

1. Abstract

Frankia are nitrogen-fixing actinomycetes that form a symbiotic association with over 200 species of woody dicotyledonous plants in eight families of angiosperms that are only distantly related to each other. The genome of *Frankia* strain EAN1pec has been sequenced and two dimensional gel electrophoresis (2DGE) is currently being investigated to map the *Frankia* proteome. Besides the ability to form a symbiotic association with plants, one of the striking features of *Frankia* is its ability to develop two unique morphological structures: vesicles and spores. Vesicles are the site of nitrogen fixation and are surrounded by a multilamellar hopanoid envelope that acts as a barrier to oxygen. Since vesicles are very resilient structures, protein extraction has historically been difficult and a significant bottleneck to discovery. 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. The effective and accurate release of proteins from cells is therefore a crucial initial step in any analytical process, and is essential to reliable proteomic analyses. 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. Pressure cycling technology (PCT) uses alternating cycles of high and low hydrostatic pressure to effectively disrupt tissues and cells in preparation for 2DGE and other analytical or preparative methods. PCT has been shown to be more disruptive than sustained high pressure. While French press treatment effectively lyses hyphae, it fails



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

2. Introduction

Frankia are gram-positive filamentous actinobacteria that are symbiotic with over 200 different species of plants which are only distantly related to each other [1]. *Frankia* are developmentally complex and produce three cell types: vegetative hyphae, spores located in sporangia and the unique lipid-enveloped cellular structures, termed 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. Their

shape is strain-dependent and host-plant-influenced. Vesicles are formed terminally on short side branches of hyphae that have a septum near their base. The mature vesicle is surrounded by 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 [2,3]. These purified vesicles retain nitrogenase activity. Initial investigations on the properties of purified vesicles have focused on nitrogen metabolism [3,4]. Vesicles are very resilient structures that are difficult to disrupt. Protein extraction from vesicles has historically been difficult and a significant bottleneck to proteomic approaches.

An investigation was initiated to determine if PCT would facilitate vesicle breakage and protein extraction. This communication reports the results of that study.

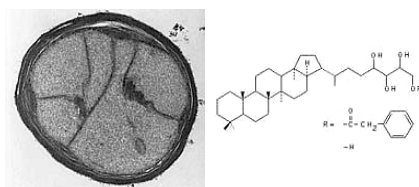


Figure 2. Electron micrograph showing multilamellar lipid outer structure of *Frankia* vesicles (left) and hopanoid chemical structure (right). Adapted from [1].

3 Materials and Methods

3.1 *Frankia* Growth Conditions

Cultures were grown and maintained in basal growth medium under nitrogen repressed conditions with NH_4Cl as the nitrogen source as described previously [4,5]. 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 [4].

3.2 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 20° C. Vesicles were isolated by passing the washed culture through a French pressure cell at 10,000-12,000 psi at 4° C. This treatment completely disrupts vegetative hyphae, but the vesicle remains intact. Vesicles were purified from the cellular debris by a series of low speed centrifugations at 20° C.

3.3 Pressure Cycling Technology

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 PCT for 80 pressure cycles in the Barocycler NEP-3229 instrument (Pressure BioSciences, West Bridgewater, MA). Each cycle consisted of 10 seconds at 35,000 psi followed by instantaneous depressurization and return to ambient pressure for 5 seconds.

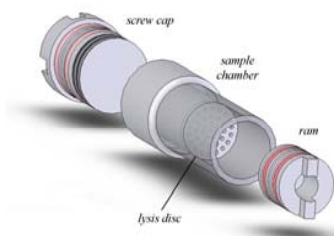


Figure 3. Exploded view showing the components of the PULSE Tube FT-500. Under high pressure, the ram forces tissue and fluid through the perforated lysis disc. Upon return to ambient pressure, the ram retracts pulling in solvent from the other chamber.

The PULSE Tubes were evacuated by centrifugation at 1,000 RCF for 1 min. The samples were then centrifuged at 25,000 RCF for 15 min to pellet cellular debris. The supernatants were precipitated in 85% acetone at 4° C 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].

3.4 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 min in SDS equilibration buffer containing 4% SDS, then 10 min 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).

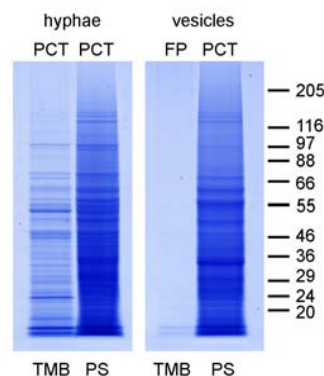


Figure 4. SDS PAGE showing *Frankia* EAN1pec intact hyphae (left) and isolated vesicle fraction proteins (right). Hyphae or vesicles were lysed by PCT or French press (FP) in either Tris-mannitol buffer (TMB) or the ProteoSOLVE Lysis Reagent.

4. Results and Discussion

Vesicles have proven to be structures recalcitrant to lysis and therefore require harsh treatments (glass bead disruption) or permeabilizing agents (detergents or lysozyme) for extraction of 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) and is used as part of vesicle purification process. The use of ProteoSOLVE Lysis Reagent and PCT greatly facilitated the release of proteins from intact vesicles. When *Frankia* hyphae were subjected to PCT at 35,000 psi vesicles and other structures were indiscriminately disrupted (Figure 3, left). The quantity and quality of the extract protein was sufficient to generate a proteome profile for the isolated vesicles and will enable the identification of vesicle-specific proteins that are involved in the functional operation of the vesicle. Figure 5 shows proteins on a 2DGE derived from the disruption by PCT of the vesicle fraction. Although it was not tested, it is presumed that initial cell lysis using PCT at lower pressure could yield intact vesicles. The lack of mechanical shearing, which is intrinsic to PCT, appears ideal for the isolation of organelles, particularly when biological function must be retained.

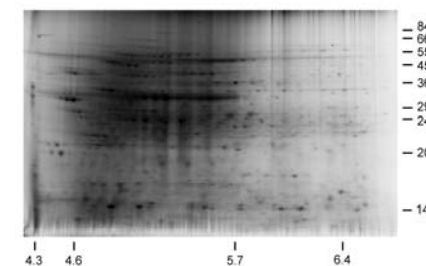


Figure 5. 2DGE showing proteins derived from PCT disruption of *Frankia* vesicle fraction. pI is estimated on the abscissa. Molecular mass (kDa) is indicated on the ordinate.

5. References

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For more information on Pressure Cycling Technology, visit www.pressurebiosciences.com.

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