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An Improved Sample Preparation Method for Analyzing Mycobacterial Proteins in Two-Dimensional Gels

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Abstract—Two-dimensional gel electrophoresis (2-DE) is currently a widely used analytical method for resolving complex mixtures of proteins. Sample preparation has a marked influence on 2-DE pattern. To reduce impurities and to increase the low-abundance proteins, protein precipitation is often used for the preparation of samples before 2-DE. In this study, we revealed that addition of SDS *prior to* TCA precipitation of mycobacterial cell extract proteins increases the resolution of the 2-D gel pattern.

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Despite landmark progress made in the development of alternative protein separation techniques, two-dimensional gel electrophoresis (2-DE) of proteins is still the most powerful analytical technique for the study of protein expression patterns. 2-DE separates proteins based on two parameters, isoelectric point (p*I*) in the first dimension and molecular size in the second dimension. 2-DE using an immobilized pH gradient (IPG) strip in the first dimension has proven to be a boon for protein analysis as it is highly flexible. Using 2-DE based approach, several workers have made significant contributions to identification of proteins from various organisms [1-4]. The majority of proteins resolved on 2-DE gels are highly abundant proteins, whereas lowly expressed proteins are usually not detected and hence identification of all proteins expressed in a given cell type is one of the major challenges to proteomics researchers. Various pre-fractionation procedures have been proposed to overcome this problem, and protein precipitation is the most commonly used method for the preparation of samples for 2-DE. Of several procedures available for sample preparation, protein precipitation with TCA followed by acetone washing is usually preferred for preparation of mycobacterial whole cell extract protein samples [5, 6]. The aim of the present study was to improve the recovery and resolution of mycobacterial proteins on 2D gels by modifying the TCA precipitation procedure. In this study, we found that addition of SDS to mycobacterial cell extract *prior to* TCA precipitation increased the resolution of protein spots on 2-D gels.

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MATERIALS AND METHODS

Mycobacterial cell extract proteins. *Mycobacterium bovis* BCG (Danish) procured from Mycobacterial Repository Centre at National JALMA Institute for Leprosy and Other Mycobacterial Diseases (Agra, India) were grown in Sauton's liquid medium at 37°C and cells were harvested in late exponential phase. Whole cell extracts were prepared according to the recommended protocol for subcellular fractionation [7] and clarified by centrifugation (12,000*g*, 30 min, 4°C). The protein content of the supernatant was assayed by Bradford method. Three equal volumes of cell protein extracts were taken into vials. Aliquot (A) was used for 2-DE without protein precipitation while the remaining two aliquots (B and C) were treated as follows for protein precipitation and further processing: trichloroacetic acid (TCA) (100% w/v) (Sigma, USA) was added to aliquot (B) at final concentration of 10% (w/v); to aliquot (C) 10% SDS (w/v) was added to achieve final concentration of 0.1% (w/v) and after boiling for 2 min TCA was added to get 10% (w/v) final concentration as in the case of aliquot (B). All mixtures were incubated at −20°C overnight and precipitated protein pellets were collected by centrifugation (18,000*g*,

15 min, 4°C). The pellets were washed with one volume of 100% ice-cold acetone and allowed to air dry. The protein pellets were suspended in appropriate volume of 2-DE rehydration buffer (Bio-Rad, USA) and the samples were subjected to 2-DE.

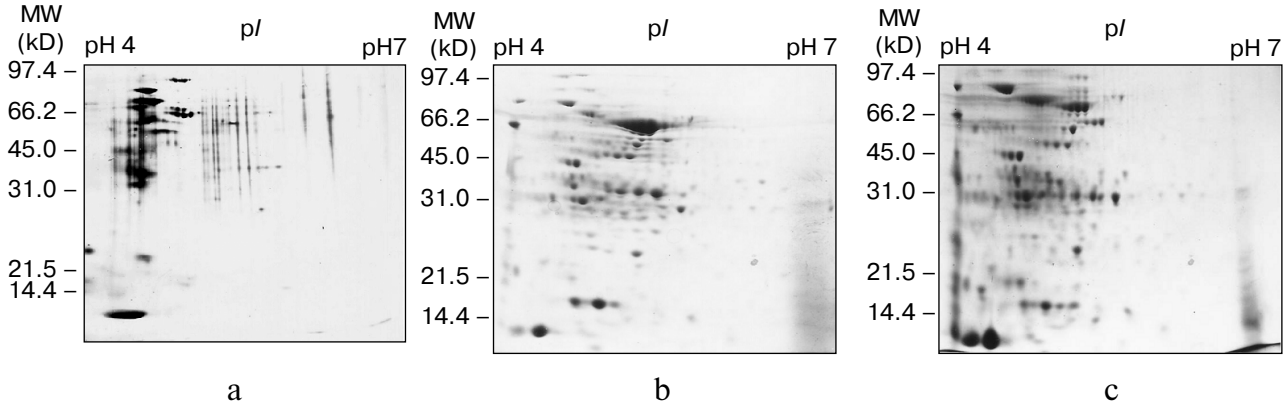
Two-dimensional gel electrophoresis. IEF (isoelectric focusing), the first dimension, was carried out employing the method of Gorg et al. [8]. IPG strips (Bio-Rad) of pH range 4-7 and length 7 cm were rehydrated with 100 μg protein suspended in rehydration buffer. The strips were focused on a Protean unit (Bio-Rad) at 20°C using the following four-step program: a) 0-250 V, 1 h; b) 250 V constant for 1.5 h; c) 250-3000 V, 4 h; and d) 3000 V constant until 15 kVh. The current limit was set at 50 μA/strip. After IEF, each strip was equilibrated for 10 min in equilibration buffer I (Bio-Rad) followed by equilibration buffer II for 10 min. The second dimension was run on vertical 12% SDS-PAGE gels [9]. After the electrophoresis, gels were stained with Coomassie Brilliant Blue to visualize proteins. All experiments were carried out at least three times.

RESULTS AND DISCUSSION

The 2-dimensional electrophoregrams of the three mycobacterial cell extract protein aliquots (A-C) are presented in the figure. The addition of SDS to mycobacterial cell extract prior to TCA precipitation improved the resolution of 2-D gels (figure, panel (c)) compared to either sample without protein precipitation (figure, panel (a)) or one with TCA precipitation but without pretreatment with SDS (figure, panel (b)).

The 2-D gel without protein precipitation revealed protein spots, but with poor resolution, overlapping, and streaking. Moreover, the low number of spots detected by visualization suggests missing of lowly abundant proteins. To overcome these problems, preparation/processing of protein sample was thought ideally required before proceeding with 2-DE, which is still the most preferable technique for the analysis of protein profiles/expression under varied conditions. Systematic evaluation of 38 protocols to concentrate normal human urinary proteins *prior to* 2-DE analysis showed greatest protein recovery with ethanol and least with acetic acid [10]. The role of perchloric acid in enriching cell extracts has also been reported [11]. Sonication of sample after TCA precipitation was found to increase resolution and reproducibility of gel electrophoresis of hippocampal neurons [12]. Of several procedures employed for sample preparation, protein precipitation with TCA followed by acetone washing is usually preferred for preparation of mycobacterial whole cell extract protein samples [5, 6]. In our work also 2-DE of a sample obtained with TCA precipitation followed by acetone washing (figure, panel (b)) showed slightly better resolution compared to the non-precipitated sample, thus confirming the earlier reports. TCA usually helps by inactivating the proteases, thus minimizing protein degradation, removal of interfering compounds, and especially for the enrichment of very alkaline proteins like ribosomal proteins [13].

An ideal sample solubilization protocol for 2-DE should result in the dissociation of all noncovalently bound protein complexes and aggregates into a solution of individual polypeptides, which remain stable during the 2-D electrophoretic separation. SDS is a good solubilizing agent and can be used to increase protein solubilization. An increase in the number of protein spots was observed subsequent to solubilization of *Fasciola hepatica* whole sample with SDS [14]. This effect of SDS on the resolution of mycobacterial extract proteins has been validated in our study. Addition of SDS to mycobacterial cell extract before TCA precipitation has resulted in enhanced resolution and increased number of spots on 2-D gels (figure, panel (c)) compared to precipitation with TCA without addition of SDS (figure, panel (b)) or



Two-dimensional gel of *Mycobacterium bovis* BCG (Danish) whole cell extract proteins: a) non-precipitated; b) precipitated with trichloroacetic acid; c) sodium dodecyl sulfate treated, trichloroacetic acid precipitated, acetone washed

precipitation with both TCA and acetone without addition of SDS (data not shown). Although this study has been carried out with *Mycobacterium bovis* BCG (Danish) whole cell extract proteins, the method of sample preparation employed has been found to give identical results on 2-DE studies using *M. tuberculosis* clinical isolates (data not shown) and this would help, to a greater extent, those working on proteomics in tuberculosis.

To summarize, our results clearly indicate that SDS treatment prior to TCA precipitation is a better method of sample preparation in resolving mycobacterial whole cell extract proteins by 2-DE. This method would help in detecting, especially, low-abundance mycobacterial cell extract proteins which might be missed in 2-DE if samples are used without SDS treatment prior to precipitation with TCA or any other better precipitating agent that may be thought of.

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