Agrovoc descriptors: verticillium albo atrum, infection, electrophoresis, proteins, plant diseases

Agris category code: H20

COBISS code 1.01

2-D separation of *Verticillium albo-atrum* proteins

Polona JAMNIK[1], Sebastjan RADIŠEK[2], Branka JAVORNIK[3], Peter RASPOR[4]

Received: March 2, 2006; accepted: August 11, 2006.
Delo je prispelo 2. marca 2006; sprejeto 11. avgusta 2006.

ABSTRACT

Verticillium albo-atrum is a phytopathogenic fungus that causes wilt disease in a wide range of crops. A proteomic approach is needed to obtain a comprehensive overview of the proteins related to the infection process. We report here on the optimisation of electrophoretic analysis of total proteins of *V. albo-atrum* in terms of the extraction of fungus total proteins, removal of interfering compounds and separation of proteins by two-dimensional electrophoresis.

Key words: *Verticillium albo-atrum*, proteomics, 2-D electrophoresis

IZVLEČEK

Verticillium albo-atrum je fitopatogena gliva, ki povzroča uvelost pri različnih rastlinah. Analiza proteoma lahko nudi celosten vpogled v mehanizme, ki so vključeni v infekcijski proces. V prispevku je predstavljena optimizacija elektroforetske analize celokupnih proteinov glive *V. albo-atrum* s povdarkom na ekstrakciji celokupnih proteinov, čiščenju ekstrakta in ločevanju proteinov z dvo-dimenzionalno elektroforezo.

Ključne besede: *Verticillium albo-atrum*, proteomika, 2-D elektroforeza

1 INTRODUCTION

Verticillium albo-atrum Reinke & Berthold is an important plant pathogenic fungus causing vascular wilts in many crop species (Engelhard, 1957). In Europe, this soil borne pathogen has caused considerable economic damage on hop (*Humulus lupulus*

[1] University of Ljubljana, Biotechnical Faculty, Food Science and Technology Department, Chair of Biotechnology, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, Assist., Ph.D.

[2] Slovenian Institute of Hop Research and Brewing, Plant Protection Department, Cesta Žalskega tabora 2, 3310 Žalec, Slovenia, Ph.D.

[3] University of Ljubljana, Biotechnical Faculty, Centre for Plant Biotechnology and Breeding, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, Prof., Ph.D.

[4] University of Ljubljana, Biotechnical Faculty, Food Science and Technology Department, Chair of Biotechnology, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, Prof., Ph.D.

L.), especially due to the appearance of highly virulent pathotypes, which induce rapid plant withering, called lethal wilt (Sewell and Wilson, 1984; Radišek et al., 2003). During the colonization of the vascular tissue *V. albo-atrum* produce phytotoxins and several enzymes, including pectinases, polysaccharidases and proteinases, capable of attacking plant cells (Puhalla and Bell, 1981). The activity of these cell wall-degrading enzymes contributes to the induction of disease symptoms and failure of host resistance (Pegg, 1989; Carder et al., 1987). SDS-PAGE and isoelectric focusing (IEF) have been the most commonly used techniques for separating proteins in studies of *Verticillium* species (Mohan and Ride, 1984; Webb et al., 1972; Huang and Mahoney, 1999). A proteomic approach could be a solution to better understanding the variation in virulence in a fungal pathogen population. Comparative proteome analysis is a good strategy for discovering proteins, which undergo changes in expression level and may underlie the differences of phenotype (Chen et al., 2003).

Two-dimensional electrophoresis (2-D electrophoresis) introduced by O'Farrell (1975) is still the most established method in proteomics for studying the properties of proteins in parallel (Kniemeyer et al., 2005). Although it has shortcomings, such as a poor ability to separate proteins with high molecular weight (above 200 kDa), hydrophobic proteins and trace-quantity expressed proteins, immobilized pH-gradient (IPG) strips used in the first-dimensional gel electrophoresis provide a basis for reproducible separation according to the proteins` isoelectric points (Chen et al., 2003). It is a powerful method for the analysis of complex protein mixtures extracted from cells, tissues or other biological samples (Westermeier, 2001).

The applicability of 2-D electrophoresis in filamentous fungal studies has already been proven (Nandakumar and Marten, 2002; Grinyer et al., 2004; Kniemeyer et al., 2005; Shimizu and Wariishi, 2005), but its use has not to date to our knowledge been reported for proteome analysis of phytopathogenic *Verticillium* species.

The aim of our study was therefore to set an appropriate 2-D electrophoresis protocol that can then be used for comparative proteome analysis between different pathotypes of *Verticillium albo-atrum.*

2 MATERIALS AND METHODS

Strain and growth conditions

Verticillium albo-atrum *(hop pathotype PG1) was isolated from infected hop plants with the mild form of hop wilt and maintained in the culture collection of the Slovenian Institute of Hop Research and Brewing, Slovenia, as a monosporic culture on potato dextrose agar (PDA) at 4 °C in the dark.*

The culture was grown in general fungal medium (Weising et al., 1995) by agitation on a rotary shaker (50 rpm) at room temperature for 7 days in the dark. Mycelia were harvested by centrifugation at 2500 x g for 5 min, washed once with 0.9 % solution of NaCl and stored at -80 °C for later use.

Protein extraction

Two different cell lysis protocols were used: grinding (Kniemeyer et al., 2005), and disruption with glass beads (Nandakumar and Marten, 2005) with the following modifications.

Mycelial biomass was frozen in liquid nitrogen and ground to a fine powder. Extraction buffer (40 mM Tris-HCl, pH = 8.0; 2 % (w/v) CHAPS, 65 mM DTT) containing a protease inhibitor cocktail (Roche) (1 tablet per 10 mL of buffer) was added in the ratio of grounded mycelial biomass : buffer = 2 : 1. The mixture was then sonicated for 5 s and centrifuged at 15000 x g for 20 min at 4 °C.

Alternatively, 0.5 g mycelial biomass was suspended in 1 ml extraction buffer (40 mM Tris-HCl, pH = 8.0; 2 % (w/v) CHAPS, 65 mM DTT) containing protease inhibitor cocktail (Roche) (1 tablet per 10 mL of buffer). Cells were disrupted by vortexing with acid washed glass beads (Sigma, diameter: 425-600microns) five times, 1 min each with 1-min intervals for cooling the mixture on ice.

In both cases, the mixture was centrifuged at 15000 x g for 20 min at 4 °C.

The protein concentration in the cell extracts was determined by the method of Bradford (1976) using bovine serum albumin as standard.

The cell extract was used either purified or non-purified for further analysis. Purification was carried out by protein precipitation using a 2-D Clean Up Kit (Amersham Pharmacia Biotech) according to the manufacturer's instruction.

2-D electrophoresis

2-D electrophoresis was performed according to Görg (1991) with minor modifications. Purified or non-purified samples (96 µg protein) were mixed with rehydration solution (9 M urea, 2 % (w/v) CHAPS, 2 % (v/v) IPG buffer, 18 mM DTT, a trace of bromophenol blue) and applied on 13-cm IPG strips, pH 3 – 10 NL, 4 - 7 (Amersham Pharmacia Biotech). Rehydration of IPG strips was carried out for 13h employing an Immobiline Dry Strip Re-swelling Tray (Amersham Pharmacia Biotech). The rehydrated strips were then subjected to isoelectric focusing (IEF), which was carried out at 20 ºC on a Multiphor II (Amersham Pharmacia Biotech). For IPG strips, pH 3 - 10 NL, the following voltage program was applied: 300 V (gradient over 1 min), 3500 V (gradient over 1.5 h), 3500 V (fixed for 4 h). In the case of IPG strips, pH 4 - 7, the voltage protocol was the same, except that the last step was extended to 4.33 h. After focusing, the strips were stored at - 80 °C for later use. Prior to SDS-PAGE, the IPG strips were equilibrated in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8; 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, a trace of bromophenol blue) containing 1 % DTT for 15 min, and containing 4.8 % iodoacetamide for an additional 15 min. SDS-PAGE as the second dimension was carried out with a 12 % running gel on the vertical discontinuing electrophoretic system SE 600 (Hoeffer Scientific Instruments) at constant 20 mA/gel 15 min and then at constant 40 mA/gel until the bromophenol blue reached the bottom of the gel.

2-D gels were visualized by Coomassie G-250 stain using SimplyBlue SafeStain (Invitrogen) or by silver staining using protocol compatible with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS) (Yan et al., 2000). Stained gels were digitalized at 300 dpi and 16 bit gray scale using an Artixscan 1800f scanner (Microtek).

Image analysis, using 2-D Dymension software version 2.02 (Syngene), included spot detection, spot quantification, pattern warping and matching. Gels were done in triplicates, which were matched to create an average gel.

3 RESULTS AND DISCUSSION

The aim of this study was to set an appropriate 2-D electrophoresis protocol for the phytopathogenic fungus *Verticillium albo-atrum.*

Experimentally, 2-D electrophoresis includes many steps, including sample preparation - protein extraction, IPG strip rehydration, isoelectric focusing, IPG strip equilibration, SDS-PAGE and gel staining (Westermeier, 2001). The most critical

step is sample preparation. During this process, it is of great importance to take care of protein modification or degradation. General guidelines exist but due to the great diversity of protein sample types and origins, the optimal procedure must be determined empirically for each sample type. Different mechanical and chemical cell or tissue disruption methods are used, depending on the type and origin of the sample (Scopes, 1994). Since filamentous fungi possess an exceptionally robust and rigid cell wall (Ruiz-Herrera, 1992), effective extraction of intracellular proteins is a key step for fungal proteomic studies (Shimizu and Wariishi, 2005). Several lysis methods have been reported (Nandakumar and Marten, 2002; Kniemeyer et al., 2005; Shimizu and Wariishi, 2005). We compared two lysis methods, which have already been shown to be efficient in fungal proteomics studies; grinding (Kniemeyer et al., 2005) and disruption with glass beads (Nandakumar and Marten, 2002). Results showed that grinding is more efficient with a protein concentration of cell extract of about 3 – 4 g/l, while cell extracts obtained by cell disruption with glass beads contained 0.2 – 0.4 g/l. It has to be mentioned that protein extraction was less efficient when the culture was cultivated for more than 7 days. Grinding was therefore the method of choice for further 2-D analysis.

The ability to analyze a sample effectively by 2-D electrophoresis is often limited by the presence of non-protein impurities in the sample. Polysaccharides, lipids, nucleic acids, ionic detergents and salts can interfere with both first dimension separation and subsequent visualization of the 2-D result. Protein precipitation is therefore an optional step in sample preparation to remove contaminants. In our case, samples were either non-purified or purified using a 2-D Clean Up Kit. Application of 2-D Clean Up Kit resulted in gels of better quality compared to non-purified samples. The spots were clear with less background and very few spots displayed vertical or horizontal streaking compared to non-purified samples, which resulted in gels with streaks, a strong background and faint spots.

For the first-dimension, the IPG strips (pH 3 – 10 NL and 4 – 7) were tested to select the appropriate one, in which proteins were equally separated along the whole gradient. In the second dimension, the appropriate gel percentage was tested. Thirteen cm IPG strips (pH 4 – 7) and a second dimension covering the 7.4 – 203 kDa range were selected to get a wide insight into the fungal proteome .

The detection of proteins in the gel depends on the staining technique employed. There are several techniques, which differ in terms of the type of dye binding to the protein, sensitivity and other parameters (Westermeier, 2001). Few proteins were observed using Coomassie G-250 stain, in spite of its fairly low sensitivity (> 7 ng). It is thus not appropriate for further studies on comparative proteome analysis between mild and lethal forms of *Verticillium albo-atrum*. 2-D results obtained from silver stained gels using a protocol compatible to MALDI TOF MS showed a large number of protein spots.

In summary, the best 2-D results were obtained when we used grinding for protein extraction, cell extract purification using a 2-D Clean Up Kit, IEF with IPG strips (pH = 4 – 7), SDS-PAGE covering the 7.4 – 203 kDa range and silver staining compatible MALDI TOF MS. The spots were clear with less background and very few spots displayed vertical or horizontal streaking. In addition, no precipitation of

proteins during gel running and gel staining was observed. 2-D gel image analysis by 2-D Dymension software detected 1055 spots using a spot filtering parameter (height > 10000) (Figure 1).

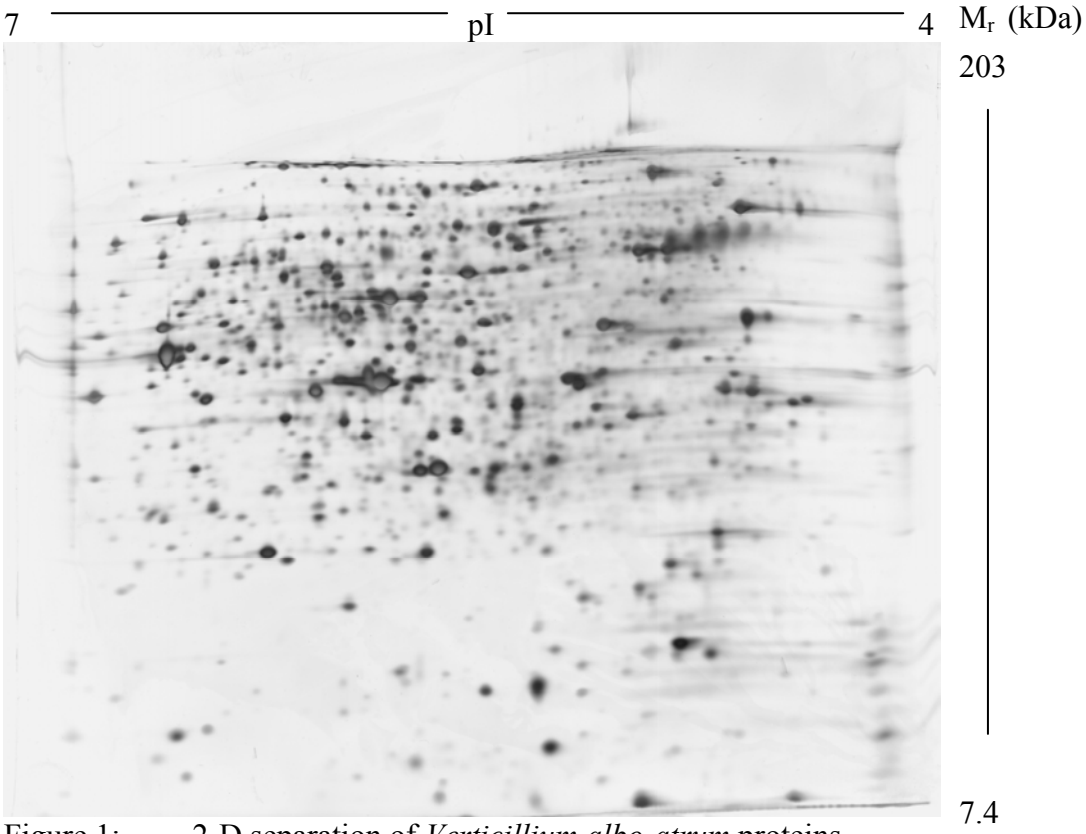

Figure 1: 2-D separation of *Verticillium albo-atrum* proteins

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