Pressure Cycling Technology Facilitates Analysis of the *Frankia* Proteome

Frankia are gram-positive, filamentous, nitrogen-fixing actinobacteria that are symbiotic with over 200 different species of plants. *Frankia* produce three cell types: vegetative hyphae, spores located in sporangia, and the unique lipid-enveloped cellular structures, termed vesicles. Vesicles are formed inside plant cell nodules, or in culture under nitrogen limiting conditions, and act as specialized structures for the nitrogen fixation process. 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. Techniques have been developed for the isolation and purification of intact vesicles from *Frankia* grown in culture [1,2,3]. Initial investigations on the properties of purified vesicles have focused on nitrogen metabolism [3,4]. Protein extraction from vesicles has historically been difficult and a significant bottleneck to proteomic studies because vesicles are resilient structures that are difficult to disrupt. Reliable and comprehensive proteomic maps of *Frankia* hyphae and vesicles can only be assured when proteins are isolated reproducibly and in a manner in which they are accurately represented in the downstream analysis. For example, 2DGE can be an accurate representation of a proteome only if the entire protein constituency of cells is recovered during the sample preparation process. While French press treatment effectively lyses hyphae, it fails to disrupt *Frankia* vesicles. Pressure Cycling Technology (PCT) used in combination with a specialized lysis reagent effectively disrupted purified vesicles enabling further investigation of the proteome of the vesicles.

Pressure Cycling Technology (PCT)

PCT uses alternating cycles of high and ambient pressures to induce cell lysis. Tissues or cell suspensions, such as bacteria, are placed in specially designed, single-use processing containers (PULSE Tubes) and are subsequently subjected to alternating cycles of high (up to 35,000 PSI) and ambient pressures in a pressure-generating instrument (Barocycler©) – together, the PCT Sample Preparation System (PCT SPS). Maximum and minimum pressures, the time at each pressure level, and the number of cycles are defined using a programmable logic controller. The reaction chamber of the Barocycler instrument can be temperature controlled using a peripheral circulating water bath. Safety features in the design of the PCT SPS significantly reduce risk of exposure to the researcher to pathogens and prevent cross-contamination of samples [5]. The PCT SPS offers a safer, more efficient method for RNA extraction than other methods in use today.

Methods

***Frankia* Growth Conditions**

Cultures were grown and maintained in basal growth medium under nitrogen repressed conditions with NH4Cl as the nitrogen source, as described previously [6,7]. Large scale batch cultures were obtained by growing cells in 15 L of medium containing 20 mM succinate or 20 mM fructose with 0.5 mM NH_4Cl, as described previously [6].

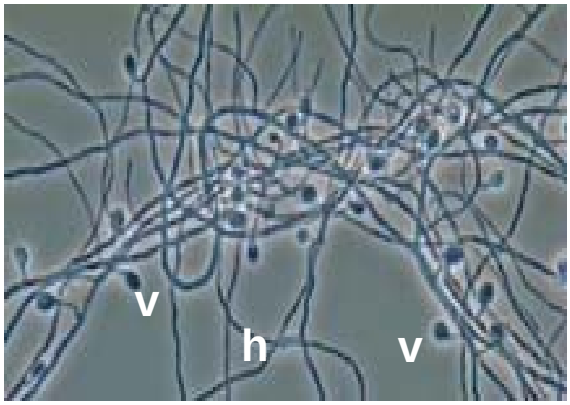

Figure 1. *Light microscopy showing generalized Frankia morphology including hyphae (h) and budding vesicles (v). Adapted from [1].*

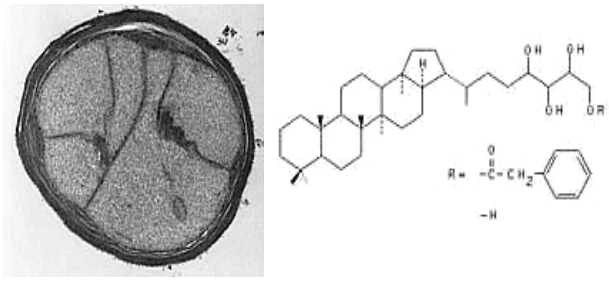

Figure 2. *Electron micrograph showing multilamellar lipid outer structure of Frankia vesicles (left) and hopanoid chemical structure (right). Adapted from [1].*

Vesicle Isolation and Purification

Vesicles were isolated and purified using a modified procedure of Tisa and Ensign [4]. Freshly harvested or frozen cells were washed twice in 25 mM Tris-HCl, 0.5 M mannitol buffer pH 7.4 at 20º C. Vesicles were isolated by passing the washed culture through a French press pressure cell at 10,000-12,000 psi at 4ºC. This treatment completely disrupted vegetative hyphae, but the vesicle remains intact. Vesicles were purified from the cellular debris by a series of low speed centrifugations at 20ºC. *Frankia* whole cells or the purified vesicle fraction were pelleted by centrifugation and suspended in ProteoSOLVE S Lysis Reagent (Pressure BioSciences, West Bridgewater, MA) supplemented with 100 mM dithiothreitol and protease inhibitor cocktail P-2714 (Sigma-Aldrich, St. Louis, MO). 1.5 mL of each suspension was placed into a PULSE Tube and subjected to pressure cycling technology (PCT) for 80 pressure cycles in the Barocycler NEP3229 instrument (Pressure BioSciences, West Bridgewater, MA). Each cycle consisted of 10 seconds

at 35,000 psi followed by instantaneous depressurization and a return to ambient pressure for 5 seconds.

The PULSE Tubes were evacuated by centrifugation at 1,000 RCF for 1 minute. The samples were then centrifuged at 25,000 RCF for 15 minutes to pellet cellular debris. The supernatants were precipitated in 85% acetone at 4ºC and the resulting flocculants were pelleted by centrifugation. The pellets were suspended in 0.75 mL ProteoSOLVE IEF Reagent (Pressure BioSciences, West Bridgewater, MA). Samples were reduced and alkylated in ultrafiltration devices (Millipore Corporation, Danvers, MA), as described by Smejkal *et al.* [7].

Electrophoresis

SDS PAGE was performed on 4-12% NuPAGE polyacrylamide gradients (Invitrogen, Carlsbad, CA). IEF was performed on IPGs pH 4-7. Following IEF, the IPGs were incubated 10 minutes in SDS equilibration buffer containing 4% SDS, then 10 minutes equilibration buffer containing 2% SDS. Second dimension PAGE was performed on 8-16% polyacrylamide gels (BioRad, Hercules, CA). Gels were stained with ProteomIQ Blue colloidal Coomassie stain (Proteome Systems, Woburn, MA).

Results and Discussion

Vesicles have proven to be structures recalcitrant to disruption and therefore require harsh lysis treatment (glass bead disruption) or permeabilizing agents (detergents or lysozyme) to extract proteins. These techniques severely limit the efficiency of protein extraction and hamper proteomic studies. Treatment by French press does not disrupt the vesicle integrity (Figure 3, right side) and can be used as part of vesicle purification process. The use of ProteoSOLVE Lysis Reagent in combination with PCT can greatly facilitate the release of proteins from intact vesicles. When *Frankia* hyphae are subjected to PCT at 35,000 psi, vesicles and other structures are indiscriminately disrupted (Figure 3, left side). The quantity and quality of the extracted protein is sufficient to generate a proteome profile for the isolated vesicles that can enable the identification of vesicle-specific proteins involved in the functional operation of the vesicle.

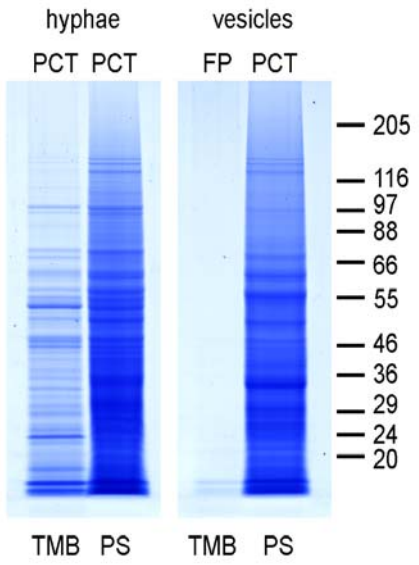

Figure 3. *SDS PAGE showing Frankia EAN1pec intact hyphae (left) and isolated vesicle fraction proteins (right). Hyphae or vesicles were lysed by PCT or French press (FP) in either Tris-mannitol buffer (TMB) or the ProteoSOLVE Lysis (PS) Reagent.*

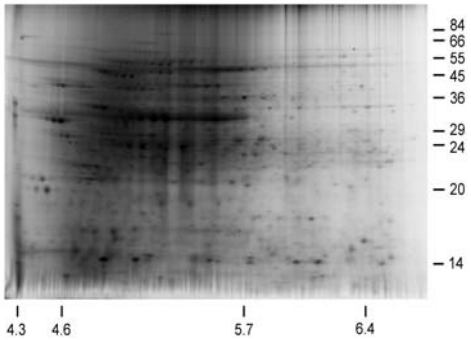

Figure 4. *2DGE showing proteins derived from PCT disruption of Frankia vesicle fraction. pI is estimated on the abscissa. Molecular mass (kDa) is indicated on the ordinate.*

Figure 4 shows proteins on a 2DGE derived from the disruption by PCT of the vesicle fraction. These data show that pressure cycling technology (PCT) used in combination with a specialized lysis reagent can effectively disrupt purified vesicles, enabling further investigation of the proteome of the *Frankia* vesicles.

Acknowledgements

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