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Comparative SELDI-TOF-MS profiling of low-molecular-mass proteins from *Lignosus rhinocerus* (Cooke) Ryvarden grown under stirred and static conditions of liquid fermentation

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ABSTRACT

Mushrooms are considered as important source of biologically active compounds which include low-molecular-mass protein/peptides (LMMP). In this study, we attempted to profile the LMMP from *Lignosus rhinocerus*, a wild medicinal mushroom, grown by static cultures (SC) and in stirred tank reactor (STR). Crude water extract (CWE) and protein fractions were profiled using H50 ProteinChip® arrays and SELDI-TOF-MS. Three protein peaks of 5.8, 6.9 and 9.1 kDa were found to be common to spectra of *L. rhinocerus* CWE from both culture conditions. Partial protein purification has resulted in detection of more peaks in the spectra of protein fractions. For protein fractions of *L. rhinocerus* cultured in STR, most peaks were observed in the range of 3–8 kDa whereas some peaks with molecular mass up to 14.3 kDa were noted in spectra of protein fractions from SC. Our results have demonstrated the optimization of profiling method using SELDI-TOF-MS for fungal LMMP.

1. Introduction

Wild mushrooms are collected mainly by the indigenous community for culinary and medicinal purposes. *Lignosus rhinocerus* (Cooke) Ryvarden, also known as the "tiger's milk mushroom", has been traditionally utilised for the treatment of cough, fever, chronic hepatitis, gastric ulcer, liver and breast cancer based on ethnobotanical knowledge (Chang and Lee, 2004; Wong and Cheung, 2008; Lee et al., 2009). Chang et al. (2005) noted that this mushroom has more than 15 uses according to different tribes of rural and indigenous communities in Peninsular Malaysia. This polyporale is characterised by the formation of a huge sclerotium hidden underneath the soil while the sporophore is supported by a hard and woody stem which emerged from the buried sclerotium (Pegler, 1997). Previously, it has been reported that extracts from *L. rhinocerus* sclerotium exhibited *in vitro* antiproliferative effect on leukemic cells (Lai et al., 2008) and immunomodulatory activity (Wong et al., 2011). The rarity of this species in nature renders the collection of sporophores and sclerotia difficult. Cultivation of this species by solid-substrate fermentation takes a long time and results in low yield.

Liquid fermentation has been proposed as an alternative method for the production of mycelia biomass and fungal metabolites. Mycelia will be grown in vessel and optimum condition necessary for maximum yield e.g. suitable carbon and nitrogen sources, pH and temperature will be provided. Large-scale industrial fungal fermentation is carried out via shallow fermentation in trays under static condition or submerged fermentation in stirred tank reactor (STR). In static (or surface) cultures (SC) where aeration and agitation were absent, fungi grow in the form of mycelia mat. For submerged fermentation in STR, mycelial pellets are exposed to different degree of aeration and agitation (Rhodes and Fletcher, 1966).

Mushrooms are regarded as good source of proteins; previous work has shown that fungal protein/peptide(s) which exhibited biological activities usually comprise of those with low molecular mass (Lee et al., 2004; Wang and Ng, 2004; 2006). The ability of fungi to colonise diverse substrates and their saprotrophic nature are attributed to the secretion of a broad array of hydrolytic enzymes for digestion of nutrient sources. While protein secretion is a crucial physiological process in all fungi/mushrooms, there is little information available on the molecular aspects. Though production of fungal enzymes by fermentation is routinely used in the industry, there is little work done to systematically study the protein expression in fungi grown under different growth conditions.

Proteomic approaches are suitable for the study of how protein expression in an organism changes under various environmental conditions. Surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) which combines chromatography and mass spectrometry, allows "on-chip profiling" on ProteinChip® arrays with different surface chemistries. Hence, only proteins that interact with chemical surface of arrays are retained for analysis. This permits partial characterisation of proteins and rapid analysis of large

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number of samples. In recent years, the use of SELDI-TOF-MS to investigate biological phenomena has increased and diversified. While SELDI-TOF-MS has been extensively used for biomarker discovery in the medical field, there are also some reports on its application in microbial protein expression (Hodgetts et al., 2004), bioprocess monitoring (Berrill et al., 2010) and food analysis (Kosters et al., 2010).

Owing to its high sensitivity, it might be feasible to use SELDI-TOF-MS for analysis of low-molecular-mass proteins (LMMP) from mushrooms. Hence, the objective of this study was to develop a suitable method for SELDI-TOF-MS profiling of LMMP from *L. rhinocerus* grown by liquid fermentation under different culture conditions.

2. Materials and methods

2.1. Materials and chemicals

The axenic culture of *L. rhinocerus* (KUM61075) used in this study was obtained from tissue culture of a sclerotium collected from its natural habitat in Kenaboi Forest Reserve, Negeri Sembilan, Malaysia. Mycelia cultures were maintained by periodic subculture on malt extract agar (Oxoid) and deposited in Mushroom Research Centre, University of Malaya. Spent yeast was obtained from a local brewery while brown sugar was purchased from a supermarket in Petaling Jaya, Selangor, Malaysia. The energy absorbing molecules (EAM) solution used in sample preparation for SELDI-TOF-MS, α-cyano-4-hydroxy-cinnamic acid (CHCA), was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Other chemicals such as ammonium sulfate, trifluoroacetic acid (TFA), methanol and acetonitrile (gradient grade for liquid chromatography) were purchased from Merck (Darmstadt, Germany).

2.2. Liquid fermentation of L. rhinocerus

For SC of *L. rhinocerus*, 100 mL of liquid media consisting of brown sugar (4.0%), spent yeast (2.0%) and malt extract (Oxoid) (2.0%) was prepared in 500 mL Erlenmeyer flasks, sterilised and inoculated with 10 mycelia plugs measuring 9 mm in diameter cut from the periphery of a 14 days-colony using sterile cork borer. Flasks were incubated at 25 °C under static condition. Inoculum for STR was prepared by inoculating 6 mycelia plugs into 100 mL Erlenmeyer flasks containing 50 mL sterile liquid media as above. The flasks were stoppered with non-adsorbent cotton plugs and incubated at room temperature on a rotary shaker shaking at 150 rpm for 7 days. A 5 L STR containing 3 L of the same media was prepared and sterilised before inoculated with 10% (v/v) liquid inoculum. Fermentation in STR was carried out at room temperature under sufficient aeration at 200 rpm.

2.3. Preparation of crude water extracts

Mycelia biomass cultured in SC and STR were harvested after 14 days. Contents of the cultures were first homogenised in distilled water at a ratio of 1:2 (v/v) for 10 s using a commercial Waring blender. The suspension was then vacuum filtered using a muslin cloth of pore size 25 μm for separation of mycelia biomass from the water extract. The crude water extract (CWE) was then lyophilized (Labconco) and kept at 4 °C for analysis.

2.4. Partial protein purification

The CWEs of *L. rhinocerus* were redissolved in distilled water at the ratio of 1:10 (w/v) and centrifuged at 5000 rpm for 5 min to remove solid impurities. Appropriate amount of solid ammonium sulfate to achieve desired saturations (0–100%) was weighed. Protein precipitation was carried out by gradual addition of salt to the redissolved CWEs under stiring condition at 4 °C until the salt was fully dissolved. After equilibrium was reached, CWEs containing dissolved salts were centrifuged at 8000 rpm for 15 min at 4 °C. The pellets were resuspended in 4 mL of distilled water and subjected to dialysis using SnakeSkin™ Pleated Dialysis Tubing (Thermo Scientific). These protein fractions were dialysed against distilled water under stirring condition for 48 h. Dialysed protein fractions were stored at −20 °C.

2.5. Protein estimation

Protein content of the fractions was determined using Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) according to the recommended protocols from the manufacturer. Absorbance readings were taken with Sunrise™ ELISA reader (Tecan, Switzerland). Protein content for each protein fraction was estimated from the standard curve of albumin.

2.6. SELDI-TOF-MS analysis

CWEs and protein fractions of *L. rhinocerus* were analysed using reversed-phase or hydrophobic H50 ProteinChip® arrays (Bio-Rad Laboratories, Inc.). Initially, the arrays were equilibrated in binding buffer, 0.5% TFA in 50% acetonitrile. Samples (5 μL) containing 0.5 μg of protein were then spotted on the arrays. The arrays were air-dried before 2 μL of saturated EAM solution, CHCA in 0.5% TFA (v/v) in 50% acetonitrile (v/v), was added. Again, arrays were allowed to air-dry before analysed with the ProteinChip SELDI System (PSC 4000) (Bio-Rad Laboratories, Inc.). Data collection was carried in positive ion mode using the following acquisition settings: mass range of 0 to 20 kDa, focus mass of 10 kDa, matrix attenuation of 500 Da and sampling rate of 800 MHz. Laser energy used for shot sequences were based on the following settings: warming shot 1 000 nJ (1) and data shots 910 nJ (10). Spectra were calibrated using external calibration against a mixture of standards consisting of somatostatin (1 637.9 Da), arg-insulin (5 963.8 Da) and cytochrome c (bovine) (12 230.92 Da).

2.7. Statistical analysis

Statistical analysis was performed using the ProteinChip Data Manager 3.5 (Bio-Rad Laboratories, Inc). Baseline subtraction and normalisation on total ion current were performed for all spectra according to default protocol. Peaks with m/z values <2.5 kDa were excluded from analysis as these peaks were mainly ion noise from the matrix. For Expression Difference Map (EDM) analysis, spectra were compiled and qualified peak mass peaks with mass-to-charge ratios (m/z) between 2.5 and 15 kDa were auto-detected. Peak clusters were completed using second-pass peak selection with S/N >2, within 0.1% of mass window and allowing estimated peaks to be added. *p*-values were determined using mean peak intensities from triplicate samples with $p<0.05$ was considered as statistically significant.

3. Results

3.1. Comparison of mycelia growth under different culture conditions

In SC, *L. rhinocerus* pellets fused to form a flat, white pellicle with brown colouration in the centre which eventually became convoluted. Entire surface of the media was covered by mycelia mat in approximately 14 days. Growth in the form of small pellets was observed when *L. rhinocerus* was cultured in STR.

3.2. Comparison of yield of protein fractions

From the lyophilized CWEs of *L. rhinocerus*, a total of twenty protein fractions were obtained using different salt saturations (0–100%). For each fraction, protein content was estimated by the BCA protein assay and its yield (expressed as μg of protein precipitated per g of extract) was determined. As shown in Fig. 1, protein yield of the fractions varied