Protein profile analyses of healthy and root rot disease infected pseudo-ginseng (*Panax pseudo-ginseng* var. *notoginseng*) roots

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Abstract:

Panax pseudo-ginseng var. *notoginseng* is a traditional Chinese medicinal herb cultivated on a large scale in Yunnan and Guangxi Provinces, China. Pseudo-ginseng is often attacked by *Fusarium solani*, causing pseudo-ginseng root rot disease. In order to facilitate the development of root rot disease resistant cultivars, the protein profiles of healthy roots and infected roots were generated and compared. Silver stained SDS-PAGE gels and Coomassie brilliant blue stained gels were analyzed. The results indicated that the infected pseudo-ginseng tissue samples had different protein profiles and less number of protein bands than that of the healthy tissues. The healthy hairy side roots had less protein bands than the mature tap root tissues.

Keywords: protein profiles, pseudo-ginseng, root rot disease, SDS-PAGE, silver stained gel, proteomics.

Introduction:

Panax pseudo-ginseng var. *notoginseng* is a very valuable traditional Chinese medicinal herb which is rarely found in the wild. It is mainly found in Bhutan and northeastern India. However, this herb is domestically cultured on a large scale in southern China (Yunnan and Guangxi Provinces). Pseudo-ginseng is closely related to *Panax ginseng* and has been known to the Chinese as "the miracle root for the preservation of health." It has a mild to bitter taste and works on the Heart and Kidney Meridians and serves to build Yin and blood in Traditional Chinese Medicine (TCM). Its root is rich in iron, calcium, proteins, saponins, flavonoids and many other phyto-chemicals. One of China's most famous traditional Chinese medicine doctors (famous for his internationally known traditional Chinese medicinal herb classification system: Bencao Gangmu) in the sixteen century, Li Chih Shen, said that "pseudo-ginseng is more valuable than gold." It has long been recognized as one of China's best tonic herbs for building the blood (to stop bleeding and release stagnation), improving the circulation and preventing anxiety (to invigorate blood circulation and stop pain). Pseudo-ginseng increases blood flow in the coronary artery (the artery that supplies the heart with blood) and increases the consumption of oxygen in the middle muscular layer of the heart. Pseudo-ginseng can relieve chest pain or the feeling of oppression in the chest due to angina pectoris induced by coronary insufficiency. Pseudo-ginseng has also been found to stop internal and external bleeding, while being able to disperse blood clots. Pseudo-ginseng (known in Chinese as Yunnan Baiyao) has been distributed to members of the armed services of several Asian countries to be used in case of injury (http://www.healthymagnets.com/cgi-local/SoftCart.exe/tienchi.htm?E+scstore).

Pseudo-ginseng is often attacked by *Fusarium solani*, causing pseudo-ginseng root rot disease (Figure 1). The spread of pseudo-ginseng root rot disease has dramatically reduced the pseudo-ginseng farming industry in China. Pathogen identification and fungicide control of the disease has contained the disease to a certain extent but environmental concerns about the large-scale application of chemicals were recently raised. Recently, screening for antagonistic microorganisms as potential biological control alternatives was initiated (Cai et al. 1996, 2000). A yeast strain, Rp-6, was found to be antagonistic to *F. solani* and field experiments demonstrated that application of Rp-6 inhibited the Pseudo-ginseng root rot incidence by 61.5% (Cai et al. 1996, 2000).

Since the late 1980's, Ekramoddoullah et al. have studied the white pine blister rust and Douglas-fir root rot pathosystems extensively with both proteomics and genomics approaches (Ekramoddoullah et al. 1993, 1999, 2000; Yu et al. 2000, 2001). In order to facilitate the downstream development of root rot disease resistant pseudo-ginseng cultivars, an approach similar to that successfully used in white pine blister rust and Douglas-fir root rot pathosystem was used in the pseudo-ginseng root rot pathosystem. The protein profiles of healthy pseudo-ginseng roots and infected pseudo-ginseng roots were studied in this report. Our objectives in this study were: (1) to extract total

proteins from healthy pseudo-ginseng roots and infected pseudo-ginseng roots and cultivated fungal culture; (2) to compare the integrity of the extracted total proteins by visualizing the SDS-PAGE gel stained with Coomassie blue; (3) to study the protein profile by one-dimensional SDS-PAGE stained with Coomassie blue and refine the profile by studying the silver-stained gel and (4) to analyze in detail the protein profiles by using the PDI Quantity One software package.

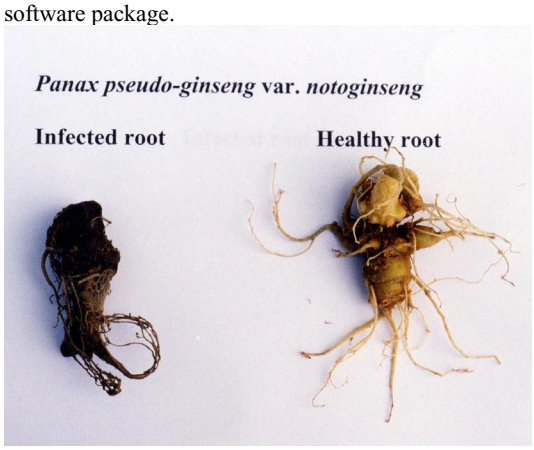

Figure 1. The healthy and infected pseudo-ginseng roots imported from Yunnan, China in May 2000.

Materials and methods:

Healthy and infected pseudo-ginseng roots and fungal cultures: Fresh pseudo-ginseng roots were collected in Yunnan, China and imported to Canada in May 2000 under the Chinese and Canadian CFIA quarantine permits. The roots were packaged in moisture preserving moss packaging materials. Upon arrival at Pacific Forestry Centre, Victoria, Canada, the healthy and infected roots were photographed (Fig. 1) and the fungal pathogen was isolated from the infected roots to generate the fungal axenic culture. The remaining pseudo-ginseng roots were freeze-dried and stored at -20^{o}C freezer until protein extraction. Infected roots were surface treated with 70% ethanol and dissected under sterile condition to get small pieces of infected bark tissues. The dissected infected root tissue was placed on potato extract agar plates for room temperature incubation. After two weeks of incubation, the fungal culture was subcultured and fungal tissue was scraped from the agar plates for lyophilization and -20^{o}C storage until protein extraction.

Extraction of proteins: Proteins were extracted as previously described (Ekramoddoullah and Hunt 1993; Ekramoddoullah and Taylor, 1996) with minor modifications. Samples of freeze dried healthy and infected pseudo-ginseng roots and fungal mycelia were ground to powder in liquid nitrogen after which 50 mg of powder was extracted with 0.7 mL of extraction solution I (ES I) (4% SDS, 5% sucrose, 5% mercaptoethanol) for 10 min at room temperature with vortexing. The extract was centrifuged at 10,000g for 15 min. The clear supernatant was heated to 100¡C for 3 min and then cooled to room temperature. Proteins were precipitated by adding cold (-20¡C) acetone (8X volume of the supernatant). Precipitation was allowed to continue for one hour (at -20¡C), after which the sample was centrifuged at 10,000g. The pellet was resuspended in 0.2 mL of extraction solution II (ES II) (4% SDS, 5% sucrose, 5% mercaptoethanol and 1% NP-40), centrifuged at 10,000*g* for 15 min and the residue was discarded. The protein content of all extracts was determined (Ekramoddoullah and Davidson 1995) using bovine serum albumin as a standard. Briefly, the protein solution and standard were spotted on a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Canada Ltd., Toronto, Canada). The membrane was stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA, USA) in 50% methanol for 8 min, then destained in 50% methanol:10% acetic acid for 8 min at room temperature. The membrane was then rinsed with water for 10 min and scanned with the PDI 420oe light scanner interfaced with PDI Quantity One software (version 3.0) (PDI, Huntington Station, NY, USA). Scanning, detection and quantification were performed according to the PDI instruction manual.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): Individual protein extracts were separated on an equal total protein amount basis by discontinuous SDS-PAGE as described by Laemmli (1970) with a Mini-PROTEAN 3 vertical slab gel apparatus (Bio-Rad). Either "Ready gel precast gels (Tris-HCl gels for SDS-PAGE)" from Bio-Rad or manually cast minigels were used in the protein profile analyses. The manually cast separation gels contained 12% acrylamide with a dimension of 100 mm X 80 mm X 0.75 mm. All reagents were of electrophoresis pure grade and supplied by Bio-Rad. To examine differences between the healthy and infected tissues, 40 micrograms of each protein sample was loaded per lane for Coomassie blue staining. For silver staining, five micrograms of each protein sample was loaded per lane. Electrophoresis gels were calibrated with low molecular weight protein standards (Bio-Rad) ranging from 14.4 to 97.4 kDa or rainbow-coloured protein molecular weight markers from Amersham Pharmacia Biotech. Electrophoresis was carried out at a constant current of 10 mA per stacking gel and 15 mA per separation gel using a Model 3000/300 power source (Bio-Rad). Following electrophoresis, gels were stained either with Coomassie brilliant blue or silver according to the method of Hochstrasser et al. (1988).

Protein profile data analyses: After the Coomassie or silver stained gel were successfully run and stained, gel scanning, band detection, molecular weight

estimation, and quantification of protein bands were performed with the PDI 420oe light scanner interfaced with PDI Quantity One as previously described (Ekramoddoullah and Taylor, 1996). In brief, the gels were scanned into one-dimensional scan format files. The image files were then analyzed by 10 steps of analysis including frame lanes, background subtraction, band detection, molecular standards application and band matching. The image files were finally exported as tiff-formatted files for graphics.

Results:

Total protein extraction from infected pseudo-ginseng roots, healthy pseudo-ginseng roots and dissected healthy pseudo-ginseng root tissues: Total proteins were extracted from infected pseudo-ginseng roots, fungal culture and whole healthy pseudo-ginseng roots (Fig. 1) and different tissues of the whole healthy roots including outer protective layers, inner root tissues (mixture of cortex and pith) and hairy side roots. Forty micrograms of extracted proteins from each extraction were loaded onto a SDS-PAGE. After running, the gel was stained with Coomassie blue (Fig. 2). All the protein samples were resolved well on the SDS-PAGE. Equal protein loads were verified by the intensity of the banding pattern among the healthy roots and the dissected healthy tissues (Fig.2 lane 1, 3-5). The infected roots showed less bands and slightly different banding pattern compared with the healthy roots and tissues (Fig. 2 lane 2). The extracted proteins were further used in protein profile analyses.

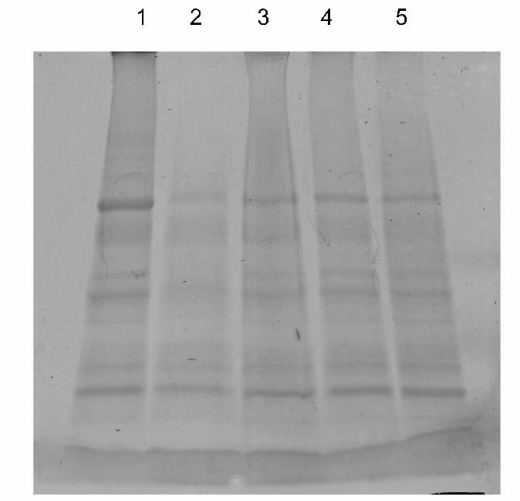

Figure 2. Scanned image of Coomassie stained mini-SDS-PAGE. Forty micrograms of total proteins was loaded in each lane. Lane 1: total protein extracts from whole healthy pseudo-ginseng roots; lane 2: total protein extracts from infected whole pseudo-ginseng roots; lane 3: total protein extracts from the outer protective layer of healthy pseudo-ginseng roots; lane 4: total protein extracts from the inner tissues of healthy pseudo-ginseng roots; lane 5: total protein extracts from the hairy side roots of healthy pseudo-ginseng roots.

Protein profile analyses by Coomassie blue stained gels: Figure 3 showed another round of total protein extraction from the infected, healthy roots and healthy root tissues. A fungal culture total protein sample was also loaded on this SDS-PAGE (Fig. 3 lane 1). With the autoband detection function of the Quantity One, eleven bands were detected from the fungal culture total protein (Fig. 3 lane 1). Only four bands were detected by the same setting from the infected root sample indicating some degradation of this particular extract (Fig. 3 lane 2). Thirteen bands were detected from the healthy whole roots and the inner root tissues (Fig. 3 lane 3 and 5) whereas twelve bands were detected from the outer protective layer (Fig. 3 lane 4). On the other hand, only seven bands were detected from the hairy side root sample (Fig. 3 lane 6). Of interest, a 28.9 kDa protein band was present in large amount only in healthy tussues (Fig. 3 lanes 3-5). Also an unique band (25 kDa) band was detected in infected tissues.

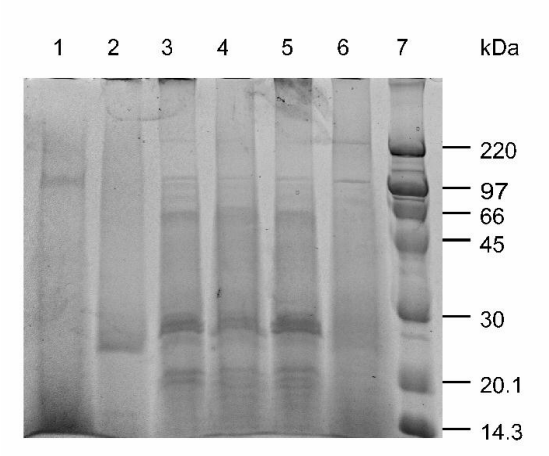

Figure 3. Scanned image of Coomassie stained mini-SDS-PAGE. Forty micrograms of total proteins was loaded in each lane. Lane 1: total protein extracts from fungal mycelial culture isolated from infected pseudo-ginseng roots; lane 2: total protein extracts from infected whole pseudo-ginseng roots; lane 3: total protein extracts from whole healthy pseudo-ginseng roots; lane 4: total protein extracts from the outer protective layer of healthy pseudo-ginseng roots; lane 5: total protein extracts from the inner tissues of healthy pseudo-ginseng roots; lane 6: total protein extracts from the hairy side roots of healthy pseudo-ginseng roots; lane 7: rainbow-coloured protein molecular weight markers from Amersham Pharmacia Biotech.

Figure 4 showed a scanned Coomassie blue stained SDS-PAGE image. The low range protein molecular weight markers for Coomassie and silver stained gel from Bio-Rad was used instead of the slightly different rainbow coloured protein molecular weight markers from Amersham Pharmacia Biotech. More bands were detected on this Coomassie blue stained gel due to better resolution. Due to the limited amounts of total protein extracted from the fungal culture, subsequent analyses were conducted without the fungal culture. Sixteen bands were detected in the healthy whole root, protective layer and the inner tissues (Fig. 4 lane 2, 3 and 4). Fewer visible bands were detected from the infected root sample (three bands, Fig. 4 lane 1) and the healthy side root sample (five bands, Fig. 4 lane 5).

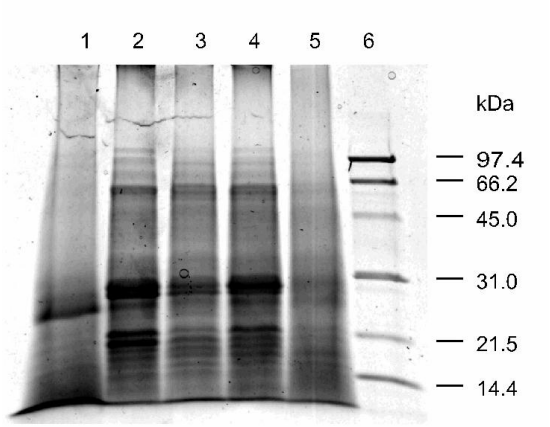

Figure 4. Scanned image of Coomassie stained mini-SDS-PAGE for protein profile analyses. Forty micrograms of total proteins was loaded in each lane. Lane 1: total protein extracts from infected whole pseudo-ginseng roots; lane 2: total protein extracts from whole healthy pseudo-ginseng roots; lane 3: total protein extracts from the outer protective layer of healthy pseudo-ginseng roots; lane 4: total protein extracts from the inner tissues of healthy pseudo-ginseng roots; lane 5: total protein extracts from the hairy side roots of healthy pseudo-ginseng roots; lane 6: low range protein molecular weight markers for Coomassie and silver stained gel from Bio-Rad.

Protein profile analyses by silver stained gel: Silver stained SDS-PAGE gel resolved much more bands than the same set of samples stained with Coomassie blue as shown in Figure 5. Only 5 micrograms of total protein was loaded on each lane. Nineteen bands were detected on the infected root sample (Fig. 5 lane 1) of which two protein bands were unique (25.6 kDa and 28.5 kDa). Twenty-eight bands were detected from the healthy whole root and its protective layers (Fig. 5 lane 2 and 3) whereas thirty-four bands were detected from the healthy root inner tissues (Fig. 5 lane 4). Eighteen bands were detected from the healthy side root sample (Fig. 5 lane 5).

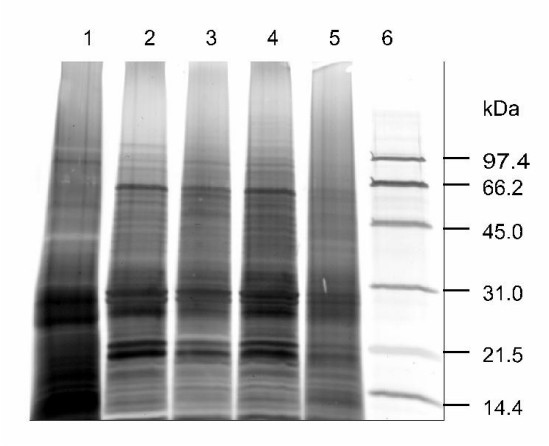

Figure 5. Scanned image of silver stained mini-SDS-PAGE for protein profile analyses. Five micrograms of total proteins was loaded in each lane. Lane 1: total protein extracts from infected whole pseudo-ginseng roots; lane 2: total protein extracts from whole healthy pseudo-ginseng roots; lane 3: total protein extracts from the outer protective layer of healthy pseudo-ginseng roots; lane 4: total protein extracts from the inner tissues of healthy pseudo-ginseng roots; lane 5: total protein extracts from the hairy side roots of healthy pseudo-ginseng roots; lane 6: low range protein molecular weight markers for Coomassie and silver stained gel from Bio-Rad.

Discussion:

In this study, total proteins were successfully extracted from infected pseudo-ginseng roots, healthy pseudo-ginseng roots and dissected healthy pseudo-ginseng root tissues with the methodology previously used for conifer pathosystems (Ekramoddoullah et al. 1993, 1999, 2000). Fresh tissues and their proteins survived the long distance international trip. Subsequent freeze-drying of the tissues and storage the tissues at -20 ^{o}C preserved the proteins. The extracted proteins were used in the one-dimensional SDS-PAGE protein profile analyses as the first step in proteomics study in pseudo-ginseng root. In a related study, the protein samples were used extensively in a series of Western immunoblot analyses (Cai et al. manuscript submitted). Based on these analyses, two-dimensional analyses could be conducted. Furthermore, the preserved samples were successfully used in total RNA extraction and reverse transcription (Cai et al. unpublished data). The reverse-transcribed cDNA could be used in further cloning of interested target proteins if N-terminal sequence could be determined or partial peptide sequences could be derived from two-dimensional spot sequencing.

Infected pseudo-ginseng roots showed a different banding pattern compared with the healthy roots and the dissected healthy tissues. Some of the protein banding differences could be due to protein degradation suggesting that the fungal pathogen had an impact on the infected tissues (Fig. 3 lane 2). The two unique protein bands (25.6 kDa and 28.5 kDa) detected in infected tissue are of interest for further study to examine whether they relate to pathogenicity during infection (Fig.5 lane 1). The healthy whole root showed basically the same banding pattern as the dissected protective layer and the inner tissues (Fig. 5 lane 2, 3 and 4). However, the hairy side roots had a different banding pattern from the other parts of the healthy roots suggesting that the mature tap root contained a different set of proteins than the side roots (Fig. 5 lane 5).

Recently, a large-scale survey and parallel lab analyses resulted in several yeast isolates that were promising in biocontrol of pseudo-ginseng root rot disease (Cai et al. 1996). One of the yeast strains, Rp-6, was found to be antagonistic to *F.solani* and field experiments demonstrated that application of Rp-6 inhibited the pseudo-ginseng root rot incidence by 61.5%. It was also found that some of these yeast strains increased the rate of pseudo-ginseng seed germination, especially isolates Rp-4 and Rp-6 (20% or 18%, respectively, comparing with water). The un-germinated seeds were found rotten or infected by the root rot disease. Analysis of superoxide dismutase (SOD), a key enzyme involved in an antioxidant reaction which may be involved in the stress reaction resulting from infection, revealed higher activity in the treated pseudo-ginseng roots (77.5u/g) than in the control roots (55u/g), but the reverse effect was seen on leaves (Cai et al. 2000). It would be interesting to carry out similar one-dimensional protein profile studies as performed in this study of Rp-6 treated seedlings. At the time this study were conducted, those treated samples were not available in Canada. Follow-up studies and further characterization of selected targeted sequences could be jointly conducted in China and Canada in the future.

Both proteomics and genomics approaches were used in a series of studies of the white pine blister rust pathosystem and the Douglas-fir root rot pathosystem (Ekramoddoullah et al. 1993, 1999, 2000; Yu et al. 2000, 2001). By applying the successful approaches from other plant-disease pathosystems studies to pseudo-ginseng root rot pathosystem could save time and increase the opportunities for developing effective disease control methods. A root-specific promoter was recently isolated from western white pine (Liu and Ekramoddoullah, unpublished data). By incorporating this kind of promoter with antifungal genes such as antimicrobial peptide genes and genetically transforming pseudo-ginseng, a multi-resistant pseudo-ginseng cultivar could be developed. At that time, the protein profiles generated in this study could be used in the characterization of those transgenic pseudo-ginseng plants.

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