African Journal of Microbiology Research Vol. 6(4) pp. 757-763, 30 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.901
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Protein extraction methods for the two-dimensional gel electrophoresis analysis of the slow growing fungus *Undifilum oxytropis*

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Accepted 24 October, 2011

The fungus *Undifilum oxytropis* produces the toxin swainsonine and is symbiotic with locoweeds, which are toxic Oxytropis and Astragalus species. The genus Oxytropis includes perennial legumes that are widespread in many rangeland regions around the world. Consumption of locoweeds causes significant livestock poisoning and severe economic losses. Information about swainsonine synthesis by the *U. oxytropis* endophyte in locoweeds is limited and the interactions between the fungus and locoweed plant are poorly understood. Since *U. oxytropis* is a slow growing fungus that does not readily sporulate, its genetic characterization has been limited. An understanding of its proteome can be an important component in its biological characterization. The goal of this study was to develop an efficient protein extraction method for *U. oxytropis*. To develop an optimized protein extraction protocol for *U. oxytropis*, five protein extraction methods were evaluated. Of the five procedures assessed, trichloroacetic acid (TCA) in acetone was shown to be the best method for the fungus. The *U. oxytropis* proteins extracted using the TCA-acetone method were further characterized using two dimensional gel electrophoresis (2-DE) followed by mass spectrometry. The high resolution of the 2-DE reference map provided a useful approach for proteomic analysis of slow growing fungi.

Key words: Endophytes, mass spectrometry, protein extraction method, swainsonine, two-dimensional polyacrylamide gel electrophoresis, *Undifilum oxytropis*.

INTRODUCTION

Livestock poisoning by toxic plants causes significant economic losses to the livestock industry. Losses from death and reduced reproduction of cattle in the western United States are estimated at $340,000,000 (Nielsen, 1988). The major cause for the poisonings is the consumption of the locoweed plant genera Astragalus and Oxytropis. *U. oxytropis* is a newly described endophytic fungus isolated from Oxytropis locoweed species (Pryor et al., 2009; Yu et al., 2010).

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Toxic species of Oxytropis can produce the alkaloid indolizidine swainsonine (1,2,8-trihydroxyoctahydroindolizidine), which inhibits *lysosomal mannosidase*, *Golgi mannosidase* (Tulsiani et al., 1984), and causes the chronic neurological disease (locoism) in horses, cattle, pig, and sheep (McLain-Romero et al., 2004; Oldrup et al., 2010; Tulsiani et al., 1984). The toxic effect of *U. oxytropis* fungi on rats is indistinguishable from that induced by locoweeds (McLain-Romero et al., 2004).Although several studies described the toxicology and pathology of locoism (Obeidat et al., 2005; Stegelmeier et al., 1999; Taylor and Strickland, 2002), the mechanism by which swainsonine is synthesized in *U. oxytropis* is not known. The swainsonine biosynthesis

pathway has been partially elucidated in the fungi *Metarhizium anisopliae* and *Rhizoctonia leguminicola* (Guengerich and Broquist, 1976; Harris et al., 1988; Wickwire et al., 1990a, b).

U. oxytropis is a very slow growing endophytic fungus (Mukherjee et al., 2010; Pryor et al., 2009). The endophyte does not readily sporulate or produce external mycelium on its host plants. The fungus is present within the coat and not the embryo of locoweed seeds, and the relationship with its host plant is thought to be mutualistic (Ralphs et al., 2002). When Pryor et al. (2009) cultured *U. oxytropis in vitro* on potato carrot agar, water agar, or potato dextrose agar (PDA), the fungus grew very slowly. To attain a colony diameter of at least 5 mm required incubation of cultures for more than 30 d. After 30 d, continued radial growth was extremely slow or arrested.

Proteomics provide some of the most powerful tools for protein expression profiling.

The initial step is to separate proteins, and effective protein sample preparation is critical for high resolution of two dimensional gel electrophoresis (2-DE) analyses. Unfortunately, there is no single protein extraction method that can be universally applied for all kinds of organisms to be analyzed by 2-DE. Protein extraction and sample preparation are challenging steps which have a significant impact on both the first dimension of a 2-DE gel for the intrinsic isoelectric point (pI) using isoelectric focusing (IEF), and on the second dimension. Moreover, protein preparation of a fungus like *U. oxytropis* is difficult, not only because it is so slow growing, but also because it contains various interfering substances, like polysaccharides, polyphenols, lipids, organic acids, alkaloids and other secondary metabolites. The interfering compounds can cause horizontal and vertical streaking, smearing, and reduce the number of distinctly resolved protein spots in 2-DE gels (Gomez-Vidal et al., 2008; Gorg et al., 2004). While protein extraction protocols have been published for other fungi (Isola et al., 2011; Fernandez-Acero et al., 2006; Kim et al., 2007; Rampitsch et al., 2006), the protein extraction methods needed to be optimized for *U. oxytropis*. The fungus is slow growing and this characteristic causes limitations in the availability of starting material.

To the present time, the proteomic investigations of *U. oxytropis* have been limited by the lack of efficient protein extraction methodology.The main objective of this study was to develop a protein extraction protocol suitable for 2-DE analysis of *U. oxytropis*. We compared five protein extraction methods for *U. oxytropis*: (I) trichloroacetic acid (TCA) precipitation, (II) acetone precipitation, (III) precipitation with ammonium acetate in methanol following phenol extraction, (IV) precipitation with TCA in acetone, and (V) ammonium sulfate precipitation. The five protein extraction methods were evaluated for protein yield, 2-DE patterns, and the number of spots clearly visible on the 2-DE gels. Mass spectrometry was performed to identify protein spots in the gels.

MATERIALS AND METHODS

Fungal material and growth conditions

U. oxytropis was isolated from *Oxytropis lambertii* plants (Winston, NM) as previously described (Oldrup et al., 2010). *U. oxytropis* was grown on potato dextrose broth (PDB) medium at 25°C with agitation (170 rpm) for 1 month. Mycelia were recovered by vacuum filtration, rinsed with de-ionized water, frozen with liquid nitrogen and stored at -80°C until further processing.

Protein extraction

Proteins were extracted from fungi by grinding mycelia in a precooled mortar with a pestle in the presence of liquid nitrogen. Five different extraction procedures were compared: (i) trichloroacetic acid (TCA) precipitation, (ii) acetone preciptation, (iii) precipitation with ammonium acetate in methanol following phenol extraction, (iv) precipitation with TCA in acetone, and (v) ammonium sulfate precipitation.

Trichloroacetic acid (TCA) precipitation

Powdered mycelia (100 mg) were homogenized in 2 ml of extraction buffer (0.1 M KCL, 0.5 M tris-base, 0.5 M sucrose, 50 mM ethylene diamine tetraacetic acid (EDTA), 30 mM HCL, 50 mM dithiothreitol) for 30 min on a shaker. The supernatant was collected after centrifugation at 13200 rpm for 30 min at 4°C. Five volumes of 20% w/v TCA was added to the supernatant and proteins were allowed to precipitate overnight at -20°C. Total proteins were recovered by centrifugation at 13200 rpm for 30 min at 4 °C. The pellet was rinsed twice with ethanol, dried under vacuum for 1 h and resuspended in lysis buffer (9.8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS), 0.5% ampholytes, 0.002% bromophenol blue). Cell debris was removed by centrifugation as described previously.

Acetone precipitation

The powdered mycelia (100 mg) were vortexed in 2 ml of extraction buffer and the supernatant was collected by centrifugation as described above. Five volumes of ice-cold acetone were added to precipitate the proteins overnight at -20°C. After centrifugation at 13200 rpm for 30 min at 4°C, the collected pellets were lyophilized for 30 min and resuspended in lysis buffer as described previously. Insoluble material was removed by centrifugation.

Precipitation with trichloroacetic acid (TCA) in acetone

Powdered mycelia (100 mg) were homogenized in 10% TCA in acetone with 0.07% 2-mercaptoethanol for 10 min and incubated overnight at -20 °C. Proteins were concentrated by centrifugation at 13200 rpm for 30 min at 4°C and the pellet washed with cold acetone containing 0.07% 2-mercaptoethanol. Residual acetone was removed by lyophilization. The pellet was resuspended in lysis buffer and insoluble material was removed by centrifugation as described previously. Samples were stored at -80°C for further analysis.

Phenol extraction

Powdered mycelia (100 mg) were extracted by the addition of a weight-equivalent volume of the extraction buffer described

previously. The homogenate was vortexed for 10 min and subjected to ultrasonic waves for 30 min at 4°C. The proteins in the supernatant were collected by centrifuging as mentioned previously, transferred to a microfuge tube, mixed with an equal volume of phenol, pH 8.0 and vortexed for 15 min at 4°C. Samples were centrifuged as mentioned previously, the phenol phase was transferred to another tube, and the extraction procedure was repeated three times by adding extraction buffer (equal v/v) to the phenolic phase. Proteins were precipitated by adding 5 volumes of cold (-20 °C) 0.1 M ammonium acetate in methanol to the phenolic phase. The mixture was then incubated at -20 °C overnight. The precipitate was collected by centrifugation as mentioned previously, and the pellet was washed several times with 0.1 M ammonium acetate in methanol and then twice with ice-cold acetone. The final pellet was vacuum-dried and residual acetone was evaporated (Granier, 1988; Hurkman and Tanaka, 1986; Meyer et al., 1988; Usuda and Shimogawara, 1995). Proteins were solubilized in lysis buffer as previously described by pipetting and vortexing at 28°C. The samples were incubated for 1 h at room temperature under agitation. Insoluble material was removed by centrifugation as mentioned previously and the supernatant was stored at -80°C for further analysis.

Ammonium sulfate precipitation

Powdered mycelia (100 mg) were extracted by the addition of an equivalent volume of extraction buffer (0.2 M potassium phosphate buffer, pH 7.0). Cell debris was removed by centrifugation as mentioned previously and the supernatant collected. Ammonium sulfate was slowly added to the desired percent (100%) saturation (Englard and Seifter, 1990) and the mixture was stirred for 30 min at 4°C. Proteins were concentrated by centrifugation and dissolved in lysis buffer and insoluble material was removed by centrifugation as described previously. The protein concentration was determined by the Bradford method according to the manufacturer's (Sigma) instructions (Bradford, 1976). Concentration of proteins in each sample was calculated against a standard curve of bovine serum albumin.

Two-dimensional electrophoresis

Each sample was subjected to three replicates. For each replicate, 100 µg of protein (resuspended in 340 µl rehydration solution, GE Healthcare) was loaded onto an 18 cm IPG Strip with pH 3 to 11 (GE Healthcare) that had been rehydrated for 20 h. The immobilized pH gradient (IPG) strips were then subjected to IEF at 20°C with a current of 50 µA/strip in an Ettan IPGphor isoelectric focusing apparatus (Amersham Biosciences). The voltage settings for IEF were 500 V for 3 h, 1000 V for 3 h, 8000 V for 4 h and 8000 V for 2 h.

The focused strips were prepared immediately for the 2^{nd} dimension of the 2-DE gel electrophoresis. For the 2-DE gel electrophoresis, the gel strips were equilibrated for 15 min in 10 ml equilibration buffer (50 mM Tris-HCL, [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT). The strips were washed twice with distilled water and further equilibrated with buffer (50 mM Tris-HCL, [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 2.5% iodoacetamide). For the second dimension, IPG strips were loaded onto precast 12% polyacrylamide gel (20×20 cm) with Tris-glycine buffer system as described (Laemmli, 1970). Strips were overlayed with agarose sealing solution (25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.001 bromophenol blue). The gels were run on an electrophoresis unit (Hoefer SE 600 Ruby) at 200 V at 4°C according to the manufacturer's instructions.

Gel staining and image analysis

The 2-DE gels were visualized by silver staining as described by Shevchenko et al. (1996b) with minor variations. After electrophoresis, the gels were rinsed with distilled water and fixed overnight in 50% methanol and 5% acetic acid solution. The gels were then washed with 50% methanol and distilled water for 10 min each. The gel was treated with 0.02% sodium thiosulfate for 1 min and distilled water for 10 min. Gels were finally incubated with 0.1% silver nitrate for 1 h at 4°C on an orbital rotator. The gels were washed with distilled water and then developed three times (each time 10 min with fresh solution) in 0.04% formaldehyde, in 2% sodium carbonate with intensive shaking. The stained 2-DE gels were scanned with an Image Scanner (Bio-Rad, 300 DPI, 256 gray levels).

Protein identification

Protein spots were manually excised from silver stained 2-DE gels. The digestion protocol used was based on the methods of Shevchenko et al. (1996b) with some modification. The protein spots chosen for identification were precipitated from the gel with TCA in acetone. The excised gel was cut into approximately 1 to 2 mm^3 pieces, washed with distilled water to remove ammonium sulfate, dehydrated with acetonitrile, and vacuum dried (Speed Vac, Ecospin). The proteins were reduced with 50 mM DTT at room temperature for 1 h and alkylated with acetonitrile at room temperature for 0.5 h. The gel pieces were rehydrated in 200 µl of 100 mM ammonium bicarbonate for 30 min at room temperature and dehydrated with acetonitrile for 10 min at room temperature. The samples were digested with 50 µl of 20 ng/µl sequencing-grade modified trypsin in 50 mM ammonium bicarbonate overnight at 37°C. The peptides produced by the digestion were extracted once with 50 mM ammonium bicarbonate and twice with 50% [v/v] acetonitrile in 5% [v/v] formic acid. The volume of the extract was reduced to <20 µl by evaporation in a vacuum centrifuge and frozen for later mass spectrometry analysis.

Tandem mass spectrometry (MS-MS) and database searching

Tryptic peptides were infused into a hybrid linear ion trap Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (LTQ-FT, Thermo, San Jose, CA) through a microelectrospray ion source from an ultra-performance liquid chromatography (UPLC) (Acquity, Waters Corp., Milford, MA). Chromatography consists of an isocratic elution of 5% solvent A for 5 min and 40 min linear gradient to 70% solvent B [A: 0.1% formic acid (FA) in H_2O, B: 0.1% FA in acetonitrile (CAN)] with a 1.0 x 150 mm 5 µm reversed phase trapping column (Zorbax, 300SB-C18). FT-ICR mass spectra were collected at a mass resolving power of 100,000 (m/z = 400) and data dependent linear ion trap tandem mass spectra were collected for the five most abundant ions observed in each FT-ICR parent ion mass spectrum.

Mass spectral data was searched against the swiss prot fungi database for protein identification using the Bioworks browser. Protein identifications with an xcorr value > 2 were selected. The protein identification and data documenting the identifications from 2-DE gels are listed in (Table 1).

RESULTS

In this study five different protein extraction methods were evaluated for proteomics studies of *U. oxytropis*.

Table 1. MS identification of proteins from *U.* oxytropis.

Spot No.	Organism	Protein name	Accession number	Molecular weight (kDa)	pI	Peptide sequence	P-Value
1	*Trichoderma koningii*	Glyceraldehyde-3- phosphate dehydrogenase 2	P17730	36083.5	6.25	R.VPTANVSVVDLTVR.I	6.56E-65
2	*Prochlorococcus marinus*	Aspartate semialdehyde dehydrogenase	P49420	37533.97	6.38	R.KILNQSELAITATCVR.V	5.45E-70
3	*Humicola lutea*	Superoxide dismutase [Cu-Zn]	P83684	15853.9	6.36	R.TLVVHAGTDDLGR.G	7.10E-50
4	*Neurospora crassa*	Woronin body major protein	P87252	19114.6	6.43	R.LGDILILQGRPCQVIR.I	6.73E-25
5	*Bordetella avium (strain 197N)*	Homoserine O-acetyltransferase	Q2KU63	45061.5	5.62	R.NTTSPDTTSHR.P	3.99E-58

Major emphasis was laid on each method of extraction, gel background, 2-D patterns, intensity of protein spots, and total number of spots for *U. oxytropis* species protein analysis (Table 2).

For precipitation with TCA extraction procedure (I), an average protein yield of 0.23 µg/µl were obtained, whereas, in the acetone precipitation (II) an average protein yield of 0.29 µg/µl were obtained. For precipitation with TCA in acetone (III) and precipitation with ammonium acetate in methanol following phenol extraction (IV), the protein concentrations and yield were lower, 0.20 µg/µl and 0.19 µg/µl respectively. Ammonium sulfate precipitation (V) gave a high protein concentration about 0.44 µg/µl. The protein yield comparisons of the five extraction procedures showed that ammonium sulfate precipitation gave the greatest yields among the five methods.

The 2-DE protein profiles for samples prepared from the five different protein extraction methods for *U. oxytropis* are shown in Figure 1. Compared with the other four protein extraction methods 2-DE protein profiles, the precipitation with TCA in acetone method produced the best resolution of separated proteins, lower streaking, and a clear background (Figure 1D). The TCA precipitation method provided clear protein profiles, but the separation and intensity of protein spots was less visible than for the other four extraction methods (Figure 1A). Precipitation with ammonium acetate in methanol following phenol extraction method, gave a similar 2-DE profile pattern with TCA in acetone, but the phenol extraction method exhibited apparent vertical streaks in 2-DE gels (Figure 1C). The protein pattern with acetone precipitation did not provide a clear separation (Figure 1B). However, the ammonium sulfate precipitation method did not give a clear background, and gave several large smears (Figure 1 E). These results suggest that precipitation with TCA in acetone is the most efficient protein extraction method, among the five methods, for *U. oxytropis*. It is expected to be a suitable protein extraction method for application in proteomic studies of *U. oxytropis*.

DISCUSSION

In this study, of the five protein extraction methods compared, the precipitation with TCA in acetone seems to be the most efficient, reliable method for 2-DE protein separation for *U. oxytropis*. Precipitation with TCA in acetone is one of the most commonly used procedures for preparation of 2-D electrophoresis samples, and can be more effective than either TCA or acetone alone. TCA in acetone provided a high quality protein profile with an intermediate number of spots and spot intensity with a clear background, indicative that it is a powerful method for further proteomics studies of *U. oxytropis*. The protein spots selected from the gel showed that is compatible with mass spectrometry for proteomic research of *U. oxytropis*.

To determine the identity of proteins that were extracted from 2-DE gels, 5 spots were selected from gels run with samples extracted using the precipitation with TCA in acetone method (Figure 1D). Due to the absence of genomic and proteins databases for *U. oxytropis*, proteins mass spectral data were searched against the swiss prot fungi database for protein identification with the Bioworks browser. Only protein identifications with an xcorr value > 2 were selected (Table 1).

Spot 1 was identified as a glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a multifunctional protein that has been identified to be a prominent target of oxidative stress modifications on the surface of several fungi and Gram-positive pathogens (Bergmann et al., 2004; Pancholi and Fischetti, 1992). GAPDH is not only found in the cytoplasm, but also found in the plasma membrane (Rogalski et al., 1989). It plays a pivotal role in glycolysis to catalyze the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. Furthermore, GAPDH associates tightly with Enolase 2 and Hspa8, as well as in oxidative stress-induced cell death (Bulliard et al., 1997; Nakajima et al., 2009).

Table 2. Comparison of five different protein extraction methods.

	A	B	C	D	E
Protein yield (µg/g fresh wt. fungi)	2300	2900	1900	2000	4400
Total number of spots	156±2.45	254±3.26	292±2.91	357±3.77	192±2.21
Streaking or smearing	None	Not clear	Vertical streaks	Low streaking	Big smear
Separation	Good	Poor	Good	Good	Poor

(A) TCA precipitation, (B) acetone precipitation, (C) precipitation with ammonium acetate in methanol following phenol extraction, (D) precipitation with TCA in acetone, (E) ammonium sulfate precipitation.

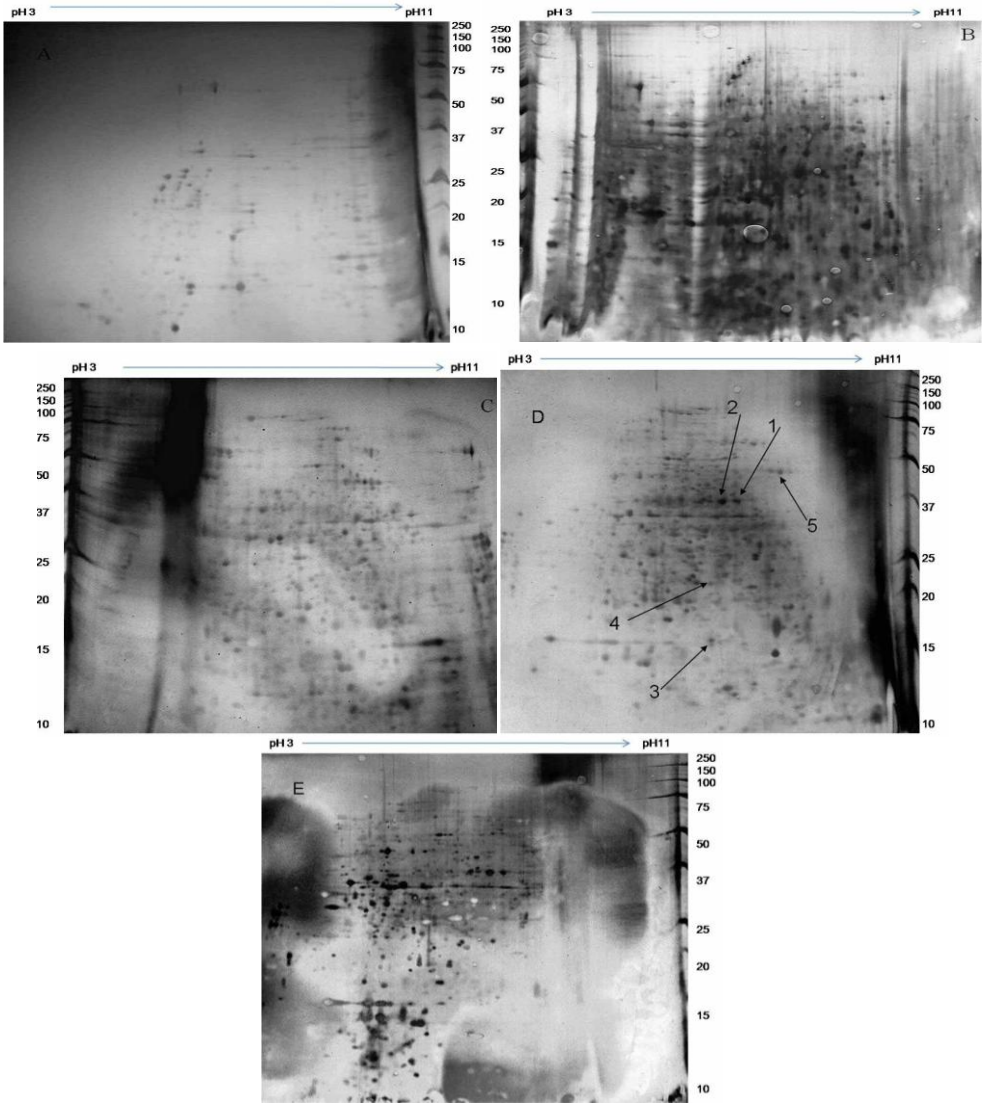

Figure 1. Comparison of the two-dimensional gel electrophoresis images from *U. oxytropis* using different protein extraction methods: (A) TCA precipitation, (B) acetone precipitation, (C) precipitation with ammonium acetate in methanol following phenol extraction, (D) precipitation with TCA in acetone, (E) ammonium sulfate precipitation.

Spot 2 was identified as aspartate semialdehyde dehydrogenase. In *Escherichia coli* aspartate semialdehyde dehydrogenase is active as a homodimer and with a subunit molecular mass of 39 kDa

(Hadfield et al., 2001). This enzyme is involved in lysine biosynthesis which catalyzes the formation of L-2,3-dihydrodipicolinate, and the aspartate semialdehyde can be further reduced to homoserine, which finally leads to decarboxylation to produce lysine (Cahyanto et al., 2006; Hadfield et al., 2001). Early studies on the enzyme indicated that ribonucleic acid (RNA) binding by glyceraldehyde-3-phosphate dehydrogenase, which catalyzes a similar reaction to GAPDH (Biellmann et al., 1980; Faehnle et al., 2005; Holland and Westhead, 1973). Aspartate semialdehyde dehydrogenase is an essential enzyme found in many organisms such as bacteria, fungi, and higher plants. The aspartate pathway is responsible for the biosynthesis of L-lysine; aspartate semialdehyde dehydrogenase catalyzes the initial reactions in the pathway.

Spot 3 identified as superoxide dismutase. The enzymes superoxide dismutases (SODs) play a major role in the defense of aerobic organisms against oxidative stress, and catalyze the breakdown of superoxide into hydrogen peroxide and water (Fridovich, 1995). Cu and Zn SOD are usually found in the cytosol of eukaryotic cells, in the outer mitochondrial space and for plant cells, in the chloroplast (Kawamata and Manfredi, 2008; Krumova et al., 2008; Sturtz et al., 2001). SODs have been reported to play a role in oxidative stress due to heavy metal toxicity in plants. In the context of a symbiotic system, SOD enzymes could influence the plant symbiont (Lanfranco et al., 2005). Information about this enzyme in filamentous fungi is scarce, however, some research on the response of filamentous fungi to oxidative stress has been carried out (Lanfranco et al., 2005; Vallino et al., 2009

Spot 4 was identified as a Woronin body major protein, a specialized peroxisome that is an organelle that prevents cytoplasmic bleeding after hyphal injury (Leal et al., 2009; Tenney et al., 2000). Woronin bodies have been identified in several fungi and are located on the cytoplasmic side of the cell envelope (Collinge and Markham, 1992). Its major function is to maintain the seal of the septal pore in response to cellular damage (Jedd and Chua, 2000; Tenney et al., 2000). Additional, studies will be required to define the exact role of Woronin body in *U. oxytropis*.Spot 5 was identified as homoserine O-acetyltransferase. Homoserine O-acetyltransferase is the first enzyme in the methionine biosynthetic pathway in several organisms such as in gram-positive bacteria of the genus *Bacillus*, fungi, yeast (Anderson and Anderson, 1998; Langin et al., 1986; Saint-Girons et al., 1988).

This enzyme catalyzing the transfer of acetyl group from acetyl-CoA to homoserine (Wang et al., 2007).he complete proteomic analysis in *U. oxytropis* is under progress in our laboratory. It is expected that proteomic studies of *U. oxytropis* will provide insight into our better understanding of the genetics, physiology, biology, ecology, mechanism of swainsonine biosynthesis in fungi and locoweed, and interaction between *U. oxytropis* and its plant host.

ACKNOWLEDGEMENTS

The authors thank Champa Senqupta-Gopalan, Soum Sanogo, Deanna Baucom, Rich Richins, and Jinfa Zhang for equipment and technical assistance. This study was financed in part by USDA Special grant 59-5428-327, New Mexico State University Agricultural Experiment Station, National Natural Science Foundation of China (No. 3087190).

Abbreviations: PDA, Potato dextrose agar; **2-DE,** two dimensional gel electrophoresis; **IEF,** isoelectric focusing; **PDB,** potato dextrose broth; **TCA,** trichloroacetic acid; **EDTA,** ethylene diamine tetraacetic acid; **IPG,** immobilized pH gradient; **UPLC,** ultra-performance liquid chromatography; **FA,** formic acid; **CAN,** acetonitrile; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **SODs,** superoxide dismutases; **RNA,** ribonucleic acid; **FT-ICR,** Fourier transform ion cyclotron resonance.

REFERENCES

Anderson NL, Anderson NG (1998). Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis., 19(11): 1853-1861.

Bergmann S, Rohde M, Hammerschmidt S (2004). Glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pneumoniae is a surface-displayed plasminogen-binding protein. Infect Immun., 72(4): 2416-2419.

Biellmann JF, Eid P, Hirth C, Jornvall H (1980). Aspartate-beta-semialdehyde dehydrogenase from *Escherichia* coli. Purification and general properties. Eur. J. Biochem., 104(1): 53-58.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Bulliard C, Zurbriggen R, Tornare J, Faty M, Dastoor Z, Dreyer JL., (1997). Purification of a dichlorophenol-indophenol oxidoreductase from rat and bovine synaptic membranes: tight complex association of a glyceraldehyde-3-phosphate dehydrogenase isoform, TOAD64, enolase-gamma and aldolase C. Biochem. J., 324 (Pt 2): 555-563.

Cahyanto MN, Kawasaki H, Nagashio M, Fujiyama K, Seki T (2006). Regulation of aspartokinase, aspartatesemialdehyde dehydrogenase, dihydrodipicolinate synthase and dihydrodipicolinate reductase in Lactobacillus plantarum.Microbiology, 152(Pt1): 105-112.

Collinge AJ, Markham P (1992). Ultrastructure of hyphal tip bursting in Penicillium chrysogenum. FEMS Microbiol Lett., 70(1): 49-54.

Englard S, Seifter S (1990). Precipitation techniques. Methods Enzymol., 182: 285-300.

Faehnle CR, Ohren JF, Viola RE (2005). A new branch in the family: structure of aspartate-beta-semialdehyde dehydrogenase from *Methanococcus jannaschii*. J Mol Biol., 353(5): 1055-1068.

Fernandez-Acero FJ, Jorge I, Calvo E, Vallejo I, Carbu M, Camafeita E, Lopez JA, Cantoral JM, Jorrin J (2006). Two-dimensional electrophoresis protein profile of the phytopathogenic fungus *Botrytis cinerea*. Proteomics., 6 Suppl 1: S88-96.

Fridovich I (1995). Superoxide radical and superoxide dismutases. Ann. Rev. Biochem., 64: 97-112.

Gomez-Vidal S, Tena M, Lopez-Llorca LV, Salinas J (2008). Protein extraction from Phoenix dactylifera L. leaves, a recalcitrant material, for two-dimensional electrophoresis.Electrophoresis., 29(2): 448-456.

Gorg A, Weiss W, Dunn MJ (2004). Current two-dimensional electrophoresis technology for proteomics. Proteomics, 4(12): 3665-3685.

Granier F (1988). Extraction of plant proteins for two-dimensional

electrophoresis. Electrophoresis., 9(11): 712-718.
Guengerich FP, Broquist HP (1976). Lysine catabolism in *Rhizoctonia leguminicola* and related fungi. J. Bacteriol., 126(1): 338-347.
Hadfield A, Shammas C, Kryger G, Ringe D, Petsko GA, Ouyang J, Viola RE (2001). Active site analysis of the potential antimicrobial target aspartate semialdehyde dehydrogenase. Biochemis. 40(48): 14475-14483.
Harris CM, Schneider MJ, Ungemach FS, Hill JE, Harris TM (1988). Biosynthesis of the Toxic Indolizidine Alkaloids Slaframine and Swainsonine in *Rhizoctonia-Leguminicola* - Metabolism of 1-Hydroxyindolizidines. J. Am. Chem. Soc., 110(3): 940-949.
Holland MJ, Westhead EW (1973). Chemical reactivity at the catalytic sites of aspartic -semialdehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. Biochemis., 12(12): 2276-2281.
Hurkman WJ, Tanaka CK (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol., 81(3): 802-806.
Isola D, Marzban G, Selbmann L, Onofri S, Laimer M, Sterflinger K (2011). Sample preparation and 2-DE procedure for protein expression profiling of black microcolonial fungi. Fungal Biol., 115(10): 971-977.
Jedd G, Chua NH (2000). A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. Nat. Cell Biol., 2(4): 226-231.
Kawamata H, Manfredi G (2008). Different regulation of wild-type and mutant Cu,Zn superoxide dismutase localization in mammalian mitochondria. Hum. Mol. Genet., 17(21): 3303-3317.
Kim Y, Nandakumar MP, Marten MR (2007). Proteomics of filamentous fungi. Trends Biotechnol., 25(9): 395-400.
Krumova E, Dolashki A, Pashova S, Dolashka-Angelova P, Stevanovic S, Hristova R, Stefanova L, Voelter W, Angelova M (2008). Unusual location and characterization of Cu/Zn-containing superoxide dismutase from filamentous fungus *Humicola lutea*. Arch. Microbiol., 189(2): 121-130.
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat., 227(5259): 680-685.
Lanfranco L, Novero M, Bonfante P (2005). The mycorrhizal fungus Gigaspora margarita possesses a CuZn superoxide dismutase that is up-regulated during symbiosis with legume hosts. Plant Physiol., 137(4): 1319-1330.
Langin T, Faugeron G, Goyon C, Nicolas A, Rossignol JL (1986). The MET2 gene of Saccharomyces cerevisiae: molecular cloning and nucleotide sequence. Gene., 49(3): 283-293.
Leal J, Squina FM, Freitas JS, Silva EM, Ono CJ, Martinez-Rossi NM, Rossi A (2009). A splice variant of the Neurospora crassa hex-1 transcript, which encodes the major protein of the Woronin body, is modulated by extracellular phosphate and pH changes. FEBS Lett., 583(1): 180-184.
McLain-Romero J, Creamer R, Zepeda H, Strickland J, Bell G (2004). The toxicosis of *Embellisia* fungi from locoweed (*Oxytropis lambertii*) is similar to locoweed toxicosis in rats. J Anim Sci., 82(7): 2169-2174.
Meyer Y, Grosset J, Chartier Y, Cleyet-Marel JC (1988). Preparation by two-dimensional electrophoresis of proteins for antibody production: antibodies against proteins whose synthesis is reduced by auxin in tobacco mesophyll protoplasts. Electrophoresis., 9(11): 704-712.
Nakajima H, Amano W, Kubo T, Fukuhara A, Ihara H, Azuma YT, Tajima H, Inui T, Sawa A, Takeuchi T (2009). Glyceraldehyde-3-phosphate dehydrogenase aggregate formation participates in oxidative stress-induced cell death. J. Biol. Chem., 284(49): 34331-34341.
Nielsen DB (1988). Economic impact of poisonous plants on the rangeland livestock industry. J. Anim. Sci., 66(9): 2330-2333.
Obeidat BS, Strickland JR, Vogt ML, Taylor JB, Krehbiel CR, Remmenga MD, Clayshulte-Ashley AK, Whittet KM, Hallford DM, Hernandez JA (2005). Effects of locoweed on serum swainsonine and selected serum constituents in sheep during acute and subacute oral/intraruminal exposure. J. Anim. Sci., 83(2): 466-477.
Oldrup E, McLain-Romero J, Padilla A, Moya A, Gardner D, Creamer R (2010). Localization of endophytic Undifilum fungi in locoweed seed and influence of environmental parameters on a locoweed in vitro culture system. Botany-Botanique., 88(5): 512-521.
Pancholi V, Fischetti VA (1992). A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J Exp. Med., 176(2): 415-426.
Pryor BM, Creamer R, Shoemaker RA, McLain-Romero J, Hambleton S (2009). Undifilum, a new genus for endophytic *Embellisia oxytropis* and parasitic *Helminthosporium bornmuelleri* on legumes. Botany-Botanique., 87(2): 178-194.
Ralphs MH, Gardner DR, Turner DL, Pfister JA, Thacker E (2002). Predicting toxicity of tall larkspur (Delphinium barbeyi): measurement of the variation in alkaloid concentration among plants and among years. J Chem Ecol., 28(11): 2327-2341.
Rampitsch C, Bykova NV, McCallum B, Beimcik E, Ens W (2006). Analysis of the wheat and *Puccinia triticina* (leaf rust) proteomes during a susceptible host-pathogen interaction. Proteomics., 6(6): 1897-1907.
Rogalski AA, Steck TL, Waseem A (1989). Association of glyceraldehyde-3-phosphate dehydrogenase with the plasma membrane of the intact human red blood cell. J. Biol. Chem., 264(11): 6438-6446.
Saint-Girons I, Parsot C, Zakin MM, Barzu O, Cohen GN (1988). Methionine biosynthesis in Enterobacteriaceae: biochemical, regulatory, and evolutionary aspects. CRC Crit Rev Biochem., 23 Suppl 1: S1-42.
Shevchenko A, Wilm M, Vorm O, Mann M (1996). Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal. Chem., 68(5): 850-858.
Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Schoch TK, Coulombe RA, Molyneux RJ (1999). Pyrrolizidine alkaloid plants, metabolism and toxicity. J. Nat. Toxins., 8(1): 95-116.
Sturtz LA, Diekert K, Jensen LT, Lill R, Culotta VC (2001). A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. J. Biol. Chem., 276(41): 38084-38089.
Taylor JB, Strickland JR (2002). Appearance and disappearance of swainsonine in serum and milk of lactating ruminants with nursing young following a single dose exposure to swainsonine (locoweed; *Oxytropis* sericea). J. Anim. Sci., 80(9): 2476-2484.
Tenney K, Hunt I, Sweigard J, Pounder JI, McClain C, Bowman EJ, Bowman BJ (2000). Hex-1, a gene unique to filamentous fungi, encodes the major protein of the Woronin body and functions as a plug for septal pores. Fungal Genet. Biol., 31(3): 205-217.
Tulsiani DR, Broquist HP, James LF, Touster O (1984). The similar effects of swainsonine and locoweed on tissue glycosidases and oligosaccharides of the pig indicate that the alkaloid is the principal toxin responsible for the induction of locoism. Arch. Biochem. Biophys., 232(1): 76-85.
Usuda H, Shimogawara K (1995). Phosphate Deficiency in Maize .6. Changes in the 2-Dimensional Electrophoretic Patterns of Soluble-Proteins from 2nd-Leaf Blades Associated with Induced Senescence. Plant Cell Physiol., 36(6): 1149-1155.
Vallino M, Martino E, Boella F, Murat C, Chiapello M, Perotto S (2009). Cu,Zn superoxide dismutase and zinc stress in the metal-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn. FEMS Microbiol. Lett., 293(1): 48-57.
Wang M, Liu L, Wang Y, Wei Z, Zhang P, Li Y, Jiang X, Xu H, Gong W (2007). Crystal structure of homoserine O-acetyltransferase from Leptospira interrogans. Biochem. Biophys. Res. Commun., 363(4): 1050-1056.
Wickwire BM, Harris CM, Harris TM, Broquist HP (1990a). Pipecolic acid biosynthesis in *Rhizoctonia leguminicola*. I. The lysine saccharopine, delta 1-piperideine-6-carboxylic acid pathway. J. Biol. Chem., 265(25): 14742-14747.
Wickwire BM, Wagner C, Broquist HP (1990b). Pipecolic acid biosynthesis in *Rhizoctonia leguminicola*. II. Saccharopine oxidase: a unique flavin enzyme involved in pipecolic acid biosynthesis. J. Biol. Chem., 265(25): 14748-14753.
Yu Y, Zhao Q, Wang J, Wang Y, Song Y, Geng G, Li Q (2010). Swainsonine-producing fungal endophytes from major locoweed species in China. Toxicon., 56(3): 330-338.