Pressure enhanced processes (PrEP) enabling high quality two-dimensional gel electrophoresis of *Frankia* mycelia and vesicle structures

Louis S. Tisa[1], Gary B. Smejkal[1,2], Tom Berkelman[3], Chiara Rivas-Morello[2], Alexander Trachtenberg[2] and Winston P. Kuo[2]

[1] Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, NH.
[2] Harvard Clinical and Translational Science Center, Harvard Medical School, Boston, MA; [3] BioRad Laboratories, Hercules, CA.

ABSTRACT

The actinobacteria, *Frankia*, are particularly challenging in terms of sample preparation, IEF, and 2-DE. While French Press is typically used for the disruption of *Frankia* mycelia, protein yields are typically very low and biased toward cytoplasmic proteins. Operating at a maximum pressure of 20,000 psi, the French press fails to disrupt vesicles such that this recalcitrant organelle has remained largely uncharacterized in terms of its protein constituency. Alternatively, a pressure cycling technology (PCT) that recycles the sample at 45,000 psi maximum pressure effectively disrupts both hyphae and the resilient vesicles. However, increased protein yield from *Frankia* strain EAN1pec corresponds to increased release of an indigenous red pigment which interferes with both protein assay and IEF. The pigment is lessened, but not removed by acetone precipitation and concentrates with proteins in ultrafiltrative retentates, suggesting the pigment exists as high molecular weight aggregates which are not dissociated in the presence of chaotropes, detergents and reducing agents. If not removed, this pigment precipitates on the surface of the immobilized pH gradient (IPG) and prevents proteins from being fully imbibed into the strip. This is evidenced by the preponderance of horizontal streaking in second dimension gels. Pigment that is imbibed in the IPG rapidly focuses in the pH 4-5 region of the gradient, creating a highly conductive zone where a localized voltage drop prevents proteins from properly focusing. Hence, it is ambiguous as to whether proteins that are aligned with this zone are due to their association with the pigment or as an electrophoretic phenomenon. Ultrafiltration of mycelia and vesicle homogenates with porous membranes of 100 kDa nominal molecular weight limit (NMWL) partitioned 80% of the initial sample volume containing approximately 60% of the protein mass into the filtrate, whereas most of the offending pigment was accumulated in the retentate. Consequently, more than 500 proteins were resolved in two-dimensional gels of vesicle filtrates.

1. INTRODUCTION

Frankia infect roots and induce nodule formation in a variety of woody dicotyledonous plants, leading to a symbiotic nitrogen-fixing association [1,2]. These bacteria are developmentally complex forming three cell types: vegetative hyphae, spores located in sporangia, and unique lipid-enveloped organelles called vesicles. Vesicles are formed inside of the plant cells of the nodule or in culture under nitrogen limiting conditions and act as specialized structures for the nitrogen fixation process [3]. Their shape is strain-dependent and host-plant-influenced. Vesicles are formed terminally on short side branches of hyphae and have a septum near their base. The mature vesicle is surrounded by an envelope that extends down the stalk of the vesicle past the basal septum which separates the vesicle from the hyphae. One of the functions of the vesicle is to protect the nitrogenase enzyme from oxygen inactivation.

Vesicles are very resilient structures that are difficult to disrupt and protein extraction from vesicles has historically been difficult and a significant bottleneck to proteomic approaches. Methods have been described for the isolation and purification of intact vesicles from *Frankia* grown in culture [4,5]. These purified vesicles retain nitrogenase activity. Initial investigations on the physiological properties of purified vesicles have focused on nitrogen and carbon metabolism, and bioenergetics [6,7].

In these studies, maximal protein recoveries were attained using a pressure cycling technology (PCT) that recycled the samples at 45,000 psi maximum pressure in combination with optimized chemistries. Interference with IEF of proteins was minimized in 100 kDa filtrates enabling unprecedented numbers of proteins to be separated by 2DE.

2. MATERIALS AND METHODS

2.1 Growth Conditions

Frankia strain EAN1pec was grown and maintained in basal growth medium with NH_4Cl as the nitrogen source, as described previously [8,9]. Large scale batch cultures of strain EAN1pec were obtained by growing cells in a carboy with 15 liters of medium containing 20 mM succinate or 20 mM fructose with limited 0.5 mM NH_4Cl as described previously [5].

2.2 Vesicle isolation and purification

Vesicles were isolated and purified as described previously [5,7]. Freshly harvested or frozen cells were washed twice with 25 mM Tris-HCl, 0.5 M mannitol buffer pH 7.4 at 20C. Vesicles were isolated by passing the washed culture through a French pressure cell at 10,000-20,000 psi at 4C. Prior to passage through the French pressure cell, protease inhibitors (Sigma-Aldrich Chemical, St, Louis, MO, USA) were added to the cell suspension to prevent protein degradation. As a result of this treatment the mycelia are completely disrupted but the vesicles remain intact. Vesicles were purified from the cytosolic and membrane fractions by a series of low speed centrifugations at 20C. The numbers of vesicles were counted by using a Petroff-Hausser counting chamber with a phase-contrast microscope at a 400X magnification.

2.3 Pressure Cycling Technology (PCT)

Proteins were extracted by PCT as described by Ringham *et al.* [10, 11] using components from the ProteoSOLVE CE, IEF, and SB Kits (Pressure BioSciences, South Easton, MA, USA). For this, mycelium and purified vesicle suspensions were collected by centrifugation at 10,000 x g for 10 minutes and 100 mg of each pellet was transferred to a PULSE Tube. One hundred milligrams of CE SiC abrasive from the ProteoSOLVE CE Kit and 50 uL of concentrated protease inhibitors were added and the samples were mixed thoroughly and flash frozen. This frozen amalgam impregnated with the abrasives was then extruded through the perforated lysis disk of the PULSE Tube, followed by the addition of 1300 uL of ProteoSOLVE IEF Reagent and 35 uL of 200 mM tributylphosphine. Following 30 pressure cycles at 35,000 psi maximum pressure, the PULSE Tubes were coupled to the insert of an Ultrafree CL microfilter (Millipore Corporation, Danvers, MA, USA) and evacuated by centrifugation at 500 RCF for two minutes. The filtrates were alkylated by the addition of 10 mM acrylamide monomer. The alkylation reaction was quenched after two hours by the addition of 50 mM dithiothreitol [12]. The samples were concentrated using an Ultrafree centrifugal ultrafiltration device with 10 kDa NMWL. Protein concentrations of the retentates was determined with the Bradford reagent (BioRad Laboratories, Hercules, CA, USA).

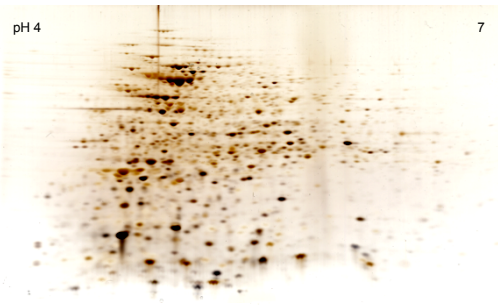

Figure 1. *2DE of 100 kDa filtrate of Frankia EAN1pec total cell lysate from NH_4Cl nitrogen source.*

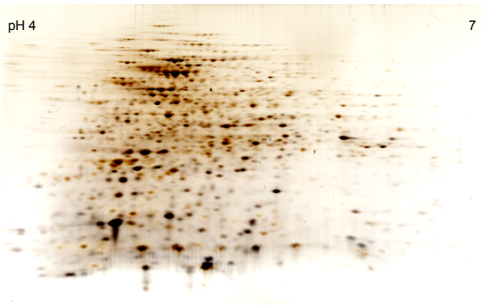

Figure 2. *2DE of 100 kDa filtrate of Frankia EAN1pec total cell lysate from N_2 nitrogen source.*

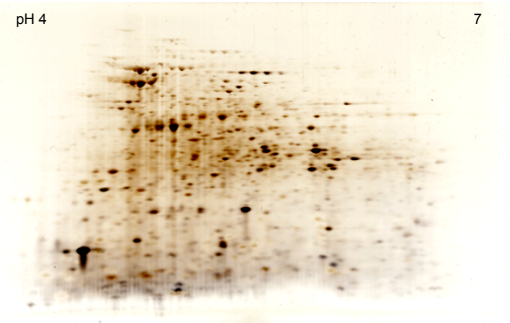

Figure 3. *100 kDa filtrate of purified vesicle fraction from Frankia EAN1pec.*

2.4 Two-Dimensional Polyacrylamide Gel Electrophoresis

Prior to IEF, the red pigment was removed from the samples using an Ultrafree centrifugal ultrafiltration device with 100 kDa NMWL and the filtrates reserved for analysis. ReadyStrip IPGs pH 4-7 were hydrated with 200 uL of each sample for four hours prior to IEF for 100,000 Vh at 24°C. The IPGs were equilibrated twice for ten minutes in 375 mM Tris HCl pH 8.8 containing 2.5% SDS, 50 mM DTT, 0.005% bromophenol blue, and 0.005% phenol red followed by PAGE on 8-16% Criterion Tris-HCl gradient gels. Gels were sequentially stained with ProteomIQ Blue Stain [13] and Silverquest Silver Stain (Invitrogen, Carlsbad, CA, USA) and analyzed using PDQuest Image Analysis Software. ReadyStrips, the Criterion System, and PDQuest were from BioRad Laboratories (Hercules, CA, USA).

3. RESULTS AND DISCUSSION

Pressure Cyling Technology (PCT) yielded more total protein from *Frankia* mycelia than French Press, which failed to isolate proteins from the more recalcitrant vesicles in isotonic buffers. The ProteoSOLVE IEF reagent yielded three times more protein from mycelia and six times more protein from purified vesicle fractions than the ProteoSOLVE SB Reagent. The increased protein yields corresponded to increased recoveries of a red pigment intrinsic to *Frankia* EAN1pec which interferes with protein assay and IEF. The pigment was effectively removed by ultrafiltration enabling high quality 2DE.

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