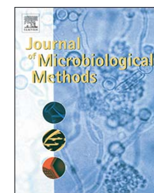
Journal of Microbiological Methods 142 (2017) 63–70

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth



Preparation of intracellular proteins from a white-rot fungus surrounded by polysaccharide sheath and optimization of their two-dimensional electrophoresis for proteomic studies

Takahito Watanabe[a,*], Koichi Yoshioka[a,b], Ayako Kido[a], Junseok Lee[a], Hikari Akiyoshi[a], Takashi Watanabe[a]

[a] Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Japan
[b] Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

A R T I C L E I N F O

Keywords:
Ceriporiopsis subvermispora
Polysaccharide sheath
Proteomics
Two-dimensional electrophoresis
White-rot fungi

A B S T R A C T

The functions and properties of fungal sheath, an extracellular polysaccharide produced by many white-rot fungi, have been studied. However, the strong adherence of the sheath to fungal hyphae had been a major impediment in preparing intracellular proteins from the fungi and analyzing their cellular responses. To overcome this issue, we developed a rapid and easy method to remove the polysaccharide sheath using a selective lignin degrader, *Ceriporiopsis subvermispora*, which produces large sheath amounts in the presence of a lignin-derived aromatic compound. Using this approach, we achieved thorough removal of sheath and cell disruption using beads and a solution with a high protein-solubilizing power, which enabled the efficient extraction of intracellular proteins from *C. subvermispora* surrounded by sheath. In addition, for proteomic analysis, we investigated whether these extracted proteins were compatible with two-dimensional electrophoresis. By efficiently concentrating on protein solubilization in the first dimension and using a stacking gel in the second dimension, we successfully obtained a high-resolution proteome map of *C. subvermispora*. We also used the same proteins for fluorescence two-dimensional difference gel electrophoresis to obtain the quantitative protein expression profiles. These steps demonstrated that two-dimensional electrophoresis-based proteomics can be used to clarify the composition of intracellular proteins from sheath-producing white-rot fungi.

1. Introduction

White-rot fungi are basidiomycetes that can degrade and mineralize lignin. The importance of molecular characterization of ligninolysis and its biotechnological application in white-rot fungi have been previously studied (Teeri, 2004). For the past several decades, studies on lignin degradation of white-rot fungi have focused on extracellular proteins such as ligninolytic and cellulolytic enzymes (Hammel and Cullen, 2008; Manavalan et al., 2015). Although the characterization and expression analyses of these extracellular enzymes are important, intracellular metabolic systems are also directly or indirectly involved in the complete mineralization of lignin. For example, the generation of high redox potentials and the biosynthesis of low-molecular-weight compounds such as organic acids and lipid-related metabolites play a significant role in mediating the triggering of wood decay and behave synergistically with ligninolytic enzymes (Wariishi et al., 1992; Kuan and Tien, 1993; Galkin et al., 1998; Urzua et al., 1998; Enoki et al., 1999; Gutierrez et al., 2002; Watanabe et al., 2002; Rahmawati et al., 2005; Nishimura et al., 2008, 2012a, 2012b). Therefore, the characterization and expression of intracellular enzymes in white-rot fungi can also provide us with a better understanding of the molecular mechanism of lignin degradation.

Many studies adopted proteomic approaches for obtaining expression profiles of intracellular and extracellular enzymes produced by white-rot fungi and for identifying proteins, peptides, or their post-translational modifications (Abbas et al., 2005; Shimizu et al., 2005; Sato et al., 2007; Matsuzaki et al., 2008; Vanden Wymelenberg et al., 2009; Mahajan and Master, 2010; Hori et al., 2014a, 2014b). These proteomic-based findings have contributed to a more detailed understanding and better use of the biological degradation of lignocellulose in white-rot fungi. However, as described above, extracellular expression profiles, also referred to as secretomes, have been focused on, rather than intracellular ones. The reasons for this may include the fact that secretomes contain many proteins such as ligninolytic and cellulolytic enzymes that are involved in lignocellulose degradation and that there are now proteomic methods that can be used to investigate

* Corresponding author at: Laboratory of Biomass Conversion, Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan.
E-mail address: takahito@rish.kyoto-u.ac.jp (T. Watanabe).

http://dx.doi.org/10.1016/j.mimet.2017.09.009
Received 21 July 2017; Received in revised form 11 September 2017; Accepted 11 September 2017
Available online 12 September 2017
0167-7012/ © 2017 Elsevier B.V. All rights reserved.

basidiomycete secretomes (Alfaro et al., 2014). The preparation of intracellular proteins from white-rot fungi can be challenging with respect to the quantity and quality, compatibility with two-dimensional electrophoresis (2-DE), and downstream applications, depending on the species employed, growth medium, and growth conditions. Some studies (Shimizu and Wariishi, 2005; Fragner et al., 2009) the following possible causes of these challenges: i) many white-rot fungi possess exceptionally robust cell walls; ii) contamination by fungal metabolites, salts, lipids, nucleic acids, and polysaccharides, including cell walls and polyphenols from wood meal cultures, can occur, some of which cause various problems when extracellular proteins are prepared; and iii) some membrane proteins and posttranslationally modified proteins remain insoluble during the entire 2-DE procedure.

Some white-rot fungi grown on natural substrates and/or glucose-containing medium under submerged conditions produce large amounts of extracellular polysaccharide—also referred to as adherent glucan, exopolysaccharide, extracellular mucilaginous material, fungal sheath, and hyphal sheath—covered with fungal hyphae; many researchers have reported and discussed the structures, physiological properties, and function of this extracellular polysaccharide, hereinafter referred to as "sheath" (Evans et al., 1991; Burns et al., 1994; Gutiérrez et al., 1995; Manzoni and Rollini, 2001; Rosado et al., 2003; Wagner et al., 2004; Vesentini et al., 2007; Scherba and Babitskaya, 2008). Because sheath contains cellulolytic and ligninolytic enzymes, organic acids such as metal chelators and lipid-related metabolites, it has been indicated that, in some species of white-rot fungi, their sheaths might play an important role in their ligninolysis (Evans et al., 1991; Gutiérrez et al., 1995). In the extraction/preparation of intracellular proteins from the sheath-producing fungi, the sheath needs to be completely removed because fungal mycelia surrounded by the sheath are difficult to disrupt and also the failure of cell disruption results in poor protein yield. However, to the best of our knowledge, no reports have described the effective preparation of intracellular proteins from white-rot fungi producing large amounts of sheath.

We recently reported that a selective lignin-degrading white-rot fungus, *Ceriporiopsis subvermispora*, produces polysaccharide sheath when it grows on a glucose-containing medium supplemented with wood blocks (Suzuki et al., 2017). In this study, we aimed to develop a rapid method of removing adherent sheath from this fungus, enabling the effective preparation of its intracellular proteins. We also examined whether the same proteins were fully separated on as part of a 2-DE-based proteomic set-up for *C. subvermispora*.

2. Materials and methods

2.1. Fungal cultures

Ceriporiopsis subvermispora ATCC 90467 was purchased from American Type Culture Collection and grown on a potato dextrose agar (PDA) plate at 28 °C for 5 d. Using a cork borer (7.0-mm diameter), four pieces from the PDA plate, which was completely covered with the fungus, were cut off and added into 200 mL of synthetic dextrose (SD) medium [2% glucose and 0.67% yeast nitrogen base without amino acids (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA)] and the SDW medium [an SD medium containing eight wood blocks (20 × 10 × 10 mm) of Japanese beech (*Fagus crenata*)], respectively. These cultures were incubated at 28 °C and 70% relative humidity in the dark under static conditions for 2 weeks.

Two milliliters of sterilized water was added directly to a PDA plate completely covered with the fungus, and the mycelium was gently scraped from the surface of the plate using a rubber scraper. Five hundred microliters of the mycelial suspension was inoculated on a 300-mL Erlenmeyer flask containing 50 mL of BIII medium and was then statically preincubated at 28 °C in the dark for 1 week. The recipe of the BIII medium as previously described (Kirk et al., 1986) was modified, and it contained the following per liter: D-glucose, 10.0 g; $(NH_4)_2SO_4$, 4.0 g; KH_2PO_4, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.53 g; $CaCl_2{\cdot}2H_2O$, 0.101 g; nitrilotriacetic acid, 15 mg; NaCl, 10 mg; $MnSO_4{\cdot}5H_2O$, 5 mg; $CoSO_4{\cdot}7H_2O$, 1 mg; $CuSO_4{\cdot}5H_2O$, 1 mg; $FeSO_4{\cdot}7H_2O$, 1 mg; thiamine-HCl, 1 mg; $ZnSO_4{\cdot}7H_2O$, 1 mg; $AlK(SO_4)_2{\cdot}12H_2O$, 0.1 mg; H_3BO_3, 0.1 mg; and $NaMoO_4{\cdot}2H_2O$, 0.1 mg. One milliliter of the pre-culture was added to 47 mL of fresh liquid BIII medium and incubated statically at 28 °C and 70% relative humidity in the dark for 1 week. After the 1-week incubation, 2 mL of filter-sterilized 50 mM vanillin (4-hydroxy-3-methoxybenzaldehyde) was added to the culture, and the incubation was continued for an additional week under the same conditions.

2.2. Preparation of intracellular proteins from a sheath-producing fungus, C. subvermispora

Fungal hyphae thickly covered with sheath—they looked like gelatinous slime—were harvested by filtration through one layer of Miracloth (Merck Millipore, Darmstadt, Germany). The harvested slime was transferred into a 500-mL glass beaker, and then supplemented with distilled and deionized water until the total volume reached 250 mL; this was followed by the addition of 250 mL of ethanol. After the mixture had been thoroughly stirred to remove sheath from the slime, the resultant mycelia were harvested by filtration through one layer of Miracloth again. The Miracloth was placed mycelium-side-up onto a large absorbent paper towel to absorb maximum liquid. The final mycelial pellet was weighed and stored at − 80 °C until further use.

Approximately 50 mg of fungal mycelia (cells) was transferred into a 2-mL self-standing screw cap tube, and then three zirconia beads (5-mm diameter) and 300 μL of a solubilization solution as described below were added. Using the Beads crusher μT-01 (TAITEC Corporation, Saitama, Japan), the fungal mycelia were disrupted at 4600 rpm for 1 min at room temperature. After centrifugation at 12,000 × g for 15 min at 16 °C, the supernatant was treated with the polyacrylamide gel electrophoresis (PAGE) clean-up kit (Nacalai Tesque, Kyoto, Japan). The proteins were dissolved in two solubilization solutions, respectively: i) standard solubilization solution, 7 M urea, 2 M thiourea, 3% CHAPS, 1% Triton X, 0.5% IPG buffer (GE Healthcare, Buckinghamshire, England, UK), and 18 mM DTT; and ii) optimized solubilization solution, 7 M urea, 2 M thiourea, 2% CHAPS, 1% ASB-14 (Merck Millipore), 2% IPG buffer, and 100 mM DTT. The protein concentration was determined using the 2D Quant Kit (GE Healthcare).

2.3. The optimized 2-DE protocol

An Immobiline DryStrip [pH 4–7, 11 cm] (GE Healthcare) was rehydrated overnight by 200–400 μg of protein dissolved in 220 μL of the optimized solubilization solutions containing 0.002% bromophenol blue (BPB). Isoelectric focusing (IEF) was performed at 20 °C with Multiphor Electrophoresis Unit (GE Healthcare). The running conditions were as follows: 300 V for 1 min, followed by 300–3500 V for 1.5 h, and finally 3500 V for 6 h. For the reduction and alkylation of proteins, the focused strips were equilibrated with a high SDS-equilibration buffer [50 mM Tris-HCl (pH 6.8), 6 M urea, 100 mM DTT, 30% glycerol, 4% SDS, and 0.002% BPB] and then with the same buffer but replacing DTT with 135 mM iodoacetamide. These equilibrations were performed with gentle shaking at 30 °C for 20 min. After the reduction and alkylation steps, the strips were loaded onto commonly used slab gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and enclosed by 1% low-melting agarose gels in an SDS-PAGE running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The polyacrylamide concentrations of stacking and separating gels were 4.5% and 12.5%, respectively. SDS-PAGE was performed at 20 mA for approximately 4 h at room temperature. Gels were stained in Coomassie Brilliant Blue (CBB) Stain One (Nacalai Tesque).

Download English Version:

https://daneshyari.com/en/article/5522162

Download Persian Version:

https://daneshyari.com/article/5522162

Daneshyari.com