Isolation protocol for two-dimensional-polyacrylamide gel electrophoresis analysis of *Haloferax volcanii* proteome

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High-resolution two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE) is an important step for elucidating the global view of proteins present in a cell or tissue at any given time, including those with posttranslational modifications and rapid turnover rates (1). The isolation of the majority of proteins produced under defined conditions is one of the most important steps in this type of proteomic analysis.

Isolating proteins suitable for 2D-PAGE from salt-loving microorganisms represents a significant challenge. Halophilic archaea such as *Haloferax volcanii*, a member of the Halobacteriaceae family, require 2–5 M salt for viability; at lower salt concentrations, their cells become morphologically distorted, and cell lysis occurs. The cytoplasm of halophiles contains high internal concentrations of K⁺ and Na⁺, which make it iso-osmotic with its environment. Such high levels of salt present in the cytoplasm interfere with isoelectric focusing and subsequent 2D-PAGE. Although Mojica et al. (2) previously reported a 2D-PAGE analysis of *H. volcanii* proteins, their study examined [³⁵S] methionine-labeled, newly synthesized *H. volcanii* proteins. Our goal was to examine unlabeled accumulated proteins from crude extracts so that the gels would be ultimately suitable for matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS) analysis. Using *H. volcanii* DS2 as a model organism, our laboratory developed an efficient protein isolation protocol that generates high-resolution two-dimensional gels for proteomic analysis.

Culture medium was prepared from two stock solutions. Solution 1 contained 2.14 M NaCl, 0.25 M MgCl₂,

30 mM KCl, and 15 mM CaCl₂ (all chemicals from Sigma, St. Louis, MO, USA). Solution 2 contained 2% (w/v) tryptone and 2% (w/v) yeast extract (Fisher Scientific, Pittsburgh, PA, USA). Both solutions were adjusted to pH 6.8 and then autoclaved for 30 min at 120°C. Three volumes of solution 1 were mixed with one volume of solution 2 to a final volume of 1 L. A 1.8-mL glycerol stock of *H. volcanii* DS2 was then added to the medium and incubated with shaking at 37°C at 150 rpm. Growth was allowed to occur until the stationary phase was reached, as indicated by an A₄₂₀ of 1.8–2.3. The cells were then concentrated by centrifugation at 3540× g at 4°C for 5 min.

From the recorded absorbance of the cultured cells at 420 nm, we calculated the sample equivalent, or volume of cells, required to obtain an absorbance of 1.0 at 420 nm. Ten sample equivalents of cells were taken and added to a 15-mL Falcon® Polypropylene Tube (Fisher Scientific). Forty microliters of sample buffer I [0.3% sodium dodecyl sulfate (SDS), 0.2 M dithiothreitol (DTT), 50 mM Tris-HCl, pH 8.0, and protease inhibitors; Genomic Solutions, Ann Arbor, MI, USA] were added per equivalent, and the mixture was placed on ice for 10 min. Just prior to use, a protease inhibitor tablet was dissolved in sample buffer I, per the manufacturer's instructions (Complete™ Protease Inhibitor Cocktail; Roche Applied Science, Indianapolis, IN, USA). The samples were placed in a 3500 molecular weight cut-off (MWCO) Slide-A-Lyzer® Dialysis Cassette (Pierce Endogen, Rockford, IL, USA) and dialyzed against 20 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Pierce Endogen)

in 50 mM Tris-HCl, pH 8.0, containing 0.3% SDS. The use of dialysis tubing instead of cassettes yielded incomplete focusing (data not shown), probably due to residual salts in the sample. Dialysis was conducted in three stages of 2, 4, and 17 h, with the buffer changed between each step. These dialysis steps were critical because crude, undialyzed samples consistently caused the immobilized pH gradient strips (IPG; Amersham Biosciences, Piscataway, NJ, USA) to overheat during isoelectric focusing, which resulted in incomplete focusing (data not shown). One 4-μL aliquot per sample equivalent of sample buffer II (50 mM MgCl₂, 8 U DNase I, 3 U RNase A, 0.5 M Tris-HCl, pH 8.0; Genomic Solutions) was added to the mixture, which was set on ice for an additional 10 min.

Protein concentrations were determined as previously described (3), using the Bio-Rad Protein Assay Stain (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin (BSA) was used to generate a standard curve. All protein samples and BSA standards were suspended in 20 μL of a 10:1 (v/v) mixture of sample buffers I and II.

Proteins were isolated using the protocol of Rafie-Kolpin et al. (4), with slight modifications. All sample preparations were performed on ice with ice-cold reagents. All centrifugations were for 3 min at 4°C at 10,285× g. Protein extracts (100 μg) were mixed with an equal volume of 10% trichloroacetic acid (TCA), incubated on ice for 5 min, and then centrifuged. The pellet was resuspended in the same volume of 5% TCA and incubated on ice for 5 min, followed by centrifugation. The pellet was washed twice with an equal volume of acetone, centrifuged, and allowed to air-dry for 10 min. The TCA precipitation and acetone wash sequence described earlier were then repeated, except that a single acetone wash was used. Then, 165 μL loading buffer (Genomic Solutions) containing 8 M urea, 4% 3-(3-cholamidopropyl dimethylammonio)-1-propanesulfonate (CHAPS), 40 mM Tris, pH 8.0, 65 mM DTT, and 0.01% bromophenol blue were added and vortex mixed briefly. Lastly, 205 μL rehydration buffer (Genomic Solutions) containing 8 M urea, 2% CHAPS, 10 mM DTT, 2% carrier

ampholytes, and 0.01% bromophenol blue were added, and the mixture was vortex mixed briefly.

IPG strips of linear pH gradients (3.5–4.5, 4–5, 4.5–5.5, 5–6, 6–11, and 4–7) were rehydrated overnight at 18°C with 360 μ L prepared sample containing 100 μ g protein. Isoelectric focusing was then performed at 20°C for 24 h. A total of 80,000 Vh were delivered to the gels using the Investigator™ Power Supply (max voltage of 5000 V; max current of 80 μ A/gel; and end-of-run voltage hold at 125 V; Genomic Solutions) (5).

Each IPG strip was washed for 15 min in 10 mL equilibration buffer I (6 M urea, 133 mM DTT, 30% glycerol, 50 mM Tris-acetate, pH 7.0; Genomic

Solutions) and then for 15 min in 10 mL equilibration buffer II (6 M urea, 2.5% iodoacetamide, 30% glycerol, 50 mM Tris-acetate, pH 7.0; Genomic Solutions). The IPG strips were loaded onto 10% precast Duracryl® Gels (Genomic Solutions) (22 cm $\times$ 23 cm $\times$ 1 mm; Tris/tricine/SDS chemistry). Electrophoresis was carried out for 18–19 h (500 V; 1600 mW/gel) at 4°C (6).

We fixed and stained the gels in an Investigator Gel Processor (Genomic Solutions). After 2D-PAGE, the gels were fixed for 30 min in 40% methanol and 10% acetic acid and rinsed for 5 min with deionized water to remove excess fixative. The gels were stained with SYPRO® Ruby (Molecular Probes, Portland, OR, USA) for 12 h in

the dark with gentle rocking. The gels were then submerged in deionized water for 5 min, destained for 30 min with 10% methanol and 6% acetic acid, and stored in 2% glycerol in the dark. The gels were imaged under 470 nm light with a LAS-1000 Plus Imager (FUJIFILM Medical Systems, Stamford, CT, USA) (6).

Using this method for the preparation of protein samples, we analyzed two-dimensional gels prepared with overlapping IPG strips from pH 3.5 to 11.0. In total, we detected approximately 600 proteins. However, the majority of proteins are toward the acidic region, pI (isoelectric point) 3.5–5.0.

We observed that there were significant qualitative and quantitative spot differences between samples isolated during the mid-log phase as opposed to the stationary phase. Using IPG strips of pH 4.0–5.0, we observed a 6.7-fold increase in the number of protein spots between the stationary and mid-log phases. The average number of spots detected during stationary phase was 310, whereas 47 spots were observed in the mid-log phase (Figure 1). It is possible that during mid-log phase, the growth may still be underdeveloped, while during the stationary phase, the organism is in an active metabolic state. The differential expression of these genes may be growth-phase specific and reflects metabolic differences in response to increasing population density, accumulated metabolic products, and nutrient depletion in the media.

Gel resolution was also affected by the bacterial growth phase. Samples isolated from the mid-log phase displayed a large amount of streaking within the molecular mass range of 66–205 kDa. This observation was made using IPG strips of varying pH ranges. Although some streaking was still evident in the samples isolated from the stationary phase, the resolution was dramatically improved. This allowed for more accurate spot detection and a more precise evaluation of the spot volume (Figure 1).

Washing conditions also proved critical to high-quality results. Impurities in the samples led to bow-shaped protein spots in the gel. Repeating the TCA precipitation-acetone wash sequence considerably reduced the

Figure 1. Two-dimensional gels of *Haloferax volcanii* proteins isolated from mid-log (left panel) and stationary phase (right panel) cultures. MW, molecular weight.

Figure 2. Two-dimensional gels of *Haloferax volcanii* proteins prepared without (left panel) or with (right panel) two additional trichloroacetic acid (TCA) precipitations and an acetone wash. MW, molecular weight.

streaking and slightly increased the number of visible spots (Figure 2). This observation is consistent with another report that suggests that additional precipitations/washes reduce sample impurities, allowing for subsequent isoelectric focusing to achieve greater separation of proteins in the first dimension, with an increased overall resolution of proteins on two-dimensional gels (7). Additional TCA precipitations limited streaking between 81–97 kDa but were accompanied by spots of less intensity. Washing of samples using ammonium bicarbonate and acetone were also tested. Although these additional washing steps considerably reduced streaking, the total number of protein spots significantly decreased. Therefore, these steps were not incorporated into our final protocol.

In conclusion, we have developed an improved protein isolation protocol for *H. volcanii* that can be used successfully for 2D-PAGE. One major advantage of this method is that it is compatible with the global identification of proteins using MALDI-TOF MS because proteins are not labeled. Significantly more protein spots were noted at the stationary phase versus the mid-log phase. Additional TCA washes during sample preparation improved the spot resolution and decreased streaking on the gels, which, in turn, resulted in enhanced spot detection. The future analysis of the *H. volcanii* proteome may yield valuable information about the organism's ability to tolerate and survive extremely high salt conditions. In addition, this procedure may prove useful for the proteomic analyses of other halophiles.

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Rapid selection of *Drosophila* S2 cells with the puromycin resistance gene

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The *Drosophila melanogaster* Schneider 2 (S2) cell line was established in 1972 (1). These cells grow rapidly in culture at room temperature without a need for CO₂. Various expression vectors have been constructed that facilitate both constitutive (2) and inducible expression (3). Many studies have indicated that the generation of recombinant proteins with S2 cells is more desirable than other methods, since native proteins derived from S2 cells do not usually interact with those derived from mammalian cells. Despite these advantages, only two markers (i.e., hygromycin and blastcidin) are commercially available for establishing stable transformants (Invitrogen, Carlsbad, CA, USA). Additionally, approximately 2 weeks are required to establish a stable transformant with blastcidin, and more than 3 weeks are needed with hygromycin.

Figure 1 illustrates the structure of a co-expression vector for S2 cells, pCOPURO, which contains the Copia pro-

motor, puromycin N-acetyl-transferase (*pac*), and the simian virus 40 (SV40) poly(A) sequence. The cDNA coding for the *pac* sequence was digested from the puromycin resistance module (Stratagene, La Jolla, CA, USA) with restriction enzymes *Hind*III and *Xba*I (both from Promega, Madison, WI, USA). This *pac* sequence was blunted with T4 polymerase (Promega) and ligated with T4 ligase (Promega) to the *Eco*RV site of pBSIISK+ (Stratagene). After determination of the correct orientation of the insert with several restriction digestions, the *pac* sequence in pBSIISK+ was digested with *Bam*HI and *Xho*I (both from Promega) (Figure 1, Fragment 2). The plasmid, pCo-HYGRO, which contains the Copia promoter, the hygromycin resistance gene, and the SV40 poly(A) sequence (Invitrogen) was digested with *Xba*I and *Xho*I to remove the Copia promoter and the hygromycin resistance gene (Figure 1, Backbone 1).

The sequence of the Copia promoter