

Full Length Research Paper

Isolation and characterization of differentially expressed genes in the mycelium and fruit body of *Pleurotus ostreatus*

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The fruiting body of one of the most widely cultivated mushrooms, the oyster mushroom (*Pleurotus ostreatus*) is highly interesting, both commercially and scientifically. In the present study, we performed comparative proteomic profiling of *P. ostreatus* at two unique developmental stages; mycelium and fruit body, using two-dimensional gel electrophoresis (2-DE). Seven hundred fourteen (714) spots were detected and 29 spots (showing a high level of difference in their expressions) were identified by tandem mass spectrometry and basic local alignment search tool (BLAST) searching of an expressed sequence tag (EST) database of *P. ostreatus*. Among them, six proteins (putative fatty acid oxygenase, heat shock *sks2*, *PriA* homologue, Ap-1 like transcription factor YAP7, mung bean seed albumin, and C2H2 Zinc finger domain protein) and one protein (peroxisomal biogenesis factor 6) showed increased expression levels at the fruiting process and the mycelial stage, respectively. Through reverse transcriptase-polymerase chain reaction analysis, *priA* homologue and AP-1 like transcription factor *yap7* showed gradually increased expression from mycelia to fruit body, whereas putative fatty acid oxygenase and heat shock protein *sks2* were expressed only in the fruit body. These results provide useful information for future studies of mushroom development of *P. ostreatus*.

Key words: Developmental stage, mushroom fruiting, *Pleurotus ostreatus*, protein, two-dimensional gel electrophoresis.

INTRODUCTION

The oyster mushroom, *Pleurotus ostreatus*, and white button mushroom, *Agaricus bisporus*, are the most widely cultivated edible mushrooms worldwide (Kues and Liu, 2000; Sánchez 2010). Efficient production of edible

mushrooms relies on the precise control of fruiting body development, and hence identification of the molecular mechanism of fruiting body development has commercial and scientific significance. The functions and medicinal

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Abbreviations: ESTs, Expressed sequence tags; 2-DE, two-dimensional gel electrophoresis; IPG, immobilized pH gradient; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; CSF, cerebrospinal fluid; RT-PCR, reverse transcriptase-polymerase chain reaction.

effects of secretomes and enzymes of *P. ostreatus* on human diseases have been widely studied (Cohen et al., 2002; Kurashige et al., 1997; Rop et al., 2009; Ruiz-Dueñas et al., 2001; Sarangi et al., 2006; Zorn et al., 2005). It is noteworthy that studies of the mushroom itself are rare despite its importance in mycology and its physiology. We previously analyzed and classified 11,761 expressed sequence tags (ESTs) and 4,060 unigenes corresponding to the developmental stages of *P. ostreatus* (Joh et al., 2007; Lee et al., 2002). As shown in other organisms, the EST analyses (Joh et al., 2007; Lee et al., 2002), genetic display (Sunagawa and Magae, 2005), and linkage mapping (Larraya et al., 1999; Larraya et al., 2001; Park et al., 2006) methods have also been reported as useful methods for characterizing genomic contents (for example, coding regions) and identifying the genes involved in *P. ostreatus* development. The physiological functions and putative roles of each significant gene discovered using various approaches have been investigated. However, the current functional and proteomic knowledge is still insufficient to explain mushroom development.

Hydrophobic, ligninolytic, antitumor, anti-inflammatory, and antiviral functions and effects of several extracellular proteins have been studied extensively in *P. ostreatus*. For instance, laccases (Lettera et al., 2010; More et al., 2011; Pakhadnia et al., 2009), lectin (Jedinak et al., 2011; Li et al., 2008), and beta-glucan (Bobek et al., 2001; Kurashige et al., 1997; Rop et al., 2009) obtained from *P. ostreatus* have been frequently analyzed for their ligninolytic activity and medicinal effects. Hydrophobins (Asgeirsdottir et al., 1998; Penas et al., 2002) and metalloprotease (Joh et al., 2004) have been analyzed as the differentially regulated vegetative or fruiting-specific proteins. However, no large-scale proteomic study has been conducted to enhance the information available about *P. ostreatus*, apart from a protein identification study by tandem mass spectrometry for *de novo* sequencing with a database search with different programs; in that study, however, only four proteins of interest were characterized because of low homology with sequence data from different microorganisms (Matis et al., 2005).

In the present study, we generated two proteomic maps of *P. ostreatus* using two-dimensional gel electrophoresis (2-DE) analyses and thereby isolated proteins that were differentially expressed in developmental stages. In order to supplement the current lack of information, protein identification was performed by combining surveys with *P. ostreatus* EST, yeast genome, and Genbank protein databases.

MATERIALS AND METHODS

Strain and culture conditions

P. ostreatus strain ASI 2029 was obtained from Mushroom Research Division, Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 404-707, Korea, and used for all the study experiments. Ve-

getative growth was performed on MacConkey agar supplemented with meropenem (MCM) agar plates (Lee et al., 2002). To obtain fruiting bodies, ASI 2029 strain was inoculated on a growth medium consisting of 570 g poplar sawdust, 120 g rice bran, and 65% water in a 1000-cm³ disposable bottle. The cultures were incubated at 25°C in the dark for 25 to 40 days and then transferred to conditions that induced fruiting (light, 12 to 15°C, 85% humidity). All samples for analysis were immediately frozen in liquid nitrogen and stored at 80°C until use.

Sample preparation

Mushroom samples were ground to a fine powder under liquid nitrogen using a mortar and pestle and stored at -80°C. Protein samples were prepared from mushroom powder using the method of Lametsch et al. (2001). Aliquots of samples (100 mg each) were suspended in 1 mL of lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris, 1% DTT, and protease inhibitor cocktail (Roche Applied Science, Germany). After incubating for 40 min, samples were centrifuged at 10 000 × *g* for 1 h and the resulting supernatants were used as the total protein extract. Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard.

2-DE and image analysis

Approximately, 200 µg of proteins were applied on 17-cm immobilized pH gradient (IPG) strips (3–10 nonlinear, Bio-Rad). IPG strips were rehydrated overnight with a rehydration solution containing 7 M urea, 2 M thiourea, 2% CHAPS, bromophenol blue (a few grains), and 2 mM tributylphosphine. After rehydration, isoelectric focusing (IEF) was performed for total 45,000 Vh using a PROTEAN IEF Cell unit (Bio-Rad) at 100, 200, 500 and 1000 V for 1 h per step, respectively and then the voltage was gradually increased to 8000 V. The current limit was adjusted to 50 mA per strip, and the run was conducted at 20°C. After IEF, IPG strips were incubated for 20 min with 10 mL of equilibration solution consisting of 50 mM Tris-Cl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 5 mM tributylphosphine. The IPG strips were transferred onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12% T, 2.67% C) with a run of 10 mA per gel for 1 h followed by 20 mA per gel until the dye front reached the bottom of the gel. Gels were fixed for 1 h in 50% methanol and 5% acetic acid, and stained with silver using the method of Shevchenko et al. (1996). Stained gels were matched and analyzed with the PDQuest software (Bio-Rad). The maps of mycelia and fruit body were evaluated in separate match sets. In addition, spots consistently present in the remaining gels were added to the master set. Fifteen replicate gels obtained for each group were normalized by the total quantity in valid spots and analysis. Protein quantities were given as parts per million (PPM) of the total integrated optical density of spots in the gels, according to PDQuest. The statistical effects of stages and replication of experiments on the average spot densities were submitted to two-way analysis of variance (ANOVA) using the R statistical package. The intensities were considered statistically significant at the $p < 0.05$ level.

Protein identification

For protein identification, the spots were excised from the silver-stained gels and destained (Gharahdaghi et al., 1999). Digestion was performed with 15 µL of trypsin solution (10 mg/mL trypsin in 25 mM NH₄HCO₃) and incubated overnight at 37°C. After enzymatic digestion, the extraction was repeated twice with 20 µL of 5% trifluoroacetic acid (TFA) in 50% acrylonitrile (ACN) and the three

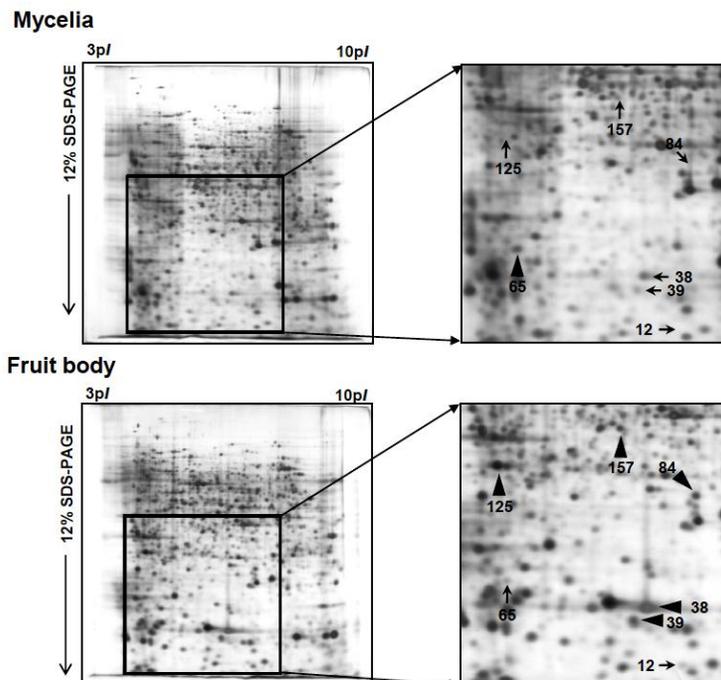


Figure 1. Expression patterns of representative 7 spots in mycelia and fruiting body of *P. ostreatus*. 12, Heme peroxidase; 38, heat shock protein; 39, PriA; 65, peroxisomal biogenesis factor 6; 84, hypothetical protein; 125, mung bean seed albumin; 157, AP-1 like transcription factor YAP7. Arrow heads indicate increased spots; arrows indicate decreased spots.

extracts in the same solution were concentrated through vacuum centrifugation. Sample peptide masses were obtained using the Applied Biosystems 4700 Proteomics analyzer matrix-assisted laser desorption/ionization- time-of-flight (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems, Framingham, MA, USA) in the positive ion reflector mode. MS/MS analysis was performed on the five most abundant ions and the proteins identified by searching the SWISS-PROT and National Center for Biotechnology Information databases using the Mascot programs (Matrix Science, London, UK). Mass accuracy was considered to be within 50 ppm for peptide mass analysis and within 100 ppm for MS/MS analysis. The partial amino acid sequences were obtained by *de novo* peptide analysis methods. These partial sequences were used in searches for homologous sequences in the EST database of *P. ostreatus* (Joh et al., 2007) followed by further analysis with BLAST searches (NCBI BLAST).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from a tissue sample (100 mg) using a TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen Life Technologies, USA). Total RNA (10 µg) was further processed with RQ1 RNase-free DNase (Promega, USA) in accordance with the manufacturer's instructions for RNA extraction. For RT-PCR analysis, the reverse transcription of RNA (1 µg) in a 20-µl reaction volume was performed using oligo-dT18 and ImProm-II™ reverse transcriptase (Promega, USA). Reactions were incubated at 25°C for 5 min, at 42°C for 60 min, and then for 10 min at 70°C to inactivate the reverse transcriptase. The PCR reaction was conducted in a 50-µl reaction mixture containing 10 mM dNTP mixture, 10 pmol of each specific primer, 1 unit Taq-polymerase (TaKaRa

Korea Biomedical Inc., Seoul, Korea), 10 × PCR buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl and 25 mM MgCl₂), and 1 µl cDNA product or 0.5 µg total RNA sample as a control reaction. The mRNA levels of genes were normalized according the mRNA level of the beta-actin gene.

RESULTS

Protein characterization and identification

Seven hundred fourteen spots were counted from each gel using a PDQuest image analyzer. Various parameter settings were tested; however, all results reported approximately the same numbers of spots for nondepleted cerebrospinal fluid (CSF), microbead depleted CSF, and column depleted CSF. All the spots were characterized by measuring their positions and comparative intensities on all the gels using the PDQuest image analyzer; we then selected selected proteins that showed differential expression levels (at least two times more) between the mycelia and the fruiting body (Figure 1). Of the seven proteins, one protein showed a higher expression level in the mycelia sample, whereas the other six proteins showed a higher level of expression in the fruiting body. Expression levels of proteins were evaluated by comparing the densities of silver staining using an image analysis program (PDQuest software).

The expression of protein spot #65 (peroxisomal biogenesis factor 6) expression was 2.9-fold higher in mycelia

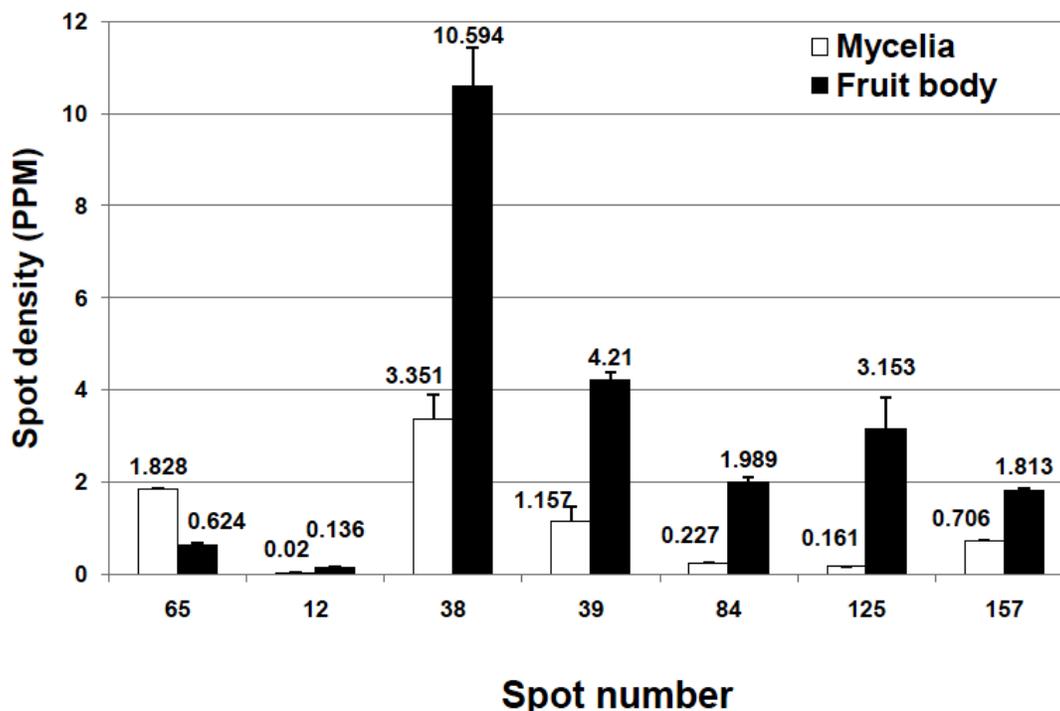


Figure 2. Expression values of 7 representative spots of the mycelia and fruiting body of *P. ostreatus*. 12, Heme peroxidase; 38, heat shock protein; 39, *PriA*; 65, peroxisomal biogenesis factor 6; 84, hypothetical protein; 125, mung bean seed albumin; 157, AP-1 like transcription factor YAP7. The spots were normalized using the total spot volume normalization method. Intensities were considered significant at the $p < 0.05$ level.

than in the fruiting body. The other 6 proteins showed higher expression in the fruiting body with 2.6 to 19.6-fold changes. Spot #125 (Mung bean seed albumin) showed highest (19.6-fold) change in expression in the fruiting body compared to mycelia (Figure 2). Peroxisomal biogenesis factor 6 is the only spot that showed a higher expression in the mycelia stage, although its function is not yet characterized in mushroom development. In the case of humans, peroxisomal biogenesis factor 6 has a role in peroxisomal protein import and is associated with human disease (Matsumoto et al., 2001). To identify other proteins, the derived peptide sequences from mass spectrometry were investigated through translated sequences of all ESTs (Joh et al., 2007) using a local TBLASTN program and BLASTN on Genbank database. Table 1 presents the identified proteins. Protein #39 was associated with the *priA* gene, which was previously reported with higher expression in the immature fruiting stage, and was reported to play a role during the beginning of fruiting of *Lentinus edodes* (Kajiwara et al., 1992). The other 5 proteins are identified as heme peroxidase, hypothetical protein, Mung bean seed albumin (Liu 1993), AP-1 like transcription factor YAP7 (Fernandes et al., 1997), and heat shock protein. Among them, Mung bean seed albumin and AP-1 like transcription factor YAP7 are putatively implicated in the environmental stress response, reproduction process, and cellular meta-

bolism and functions, but with no clear function in mushroom development.

RT-PCR

The primers based on the EST sequences corresponding to proteins were used for quantitative RT-PCR to test mRNA expressions in the two developmental stages (Table 2). The mRNA purifications were normalized by beta-actin gene analysis of the two stages. The *priA* homologous gene exhibited high expression in the mature fruiting process, but low expression in the mycelial stage. AP-1 like transcription factor YAP7 showed increased expression in fruit body. Although these two genes showed low expressions in the mycelia, heme peroxidase and heat shock protein were expressed only in the fruit body. Mycelia-specific protein (the peroxisomal biogenesis factor 6) could not be tested because its DNA sequences were not available in the database. The other two fruiting-specific proteins failed the RT-PCR assays. This might be due to the sequence differences between genes in *P. osteratus* and genes in the database. Nevertheless, mRNA expression analyses of 4 proteins showed the same expression profiles as those of the protein expressions by 2-DE analyses in the developmental stages of *P. ostreatus*.

Table 1. Identification and characterization of differentially expressed proteins between the mycelia and fruiting body of *P. ostreatus*.

Spot no.	Accession no.	Peptide sequences	Protein	Mascot score	e-value	Theoretical MW/pI	Expression level (fold change)	Specificity (stage)
12	EIN04094.1	AAFFNLDVLCPTKKFLHT	Heme peroxidase	-	8.00E-44	-	6.8	Fruit body
38	EIW60561.1	RKWYDNWYHE	Heat shock protein	-	1.00E-23	-	3.2	Fruit body
39	gi18033194	AYAQWVVIIIHNVGSK	PriA	100	-	15126.48/5.87	3.6	Fruit body
65	PEX6_ASHGO	LRPNEGPTK	Peroxisomal biogenesis factor 6	97.8	-	113428.7/5.11	-2.9	Mycelia
84	XP_001838263	VIEPEIR	Hypothetical protein	-	0.009	-	8.8	Fruit body
125	CAA50008.1	AGFTTNKEVFASPR	Mung bean seed albumin	-	2.00E-27	-	19.6	Fruit body
157	YAP7_YEAST	LSKNWELPQR	AP-1 like transcription factor YAP7	95.543	-	27370.12/9.74	2.6	Fruit body

Table 2. Primers used for reverse transcription and polymerase chain reaction (PCR).

Spot no.	Description	Forward (5'-3')	Reverse (5'-3')
12	Heme peroxidase	TCCATTGCCCATGTAGTGAA	CCATACTCGCCCAGTCCTAA
38	Heat shock protein	ACCAAAACCTTTTCCCGACT	AGGCGACGTATGATGGTGTT
39	PriA	ACTCTTACCGTCGCACCATC	CGATCATCCACTTGGTGTTG
157	AP-1 like transcription factor YAP7	TGCATACCAGcGAGTACGAC	CACAACCTTCTTGCTCGTCA
Control	Actin	TGGACAAGTCATCACCATCG	GAAGCACTTGCGATGAACAA

DISCUSSION

The number of proteomic studies based on numerous genetic data has recently increased because of their high accuracy for elucidating the mechanisms underlying physiologic, genetic, and cellular functions. In the present study, we separated over 700 spots, using two-dimensional gel electrophoresis (2-DE). From the result of 2-DE analysis, highly expressed and stage-specific spots were selected and employed for mass spectrometry. In addition, we employed previously constructed EST database for protein identification (Joh et al., 2007; Lee et al., 2002) and supplemented it with a local BLAST program. The local program contains TBLASTN that translates all EST sequences to

corresponding amino acid (AA) sequences and surveys the translated sequences to identify matches with the peptide sequence input. Each spot provided multiple EST sequences due to the several AA sequences generated from the mass spectrometry. We chose an EST sequence that had the highest matching score and surveyed the sequences through the GenBank database (NCBI) for a final identification. Eventually, six chosen spots were identified (Table 1).

Using this methodology, *priA* homologue, which is a well-known developmentally regulated protein in mushrooms (Kajiwara et al., 1992), was identified. This identification of *priA* as a differently expressed protein during mushroom development will facilitate the verification of the methodology

used in this study. mRNA expression profiling was also conducted to verify the results obtained through proteomic profiling during the developmental stages (Figure 3). Moreover, the gene expression profiles at the two developmental stages provide additional information to help understand their roles during mushroom development.

In the present study, we first present the reference images of the expressed proteins of two developmental tissues of *P. ostreatus*. The methods for identification of the proteins using EST data were tested and verified. Eventually, four development-specific genes were identified and characterized. The results of this study indicate that protein analysis with useful genetic data provides valuable information on mushroom develop-

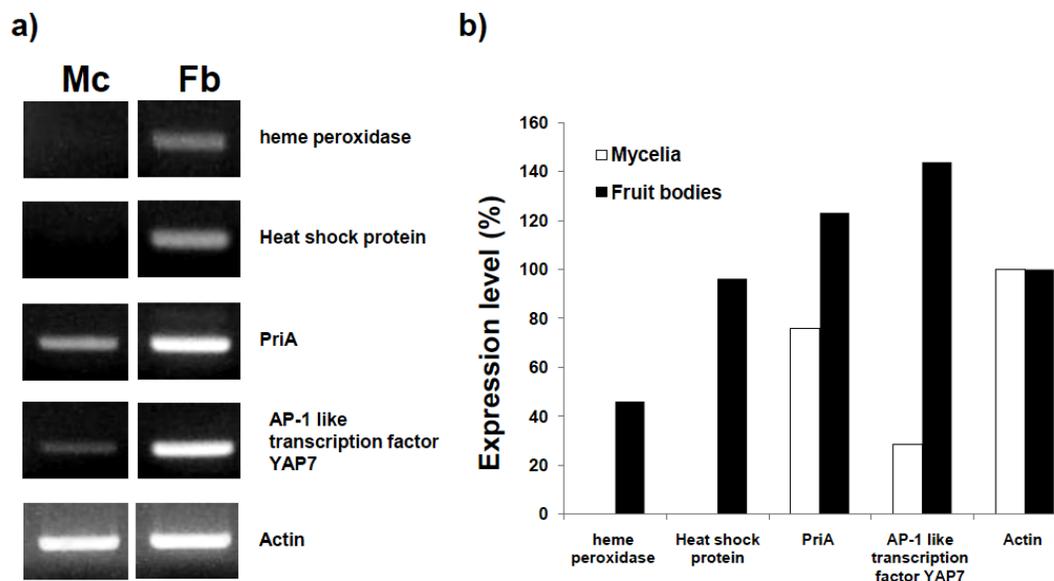


Figure 3. RT-PCR analysis (a) and mRNA expression levels (b) of 4 fruit body-specific genes at two developmental stages of *P. ostreatus*. Mc, Mycelia; Pr, primordia; Fb, fruit body.

ment at the protein level.

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