SDS-containing buffers is frequently employed for the extraction of proteins from plant tissues. However, protein yields are typically low and SDS must be removed for downstream analyses such as protein assay, 2DGE, or mass spectrometry to be possible. Alternatively, PCT was used to optimize protein extraction from phloem samples. Concomitant to increased protein yield was an increase of tannins and other polyphenolic substances that interfered with both protein assay and 2DGE.

While PVP has been described for the removal of tannins, it is soluble in aqueous solution and cannot be removed by centrifugation or filtration. Since it is insoluble in acetone, it co-precipitates when proteins are acetone precipitated. Further, the inclusion of PVP resulted in 70%-80% loss of proteins when compared to the same buffers without added PVP.

PCT using Reagents C, D, and E yielded the most total protein from phloem (Figures 1 and 4), but required downstream measures for the removal of tannins. PVPP, the insoluble form of PVP, was used to construct spin columns which effectively removed tannins. Approximately 54% of the total protein was recovered in the column flow through with a 95% decrease in measurable tannins. When the columns were washed once, and the flow through and wash combined, protein recoveries were 92%-100% concomitant with an 89% decrease in tannin concentration. Likewise, high MW tannins were effectively removed by ultrafiltration using 100,000 Da NMWLs where proteins were partitioned to the filtrate rather than the retentate (Figure 3).

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