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Sample Preparation Procedure for Cellular Fungi

Alois Harder

Summary

A crucial step in quantitative proteomics is an artefact free and reproducible sample preparation protocol, which has to be adapted and optimized to nearly all types of cells. Here we provide a sample preparation method for quantitative proteomics of cellular fungi. Two different protein extraction methods were compared with focus on reproducibility, minimized proteolytic degradation and protein losses during the sample preparation.

In the first preparation the cells were lysed by sonication followed by protein solubilization in "standard" lysis buffer. The second preparation was performed with a SDS-presolubilization step followed by sonication and further boiling, before diluting the sample with lysis buffer. We have shown that the sample preparation for cellular fungi is performed with maximum protein solubilization, higher reproducibility and a reduced proteolytic activity by including a SDS-presolubilization step in the sample preparation protocol.

Key Words: 2D-electrophoresis; fungi; proteolytic activity; sample preparation; yeast.

1. Introduction

Fungal cells have nowadays found their way in nearly all areas of biochemistry and pharmacy. Famous examples are active medicaments like Perenterol™ with *Saccharomyces boulardii* as the active ingredient ***(1,2)***, yeast host cell systems like the vaccine production cycle or diverse secondary metabolite discovering systems like the Novobiocin synthesis to name only few ***(3,4)***. To push those applications or detect new fungal targets or metabolites, a solid basis of information in cell growth, cell cycle, gene- and protein data are essential. Whereas cell growth and cycle can be researched in conceivable

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time, gene and protein data are missing for the majority of the fungi. The finished and currently running fungal genome sequencing projects are mainly concentrating on model- or pathogen strains: *Saccharomyces cerevisiae*, *Ashbya gossypii*, *Aspergillus fumigatus*, *Candida parapsilosis*, *Candida dubliniensis*, *Pneumocystis carinii*, *Phytophthora infestans*, *and Schizosaccharomyces pombe* have been sequenced or will be finished soon and are available for the community ***(5,6)***.

For identification of fungal proteins, genome data of the organism to be researched has to be available. Although the fungal protein databases are growing, the listed open reading frames are far from their complete and correct functional characterization.

However, with the sequenced fungal genomes, the available protein data and the homology – search programs with a solid foundation for further proteome analysis where eumycotic cells are present. Fungal cells are easy to cultivate, to manipulate, and in most cases are non-toxic, but the preparation of a robust and artefact-free protein extract for a proteomics approach is a challenge. Major problems in fungal proteomics result from the cells strong proteolytic activity. Most of the fungal proteases, intensively researched in the eumycotic model organism *Saccharomyces cerevisiae*, are located in the cytosol and the vacuole. Few proteases are located in the membrane, the endoplasmic reticulum, the mitochondria, and the Golgi complex ***(7,8)***. These include endoproteinases, carboxypeptidases, aminopeptidases, and dipeptidyl-aminopeptinase. By activation of these proteases protein modifications will occur and can rule out quantitative proteomics research ***(9–10,11)***.

Of the many cellular proteases, the lumenal vacuolar proteases comprise the major source of problems for protein analysis and proteome research. Endoproteinase A and B (PrA and PrB), carboxypeptidases Y and S, aminopeptidase I (LAP IV), and the aminopeptidase yscCo (ApCo) are found soluble in the vacuole ***(12,13)***. Polypeptide inhibitors of these vacuolar proteases are found in the cytosol (e.g., inhibitor for PrB is I_B; inhibitor for yscCo is $yscCo_F$). By preparing the crude extract all cell compartments are broken up, meaning that corresponding protease inhibitors will bind to their substrate by forming an inactive complex—in this state proteolytic activity is inhibited. As reported from Jones et al. the fungal proteases PrA and PrB, can be activated out of the crude extract by lowering the pH to a level of 4–5. The reason for this activation might be the hydrolysis of the polypeptide inhibitors ***(7,14)***. Further research on protease deficiency mutants showed that the addition of denaturating agents activates proteolysis by removing the corresponding inhibitor from the protease molecule. Due to the exigency in proteomics research, for working in strict

denaturation conditions, the necessity of a "non standard" sample preparation procedure for fungal cells is obvious.

The fungal sample preparation for proteomics research has to

- (BL)be carried out in strong denaturating conditions for its stability during the 2D - electrophoretic separation
- display robustness to guarantee reproducibility by repeating the experiments
- solubilize low abundant protein as well as high and low molecular weight proteins in all pH ranges
- effectively inactivate the strong proteolytic activity of fungal cells

2. Materials

2.1. Cell Culture

1. Standard aerobic incubator with an inbuilt horizontal rotary shaker. 300 mL Erlenmeyer flasks with silicone plugs are used for aerobic cell cultivation.
2. Horizontal rotary shaker (e.g., HS 260, IKA).
3. Incubation mask (e.g., Certomat H/HK).
4. *S. cerevisiae* haploid wild type strain N318C ("Deutsche Sammlung für Mikroben und Zellinien", DSMZ Braunschweig, Germany).
5. Defined synthetic YNB medium (Difco Labs, San Francisco CA, USA) is dissolved to a concentration of 6.7 g/L in distilled water (<18.4 μS).
6. The medium then is supplemented with 20 g/L (w/v) glucose and buffered with sodium hydroxide (6 g/L, w/v) / succinic acid (10 g/L, w/v) to pH 5.8.
7. For a control smear a Sabouraud agar plate is used.

2.2. S - 35 In Vitro *Cell Labeling*

1. S 35 - methionine from GE Healthcare (SJQ0079-2.5MCi).
2. 2 × 4 cm cut filter paper (GE Healthcare, 80-110619) is used for drying and counting the labelled protein.
3. Scintillation cups (12 mL), e.g., from Sigma.
4. Liquid scintillation counter (e.g., Beckman LS 6000 IC / 1801). For detailed information in working prescriptions with radioactivity, calibrating of the scintillation counter, waste disposal, radioactive sample storage etc. please refer to reference ***(16)***.

2.3. Cell Disruption and Protein Solubilization

1. Lysis buffer: 7*M* urea, 2*M* thiourea, 4% CHAPS (w/v), 1% DTT (w/v), 0.5% Pharmalytes, pH 3–10 (v/v) and 10 m*M* Pefabloc protease inhibitor.
2. Presolubilization SDS-buffer (95°C): 1% (w/v) SDS, 100 m*M* Tris-HCl, pH 7.0.
3. Sonifier (Bandelin 60W, HD 2070 UW).

2.4. Scintillation Counting and Protein Assay

1. For S35-labelled cells, a scintillation counting is performed: 4.5 mL POPOP solution is required for one vial; 2 × 4 cm cut filter paper is used for the counting (*see* **Note 1).** As a calibration standard a C12 isotope (GE Healthcare) is applied.
2. Lowry protein assay kit for SDS containing samples.
3. The Bradford protein assay kit (Bio-Rad) for samples dissolved in lysis buffers.
4. BSA (Bio-Rad) is used as a protein standard for both protein assays.

UV-spectral photometer e.g., Beckman DU-64 (*see* **Note 2).**

3. Methods

3.1. S. cerevisiae Cell Culture

1. The *S. cerevisiae* haploid wild type strain N318C is cultivated at 30 °C in defined synthetic YNB medium. First a preculture is grown, in which about 10^5 cells are inoculated in 30 mL culture medium (see previous section) and grown to a cell density of OD 0.6 measured at λ=610nm (*see* **Note 3).**
2. Then the main culture (30 mL, 300-mL Erlenmeyer flasks) is started with an inoculum from 10 µL out of the preculture. The cells are harvested by centrifugation (5,000*g*, 15 min) in the late midlogarithmic phase, when the culture reached an OD of 1.1 followed by washing in ice-cold sterilized water (*see* **Note 4).**
3. A control smear is performed on a Sabouraud agar plate to check for cellular contaminations. The incubation of the agar plate was done for 48 h at 37 °C in sterile conditions.
4. Harvested yeast cells are then transferred into individual 1.5 mL Eppendorf tubes and stored at –78 °C, if not processed immediately.

3.2. *S-35* In Vitro *Labeling (optional)*

1. The S-35 in vitro labelling can be done additionally to the standard cell growth procedure. For the in vitro S 35 - labelling experiment the main culture is grown to an OD of 1.0 (λ=610 nm)
2. Then 10 mL of the cell suspension is inoculated with 100 µCi and kept shaking for a quarter of the fungal generation time (for *S. cerevisiae* in YNB – medium→ 30min).
3. After the S-35 incubation the cells are harvested by centrifugation (5 min, 5,000*g*) and the supernatant is discarded to the radioactive waste. The amount of protein, which will be extracted out of the obtained S-35 labeled pellet will be approx 5 mg protein solubilized in lysis buffer. Only methionine deficient mediums are suited for S-35 in vitro labeling.

3.3. Cell Disruption and Protein Solubilization

1. The yeast cell pellet from 10 mL culture OD 1.1 (see Section 21.3.1) is resuspended in 200 µL hot (95 °C) SDS buffer for presolubilization (*see* **Note 5 and 6**).

2. After brief vortexing the hot suspension is sonicated 10 times for 1s (60 W, 20 kHz). The sonication has to be performed in such way that the probe is dipping as deep as possible in the suspension and not touching the sample cup during the agitation time. A 1.5-mL cup (conic bottom) is used for maximum abrasive agitation. Avoid foaming of the sample during this process **(***see* **Note 7)**.
3. After cell lyses the sample is boiled additionally for 5 min and then cooled down in an ice cold water bath to a sample temperature of maximum 20 °C **(***see* **Note 8 and 9)**.
4. Then the suspension is diluted with 500 µL lysis buffer and kept shaking for 20 min at room temperature.
5. After spinning down for 5 min at 10,000*g*, the protein concentration is measured from the clear supernatant (see Section 21.3.4).
6. The clear supernatant is stored at –78 °C or subjected to 2D-electrophoresis. An example of four replicate gels (IPG 4-7, 250 µg protein load, *S. cerevisiae*, SDS presolubilisation) is shown in **Fig. 2** compared to a "standard preparation protocol" without the SDS presolubilization step **(Fig. 1)**.

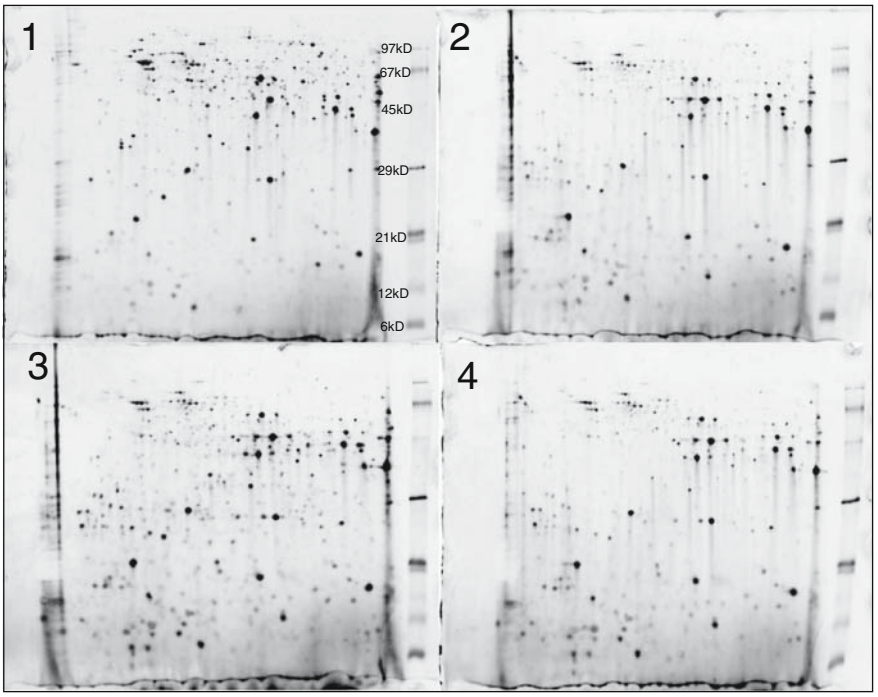

Fig. 1. Displaying four replicate 2D gels (IPG 4-7L, 250 µg protein load, fluorescence stain) from the yeast proteome (*S. cerevisiae*) performed without the SDS - presolubilization step **(see Note 12 and 13).**

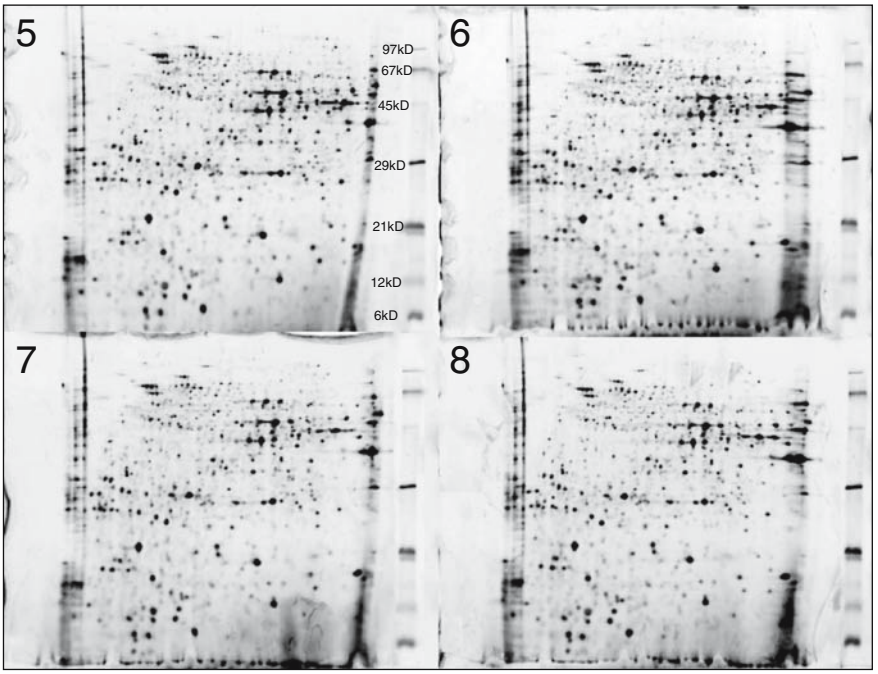

Fig. 2. Displaying four replicate 2D gels (IPG 4-7L, 250 µg protein load, fluorescence stain) from the yeast proteome (*S. cerevisiae*) performed with the SDS - presolubilization step (*see* **Note 14).**

7. An approximate 7 µg/µL protein concentration can be expected in that obtained 700-µL sample volume (*see* **Note 10**)

3.4. Scintillation Counting and Protein Assay

1. 1. For S35 - labeled cells, the protein concentration is measured by removing 1 µL of the lysis buffer extract to a filter. After drying the sample, the filter is inserted into a counting vial, which is filled with 4.5 mL POPOP - solution. The scintillation counter is calibrated to a C12 – standard, which was first solubilized in lysis buffer. The scintillation counting should be repeated three times for each sample. Approximately 3×10^6 cpm should be loaded onto an IPG strip 4–7, 2.5×10^6 counts onto the gradient 3–10. This method is working with and without the SDS presolubilization step (*see* **Note 11**).
2. 20 µL of the SDS extract are used for the Lowry protein assay. 5 µL of the lysis buffer extract are taken for the Bradford protein assay.

4. Notes

1. For S35 counting: after an appropriate amount of sample is pipeted onto the filter, the sample has to be dried for at least 30 min, due to water residues suppress the radio signal significantly.
2. The determination of the protein content is optional, but an optimum and reproducible protein load is essential for successful protein quantification.
3. Significantly increased proteolytic activity in fungi is observed in cells grown in minimal medium compared to cells grown in full medium, in cells harvested in their stationary phase (increases proteases activity to a level at least 100 times that of log phase cells), in vital cells stored or grown in nitrogen starvation and in the presence of peptone in the culture medium.
4. When you are researching fungal stress responses, washing the pellet with ice cold water will induce a deficient cell response. In that case the cell washing can be performed with 15 °C water as well.
5. The whole procedure is carried out in one single Eppendorf tube. By establishing the yeast sample preparation procedure main focus was given to its reproducibility, robustness, and maximum protein solubility with respect to the yeast's peculiarities like proteolytic activity and cell wall constitution.
6. Dried pellets (for measuring the amount of cells) are not suited for further proteome analysis, due to heat shock responses of the cells. Homologue pellets should be used for protein extraction and 2D separation.
7. The correct sonication will provide a quantitative cell disruption and protein disaggregating by simultaneously cracking the fungal DNA and RNA strands, which otherwise can produce horizontal streaks in the 2D PAGE. The DNA and RNA fragments should become visible as a thin white foam after the sonication step.
8. By presolubilization in hot SDS buffer fungal proteases are denatured and inactivated during the cell breakage. Boiling the sample in SDS will increase protein solubility, especially for the high molecular weight and hydrophobic proteins, due to the high affinity of SDS to proteins in solution.
9. It is crucial to cool down the SDS-boiled sample below 20 °C, to avoid carbamylation reactions with the urea containing lysis buffer. The final SDS concentration should not exceed 0.25% in the extract to be applied onto the IPG strip, due to the interference of the SDS with the isoelectric focussing, so be sure that during the SDS boiling step the total volume is kept.
10. By starting with about 1×10^8 cells *(S. cerevisiae)* the obtained protein concentration will be approximately 7µg/µL in a 700 µL volume.
11. The protein concentration within this preparation method can be measured with the Lowry Kit after the SDS-presolubilization or with a scintillation counter (by S-35 labeled cells) out of the final extract. Determination of the dry weight or cell counting after cell harvesting should be done at least one time to check purity and approximate amount of cultivated cells.

12. A major problem in fungal protein extraction is the strong fungal proteolytic activity. By applying a standard preparation protocol significant degradation can occur (*see* **Fig. 1**) and consequently a protein quantification is useless.
13. In many cases proteolytic activity is not as obvious as in the gels 1–4 (**Fig. 1**), especially when degradation only begins. It can show up in disappearance of single spots or in the decrease of the quantities of some spots, which can be spuriously interpreted as a cell response. Therefore the complete and irreversible inactivation of the mycotic proteome is essential.
14. Note, that the obtained "crude extract" will include proteins from the cytoplasm, the organelles, membrane bound proteins and surface proteins. Integral plasma membrane proteins as well as integral cell wall proteins are not extracted with that recipe (*see* **Fig. 2**).

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