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Short Communication

A simple technique to improve the resolution of membrane acidic proteins of the haloarchaeon *Haloferax volcanii* by 2D electrophoresis

Proteins present in the archaeal cell envelope play key roles in a variety of processes necessary for survival in extreme environments. The haloarchaeon *Haloferax volcanii* is a good model for membrane proteomic studies because its genome sequence is known, it can be genetically manipulated, and a number of studies at the "omics" level have been performed in this organism. This work reports an easy strategy to improve the resolution of acidic membrane proteins from *H. volcanii* by 2DE. The method is based on the solubilization, delipidation, and salt removal from membrane proteins. Due to the abundance of the S-layer glycoprotein (SLG) in membrane protein extracts, other proteins from the envelope are consequently underrepresented. Thus, a protocol to reduce the amount of the SLG by EDTA treatment was applied and 11 cm narrow range pH (3.9–5.1) IPG strips were used to fractionate the remaining proteins. Using this method, horizontal streaking was substantially decreased and at least 75 defined spots (20% of the predicted membrane proteome within this p*I*/Mw range) were reproducibly detected. Two of these spots were identified as thermosome subunit 1 and NADH dehydrogenase from *H. volcanii*, confirming that proteins from the membrane fraction were enriched. Removal of the SLG from membrane protein extracts can be applied to increase protein load for 2DE as well as for other proteomic methods.

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Membrane proteins play key roles in processes necessary for cell survival. Archaea are cosmopolitan but predominant in extreme environments [1]. Archaeal cell envelopes are composed of a cytoplasmic membrane containing repeating isoprenyl groups linked to a glycerol backbone, surrounded by the S-layer [2]. Haloarchaea grow optimally in extremely high salt concentrations (>2 M NaCl). Within this group, *Haloferax volcanii* lives over a wide range of salinities (1.5–4 M NaCl) and temperatures (25–50°C) [3]. To balance the osmotic pressure haloarchaea accumulate equimolar amounts of intracellular KCl [4], and to avoid precipitation they have adapted their proteins by increasing their relative number of D and E residues making the p*I* of their proteome more acidic (p*I* 3–5) than that of nonhalophiles [4]. The cell envelope and processes occurring within are essential for adaption of *H. volcanii* to the harsh environments where it grows (high salt concentration, nutrient limitation, day/night temperature variation), thus the study of membrane proteins turns very interesting [2,5,6]. On the other hand, the availability of its genome sequences, the variety of molecular/genetics tools, and a number of studies at genomic, transcriptomic, proteomic, and metabolomic levels makes *H. volcanii* a good model for membrane proteomic studies [7,8].

Membrane proteins are attractive targets in proteomics research. However, classical methods have failed to solve most of them probably due to their hydrophobic nature [9–11]. During the last decade, a number of protocols were proposed to optimize total protein preparations of *H. volcanii* for IEF/SDS-PAGE [12–14]. However, to the best of our

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Abbreviation: SLG, S-layer glycoprotein

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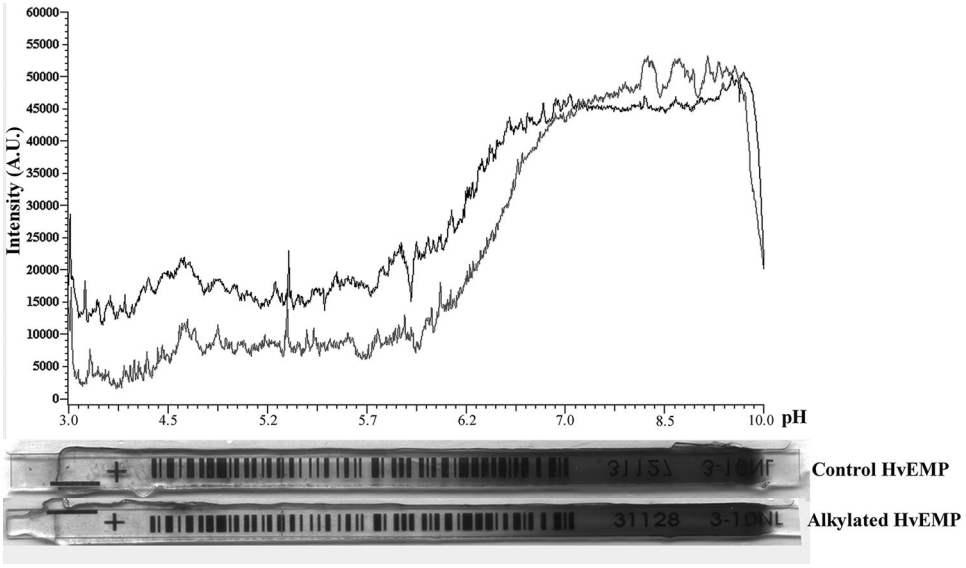

Figure 1. Analysis of proteins retained in IPG strips after the second dimension. Equal amounts (200 μg) of control (black) or alkylated (gray) HvEMP were delipidated. IEF was performed on 3–10 NL, 7-cm IPG strips and proteins were visualized by colloidal CBB stain. Densitometric analysis of proteins was performed using ImageQuant software.

knowledge, there are no reports on separation of *H. volcanii* membrane proteome by this method. The aim of this work was to develop a protocol to perform reproducible 2D maps of *H. volcanii* membrane proteins that would allow the identification of proteins relevant to understand its halophylic physiology.

Despite the acidic nature of haloarchaeal proteomes, in silico 2D analysis of the membrane proteome of *H. volcanii* predicts proteins with p*I*s from 3 to 11 (www.halolex.mpg.de) gathered in two groups: one comprising protein spots with p*I*s 3–7 and the other with p*I*s >7–11. A previous work showed that alkaline membrane proteins of *Halobacterium salinarum* were retained on IPG strips during transfer to the second dimension, suggesting protein precipitation as a possible reason [15]. Oxidation of thiol groups and formation of inter- and intrachain disulfide bonds are known to induce protein precipitation [16, 17]. These reactions are prevented by adding reducing agents such as DTT or DTE during IEF, however, as these compounds are negatively charged at alkaline pH and migrate to the basic end, their effect is likely negligible at this pH range. To overcome this problem, reduction/alkylation of proteins before 2DE was assayed to prevent oxidation of *H. volcanii* membrane proteins.

To obtain an *H. volcanii* enriched membrane protein fraction (HvEMP), this archaeon was grown in modified growth media (MGM) (2.47 M NaCl) [18] at 42°C and 200 rpm. The culture was harvested at $OD_{600} = 2.0$ (10 000 × *g*, 20 min), the cells suspended in 1/10 saline buffer (2 M NaCl, 50 mM Tris-HCl pH 7.5), and disrupted by ultrasound. The cell lysate was centrifuged at 200 000 × *g* for 2 h at 4°C and pellets (membranes) were washed with saline buffer, centrifuged and suspended in 1/3 of the original volume to obtain the HvEMP fraction. Protein concentration was determined by the bicinchoninic acid method [19]. Alkylation was performed by incubating HvEMP fraction (200 μg) with 0.2 M DTT at 70°C 10 min followed by incubation with 10 mM iodoacetamide at room temperature 30 min in darkness. Due to lipids interference with further assays and to the high salt concentration, a dual purpose protocol (lipid and salt removal) was carried out for delipidation of protein extracts [20]. Briefly, methanol (400 μL) was added to the sample (100 μL), gently mixed, chloroform (100 μL) was added, mixed, and spun down. Bidistilled water (300 μL) was added, vortexed, and samples were centrifuged (11 500 × *g* for 2 min). A protein "cake" was observed between phases. The upper aqueous phase was carefully discarded, methanol (300 μL) was added and after vortexing, the sample was centrifuged and pellets were air dried. Alkylated and control protein pellets from the HvEMP fraction were suspended in rehydratation solution (8 M urea, 2% CHAPS, 20 mM DTT, 1% IPG buffer, and traces of bromophenol blue) before loading the pH 3–10 NL, 7-cm IPG strips (GE Healthcare, USA). The strips were rehydrated in a reswelling tray overnight at room temperature. IEF was performed on an Ettan IPGphor 3 (GE Healthcare) using the following program: 250 V for 2.5 h, raised in a linear gradient first to 4000 V, and then to 8000 V in a 2 h step each. The 8000 V was maintained until a total run of 35 kV/h was achieved. The long first step was used to eliminate residual salt in the sample. Then, strips were equilibrated with 2% DTT followed by 2.5% iodoacetamide in equilibrium buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol), 30 min each step. The second dimension was performed in 10% polyacrylamide gels [21]. Proteins were visualized by colloidal CBB stain [22].

The colloidal CBB-stained IPG strips of pH 3–10 NL (Fig. 1, control) showed a colorless zone from pH 3 to 6.2 indicative of the effective transfer of proteins to the gel; in contrast, the stained alkaline zone (pH 6.2–10) represented the proteins that were retained in the strips. This result agrees with the pattern generated by fluorescent-labeled membrane proteins of *H. salinarum* [15]. Alkylation slightly improved the transference of acidic proteins; however, this treatment

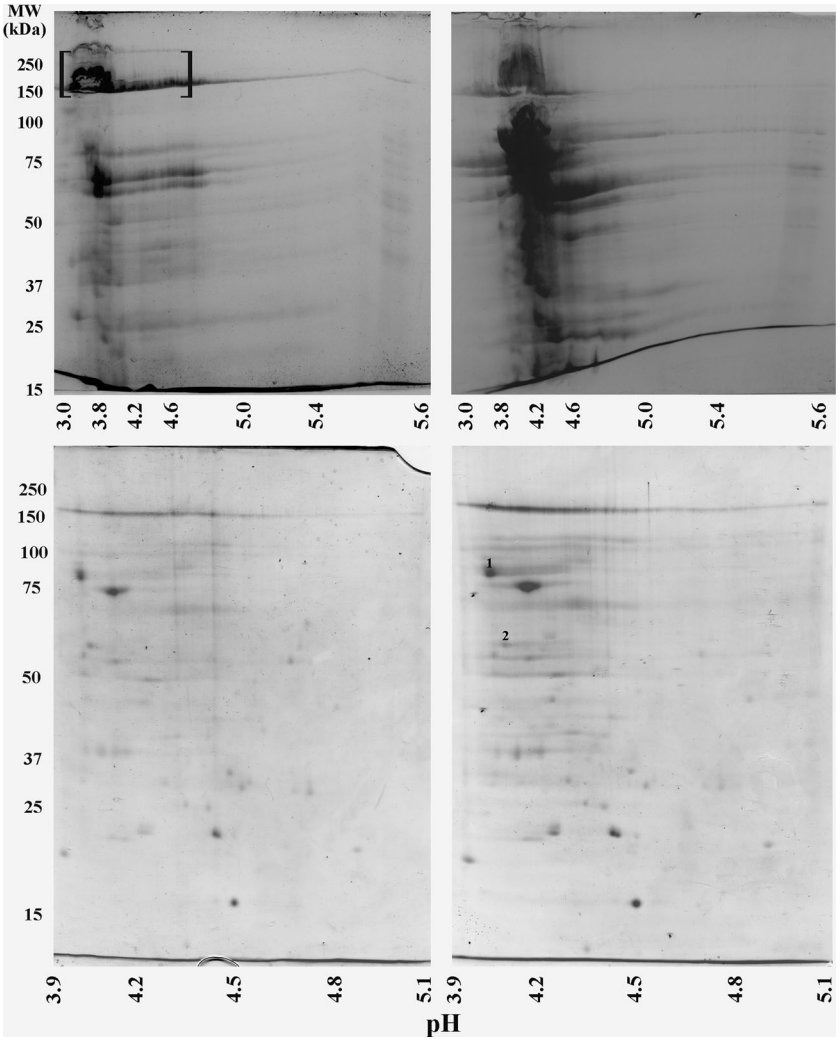

Figure 2. 2D separation of enriched membrane proteins of *H. volcanii*. Delipidated HvEMP fraction (160 μg) with (A) or without (B) SLG were fractionated on IPG strips pH 3–5.6 NL, 7 cm. Position of SLG is indicated between brackets. Note the decrease in the amount of SLG relative to the lower molecular mass proteins. Two amounts (50 or 100 μg) of delipidated SLG-free HvEMP fractions (C or D, respectively) were fractionated on micro range IPG strips pH 3.9–5.1, 11 cm. Proteins were visualized by colloidal CBB stain. Spot 1: thermosome subunit 1, spot 2: NADH dehydrogenase.

did not have any effect on the transference of basic proteins (Fig. 1). Based on our result and those obtained by Klein et al. [15], who attempted to solubilize the *H. salinarum* proteins in the strip, it can be suggested that the main factors producing retention of the proteins are the gel matrix–protein interaction more than protein precipitation per se. This observation points out that a different inert support should be developed for the IEF strip to improve transference of alkaline membrane proteins to polyacrylamide gels.

In spite of the difficulties found to analyze the alkaline protein fraction, the large amount of acidic proteins present in the membrane proteome of haloarchaea in addition to the simplicity, variety, and availability of the 2DE systems, made it worthy to optimize a protocol to study this particular group of proteins. Thus, obtaining peptide maps with defined spots and enough protein mass by 2DE is a prerequisite for further protein identification by MS methods.

A major problem for 2DE as well as for the current membrane proteomic strategies such as SDS-PAGE combined with LC-MS/MS, benzyldimethyl-*n*-hexadecyl ammonium chloride (BAC)/SDS-PAGE blue native, etc. is that a large number of membrane proteins are underrepresented in the total protein fraction, thus, increasing protein load without affecting protein resolution would be desirable.

As mentioned, archaeal cell envelopes contain the cytoplasmic membrane and S-layer [5]. The latter is composed of oligomeric units of S-layer glycoprotein (SLG) anchored to the membrane. SLG is the most abundant protein in the haloarchaeal acidic membrane protein fraction masking less represented polypeptides. As SLG can be partially detached by EDTA treatment [23], its removal seems a logical step to improve detection and analysis of minor proteins. To remove SLG, *H. volcanii* cells (50 mL culture) were centrifuged (7000 × *g* for 10 min) and suspended in 30 mL MGM medium (without yeast extract or peptone) containing 290 mM Mg^{+2}. Then, 0.5 M EDTA pH 6.8 (10 mL) was added and, after 30 min incubation at 37°C, the cells were harvested [23]. In further experiments, EDTA-treated and control *H. volcanii* cells were used as source of HvEMP fraction. IPG strips (pH 3–5.6 NL, 7 cm; GE Healthcare) were used to analyze the acidic membrane protein group.

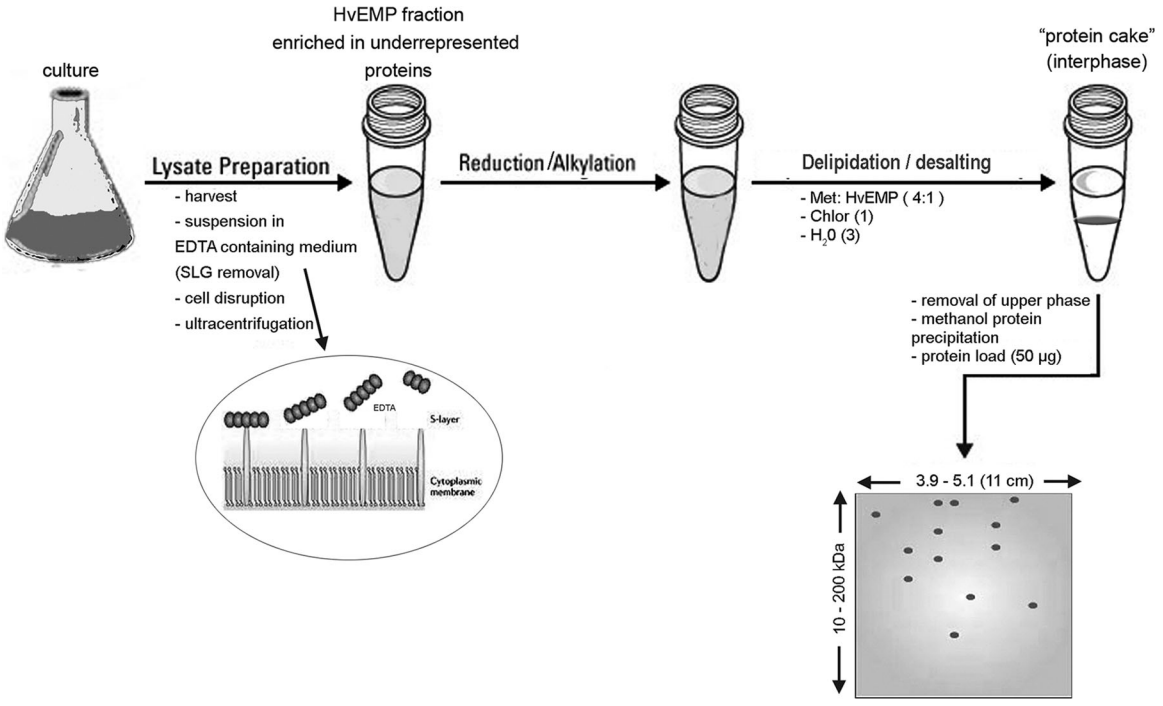

Figure 3. Sample preparation workflow.

A remarkable increase in the ratio of minor proteins to SLG (150 kDa) was observed in the EDTA-treated sample compared to untreated control (Fig. 2A and B). This simple step produced a striking improvement in the detection of less abundant proteins avoiding the risk of protein overloading.

Then, we aimed to decrease the horizontal streaks and obtain clearer, separated protein spots. As protein overloading is a common reason of horizontal streaks, two protein amounts and IPG strips (pH 3.9–5.1, 11 cm; BioRad, USA) were used. Reduction of almost 1/2 and 1/3 of the original protein load (100 and 50 µg of HvEMP, respectively) notably eliminated protein streaks allowing a better IEF of the acidic proteins present (Fig. 2C and D). In addition, the use of a longer strip and linear pH gradient generated a wider distribution of the spots. PDQuest software (v7.3.0, BioRad) under default conditions was used for semiautomated spot detection, considering spots aL, Ks, and nF as parameters (Supporting Information Fig. I). At least 75 defined spots were detected that represent 20% of the predicted membrane proteome within 3.9–5.1 p*I* range and 10–200 kDa, or a lower percentage considering that many proteins without a signal peptide or hydrophobic domain are associated to the membrane fraction [24–26]. A schematic representation of the overall procedure is summarized in Fig. 3.

Four random spots were excised from the gel and subjected to trypsin in-gel digestion, followed by PMF using a MALDI-TOF/TOF spectrometer (Ultraflex II, Bruker), at CEQUIBIEM facility, Argentina. Spectra were converted to DTA files and merged to facilitate database searching using the Mascot search algorithm v2.1 (Matrix Science, Boston, MA) against the nonredundant protein sequences of GenBank (NCBI, Bethesda, MD). Of the four spots, two were identified. Spot 1 (p*I* 4.0, 85 kDa) and spot 2 (p*I* 4.1, 60 kDa) corresponded to thermosome subunit 1 (p*I* 4.1, 58.8 kDa) and NADH dehydrogenase (p*I* 4.2, 42.7 kDa) from *H. volcanii*, respectively (Fig. 2D, Supporting Information Data spots 1 and 2). It was not surprising that the estimated apparent molecular masses of the proteins were higher than those predicted by genome sequences, as it is known that halophilic acidic proteins migrate slower in SDS-PAGE than nonhalophilic ones [27]. In agreement with our results, a similar apparent molecular mass for the *H. volcanii* thermosome subunit 1 was reported [28]. These predicted proteins did not contain motifs that would account for membrane localization; however they have been reported as membrane-associated proteins in several studies using different approaches [24–26].

Even though IEF/SDS PAGE is still far away to detect the whole membrane proteome of *H. volcanii*, this technique is still very useful to detect many proteins present in the membrane fraction, as complementary to LC-MS [29]. As previously reported, 2DE is capable to separate protein species by one charge or several hundred Daltons and for this reason is applicable when protein modifications occur at the functional level, unlike bottom-up methods [30, 31]. Most importantly, removal of SLG allowed the recovery of enough protein mass to study the underrepresented acidic membrane proteome of *H. volcanii*, which could then be identified by any existing technique in the proteomic workflow. This step can be applied to protein samples for 2DE as well as for other proteomic methods.

The authors have declared no conflict of interest.

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