FEMS Microbiology Letters 247 (2005) 17–22

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

# Development of a sample preparation method for fungal proteomics

Motoyuki Shimizu, Hiroyuki Wariishi *

*Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

Received 2 October 2004; accepted 14 April 2005

First published online 30 April 2005

Edited by G.M. Gadd

**Abstract**

Since filamentous fungi including basidiomycetous fungi possess an exceptionally robust cell wall as in microorganisms, effective extraction of intracellular proteins is a key step for fungal proteomic studies. To overcome the experimental obstacle caused by cell walls, we utilized fungal protoplasts, prepared from the brown-rot basidiomycete, *Tyromyces palustris*. The amount and quality of proteins extracted from the protoplast cells were much higher than that from the mycelial cells. Quantitative comparisons of proteome maps prepared from mycelial and protoplast cells indicated protein spots with a wider range of molecular weights and pIs in the protoplast sample. Furthermore, no streaking or tailing was observed in the protoplasts, suggesting that effective extraction of intracellular proteins from protoplasts might help suppress degradation of proteins during this process. In addition to the efficiency of protein extraction, simple and efficient subcellular fractionation was also achieved using protoplast cells.


*Keywords:* Brown-rot basidiomycetes; Protein extraction; Proteome; Protoplasts

## 1. Introduction

Very recently, the US Department of Energy and Joint Genome Institute completed the whole genomic sequence of the white-rot basidiomycete, *Phanerochaete chrysosporium* [1] and the data were made available to the public (http://www.jgi.doe.gov/programs/whit-erot.htm). Besides this basidiomycetous fungus, whole genomic sequences, or at least draft sequences, for several filamentous fungi have also been completed (http://www.broad.mit.edu/annotation/fungi/fgi/) [2]. Under such research conditions, comparative genomics and proteomics of these fungi might provide greater insight into the cellular system of eukaryotic microorganisms. Genome data are content-independent; however, proteomes are content-dependent and their quality is strongly dependent on the extraction efficiency of proteins from the cells. Filamentous fungi are known to possess an exceptionally robust cell wall [3]; thus, cell lysis is the most difficult but crucial step in sample preparation for two-dimensional gel electrophoresis (2-DE). An intracellular survey of iron-regulated proteins for the white-rot basidiomycetes *Lentinula edodes* and *P. chrysosporium* was recently reported using a 2-DE technique; however, the number of 2-DE protein spots observed for these fungi were considerably few when compared with those for other organisms [4]. To better determine fungal proteomes, a modified a sample preparation protocol was thought indispensable.

Since the cell wall is thought to cause ineffective protein extraction from basidiomycetous cells, the utilization of fungal protoplasts was of great interest. In our previous report, protoplasts of the brown-rot basidiomycete, *Tyromyces palustris*, were shown to cause metabolic activities similar to those seen with intact mycelial

* Corresponding author. Tel./fax: +81 92 642 2992.
*E-mail address:* hirowari@agr.kyushu-u.ac.jp (H. Wariishi).

0378-1097/$22.00 
doi:10.1016/j.femsle.2005.04.021

cells [5]. Thus in the present study, protoplast cells were prepared from *T. palustris* mycelial cells and utilized to omit the obstacles caused by cell walls during the extraction of intracellular proteins. Proteomic maps for intracellular proteins from protoplast or mycelial cells were also compared.

## 2. Materials and methods

### 2.1. Culture conditions

*Tyromyces palustris* (IFO 30339) was grown from hyphal inocula at 30 °C in a stationary culture under air. The MPYC medium used for the present study was supplemented with 1% malt extract, 1% peptone, 0.4% yeast extract, 0.2% casamino acid in deionized water with pH adjusted to 6.0 [6]. After a 3-day incubation, the mycelial cells were collected by filtration and rinsed with deionized water. The cells were used for either a protein extraction or protoplast preparation.

### 2.2. Protoplast preparation

Mycelial cells (1 g, wet weight) were treated with 5 mL of enzyme solution containing 2% (w/v) Novozyme 234, 0.5% (w/v) Zymolyase 20 T, 0.6 M mannitol, and 50 mM maleate (pH 6.0) [6]. After 1 h incubation at 30 °C with gentle rocking, the protoplasts were harvested by centrifugation at 750*g* for 10 min at 4 °C. The pellets were then washed by centrifugation with 50 mM potassium phosphate (pH 6.0) containing 0.6 M mannitol (buffer A). Protoplasts were counted using a hemocytometer. Yields of protoplast cells per g (wet weight of mycelia) was $(8.0 \pm 0.1) \times 10^7$.

### 2.3. Protoplast regeneration

The regeneration medium used in this study was MPYC medium containing 0.6 M mannitol and 1.5% low-temperature agarose as previously described [6]. The protoplast solution obtained as described above was diluted to a concentration of $10^5$ cells/mL using buffer A. Aliquots of suspension (100 μl) were added to the regeneration medium and placed on Petri dishes. Protoplasts were incubated at 30 °C for 2–3 days and regenerated protoplasts were counted as individual colonies.

### 2.4. Extraction of intracellular proteins

The protoplasts were suspended in SDS buffer containing 4% SDS, 2% DTT, 20% glycerol, 20 mM PMSF, and 100 mM Tris–HCl (pH 7.4). The solution was then heated for 5 min at 80 °C and insoluble material was removed by centrifugation (15,000*g* for 10 min). Four volumes of cold acetone (−20 °C) were added and the solution was incubated overnight (−20 °C). After centrifugation (15,000*g* for 10 min), the precipitate was washed with cold acetone (−20 °C) and the pellet was solubilized in urea buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 0.5% IPG buffer (pH 3–10 NL; Amersham Bioscience) and a trace of bromophenol blue. The sample was then incubated for 2 h at room temperature and insoluble material was removed by centrifugation (15,000*g* for 10 min). Samples were split in half; one was used for electrophoresis and the other for measuring protein concentration using a Bio-Rad protein assay kit based on the Lowry method.

The extraction of intracellular proteins from mycelial cells was achieved using the methods described above against a mycelial powder prepared from cells frozen under liquid nitrogen, and ground into a fine powder using a mortar and pestle.

### 2.5. Subcellular fractionation of protoplasts

For subcellular fractionation, protoplasts were collected by centrifugation, then burst via an osmotic shock by adding deionized water (0.5 mL). The supernatant and pellet were separated by centrifugation (15,000*g*), and each fraction was solubilized using SDS buffer. Sample preparation for 2-DE was as described above.

### 2.6. Two-dimensional gel electrophoresis

Isoelectric focusing was carried out with an IPGphor system (Amersham Bioscience). Immobilized pH gradient strips (pH 3–10 NL, 18 cm; Amersham Bioscience) were rehydrated for 12 h, and then 750 μg of protein in urea buffer was focused in four steps at 500 (1 h), 500–1000 (1 h), 1000–8000 (2 h), and 8000 V (8 h). After completion of focusing, strips were equilibrated with buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 2% SDS, and a trace of bromophenol blue then with a buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 2% SDS, and a trace of bromophenol blue. Strips were loaded onto precast 12.5% homogenous polyacrylamide gels (20 × 20 cm). The lower running buffer contained 385 mM tris, 50 mM glycine, 0.1% SDS, and 0.02% sodium azide, while the upper running buffer was identical except it lacked sodium azide. The system was run at 1000 V and 24 mA per gel. Gel slabs were stained in 7.5% acetic acid solution with 0.0002% SYPRO Red (Takara) and incubated with gentle rocking at room temperature for 1 h. After removal of the staining solution, gels were washed in 7.5% acetic acid solution for 30 min.

### 2.7. In-gel tryptic digestion

In-gel tryptic digestion was performed as previously described [7] with a slight modification. The target spot