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Preliminary Proteomic Characterisation of Primodia and Vegetative Dikaryotic Mycelial Cells from Tiger's Milk Mushroom (*Lignosus rhinocerus*)

(Kajian Awal Pencirian Proteomik Sel Primordia dan Sel Miselia Vegetatif Dikariot Cendawan Susu Harimau (*Lignosus rhinocerus*)

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ABSTRACT

*Tiger's Milk mushrooms (*Lignosus rhinocerus*) are polypores with three distinct parts: cap (pileus), stem (stipe) and tuber (sclerotium). The stem of this medicinal mushroom is centrally connected to the brownish woody cap that grows out from the tuber underground rather than from the wood. To date, the biotic and abiotic factors that induce the growth of this mushroom are unclear and information regarding its development is scanty. Hence, the differential protein expressions of vegetative dikaryotic mycelial and primordial cells of this mushroom were investigated. Six two dimensional-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D-SDS-PAGE) of 13 cm with pH3-10 containing the intracellular proteins of vegetative mycelial and primordial cells of* L. rhinocerus *were obtained. Analysis of 2D-SDS-PAGE using Progenesis Samespot version 4.1 yielded approximately 1000 distinct protein spots in the proteome of vegetative mycelial cells, while primordial proteome contained nearly 100 spots. Further comparison between the vegetative mycelial and primordial proteomes yielded significant up-regulation of protein expression of 5 primordial cells proteins that were labeled as P1, P2, P3, P4 and P5. These protein spots were excised, trypsin digested and submitted to mass spectrometry. Protein identification through MASCOT yielded significant identification with P1 and P2 as DnaJ domain protein, P3 and P5 as hypothetical protein while P4 as AP-2rep transcription factor. The present results suggested that P3, P4 and P5 are novel proteins that involved in the initiation of* L. rhinocerus *primordia. Our findings also suggested that stress response mechanism is present during fruitification of this mushroom.*

Keywords: Dikaryotic mycelia; Lignosus rhinocerus*; mass spectrometry; primordia; 2D-SDS-PAGE*

ABSTRAK

*Cendawan Susu Harimau (*Lignosus rhinocerus*) merupakan polipori yang terdiri daripada tiga bahagian utama iaitu payung (pileus), batang (stipe) dan umbi (sclerotium). Cendawan bernilai yang sering digunakan dalam perubatan tradisional ini mempunyai batang yang menyambungkan payung cendawan yang berwarna coklat dengan umbi yang tumbuh dari bawah tanah. Setakat ini, faktor biotik dan abiotik yang mendorong pertumbuhan cendawan ini masih tidak diketahui dan maklumat berkenaan perkembangannya adalah terhad. Maka, kajian ini dijalankan untuk mengenal pasti pengekspresan protein daripada sel vegetatif dikariot miselia dan sel primordia yang terlibat dalam pertumbuhan. Enam gel elektroforesis dua dimensi-natrium sulfat dodesil-poliakrilamid (2D-SDS-PAGE) bersaiz 13 cm dengan pH3-10 yang mengandungi protein intrasel daripada sel vegetatif dikariot miselia dan sel primordia* L. rhinocerus *telah berjaya diperoleh. Analisis 2D-SDS-PAGE menggunakan Progenesis Samespot (Versi 4.0) telah menghasilkan lebih kurang 1000 bintik protein daripada proteome sel vegetatif miselia manakala proteom sel primordia mengandungi lebih kurang 100 bintik protein. Perbandingan antara proteom sel vegetatif miselia dengan sel primordia mendapati terdapat lima bintik protein yang menunjukkan pengekspresan protein yang ketara daripada sel primordia yang dilabel sebagai P1, P2, P3, P4 dan P5. Bintik protein ini dipotong, dihadam dengan tripsin dan dihantar untuk analisis jisim menggunakan spektrometri jisim. Kelima-lima protein ini berjaya dikenal pasti melalui MASCOT dan P1 serta P2 dikenal pasti sebagai protein domain DnaJ, P3 serta P5 sebagai protein andaian manakala P4 sebagai protein faktor transkripsi AP-2rep. Hasil kajian mencadangkan bahawa P3, P4 dan P5 merupakan protein novel yang terlibat dalam inisiasi primordia* L. rhinocerus. *Di samping itu, hasil kajian juga menunjukkan kehadiran mekanisme tindak balas tekanan semasa pembuahan cendawan ini.*

Kata kunci: Dikariot miselia; Lignosus rhinocerus*; primordia; spektrometri jisim; 2D-SDS-PAGE*

INTRODUCTION

Lignosus is a macrofungal genus belongs to Polyporaceae family in phylum Basidiomycota. Genus *Lignosus* comprises of 6 species which are *L. dimiticus, L. ekombitii, L. goetzii, L. rhinocerus, L. sacer* (Douanla-Meli & Langer 2003; Núñez & Ryvarden 2001; Ryvarden & Johansen 1980) and *L. hainanensis*, which was discovered recently in tropical forest of Hainan Province, southern China (Cui

et al. 2011). *L. rhinocerus* is a type of white-rot fungus that is found in China, Malaysia, Sri Lanka, the Philippines, Australia and East Africa (Huang 1999a).

In Malaysia, *L. rhinocerus* or locally known as Tiger's Milk Mushroom, is the most popular and specifically sought after medicinal mushroom by the Semai aborigines upon request by urban middlemen (Lee et al. 2009). The mature fruit body of *L. rhinocerus* consists of 3 distinct parts which are cap (pileus), stem (stipe) and tuber (sclerotium) and this medicinal mushroom has been used for the treatment of cough, fever, chronic hepatitis, gastric ulcer, liver and breast cancer, food poisoning and as tonic to maintain body health (Lee et al. 2009). Guo et al. (2011) reported that the sclerotial polysaccharides of this mushroom demonstrate anti-inflammatory, antioxidant, anti-proliferative and immuno-modulating effects. Attempts to cultivate this mushroom have been successful (Huang 1999b; Norlidah et al. 2013). However, information regarding cellular processes leading to the initiation and development of fruit body for this mushroom is scanty. To the best of our knowledge, the proteome profiles of vegetative mycelial cells and primordial cells from *L. rhinocerus* are still unavailable. Thus, this study was conducted to develop two dimensional-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D-SDS-PAGE) reference maps of these proteomes for *L. rhinocerus* and to identify proteins that are expressed abundantly at primordia stage.

Proteomics has been employed for qualitative and quantitative measurements of large number of proteins that directly influence cellular morphology and biochemistry and subsequently provide accurate analysis of cellular protein status or changes during growth, differentiation and response to environmental factors (Böhmer et al. 2007; Kim et al. 2008, 2007a, 2007b). Due to dynamicity and specificity of functions, proteins expressed and their expression levels are changing throughout the entire life cycle of an organism. This approach has been widely applied to study cellular responses of plants and microbes under abiotic stress.

Therefore, investigation on proteins expressed in primordial cells is a crucial step towards better understanding of the fructification of this medicinal mushroom because the transition from dikaryotic mycelial cells to primordia is critical to develop a mature fruit body (Chum et al. 2008). Dikaryotic mycelial cells propagate vegetatively in the presence of adequate nutrients, and under certain environmental stresses, they aggregate to form primordia, which gradually develop into mature fruit bodies with specialized mushroom tissues (Chum et al. 2008).

Materials and Methods

Strain and media used

The fruit bodies and tubers of *L. rhinocerus* were collected from the state of Pahang, Malaysia in June, 2009. Pure cultures of *L. rhinocerus* were obtained by surface sterilized small pieces of pileus, stipe and tuber and then innoculated to potato dextrose agar (PDA) supplemented with streptomycin (200 µg/L). The innoculum were incubated in the dark condition for 15 days at 25°C.

Protein extraction

Mycelial cells and primordial cells of *L. rhinocerus* (10 g) were ground to a fine powder in liquid nitrogen using ceramic mortar and pestle. After transferred to a eppendorf tube, the cell powder (0.5 g) was suspended in 1 mL lysis solution (Urea 8M,3-[(3-Cholamidopropyl) dimethylammonio]-1- propanesulfonate (CHAPS) 4%, immobilline pH gradient (IPG) Buffer 2%, dithiothreitol (DTT) 40 mM and double-distilled water) and 20 µL protease inhibitor. The mixture was homogenized and then shaken in thermomixer (4°C, 1400 rpm, 1 h).The suspensions were then centrifuged at 4°C and 11000 rpm for 30 min. The supernatant was collected and kept in -80°C freezer. The protein concentration was determined by the method of Bradford, using bovine serum albumin (BSA) as standard.

Two-dimensional electrophoresis

For the first dimension isoelectric focusing (IEF), protein samples were solubilized in rehydration solution (8 M Urea, 2% CHAPS, 0.5% IPG buffer pH3-10, 0.28% DTT) and loaded on immobilized pH gradient (IPG) strips, (13 cm, pH3 – 10, GE Healthcare Life Sciences) with concentration of 350 µg/250 µl for in-gel rehydration. After rehydration of IPG strips for 14 h at 20°C, IEF was performed using Ettan IPGPhor II unit (GE Healthcare Life Sciences). IEF was performed following a voltage step-gradient at current of 50 µA per strip at 20°C. The initial step was started at low voltage (500 V) for 1 h, followed by 1000 V for 1 h, 8000 V for 2.5 h and subsequently 8000 V for 0.30 h. After IEF, focused IPG strips were equilibrated for 15 min in SDS equilibration buffer solution (6 M urea, 50 mM Tris-HCl pH8.8, 2% (w/v) SDS, 30% (v/v) glycerol, 60 mM DTT) and then for an additional 15 min in the same solution by substituting DTT with 5% (v/v) iodoacetamide. For second dimension SDS-PAGE, the protein samples were further separated on 12.5% SDS–polyacrylamide gels using SE600 Ruby electrophoresis system (GE Healthcare Life Sciences). The current was setting at 40 mA, 300 V and 100 W for 10 min and 200 mA, 300 V and 100 W until the bromophenol blue dye front migrated 1 cm from the bottom. To visualize the protein spots, the 2D-SDS-PAGE were stained with Coomassie Blue stain overnight. Protein patterns in the gels were recorded as digitalized images using a digital scanner (Image Scanner II Amersham Bioscience, resolution 300 dpi) and saved as melanie (MEL) and tagged image file format (TIFF) files.

Comparative image analysis

Analytical 2D-SDS-PAGE images were imported into Progenesis Same Spot version 4.1 (Nonlinear Dynamics Ltd., UK) for downstream analysis of detected protein spots.

Statistical data for spot detection, spot editing, pattern matching and quantification were performed on triplicate 2D-SDS-PAGE gel images of mycelial and primordial cells of *L. rhinocerus*. The 2D-SDS-PAGE image of mycelia cells was used as the reference gel image to match all the other gel images. Gel images were aligned with reference gel image and artifacts and mismatched spots were removed through manual editing. Normalization of images was carried out before matching with the other gel images to correct the differences in protein spot intensity. Protein spots were identified using percentage volume (% vol) and spot area. A protein spot was defined as up-regulated or down-regulated if the spot % vol changed more than two-fold with *p*-value less than 0.05. The data obtained was validated by principal component analysis.

IN-GEL TRYPTIC DIGESTION AND MASS SPECTROMETRY (MS/MS) ANALYSIS

Protein spots of interest were excised manually from Coomassie Blue stained 2D-SDS-PAGE using pipette tips and extracted from the gels. Sample preparation for MS analysis was according to the methods described by Nejad et al. (2011). After the addition of 10 µL of trypsin digestion buffer into the dried gel pieces, the mixture was incubated at 37°C overnight. For purification of digested proteins, gel pieces were washed for 10 min with 50% acetonitrile (ACN)/100 mM ammonium bicarbonate and pooled with first extract. Tryptic peptides were dried and solubilised in 0.5% formic acid. Then, tryptic peptides were fractionated by nanoflow high performance liquid chromatography (HPLC) on a C18 reverse phase column and eluted with a continuous linear gradient to 40% ACN over 20 min. The elute was analyzed by online electrospray MS/MS using a Qstar pulser (Applied Biosystems).

Four most intense ions were selected and performed in information dependent acquisition (IDA) mode (Analyst QS software, Applied Biosystems) for MS/MS analysis. Standard rolling collision energy settings was used to perform a survey scan of 400 – 1500 Da for 3 s and then followed by MS/MS scans of 50 – 2000 Da for 5 s. After that, masses were added to exclusion list for 3 min. Subsequently, generated peak list were extracted and exported to the database search program MASCOT (Version: 2.3, Matrix Science, UK) for protein identification by peptide mass fingerprinting (PMF). Search parameters within MASCOT and National Centre for Biotechnology Information non-redundant (NCBInr) database (http://www.ncbi.nlm.nih.gov/) were set with mass tolerance for parent ions and fragment ions of 1.2 and 0.6 Da, respectively. The search criteria were one missing trypsin cleavage site, carbamidomethylation of cysteine and oxidation of methionine. The significant levels of statistical analysis for protein identification were detected at $p<0.05$.

RESULTS AND DISCUSSION

Three analytical 2D-SDS-PAGE for both vegetative dikaryotic mycelial cells and primordial cells of *L. rhinocerus* were performed on IPG strips with nonlinear pH range of 3 – 10. As shown in Figure 1(a) and 1(b), representative 2D-SDS-PAGE maps for vegetative dikaryotic mycelial cells and primordial cells showed an appreciably differential protein expressions related to the initiation of fruit body.

In particular, the comparative image analysis of the 2D-SDS-PAGE maps carried out using Progenesis Same Spot version 4.1 showed approximately 1000 distinct protein spots for vegetative mycelia (Figure 1(a)) while those of primordial cells contained more than 100 spots (Figure 1(b)). A decrease in the expression level of many 14–116 kDa proteins occurred in primordial cells.

Principal component analysis (PCA) of protein spots from these two sources indicated two distinct clusters that were evident in different developmental stage of mushroom (Figure 2). These results suggested a higher metabolic activity in vegetative dikaryotic mycelial cells compared to primordial cells of *L. rhinocerus*. In addition, the expression profiles also indicate the involvement of these proteins during these two phases of fructification process. It seems possible that these results were due to fewer expressions of structural genes; increase activity of

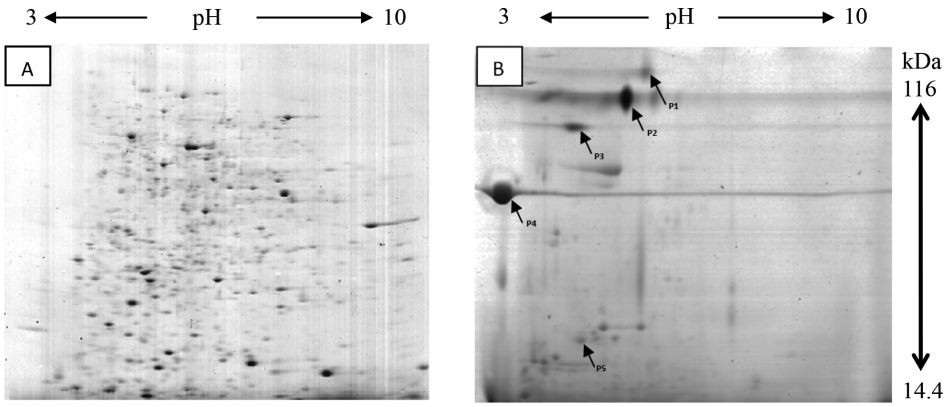

FIGURE 1. 2D-SDS-PAGE profiles of proteins from Tiger's Milk mushroom (*Lignosus rhinocerus*), (a) vegetative dikaryotic mycelial cells and (b) primordia, conducted in 12.5% and pH3-10 gels

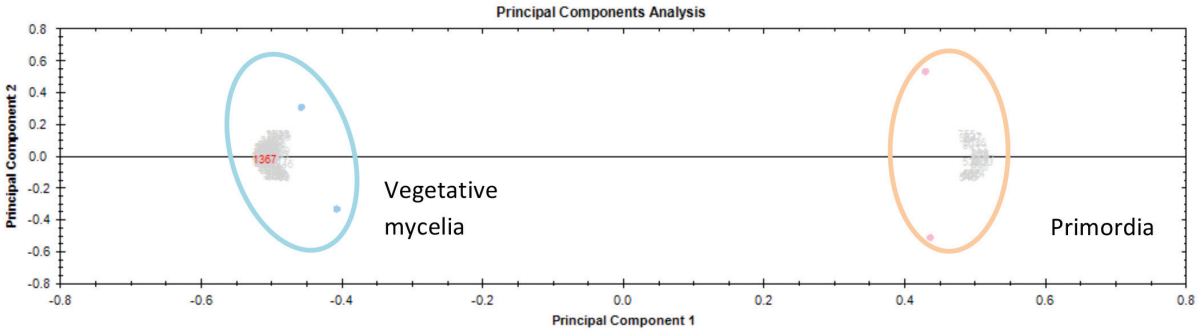

FIGURE 2. Principle component analysis of 2D-SDS-PAGE from vegetative mycelial cells and primordial cells (PCA: 96.24% ; p<0.05)

transcription and translation; and expression of different genes in intracellular trafficking and stress response (Chum et al. 2008).

Identification of proteins specific to differentiation stage had been carried out to gain more information regarding the events leading to basidiocarp development. Protein spots from primordial cells with significant 2 folds up-regulation were excised and used for protein identification through cross-species matching utilizing mass spectrometry data. Of all the protein spots being analysed through Mascot search engine, 5 spots yielded significant protein identification through peptide mass fingerprinting of targeted spectra with NCBInr database (Table 1). Data included NCBI accession number of the best match, MOWSE score, peptide sequence, sources and function of the protein.

Peptide mass fingerprinting of spots P1 and P2 yielded significant identification of protein from 24 h asexual developmental and vegetative of *Aspergillus nidulans* and the submission of P1 and P2 to NCBI protein blast through non-redundant protein sequences database yielded 45% sequence identification to DnaJ domain protein. In addition, peptide mass fingerprinting of spots P3 and P5 yielded significant identification of proteins from *Trichoderma atroviride* culture induced to conidiate by mycelial injury and a protein from *Leptosphaeria maculans* in full-length-enriched mycelium grown for 48 h in Fries liquid medium. Further NCBI protein blast analysis P3 and P5 through non-redundant protein sequences database yielded no similarity to other proteins suggesting these are novel proteins. P4 yielded significant identification of normalized cDNA Expression Library *Aspergillus flavus* cDNA clone NAFFI91 5' end similar to AP-2rep transcription factor.

DnaJ protein is a molecular chaperone that plays a variety of functional roles in cellular processes leading to the acquisition of native conformation of cellular proteins. DnaJ proteins, also known as Hsp40s (heat shock protein 40) defined by an N-terminal conserved domain of about 70 amino acids termed 'J' domain. The 'J' domain mediates the interaction of DnaJ with Hsp70s partners and essential to stimulate the ATPase activity at the ATPase domain of Hsp70. At specific subcellular locations, DnaJs may deliver specific substrates to Hsp70s and/or recruit Hsp70s (Corsi & Schekman 1997; Rudiger et al. 2001). Member of the family of Hsp70 is conserved in organisms of all kingdoms and are found ubiquitously in different cellular compartments of the living cell (Famá et al. 2007). There are several roles performed by Hsp70 chaperone machines that include folding of nascent polypeptides, refolding of denatured proteins, protein translocation across membranes and targeting of misfolded proteins to degradation. Hsp70s bind unfolded or partially folded polypeptides in an ATP-regulated cycle (Bukau & Horwich 1998). Members of the Hsp70 family are strongly up-regulated by heat stress and the current results suggested that heat stress could play a role in the initiation of primordium and presently supported by previous study (Lai et al. 2011) that optimal temperature for mycelial cells of *L. rhinocerus* is in the range 30-35°C.

TABLE 1. Peptide mass fingerprinting of protein spots

Spot number	MOWSE score	Accession number	Protein	Peptide sequence	Sources
P1	57	AA965809	DnaJ domain protein	LATVSIPR	*Aspergillus nidulans*
P2	60	AA965809	DnaJ domain protein	LATVSIPR	*Aspergillus nidulans*
P3	55	GE275168	hypothetical protein	LSIVHGGIRPR	*Trichoderma atroviride*
P4	53	CO144114	AP-2rep transcription factor	ISSPDGISNR	*Aspergillus flavus*
P5	56	FQ039434	hypothetical protein	VDGVSLPR	*Leptosphaeria maculans*

However, the reason underlying the identification of both P1 and P2 as DnaJ protein is unclear.

Peptide mass fingerprinting identify P4 as AP-2rep transcription factor, after normalization of NAFFI91 cDNA clone from *Aspergillus flavus*. AP-2 transcription factors represent a family of three closely related and evolutionarily conserved sequence specific DNA binding proteins, AP-2a, AP-2b, and AP-2g (Moser et al. 1995; Oulad-Abdelghani et al. 1996; Williams et al. 1998). AP-2 transcription factors regulate a number of genes involved in a wide range of important biological functions, including embryonic development (Schorle et al. 1996; Zhang et al. 1996), regulation of programmed cell death (Moser et al. 1997) and cell growth and differentiation (Bryne et al. 1994).

The present results suggested that P3, P4 and P5 are novel proteins that involved in the primordia initiation of *L. rhinocerus*. The existence of these primordia initiation-related enzymes ensures that a stress response mechanism is present during fruitification of this mushroom.

CONCLUSION

The comparison of 2D-SDS-PAGE maps between vegetative dikaryotic mycelial cells and primordial cells evidenced a different protein expression profiles, suggesting the initial phase of the fructification is associated with specific metabolic activity. Identification of DnaJ domain protein and AP-2rep transcription factor have casted new light on the understanding of the roles of chaperone system in regulating cellular activities in fungi and provided new evidences concerning the cellular processes that might take place during the primordia phase of *L. rhinocerus*. The current findings represent a useful step towards the comprehension of events responsible for the fruitification of *L. rhinocerus* and the established 2D-SDS-PAGE maps will be helpful in profiling protein changes during the primordia initiation and against diverse environmental factors in comparative proteomics studies.

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