OPTIMIZATION OF PROTEIN EXTRACTION FROM ORCHIDS

A Thesis
Submitted to the Honors College
By
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Abstract

Thesis: Optimization of Protein Extraction from Orchids

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There is a relatively small amount of information known about plants' responses to pathogens, and there is almost no information specifically concerning orchids' responses. One way to analyze the response of orchids to pathogens is to analyze protein expression using two-dimensional SDS polyacrylamide gels. In this research project, protein extraction from orchids was optimized to yield pure, soluble protein samples. Protein extraction from plants is notoriously difficult, so two different protein extraction kits were used to try to extract a pure protein sample. One of the kits used was CellLytic-P (Sigma, St. Louis), which is specifically made for plant protein extraction. The other kit used was ProteoPrep (Sigma, St. Louis), which may be used on any type of cell or tissue, but which allows protein extraction from specific areas of the plant cells. Protein extraction using the ProteoPrep kit not only yielded different protein fractions from the plant cell, it also yielded significantly higher protein concentrations than the CellLytic-P kit. Therefore, the ProteoPrep kit will be used for protein extraction in further research. 2-D SDS PAGE electrophoresis was attempted two times using orchid tissue, but the gels showed only minor smearing. When running future gels, Silver stain will be used instead of
less powerful Coomassie Blue stain. For future research, five orchid clones were inoculated with Tobacco Mosaic Virus-Orchid Strain (TMV-O), and one clone was mock inoculated. Tissue was harvested at different times following infection and is being stored for future protein extraction and analysis.
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Introduction

In greenhouses all over the world, rare orchid species are infected with viruses that may damage foliage and flowers or even cause plant death. Currently, there is no cure for these viruses and many infected orchids must be destroyed to inhibit the spread of these viruses. There is a relatively small amount of information known about plants' responses to pathogens, and there is almost no information specifically concerning orchids' responses. Through current research, we hope to optimize protein extraction from orchids in order to answer some of the questions regarding the biochemical response of orchids to Tobacco Mosaic Virus Orchid Strain (TMV-0).

This current research will be a continuation of research performed by Wesley Marchione (MS 2003). In his thesis, "Pathogen Resistance Genes and Proteins in Orchids," Marchione described his observation that there was a distinct difference in the number and kinds of proteins expressed in comparing infected and uninfected orchids. Infected orchids had noticeably fewer proteins expressed than did their uninfected clones. However, due to the large quantity of proteins present in all samples and significant smearing during gel electrophoresis (extraction of proteins from plants has been notoriously difficult), Marchione made only one conclusion regarding the differences in expression levels between specific proteins. He noticed that one protein (18 kDa) was expressed variably between infected and uninfected plants, and also between different time intervals following infection. This protein was never identified, but he postulated that it could be a protein involved in disease resistance. In the future, I hope to continue Marchione's protein research and discover the identity of the 18 kDa protein and its possible function in orchid pathogen resistance. By optimizing protein extraction technique, we
also hope to see clearer separation and visualization of individual proteins that will help us to identify more possible resistance proteins.

The acquisition of new knowledge regarding changes in protein expression following pathogen infection and the elucidation of signaling pathways involved in the plant's response may provide important information on host-pathogen interactions in other plants and even animals. Since plants and animals share many similar proteins with similar functions, proteomic research helps to gain insight on physiological and functional parallels, and dissimilarities. Hopefully, by completing this proteomic research, we may gather more information regarding orchid responses to viruses, and this information may some day aid in the development of virus-resistant orchid varieties produced by genetic engineering.
Review of Literature

Recently, eukaryotes (plants and animals) have been shown to have a similar mechanism that can be used to destroy invading pathogens, like bacteria and viruses. This mechanism, RNA silencing, is now believed to be a plant's equivalent of an immune system. RNA silencing is triggered by the presence of foreign double-stranded RNA (dsRNA) (Voinnet 2001). In healthy plant cells, only single-stranded RNA exists. Most plant viruses are single-stranded RNA viruses; however, in order for these viruses to replicate, they must produce dsRNA. RNA silencing is more commonly known as post-transcriptional gene silencing (PTGS), and this process describes the degradation of dsRNA following its transcription or creation (Waterhouse, et. al. 2001).

PTGS is accomplished by the activation of resistance (R) proteins. R proteins are structurally and functionally similar for nearly all types of plants, and even animals share a structurally similar counterpart that is involved in inborn immunity (Hammond-Kosack, et. al. 2003). R proteins are recognized and activated by avirulence (avr) factors of pathogens. Many different proteins are involved in the activation of the R protein response. SGT1 and RAR1 are two such proteins that are involved in signal transduction cascades that trigger R proteins (Peart, et. al. 2002). However, most protein members of the signal cascade needed to initiate the plant's response to a pathogen remain unidentified. For our research, we will be using ProteoPrep (Sigma, St. Louis), which features sequential steps that will yield cleaner protein isolations. From these isolations, we may be able to identify more proteins in the defensive signal cascade of plants.

Researchers have used the same technique that we will be using called two-dimensional SDS-polyacrylamide gel electrophoresis (2-D SDS-PAGE) to examine differences in gene
expression. Recently, Agrawal, et. al. (2002) examined the differences in protein expression between plants that were fumigated with ozone and plants that were not. Major differences were found in the proteins expressed between these two different groups of plants. The plants that were ozone stressed expressed fewer proteins involved in photosynthesis, like RuBisCO, and defense systems. These researchers expressed that this method of protein examination provided reproducible results and an excellent base for plant health comparisons.
Materials and Methods

Protein Extraction Using a CellLytic-P Kit (Sigma, St. Louis)

First, orchid leaves were ground in a sterile mortar and pestle using liquid nitrogen until a fine powder was produced. The powder was added to a microcentrifuge tube and then 2 ml of CellLytic-P reagent and 0.03 ml of Protease Inhibitor Cocktail (Sigma, St. Louis) were added per gram of leaf tissue. The mixture was then centrifuged at 12,000 x g for 10 min. The supernatant was transferred to new tubes and stored at -80°C.

Protein Extraction Using a ProteoPrep Sample Extraction Kit (Sigma, St. Louis)

Orchid leaves were ground in a sterile mortar and pestle using liquid nitrogen until a fine powder was produced. The powder was transferred to a microcentrifuge tube and 2 ml of Chaotropic Membrane Extraction Reagent 1, 2, or 3 or 2 ml of Cellular and Organelle Membrane Solubilizing Reagent was added to 250 mg of ground leaf powder. The mixture was centrifuged at 15,000 x g for 30 min at 15°C and the supernatant was transferred into sterile microcentrifuge tubes. The supernatant was reduced by adding Tributylphosphine Stock Solution (TBP) to a concentration of 5 mM (50 μl) and incubated at room temperature for one hour. The solution was alkylated by adding iodoacetamide to a concentration of 15 mM (60 μl) and incubated at room temperature for 1.5 hr. The solution was centrifuged at 20,000 x g for five min at room temperature. The supernatant was transferred to new microcentrifuge tubes and stored at -80°C.

Determination of Protein Concentration Using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA)

First, 10 μl of water was added to the first well of a micro plate. Then 10 μl of the following protein standard dilutions were added into consecutive wells: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.0625 mg/ml. Then 10 μl of each sample and 1:10 dilutions of each sample were loaded into wells. Two hundred μl of diluted Coomassie was added to each
well. The plate was read at 600 nm. A protein standard curve was used to determine the protein concentrations of the samples.

**Inoculating Orchids with TMV-O**

Orchid leaves that were previously infected with TMV-O were thoroughly ground with 3 ml of inoculation buffer using a chilled, sterilized mortar and pestle. Fine sea sand was added to the liquid and it was rubbed onto all top leaf surfaces using a sterile cotton swab. Then the plants were thoroughly sprayed with deionized water to remove sand and buffer salts. Five plants were inoculated with virus, and the one remaining plant was mock inoculated with sand and inoculation buffer.

**Tissue Collection**

One leaf from the uninfected control and 2 leaves from infected plants were cut off using sterile razor blades at each of the following times after infection: 0 hr, 8 hr, 16 hr, 24 hr, 36 hr, 48 hr, and 72 hr. The leaves were then snap frozen in liquid nitrogen and stored at -80°C.

**Performing Isoelectric Focusing (1st dimension)**

For this research we used a ZOOM IPGRunner System (Invitrogen, Carlsbad, CA) and the reagents that came with the system. First, 9.6 μl of Dithiothreitol (DTT) was added to 145.4 μl of rehydration buffer to a final volume of 155 μl. Ten μl of protein sample was added to the rehydration buffer. The solution was loaded into the wells of the ZOOM IPGRunner cassette. The ZOOM strips were inserted into the cassette and the wells were sealed with tape. The cassette was incubated at room temperature for 12 hr to rehydrate the strips. The IPGRunner system was assembled according to the protocol in the manual (pp. 21-23). The sealing tape and well covers were removed from the cassette, and buffer wicks were applied to the wells. Each
wick was wet with 750 μl of deionized water. Isoelectric focusing was performed using Protocol A (voltage ramp) for broad range ZOOM strips.

Performing SDS-PAGE (2nd dimension)

We used pre-cast NuPAGE 4-12% gradient gels to ensure that a wider range of protein sizes could be separated. The IPG strip was cut flush with the gel, and then the strip was inserted into the well. Four hundred μl of 0.5% agarose solution was pipetted on top of the strip and allowed to solidify. The IPGRunner apparatus was assembled according to the manual (page 30). Eight hundred ml of 1 X MOPS running buffer was poured into the outer chamber. Immediately prior to electrophoresis, 500 μl of NuPAGE Antioxidant was added to 200 ml of 1 X MOPS running buffer, and this solution was poured into the inner chamber. The gel was run at 200V for 50 minutes. The gels were stained over night using Coomassie Blue stain and destained.
Results and Discussion

As can be seen in the table below we determined that the ProteoPrep Sample Extraction kit yielded higher protein concentrations than the CelLytic-P kit. Also, the ProteoPrep kit allowed us to take different protein samples from different areas of the plant cell. This could be important in determining a protein's position in a signaling cascade. Therefore, the ProteoPrep kit will be used for future protein extractions from the infected and uninfected orchid tissue.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>CelLytic-P</td>
<td>0.432</td>
</tr>
<tr>
<td>1-2</td>
<td>CelLytic-P (Leaves Stored at -20°C for two days)</td>
<td>0.489</td>
</tr>
<tr>
<td>2-1</td>
<td>ProteoPrep: Chaotropic Membrane Extraction Reagent 1</td>
<td>1.849</td>
</tr>
<tr>
<td>2-2</td>
<td>ProteoPrep: Chaotropic Membrane Extraction Reagent 2</td>
<td>1.606</td>
</tr>
<tr>
<td>2-3</td>
<td>ProteoPrep: Chaotropic Membrane Extraction Reagent 3</td>
<td>3.112</td>
</tr>
<tr>
<td>2-4</td>
<td>ProteoPrep: Cellular and Organelle Membrane Solubilizing Reagent</td>
<td>1.354</td>
</tr>
</tbody>
</table>

2-D SDS-PAGE will be performed using the protein extractions from the infected and uninfected tissue. Instead of staining the gels with Coomassie Blue stain as we did in this experiment, we will use a silver staining kit for future gels. Silver stain is a more powerful stain at lower protein concentrations than Coomassie Blue. Also, silver stain should be used if mass spectroscopy is to be performed on the protein samples.

For this research, we plan to use mass spectroscopy to sequence any proteins of interest from the samples. Proteins of interest will be classified as any proteins that are expressed at differing levels between the different time intervals following infection or any protein that is
differentially expressed between the infected and uninfected samples. In the summer of 2004 we plan to visit IUPUI's new Proteomics Center to perform mass spectroscopy on the protein samples. Following protein identification by mass spectroscopy, we will try to determine the proteins' functions in orchid virus resistance.
Literature Cited


