Solubilization of Trichloroacetic Acid (TCA) Precipitated Microbial Proteins via NaOH for Two-Dimensional Electrophoresis

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Abstract: In preparing intracellular microbial samples for one- or two-dimensional electrophoresis, trichloroacetic acid (TCA) precipitation is frequently used to remove interfering compounds. Solubilization of TCA precipitate typically requires the addition of a number of chaotropes or detergents, in a multistep process, that requires hours to carry out. In this study, a simple, rapid, one-step method to solubilize TCA precipitated proteins is presented. Precipitated proteins are pretreated with 0.2 M NaOH for less than 5 min, followed by addition of standard sample solubilization buffer (SSSB). When compared to solubilization with SSSB alone, NaOH pretreatment of TCA-precipitated intracellular protein from Aspergillus oryzae and Escherichia coli shows an approximate 5-fold increase in soluble protein. In addition, two-dimensional gel electrophoresis on resolubilized proteins shows an equivalent number of proteins in samples with and without NaOH pretreatment.

Keywords: proteins • proteomics • Aspergillus oryzae • Escherichia coli

Introduction

The preparation of intracellular microbial proteins for two-dimensional gel electrophoresis (2-DE) typically includes lysis of the cell membrane or wall, inactivation or removal of interfering substances, and solubilization of sample proteins. During this process, it is of great importance to minimize protein modification or degradation, thus avoiding a quantitative loss of high molecular weight proteins, membrane proteins, and/or nuclear proteins.1 As such, three important goals in sample preparation are as follows: (i) complete dissociation of protein/protein interactions, (ii) removal of nonprotein sample components (e.g., lipids, nucleic acids, and salts), and (iii) significant reduction or elimination of protease activity.2 Although the second and third items can be addressed individually by dialysis and addition of protease inhibitors, to a certain extent, all three goals can be met in a single step: precipitation of sample proteins followed by resuspension in sample solubilization buffer.3

Precipitation of proteins using trichloroacetic acid (TCA; alone, or in combination with acetone) is a popular sample preparation method for both 1-D and 2-D electrophoresis,4,5 because it can concentrate samples, remove salts, and polysaccharides,6 and denature endogenous proteases.7,8 However, resolubilization of precipitated protein is often difficult,4,5,8 and this is especially true for proteins precipitated with TCA.9,10 For example, current protocols for resolubilization of TCA precipitate are quite time-consuming6 (Table 1), and can take up to 24 h. Alternatively, this time can be reduced to approximately 3 to 4 h by addition of a number of solubilizing agents (e.g., chaotropes, surfactants, reducing agents, etc.).5,8,11 These, however, can lead to protein modifications or aggregation, which have the potential to interfere with 2-DE.3 A better method for resolubilization of TCA precipitated protein is needed.

In resolubilizing protein precipitate for 1-D gels12 or total protein determination,13 pretreatment with alkali solution has been used to enhance solubility. In this study, we report on a modified alkali pretreatment method for resolubilization of TCA precipitated proteins for 2-DE (Table 1). Using intracellular protein from the filamentous fungus A. oryzae and the bacterium E. coli, we show simple pretreatment of TCA precipitate with mild alkali is rapid, requires addition of no potentially interfering components, leads to significant solubilization of

Table 1. Time Required for the Steps Involved in Various 2-DE Protein Preparation Protocols

<table>
<thead>
<tr>
<th>Microbial Protein Preparation Protocol</th>
<th>Standard Protocol</th>
<th>Sequential Solubilization</th>
<th>This Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest/Lyse Cells</td>
<td>45 min</td>
<td>45 min</td>
<td>45 min</td>
</tr>
<tr>
<td>TCA Precipitation &amp; Centrifugation</td>
<td>30 min</td>
<td>30 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Resolubilize Protein Precipitate</td>
<td>~ 4 hr (~24 hr)</td>
<td>~3 hr (~4 hr)</td>
<td>5-10 min</td>
</tr>
<tr>
<td>Total Preparation Time</td>
<td>~ 5 hr (~26 hr)</td>
<td>~ 4 hr</td>
<td>~ 1.5 hr</td>
</tr>
</tbody>
</table>

* Standard protocol4 involves addition of “standard sample solubilization buffer” (SSSB), followed by vortexing for 2–4 h. 5 Sequential solubilization9 requires addition of a number of solubilizing agents, some of which have been reported to interfere with 2-DE.1 The addition of NaOH for 2 min. Volume then brought to 500 μL with SSSB. Additional protein may be solubilized if precipitate is frozen overnight, thawed, and vortexed again requiring a total of approximately 24 h.

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protein precipitate, and does not reduce the number of visible proteins or the quality of 2-D gels.

Materials and Methods

**A. oryzae Growth and Cell Lysis Procedure.** Wild type Aspergillus oryzae (A1560; derived from strain IFO 4177, institute for fermentation, Osaka, Japan) was obtained from Novozymes North America, Inc. (Franklinton, NC). Storage, media, and growth conditions for this strain have been described previously.\(^1\) To lyse fungal cells, a mechanical cell disruption method was employed.\(^2\) Briefly, the harvested mycelial biomass (500 mg wet weight) and an equal amount of acid washed glass beads (0.5 mm) were added to a 2 mL Eppendorf tube containing 1 mL lysis buffer (20 mM Tris – HCl pH 7.6, 10 mM NaCl, 0.5 mM deoxycholate, and 40 \(\mu\)L/ml of protease inhibitor cocktail (Sigma Chemical Co, St. Louis, MO). The mixture was agitated in a Mini-BeadBuster\(^3\) (BioSpec, USA) at top speed, for 8 min (repeated cycles of 30 s on followed by 30 s cooling on ice). The supernatant obtained after centrifugation was then treated with DNase/RNase (Sigma Chemical Co, St. Louis, MO). Finally, the supernatant divided into two fractions (proteins from each fraction later used for different types of resolubilization procedure) was further treated with 20% \(\text{v/v}\) TCA (trichloroacetic acid, Sigma Chemical Co, St. Louis, MO) in ice for 30 min to precipitate protein. The precipitate was collected by centrifugation at 6000 g for 10 min at 4 °C. The precipitated protein was washed with acetone to remove traces of TCA and finally acetone was removed by speed vacuum treatment.

**E. coli Growth and Cell Lysis Procedure.** E. coli wild type strain W3110 was obtained from the Coli Genetic Stock Center (CGSC; New Haven, CT). Storage, media, and growth conditions for this strain have been described previously.\(^4\) Intracellular protein sample for electrophoresis was prepared according to the protocol from http://us.expasy.org/ch2d/protocols for **A. oryzae** and **E. coli**.

**Protein Resolubilization.** TCA precipitated protein pellets of microbial intracellular proteins were solubilized by two methods. In the first, TCA precipitate was resolubilized in 50 \(\mu\)L of standard sample solubilization buffer (SSSB; 8M Urea, 4% w/v CHAPS, 1% w/v DTT; Sigma Chemical Co, St. Louis, MO) and Ampholytes 2% w/v 3–10 nonlinear (NL; Amersham Biosciences, San Francisco, CA, USA) alone. After suspension in the lysis buffer, the cells were lysed by sonication (550 Sonic Dismembrator, Fisher Scientific, USA) at 40% power in ice. Cell debris was removed by centrifugation, DNase/RNase were added to the supernatant, and incubated for 30 min in ice. This protein sample was divided into two fractions (proteins from each fraction later used for different types of resolubilization procedure) and subjected to 20% \(\text{v/v}\) TCA precipitation followed by acetone washing and drying in speed vacuum similar to the **A. oryzae** protein precipitation.

**2-D Electrophoresis.** In preparation for 2-DE, 5 to 20 \(\mu\)L (depending on protein concentration) of frozen protein sample was diluted to 125 \(\mu\)L with rehydration solution.\(^5\) Rehydration of Immobiline Dry Strips (IPG strip; Amersham Biosciences, San Francisco, CA, USA) with sample was carried with Amersham dry strip re-swelling tray (Amersham Biosciences, San Francisco, CA, USA) according to manufactures instructions. IPG strips (pH 3–10 NL), 7 cm long were used for the present study. The rehydrated strips were then subjected to IEF. Isoelectric focusing was performed using Multiphor II electrophoresis unit at 18 °C in gradient mode. Briefly, 7-cm strips were focused at 0–200 V for 1 min, 200–3500 V for 1.30 h and 3500 V for 1.15 h, with a total of 8 kVh accumulated. After focusing, the strips were stored at −80 °C for later use. Prior to the second dimension SDS–PAGE, IPG strips were equilibrated for 15 min in equilibration solution (5 mL) containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS, and traces of bromophenol blue with 100 mg/10 mL (w/v) of DTT. A second equilibration was carried out for 15 min by adding iodoacetamide (250 mg/10 mL) instead of DTT in equilibration solution. A 5 mL portion of equilibration solution was used for the 7-cm strip. Second dimension vertical SDS–PAGE was performed using precast mini-gels (12% Tris-HCl, 1 mm in thickness (Bio-Rad, CA). Mini-gel 2-DE was
carried employing Mini-vertical electrophoresis system (Mini protean-II) according to the manufacturers instructions. Briefly, electrophoresis was performed at a constant current of 5 mA/gel for 20 min, followed by 12 mA/gel for 1.5 hours until the bromophenol band had exited the gel. Gels were stained with neutral silver stain as described previously.18

Electropherogram images were obtained with an imaging densitometer (GS-800; Bio-Rad) in gray scale mode. The image analysis of gels was performed using Z3 software (Compugen, Israel; http://www.2dgels.com) as described previously.19 Briefly, images of multiple gels, prepared with protein from each of the two preparation protocols, were used to construct a composite or “raw master gel” (RMG). This serves to eliminate noise and minor discrepancies between gels which should theoretically be identical. The spots on the gels were then identified, and each was assigned a spot quantity (q). The spot

Figure 2. 2-D electrophoresis of intracellular proteins from A. oryzae (A and B, 23 μg total protein loaded in each gel) and from E. coli (C and D, 20 μg total protein loaded in each gel) resolubilized after TCA precipitation either with standard sample solubilization buffer (SSSB) alone (A and C) or with NaOH pretreatment before addition of SSSB (B and D). Z3 software analysis on three-gel composites shows 312, 328, 293, 309 total spots on gels A, B, C and D, respectively. Highlighted spots or regions call attention to significant expression differences.
quantity characterizes spot size and intensity, is defined as the sum of the gray-level values of all of the pixels in a spot, and is expressed in terms of PPM (parts per million) of the total spot quantity on the gel.\(^2\) Thus, \(q\) is an approximate fractional representation of the amount of protein in a particular spot, and the ratio of \(q\) values, for the same spot on two different gels, gives an approximation of differential expression. All molecular weight and \(pL\) values on gels were estimated using protein markers obtained from Sigma Chemical Co (St. Louis, USA; Cat. No. 6539) and Bio-Rad (CA, USA; Cat. No. 161-0310), respectively.

**Results and Discussion**

**Resolubilization of TCA Precipitated Proteins via Mild Alkali Treatment.** Intracellular proteins from both *E. coli* and *A. oryzae* were extracted from whole cells and precipitated with TCA as described in the Materials and Methods section. The resulting protein precipitate was then resolubilized in one of two ways: either (i) a standard sample solubilization buffer (SSSB) was added directly to the protein precipitate or (ii) a small amount of 0.2 M NaOH was added to the precipitate first, followed by addition of SSSB. Results are shown in Table 2. In initial tests we pretreated TCA precipitate with a number of NaOH concentrations (0.2, 0.3, 0.5, 1.0 M; data not shown), and found all worked relatively well for resolubilization. For further testing, we choose the lowest concentration to avoid potential problems with 2-DE. Figure 1 shows pretreatment with 0.2 M NaOH for as little as 2 min was adequate to significantly increase solubilization of protein precipitate from both fungi and bacteria. In contrast, precipitate treated with SSSB alone was not completely solubilized after incubation at room temperature for 24 h, after freezing and thawing (i.e., overnight), or after these treatments combined with prolonged vortexing. However, when this recalcitrant precipitate was recovered via centrifugation and treated with 0.2 M NaOH, the result was a significant increase in solubilization (data not shown). Table 2 shows that compared to SSSB alone, pretreatment with NaOH resulted in approximately 4 to 5 times more soluble protein.

**Comparison of Resolubilized Proteins via 2-D Electrophoresis.** Efficiency of NaOH pretreatment for resolubilization of TCA precipitate was evaluated using 2-DE (Figure 2). In Figure 2, parts A and B, the same total amount of intracellular, *A. oryzae* protein was loaded on each gel, whereas in Figure 2, parts C and D, the same amount of intracellular, *E. coli* protein was loaded. For both organisms, gels obtained with the two different resolubilization protocols were generally comparable with respect to the number and distribution of protein spots.

To determine whether resolubilization with NaOH pretreatment affected the presence or intensity of individual spots, we used Z3 image-analysis software. To remove noise and minor interference during 1-D and 2-DE, 1 and thus, treating proteins with strong alkali or incubating for extended periods of time at elevated pH, may lead to protein alterations, (e.g., modified or precipitated) with SSSB alone. Previous studies in our laboratory (on both *E. coli* and *A. oryzae*) have shown protein expression ratios, on gels from the same sample, can exhibit up to a 3-fold difference (unpublished). Taking a conservative approach here, we note only spots showing greater than 10-fold expression level difference. Even with this constraint, Figure 3 shows for both organisms twice as many spots showed increased expression when precipitate was pretreated with NaOH than when precipitate was resolubilized with SSSB alone. It is interesting to note that for fungal proteins, many of these proteins were present in the acidic region of the gel. This was not the case for the bacterial protein, were increased expression was most strongly present in the center portion of the gel (Figure 2, parts C and D, boxed area). We conclude that pretreatment with NaOH for resolubilization of TCA precipitate yields comparable 2-D gels when compared to proteins resolubilized with SSSB alone. In addition, NaOH pretreatment may slightly increase the number of proteins resolubilized.

Others have reported that harsh treatments may lead to interference during 1-D and 2-DE,\(^3\) and thus, treating proteins with strong alkali or incubating for extended periods of time at elevated pH, may lead to protein alterations, (e.g., modified or precipitated) with NaOH for a short period (2 min) and was then immediately diluted approximately 25-fold with SSSB. Before IEF, this mixture was further diluted with rehydration buffer. As a result, the added NaOH was diluted approximately 250-fold
technical notes

before IEF, and we observed no problems typically associated with salt accumulation (e.g., swelling or burning of IPG strips, aggregation or precipitation of protein, etc.) in any of the runs. In addition, serious smearing or horizontal streaking were not evident in gels run on samples prepared with or without NaOH pretreatment. We also note that 2-DE was performed on samples from a number of different microbial growth stages, and the results were highly reproducible (data not shown).

Quantitative loss of protein during solubilization of TCA precipitate can be a problem in preparation of samples for 2-DE. Although an earlier report shows the resolubilization of precipitate can be a problem in preparation of samples for 2-DE, we also note that 2-DE was performed on samples from a number of different microbial growth stages, and the results were highly reproducible (data not shown).

We conclude that NaOH pretreatment is preferable to solubilization by SSSB alone, in that more total protein is solubilized (approximately 2 to 3 h for completion). In contrast, the NaOH pretreatment method presented here leads to a significant increase in precipitate solubilization, requires addition of only one component, and is complete in approximately 5 min, after obtaining TCA precipitated proteins from lysed cell sample. Our results with two different microbial systems imply that this method is effective for preparing intracellular protein for 2-DE. We conclude that NaOH pretreatment is preferable to solubilization by SSSB alone, in that more total protein is solubilized and preferable to "sequential solubilization" in that only one additional component is added, in a single step, which is complete in minutes.

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