Optimization of a protein extraction technique for fungal proteomics

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Abstract Protein extraction is a critical step in any proteomics study. Since most fungi possess a robust cell wall, efficient isolation of total proteins has become challenge to fungal proteomics. To circumvent this bottleneck of fungal proteomics, we standardized a protocol named as Mg/CHAPS extraction by comparing with an established method of protein extraction (Tris/EDTA extraction), using 2-DE and MALDI-TOF MS. Total mycelial proteins were isolated using both protocols from *Magnaporthe grisea* (causal agent of rice blast disease). Six hundred forty two proteins were resolved on two 2-DE gels corresponding to mycelial proteomes isolated by Mg/CHAPS and Tris/EDTA. Mycelial proteome extracted by Mg/CHAPS showed higher number protein spots than to Tris/EDTA. Quantitative analysis of mycelial proteome, histogram and MS analyses of a protein spot suggested that Mg/CHAPS extraction is more effective than the widely used protocol i.e. Tris/EDTA.

Keywords Fungal proteomics · Mg/CHAPS extraction · Tris-EDTA extraction · Protein extraction techniques · Mycelial proteome

Introduction

With the completion of the sequencing of over 40 fungal genomes [1], the monitoring of global changes in proteome is an increasingly attractive method for dissecting the molecular basis of fungal development (infection related morphogenesis like appressorium formation and maturation), fungal-plant interactions, pathogenesis and fungal colonization of plants. Now a major challenge in modern fungal biology is to understand the expression, function and regulation of the entire sets of proteins encoded by fungal genomes. This is the aim of newly emerging field of proteomics, i.e., fungal proteomics, which has been developing rapidly in recent years [2]. Two-dimensional polyacrylamide gel electrophoresis or two-dimensional fluorescence difference gel electrophoresis followed by mass spectrometry [2-DE or 2D-DIGE/MS or MS/MS (MALDI-TOF MS or MALDI-TOF/TOF MS/MS or ESI-MS/MS)] and liquid chromatography coupled with MS (LC-MS/MS) are widely used approaches in fungal proteomics. Sample preparation (extraction of proteins) is the most crucial step for both fungal proteomics approaches because it influences protein yield, biological activity, and the structural integrity of the specific target proteins [3]. Thus, care must be taken while selecting protein extraction protocol. Since most filamentous fungi possess an exceptionally robust cell wall [4], an effective strategy for extracting of proteins is required for fungal proteomic studies.

For total protein extraction, an ideal protocol would reproducibly capture all the protein species in a proteome with low contamination of other molecules. Kim and colleagues [5] used Mg/NP-40 extraction buffer (0.5 M Tris-HCl pH 8.3, 2% v/v NP-40, 20 mM MgCl₂ 2% v/v

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β -mercaptoethanol and 1mM PMSF) to extract total proteins from germinated and appressoria forming conidia of *Magnaporthe grisea*. Later on, Bhadauria and colleagues [2] modified slightly the Mg/NP-40 protocol slightly by replacing NP-40 by CHAPS and β -mercaptoethanol by DTT, and named it as Mg/CHAPS extraction.

In this study, we compared Mg/CHAPS extraction with a widely used protocol of isolating total proteins i.e. Tris/EDTA using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MALDI-TOF).

Methods and materials

Fungal strain and culture conditions The strain P131 used in this study is *M. grisea* wild-type field isolate. A 2.5 cm² square of P131 strain mycelium from the surface of oatmeal agar plate was washed with 4 ml of double distilled water and inoculated this mycelial suspension in 200 ml of complete medium (CM) liquid broth (0.6% w/v yeast extract, 0.3% w/v acid casein hydrolysate, 0.3% w/v enzymatic casein hydrolysate, 1% w/v sucrose) at 28°C for 48 hours with gentle shaking (120 rpm). The mycelia harvested by filtration were then frozen in liquid nitrogen and stored at -80°C until protein extraction.

Mg/CHAPS protein extraction Five hundred milligrams of mycelia were ground into fine powder in liquid nitrogen with a precooled mortar and pestle, and homogenized in 5 ml of ice cold Mg/CHAPS extraction buffer containing 0.5 M Tris-HCl (pH 8.3), 2% w/v CHAPS, 20 mM MgCl₂, 2% w/v DTT and 1 mM PMSF. After centrifugation at 12000×g for 15 min at 4°C, the supernatant was precipitated with 4 volumes of ice chilled 10% w/v TCA in acetone with 0.007% w/v DTT. After centrifugation at 3000×g for 10 minutes, the pellet was washed with ice chilled acetone with 0.007% w/v DTT thrice. The pellet was then resuspended in rehydration buffer [7 M Urea, 2 M Thiourea, 4% w/v CHAPS, 2%v/v IPG buffer (pH 3-10L) and 20 mM DTT].

Tris/EDTA protein extraction Five hundred milligrams of mycelia were ground into fine powder in liquid nitrogen with a precooled mortar and pestle, and homogenized in 5 ml of ice cold Tris/EDTA extraction buffer containing 10mM Tris-HCl (pH 8.0), 1 mM EDTA, 2% w/v PVPP and protease inhibitors (like 1mM PMSF, aprotinin, leupeptin and pepstatin). After centrifugation at 8000 rpm for 30 minutes at 4°C, the supernatant was precipitated with 4 volumes of ice chilled 10% w/v TCA in acetone with 0.007% w/v DTT. After centrifugation at 3000×g for 10 min, the pellet was washed with ice chilled acetone with 0.007% w/v DTT thrice. The pellet was then resuspended in rehydration buffer.

Cataloguing mycelial proteins by two-dimensional electrophoresis (2-DE) The 2-DE was performed with Ettan IPGphor system (Amersham Biosciences, Sweden) for first dimension isoelectric focusing (IEF). IPG strips (pH 3-10 Linear, 18 cm) were rehydrated overnight in reswelling tray in 340 μ l of rehydration buffer containing total proteins isolated from 500 mg of fungal mycelia. Focusing was performed through 1 hour at 300 V (Step and Hold mode), 2 hours at 500 V (Step and Hold mode), 6 hours at 1000 V (Gradient mode), 3 hours at 8000 V (Gradient mode) and 1 hour 10 minutes at 8000 V (Step and Hold mode). Before the second dimension, the gel strips were first equilibrated under gentle shaking for 15 min in equilibration buffer (Tris-HCl pH8.8, 6M Urea, 30% v/v Glycerol, 2% w/v SDS, 0.002% w/v Bromophenol Blue and 1% w/v DTT) and then in equilibration buffer containing 2.5% w/v iodoacetamide (IAA) in place of DTT. Second dimension separations (on 12.5% Polyacrylamide SDS gels) were carried out with Ettan Dalt Six (Amersham Biosciences, Sweden). The 2-D gels were silver stained.

Image scanning and analysis The silver-stained gels were scanned at 600 dots per inch (dpi) resolution using Image Scanner (Amersham Biosciences, Sweden). The transparency mode was used to obtain a grayscale image. The image analysis was performed with ImageMaster 2D-Platinum software version 5.0. The optimized parameters were as follows: saliency 25 and minimum area 10.

In-gel digestion Protein spots were excised into 1 mm pieces and placed into low-binding Eppendorf tubes. Spots were then washed with deionized water for 10 minutes with intermittent vortexing and destained twice for 15 minutes with intermittent vortexing with 200 μ l of freshly prepared 1:1 solution of 100 mM sodium thiosulfate and 30 mM potassium ferricyanide. Supernatants were discarded. Slices were washed with ultra pure (deionized) water and equilibrated for 10 minutes with intermittent vortexing in 200 μ l 25 mM ammonium bi sulfate then dehydrated twice for 10 minutes vortexing with 200 μ l 25 mM NH₄HCO₃/ 50% v/v acetonitrile (ACN). Supernatants were poured off. After drying in speed vacuum centrifuge for 10 minutes, the gel slices were then rehydrated for 15 minutes at 4°C in 25 μ l Trypsin (Sigma, Proteomics Grade) in 25mM NH₄HCO₃ (pH 8–8.5) and overlaid with minimum amount of 25 mM NH₄HCO₃ to keep them immersed throughout the digestion and incubated 16–24 hours at 37°C. Digest solution was transferred to a new low binding microcentrifuge tube. Digested peptides were extracted with one volume (40–50 μ l) 0.1% v/v TFA in 50% v/v ACN by vortexing 20 minutes at room temperature. This solution was combined with digest solution.

MALDI-TOF MS analysis The resulting peptide mixtures were desalted using Zip-Tip C18 (Millipore), and

eluted onto stainless steel MALDI target. Two μ l sample on the target were mixed with 1 μ l of 10 mg/ml CHCA (α -Cyano 4-Hydroxy Cinnamic Acid) in 0.1% v/v TFA. Samples were then allowed to dry at room temperature. The MS analysis was conducted by MALDI-TOF/TOF mass spectrometer (Auto Flex II, Bruker Daltonics) in the positive ion reflector mode. Calibration was carried out using a standard peptide mixture. The mass spectrometric data (peptide mass fingerprints) were collected from monoisotopic peaks falling in the m/z range of 750–4000 Da.

Database search Peptide mass fingerprint (PMF) data were utilized for protein identification by analyzing the sizes of tryptic fragments via the MASCOT (Matrix Science) search engine using the entire NCBI fungal protein database. For effective PMF analysis, it was assumed that peptides were monoisotopic and the possibility that methionine residues were oxidized was considered. The fingerprinting method allowed for a maximum of one missed tryptic cleavage per protein. The maximum deviation permitted for matching the peptide mass values was 100 ppm. Scores greater than 78 were considered to be significant ($p < 0.05$).

Results and discussion

A useful, interpretable, and reproducible 2-D gel electrophoresis separation rests on the appropriate selection of protein extraction strategy [3]. Because of genome sequence of rice blast fungus *M. grisea* is available in the public domain (<http://www.broad.mit.edu/annotation/fungi/magnaporthe>), so proteomic analysis/identification of the MALDI-TOF data is relatively easy to perform. Therefore, we employed proteomics approach on *M. grisea*. In this study, we standardized and established an extraction strategy by comparing a widely used protocol (Tris/EDTA) of extracting total proteins with a method (Mg/CHAPS) of Bhadauria and colleagues [2], using 2-DE and MALDI-TOF MS. For that, a 3–10 (linear mode) immobilized pH gradient in the first dimension of 2-DE was used. A total of 4 gels per extraction were run for samples representing mycelial proteome of *M. grisea* wild type strain P131. Four reproducible gels were selected for image analysis by ImageMaster 2D platinum software version 5.0 (Amersham Biosciences, Sweden). Fig. 1(a) shows two 2-DE gel images of mycelial proteomes isolated, using Mg/CHAPS extraction (A) and Tris/EDTA extraction (B).

Over 442 protein spots with molecular weight between 14.4 and 97 kDa and a pI ranging from 3 to 10 were resolved on both gels (spots were identified by ImageMaster Platinum software). However, this number was reduced to 342 by ignoring very faint spots, spots with undefined shapes and areas where electrophoresis caused irregularity in spot

pattern. Among 342 detected proteins, 200 were expressed on gel-A and rest 142 on gel-B. This shows that Mg/CHAPS extraction is more efficient to catalogue proteome on 2-DE gels than Tris/EDTA extraction. As we know that there are many difficulties encountered in the analysis of proteins in a biological sample. The most obvious is the inherent complexity of many samples. Many proteins are present in locations that prohibit easy analysis, such as membrane-bound proteins which are difficult to solubilize. Not all proteins are present in equal abundance, a concept known as dynamic range [6]. This leads to a difficulty in detection of low-abundance proteins in the presence of highly abundant ones. Unlike RNA-based methods of transcript amplification, sample protein levels cannot be increased to facilitate analysis of low-abundance proteins [7]. Membrane proteins play a crucial role in many key processes, including exchange and recognition processes. But, analysis of membrane proteins remained a major challenge for proteomics techniques based on 2-DE because of their solubilization. The introduction of zwitterionic detergents [8, 9] like CHAPS has made it possible to display membrane proteins on 2-D gels. Therefore, to address this bottleneck of resolving membrane proteins, we incorporated zwitterionic detergent like CHAPS in extraction protocol to enhance isolation and solubilization of membrane proteins. Nevertheless, no protocol is perfect, but Mg/CHAPS extraction could be more effective to resolve low abundance and membrane proteins on 2-DE gels. This might be the reason underlying the detection of 1.4-fold more protein spots on 2-DE gel (Gel A) of mycelial proteome as compared to gel B (Tris/EDTA).

To determine protein amount per spot, we selected a protein spot (spot 1) of same isoelectric point (pI) and molecular weight (Mr) on each gel and made expression histograms for both protein spots by ImageMaster 2D platinum software version 5.0 using % intensity (Figure 1b). Histogram analysis elucidated that protein spot 1 isolated by Mg/CHAPS extraction buffer showed % intensity of 0.499 as compared to 0.248 of the same protein by Tris/EDTA extraction buffer. This proves that Mg/CHAPS extraction brings about more protein output than the Tris/EDTA.

To investigate whether mycelial proteome is accurately represented by 2-DE gels, we excised spot 1 from both gels (A and B), in-gel digested with trypsin and identified by database search using peptide mass finger prints (PMF) obtained from MALDI-TOF MS. Both spots matched to trihydroxynaphthalene reductase (T3HNR), Chain A with 48% (Spot 1- Mg/CHAPS) and 40% (Spot 1- Tris/EDTA) mass fingerprint coverage (Table 1). T3HNR is a principal enzyme of melanin biosynthesis. This melanin provides *M. grisea* colony, a typical grayish colour and is required for appressorium maturation. Melanization of appressoria is

required for the penetration of rice leaf (Howard & Valent, 1996). This demonstrates that the reduction in spot number on Tris/EDTA extracted proteome gel B was due to the absence of low abundance proteins. Therefore, we conclude

that extraction procedure performed with Mg/CHAPS enhances the coverage and detection of certain groups of proteins such as membrane proteins and low abundant proteins.

Fig. 1 (a) Mycelial proteomes of *M. grisea* isolated by using Mg/CHAPS (Gel A) and Tris/EDTA (Gel B) extractions. Proteins were separated by 2-DE. In the first dimension (IEF), total proteins isolated from 500 mg of fungal mycelia were loaded on 18 cm IPG strips with a linear pH gradient of 3–10. In the second dimension, 12.5% SDS-PAGE gels were used. Proteins were visualized using silver staining. (b) Expression histogram of protein spot 1.

Table 1 Details of protein spot 1 identified by MALD-TOF MS

Spot No.	gi number	Mr (KDa) / pI	Protein name	Mascot score	Sequence coverage	Hit ratio*	Expression level (% intensity)
Spot 1 (Mg/CHAPS)	2624733	28.6 / 6.16	Trihydroxynaphthalene reductase, Chain A	110	48%	9/12	0.499
Spot 1 (Tris/EDTA)	2624733	28.6 / 6.16	Trihydroxynaphthalene reductase, Chain A	87	40%	7/10	0.248

*Hit ratio: Number of peptide masses matched/number of masses submitted to database search.

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