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Research paper

Proteome analysis provides insight into the regulation of bioactive metabolites in *Hericium erinaceus*

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ABSTRACT

Background: *Hericium erinaceus*, a famous edible mushroom, is also a well-known traditional medicinal fungus. To date, a large number of bioactive metabolites with antitumor, antibacterial, and immune-boosting effects were isolated from the free-living mycelium and fruiting body of *H. erinaceus*.

Objective: Here we used the proteomic approach to explore proteins involved in the regulation of bioactive metabolites, including terpenoid, polyketide, sterol and etc.

Results: Using mass spectrometry, a total of 2543 unique proteins were identified using *H. erinaceus* genome, of which 2449, 1855, 1533 and 690 proteins were successfully annotated in Nr, KOG, KEGG and GO databases. Among them, 722 proteins were differentially expressed (528 up- and 194 down-regulated) in fruiting body compared with mycelium. Most of differentially expressed proteins were putatively involved in energy metabolism, molecular signaling, and secondary metabolism. Additionally, numerous proteins involved in terpenoid, polyketide, and sterol biosynthesis were identified. Our data revealed that proteins involved in polyketide biosynthesis were up-regulated in the fruiting body, while some proteins in mevalonate (MEP) pathway from terpenoid biosynthesis were generally up-regulated in mycelium.

Conclusions: The present study suggested that the differential regulation of biosynthesis genes could produce various bioactive metabolites with pharmacological effects in *H. erinaceus*.

1. Introduction

Hericium erinaceus, called as lion's mane mushroom or bearded tooth fungus, is also a traditional edible and medicinal fungus in Asian countries since ancient times. Previous study suggested that many compounds with nutraceutical and pharmaceutical activities were isolated and identified from *H. erinaceus* (Lu et al., 2014). Some evidences demonstrated that this fungus could produce unusual pharmacological effects, including anti-tumor, anti-microbial, anti-oxidant, anti-aging, anti-hyperglucemic, anti-hypercholesterolemic, immune modulating and neuroprotective activity (Wang et al., 2001; Dong et al., 2006; Lee and Hong, 2010; Kim et al., 2013; Wu et al., 2018). Recently, a number of clinical evidences indicated that it had strong potential to combat Alzheimer's disease, menopause, diabetes, and cancer (Shen et al., 2014; Hiraki et al., 2017; Wu et al., 2018). Thus, this mushroom has attracted considerable attention on various pharmaceutical and clinical properties.

The isolation and identification of chemical compositions in *H. erinaceus* have been successively reported in recent years. Meanwhile, a great variety of bioactive compounds were isolated from the major tissues of *H. erinaceus*, the fruiting body and the mycelium. As high molecular weight compounds, polysaccharides in *H. erinaceus* were found mainly to enhance the immunomodulatory activity on macrophages (Ren et al., 2017; Wu et al., 2018). In general, the fruiting body contains a higher quantity of polysaccharide than the mycelium (Dong et al., 2006). In addition, > 80 kinds of bioactive metabolites have been described in the previous studies, mainly including terpenoids, polyketides, sterols and phenols (Lu et al., 2014). The diverse metabolites with low molecular weight and poor water solubility were related to antitumor, anti-microbial, hypoglycemic and neuroprotective effects. For example, both hericenones (phenols) and erinacines (terpenoids) which were respectively obtained from the fruiting body and mycelium of *H. erinaceus*, could promote nerve growth factor biosynthesis, culture astrocyte and increase neuroprotective capability (Bhandari et al., 2014; Chen et al., 2017). In particular, erinacines are also well-known unique cyathane diterpenoids. In detail, 5-6-7 tricarbocyclic, a D-xylose moiety anchored onto the cyathane framework, is present in all cyathane diterpenoids (Shen et al., 2015).

Abbreviations: DEP, differentially expressed protein; GGPP, geranylgeranyldiphosphate; TS, taxadiene synthase; ent-CPS, ent-copalyl diphosphate synthase; ent-KS, ent-kaurene synthase; PKS, polyketide synthase

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Although the chemical synthesis of bioactive compounds of *H. erinaceus* has been elucidated (Enquist Jr. and Stoltz, 2009), the pathway involved in biosynthesis of bioactive metabolites is limited by the lack of research. With the development of sequencing techniques, the genome and transcriptome of *H. erinaceus* were published for the first time last year (Chen et al., 2017). In the study, an iTRAQ proteomic approach was used to identify the protein expression in different tissues from *H. erinaceus*, including fruiting body and mycelium. Our results could provide an improved understanding of the biosynthesis pathway or key sites involved in bioactive metabolites, especially, terpenoids and polyketides. Moreover, iTRAQ-based proteomic analyses could also validate the gene expression profiles of the fruiting body and the mycelium from transcriptomic data.

2. Materials and methods

2.1. Microbiological materials

The mycelium of *H. erinaceus* (deposited in our lab) was incubated on fresh Potato Dextrose Agar (PDA: potato infusion, boiling 200 g potato in 1000 ml water; 20 g dextrose; 15 g agar powder) at 22–24 °C for 2 weeks in the dark. The fruiting body was grown on solid medium (cotton seed hull 83%; wheat bran 15%; sucrose 1%; gypsum 1%; the ratio of material to water is 1:1.4) at 20–23 °C for 5–6 weeks. The mycelium and the fruiting body were carefully collected, frozen in liquid nitrogen and immediately stored at −80 °C.

2.2. Protein extraction, digestion and iTRAQ labeling

Protein extraction, digestion, iTRAQ labeling, and mass spectrometry were conducted using protocols from previous paper (Xing et al., 2014). Briefly, total proteins were extracted from each sample using Fungus Total Protein Extraction Kit BB-3136 (Bestbio, China) according to the manufacturer's instructions. Bradford method was selected to determine the protein content. Proteins of each sample were detected by 10% SDS-PAGE. Each of the samples (75 μg in total) was deoxidized with 20 mM DTT, alkylated with 50 mM IAA and digested with trypsin.

To increase the accuracies of the project, three biological replicates were included in each sample group. The peptide samples were labeled using the iTRAQ 8-plex kit (AB sciex, USA) according to the manufacturer's protocol. Then, the fruiting bodies and the mycelium (control group) were labeled by 113–115 and 116–118 iTRAQ, respectively. After labeling, the equal amounts of each sample were mixed together and lyophilized.

2.3. SCX chromatography separation, nano-LC-MS/MS analysis

The dried peptide mixture was dissolved by buffer A (10 mM KH_2PO_4 in 25% of ACN, pH 3.0) and loaded onto Luna SCX column (250 × 4.60 mm, 5 μm, 100Å, Phenomenex, USA). Eluent was collected every minute, pooled into 16 pools, desalted on Strata-X C18 Cartridges (Phenomenex, USA) and dried under vacuum.

Desalted peptide mixture was analyzed on a nanoLC Eksigent 425 system coupled to a TripleTOF 6600 mass spectrometer (AB SCIEX). The nanoLC system comprised a Nano Trap column (350 μm × 0.5 mm ChromXP C18-CL; 3 μm; 120 Å) and an analytical column (75 μm × 15 cm ChromXP C18-CL; 3 μm; 120 Å). Peptides were separated using a linear 65 min, set flow rate to 300 nl/min. The mass spectrum conditions for Triple TOF 6600 was set as follow: The spray voltage was set at 2.4 kV and the temperature of heater was 150 °C. The mass spectrum scan range was *m/z* 350–1250 and the tandem mass spectrometry (MS/MS) scan range was 100–1500 m/z.

2.4. Database search and functional annotation

Raw proteome data were searched using the Proteinpilot™ v4.0 search engine with percolator against the *H. erinaceus* genome database (Genome project: PRJNA361338; Transcriptome project: PRJNA361340) and Russulales proteins database, respectively. In detail, 6512 protein sequences were obtained from *H. erinaceus* genome. Russulales database were downloaded from Uniprot (http://www.uniprot.org/), including 36,096 protein sequences. The average iTRAQ ratios and standard deviations were calculated for each protein using all of the available treatment control iTRAQ pairs. A 1.5-fold cutoff was used to determine up- or down-regulated proteins, with a *P*-value of < 0.05.

Functional annotation of the proteins was performed using Nr, KOG, GO and KEGG annotation. The KOG protein database is a tool for identifying ortholog and paralog proteins. Gene ontology (GO) provides the most comprehensive resource currently available for computable knowledge regarding the functions of genes and gene products. KEGG is a database for recoding the collection of high-level functions and the utility of the biological system.

2.5. Quantitative PCR

Total RNA of mycelium and fruiting body samples was extracted using RNeasy® Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Primers designed with Primer Premier 5.0 are shown in Supplementary Table S1. PrimerScript™ RT reagent Kit (TaKaRa, Japan) was used for reverse transcription. First, 1 μl RT product diluted with 20 μl ddH_2O was used as a template. Then, qPCR was performed in 15 μl reaction mixture containing 7.5 μl of 2× SYBR® Premix Ex Taq™ II (TaKaRa, Japan), 1 μl of cDNA template, and 0.3 μl of each gene specific primers and 5.9 μl ddH2O. Overall, we preformed three biological replicates and three technical replicates using the LightCycler® 480 II RT-PCR System (Roche, Switzerland). The parameters for the reactions were: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The reference 18S rRNA gene was used as an internal control. The $2^{-\Delta\Delta Ct}$ method was used for evaluating gene expression.

3. Results

3.1. Protein extraction

For efficient protein extraction, a simple and fast method was employed to guarantee minimal protein loss and degradation. As shown in Fig. 1, the mycelium and the fruiting body samples presented several well-resolved protein bands on SDS-PAGE 10% bisacrylamide gels. Most of proteins showed molecular weights ranging from 10 to 100 kDa. > 400 μg total proteins were extracted from each sample. In total, the method for *H. erinaceus* protein extraction was efficient and could be useful to apply in the following processes.

3.2. Proteome profiles

Fortunately, a total of 9895 gene models were predicted from the genome of *H. erinaceus* (39.35 Mb). Here, these gene models from *H. erinaceus* genome were used for establishing a self-built database (6512 protein sequences). In addition, all proteins from Russulales in Uniprot (36,096 protein sequences) were downloaded and formed Russulales database. In our results, 2543 and 1230 proteins were identified and quantified from our self-built database and Russulales database at a false discovery rate (FDR) of 1%. By analyzing, our self-built database was obviously suited for this proteomic analyses.

Gene annotation in *H. erinaceus* genome was applied against the NCBI Nr, KOG, KEGG and GO databases. In total, ~96% of all proteins were successfully annotated in at least one database. Among them, a total of 2449, 1855, 1533 and 690 proteins were annotated in Nr, KOG,