A simple enrichment procedure improves detection of membrane proteins by immunoblotting

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We developed a novel approach to improve detection of membrane-associated proteins in yeast cell lysates by immunoblotting. Our method consists of a simple enrichment procedure using sedimentation to remove soluble proteins and the use of an alternative electrophoresis sample buffer, which allows for protein solubilization without heating. The efficacy of this approach was demonstrated for membrane proteins in *Hansenula polymorpha* (Pho87, Gas1, and Pmr1) and *Saccharomyces cerevisiae* (Gas1). Immunoblot analysis of proteins that are not membrane-associated showed that the precipitate fraction was depleted of Sup45, carboxypeptidase Y, and Hog1; however, tubulin and, to some extent, Sup35 and Tpd3 were precipitated together with the membrane proteins. The presence of tubulin in the same fraction as the membrane proteins allows its use as a reference protein.

Immunoblotting allows the precise identification of the electrophoretic band for a particular protein in complex samples. The total amount of protein loaded per gel lane is limited, which in turn restricts detection of low-abundance proteins. Therefore, reliable detection of such proteins requires their enrichment in the samples to be analyzed. Here, we designed a simple approach to enrich membrane-associated proteins from yeast cell lysates and prepare samples for SDS gel electrophoresis. Our method markedly improved detection of certain membrane proteins.

In order to determine *Hansenula polymorpha* membrane protein Pho87 levels by immunoblotting, we initially used a conventional procedure for sample preparation. This procedure consists of

breaking cells open with glass beads and boiling the resulting cell lysates in Laemmli SDS gel electrophoresis sample buffer (1). Although the amount of cell lysate loaded per lane was quite high, the immunoblot band for full-length Pho87 was not always detectable. At the same time, we always observed a strong band corresponding to a Pho87 degradation product (data not shown).

Since Pho87 is a membrane protein, it might be concentrated by isolation of the membrane fraction of cell lysates. This could be done by centrifugation in a regular benchtop microcentrifuge. For example, a membrane fraction suitable for analysis by immunoblotting can be obtained from plant material by centrifugation at $21000 \times g$ for 2 h (2) or from yeast cell lysates

by centrifugation at $16000 \times g$ for 45 min (3). We hypothesized that increasing the salt concentration during cell disruption may protect Pho87 from degradation and stimulate membrane precipitation, thus facilitating Pho87 enrichment by centrifugation. For this purpose, different concentrations of ammonium acetate (0, 1, 2, and 3 M) during cell disruption, different durations of incubation (0.5, 1, 2, and 15 h) prior to centrifugation, and different durations of centrifugation at $16,000 \times g$ (2, 10, 20, and 40 min) were tested. Incubation in 1 M ammonium acetate for 2 h was sufficient to noticeably improve Pho87 sedimentation (data not shown).

It is well-known that sample boiling may cause aggregation of membrane proteins. In our previous experience with soluble proteins, we observed that repeated boiling of samples often reduced immunoblotting signal intensity (data not shown), indicating that sample boiling may negatively affect protein stability. At the same time, the presence of SDS in a cell lysate sample makes proteins more susceptible to hydrolysis by vacuolar proteases (4). Indeed, we could not detect the full-length Pho87 band in cell lysate mixed with regular Laemmli sample buffer if the sample was not boiled, even though the lysate contained a protease inhibitor cocktail (data not shown). To avoid boiling and ensure rapid denaturation of cellular proteins including proteases, we used sample buffer containing SDS and a high concentration of urea as a chaotropic agent, similar to that described previously (5). Thus, we have developed the following procedure for sample preparation:

Step 1. Cells (~30 mg) were suspended in 100 μ l buffer containing 1 M ammonium acetate, 150 mM NaCl, 30 mM Tris-HCl pH 7.5, 10 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and the protease inhibitor cocktail cOmplete (11836145001; Roche, Basel Switzerland) in a 2 mL Eppendorf tube. Glass beads were poured into the suspension until their level reached the bottom of the meniscus. Cells were broken by intense vortexing of the tube at 4°C for 7 min. Then, 0.5 mL of the same buffer was added, and the tube was briefly vortexed again. A 0.5 mL volume of the suspension

METHOD SUMMARY

To increase immunoblotting signal intensity of the low-abundance yeast plasma membrane protein Pho87, we modified the standard procedure of electrophoresis sample preparation from cell lysates. Our modification introduces a simple enrichment step, which reduced the amounts of most soluble proteins, and the use of an alternative electrophoresis sample buffer to avoid sample boiling.

Figure 1. Electrophoresis of protein samples prepared from yeast cells using three different methods. “E” lanes: samples prepared with our enrichment procedure; “A” lanes: samples prepared by boiling cells in regular SDS-electrophoresis buffer after alkaline treatment; “G” lanes: samples prepared by boiling cells in regular SDS-electrophoresis buffer after breaking with glass beads. Left panels: immunoblotting of *H. polymorpha* Pho87, Gas1, Pmr1, β -tubulin, Tpd3, Hog1, Sup35, Sup45, and CPY (apparent molecular weight 100, 130, 100, 50, 80, 60, 80, 50, and 70 kDa, respectively) and *S. cerevisiae* Gas1, β -tubulin, Sup45, Hsp104, Rnq1, and Sup35 (apparent molecular weight 130, 50, 50, 100, 45, and 75 kDa, respectively). Right panel: Coomassie-stained gel. Samples were prepared from the same amount of cells and adjusted to the same volume.

above the glass beads was transferred to a 1.5 mL Eppendorf tube and kept on ice for 2 h.

Step 2. Membrane proteins were precipitated by centrifugation at $16,000 \times g$ for 10 min. The precipitate was then washed with 1 M NaCl to remove residual ammonium ions.

Step 3. The pellet was dissolved in 200 μ L urea-SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 8 M urea, 1% β -mercaptoethanol, 2 mM EDTA, 5% glycerol, 0.004% bromophenol blue) at room temperature with gentle stirring. Prior to loading the samples onto a gel, insoluble material was removed by centrifugation at $16,000 \times g$ for 10 min.

Figure 2. Electrophoresis of protein samples prepared from the pellet fraction obtained after Step 2 of our procedure. “U” lanes: sample dissolved in urea-SDS sample buffer without boiling; “L” lanes: sample dissolved by boiling in the regular Laemmli SDS electrophoresis sample buffer. Left panel: immunoblotting of the indicated proteins; right panel: Coomassie-stained gel.

This approach was compared with routine methods of cell lysate preparation [boiling in the regular SDS electrophoresis sample buffer after breaking cells with glass beads (6) or after alkaline treatment (7)]. Using antibodies available in our laboratory, including a commercial antibody against β -tubulin (PA5–16863; Thermo Fisher Scientific, Waltham, MA), several proteins from *H. polymorpha* (Pho87, Sup35, Pmr1, CPY, Gas1, Tpd3, and tubulin) and *S. cerevisiae* (Sup35, Sup45, Gas1, Hsp104, Rnq1, and tubulin) were analyzed (Figure 1). Compared with the conventional methods, our procedure showed better performance for membrane-associated proteins (Pho87, Pmr1, and Gas1) and also for Tpd3 and tubulin. Precipitation of Tpd3 together with tubulin could be due to its interaction with the spindle pole body (8). At the same time, a significant portion of Tpd3 is localized to nucleus (8), which could also facilitate its precipitation. Interestingly, Sup35 was shown to be associated with the tubulin cytoskeleton (9), and it was also detected in the precipitate fraction (Figure 1). The signal intensity for most of the soluble proteins (Hog1, CPY, Sup45, Hsp104, and Rnq1) was higher if samples were prepared by one of the conventional methods. One can assume that these proteins are not precipitated during Step 2 of the procedure. Indeed, the supernatant fraction retained proteins that were poorly detected in the pellet fraction (CPY and Sup45) and was depleted of Pho87, Pmr1, Gas1, Tpd3, and tubulin, which were enriched in the pellet (data not shown). Dissolving the pellet in urea-containing buffer without boiling

greatly improved detection of the precipitated proteins, especially Pho87, as compared with boiling in regular SDS-electrophoresis sample buffer (Figure 2).

Author contributions

A.K. performed the experiments. M.A. conceived of and designed the study.

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Competing interests

The authors declare no competing interests.

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