Full Length Research Paper

A comparative evaluation of rehydration and cup-loading sample application for modified two-dimensional gel electrophoresis of human serum proteins using immobilized pH gradient

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Proteomics is a powerful technique to study proteomes extracted from biological sources. Proteome analysis classically is based on two-dimensional electrophoresis (2D-E) for protein separation and mass spectrometry (MS) for the protein identification. The serum protein analysis is a useful diagnosis that can be used as an indicator of the physiological or clinical status of a patient. One of the central and critical steps is sample application, therefore this method should be considered and optimized for 2-DE with immobilized pH gradient. The aim of this study was focused on comparative application of in-gel (rehydration loading) and in-cup (cup loading) sample application for 2-DE of human serum analysis. The results indicate that the number of spots detected with the rehydration loading was higher than the number obtained by cup-loading sample application. Also in this work, in order to obtain the best result with high resolution, a 2-DE procedure was optimized.

Key words: Proteomics, 2D-E, proteome, sample application.

INTRODUCTION

Proteomics is a novel technology for separation and analysis of proteins from tissue extract or biological fluids, reflecting alterations in gene expression profiles and posttranscriptional/translational modifications (Ahmed et al., 2003). This field is identified by separation technique, followed by identification and analysis of the separated proteins by mass spectrometry (MS) (Gygi et al., 2000). The core method of proteomics technology is two-dimensional gel electrophoresis (2-DE) that firstly has been reported in great detail by O´Farrell (1975). 2-DE is

a protein separation technique for the resolution of proteins according to their isoelectric points (pI) using isoelectric focusing (IEF) method in the first dimension and to their molecular masses (M_r) using SDS-PAGE method in the second dimension (Mi et al., 2011).

The change of the expression level of main serum proteins is an early signal of pathological states that may be indicative of diseases. Therefore, serum protein analysis using proteomics method is usually a useful diagnostic tool for disease indication (Ahmed et al., 2003). In recent years, attempts in using proteomics method were increased for the detection of potential novel biomarkers in human blood serum (Bons et al., 2007; Gong et al., 2008).

Many factors including sample preparation, sample application, rehydration method and pH range of IPG stripe, can affect protein resolution in 2-DE technology (Barry et al., 2003). The sample loading is an essential step in 2-DE with immobilized pH gradient. Sample can be applied either by applying it in the rehydration solution (rehydration loading or sample in-gel) or by applying it directly to the rehydrated immobilized gel with sample

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Abbreviations: 2-DE, Two-dimensional gel electrophoresis; **MS,** mass spectrometry; **IPG,** immobilized pH gradient; **pI,** isoelectric point; **IEF,** isoelectric focusing; **PMSF,** phenylmethylsulfonyl fluoride; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **DTT,** dithiothreitol; **TEMED,** N,N,N',N' tetramethylethylenediamine.

Table 1. IEF program for IPG strip (pH 4-7 and 18 cm).

Step	Voltage (V)	Step duration(h: min)
1	0-500 (G*)	1: 30
2	500	3:00
3	500-2500 (G)	1: 00
4	2500	3: 00
5	2500-3000 (G)	1: 00
6	3000	3: 00
7	3000-3500 (G)	2: 30
8	3500	6: 00

* Gradient.

cup (Long et al., 2006).

The aim of this study was focused on the comparative application of in-gel and in-cups loading of the serum samples for the improvement of the isoelectric focusing and 2-DE results with immobilized pH gradients.

MATERIALS AND METHODS

Immobiline dry strips (pH 4 to 7 and 18 cm), acrylamide, bis-acrylamide, CHAPS, Coomassie brilliant blue R-350 and SDS were purchased from Pharmacia. Urea, thiourea, bromophenol blue, DTT, Tris base, ammonium persulfate, glycine, TEMED, silver nitrate, PMSF and 2-mercaptoethanol, were of analytical grade and purchased from Merck (Germany).

Serum sample collection

Human serum samples were obtained from healthy donor personnel of Medical Biology Research Center (Kermanshah, Iran) by routine sampling. The blood samples (5 ml) were allowed to clot at water bath (37°C) for 30 min and then centrifuged at 3000 g. The supernatant was divided into aliquots and stored at -20°C for a maximum of 1 month.

Protein assay

The total protein concentrations of serum samples were determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Rehydration and in gel sample application

The sample containing 220 µg of proteins was mixed with a appropriate extraction buffer solution containing 8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 0.02 M Tris, 2.5% (v/v) IPG buffer (4 to 7), 1 mM PMSF and a trace of bromophenol blue to a final volume of 350 µl per strip. Then IPG strip side down into the lysis buffer and incubated at 37°C for 15 h in the strip holder. After incubation, the IPG strip was transferred in the groove of immobiline dry strip tray as the acidic end of the strip at the front anode electrode.

Rehydration and in-cup sample application

350 µl of rehydration buffer (8 M urea, 2 M thiourea, DTT, 40 mM

Tris, 2% ampholine, and a trace of bromophenol blue) were transferred in the strip holder and the IPG strip side down into it and incubated at 37°C for 15 h. After incubation, the IPG strip was transferred in the groove of immobiline dry strip tray as acidic end of strip at the in front of the anode electrode. Then the sample cup on the sample cup bar was placed near the cathodic end of the strip. After proper positioning of the sample cup, 60 to 80 µl of sample containing 220 µg proteins was applied on the strip.

First dimension separation (isoeletric focusing)

Before running isoeletric focusing, 300 ml of IPG cover fluid was poured into the tray to cover the IPG strips. The isoeletric focusing was performed using Multiphor 2 system (Pharmacia Biotech). Temperature was adjusted at 20°C by dual temperature circulator (Tamson Zoetermeer-Holl). The IEF voltage was applied using power supply (Pharmacia) according to a pre-set program (Table 1).

Equilibration of IPG strips

The focused IPG strip was incubated twice in a equilibration buffer, 0.05 M Tris-HCl (pH 8.8) containing 2% SDS, 6 M Urea, 30% Glycerol, 1% DTT and a trace of bromophenol blue. In the second equilibration step, DTT in equilibration buffer was replaced with 5% (w/v) iodoacetamide. Both equilibration steps were carried out at 37°C with gentle shaking for 30 min.

Second dimension separation (SDS- PAGE)

After isoeletric focusing, the second separation of serum proteins based on their molecular mass (KDa) was performed in a 12.5% resolving and 5% stacking poly acrylamide gels. For the second dimension, equilibrated IPG strip was placed on the upper edge of SDS-PAGE gel and sealed with 1% warm agarose dissolved in electrode buffer (25 mM Tris-base, 192 mM glycine and 3.5 mM SDS). Resolution was performed for 15 min at 50 V and 120 min at 150 V.

Silver staining

Acidic silver staining method provides a very sensitive tool for protein visualization with a detection level of 0.3 to 10 ng/spot. Silver staining was carried out according to the methods of Harvey (1997) and Mostafaie (2004) with some modifications. After electrophoresis, gels were fixed in appropriate first fixation solution (40% methanol and 7% acetic acid) for 30 min and second fixation solution (5% methanol and 7% acetic acid) for 30 min with shacking. The gel was submerged in 10% glutaraldehyde for 30 min followed by washes in deionized water. The gel was placed in deionized water containing DTT (5 ug/ul) for 30 min, and then in 1% silver nitrate for 30 min with gentle shacking and rinsed briefly in deionized water. The gel was developed in developing solution (3% w/v sodium carbonate, 0.019% w/v formaldehyde). After detection of protein spots, gel was placed in the stop solution (second fixation solution) for 30 min and finally stored in 7% acetic acid for future analysis. The 2-DE gel images were analyzed with image analysis software program, Image master 2D platinum 6.0 (Bio-Rad, USA).

RESULTS AND DISCUSSION

Proteomics, a powerful method to analyze proteome,

Figure 1. Two-dimensional pattern of human serum proteins in IPG (pH 4-7 and 18 cm) via cup-loading sample application, stained with silver.

principally is based on the two-dimensional gel electrophoresis to separate proteins and mass spectrometry for protein identification (Steven et al., 2000). 2-DE, the first step in proteomics, separates proteins based on their isoelectric points and molecular masses (Dorri et al., 2009; Mi et al., 2011). To date, several versions of 2-DE that was first reported by O΄Farrell in 1975, have been published (O΄Farrell, 1975; Issaq et al., 2008).

The new 2-D technique, introduced by Bjellqvist et al. (1982) utilizes an improved first dimension separation method that replaces the carrier ampholyte-generated pH gradients with immobilized pH gradients and replaces the tube gels with strips supported by a plastic film (Issaq and Veenstra, 2008). The immobilized pH gradient has resulted in higher resolution, improved reproducibility and larger protein loading capacity for 2-D electrophoresis (Righetti, 1990).

One of the main and critical steps for high resolution in 2-DE with immobilized pH gradients is sample application. Several methods for sample application have been developed; of these rehydration loading (sample in-gel) and cup loading (sample in cup) are the most usually employed. Although, cup loading has been found to reduce overall protein loss compared with rehydration loading, main problems with this method are leakage of proteins from the sample cup, operational variability caused by manual manipulation from rehydration to IEF, protein aggregation resulting from the presence of high concentration of proteins and formation of precipitates at the application point (Barry et al., 2003; Long et al., 2006). The sample in-gel application method used the large sample volumes without the use of sample loading cup, eliminates precipitation at the sample application point, and thus, improves uniform resolution without side effects throughout the pH range of the gel (Rabilloud et al., 1994).

In this study, in-gel and in-cup sample application methods for 2-DE of human serum proteins were evaluated. We applied the IPG strip rehydrated for overnight at 37°C for isoelectric focusing with immobilized pH gradients. After the isoelectric focusing, the gel was equilibrated and transferred to SDS-PAGE for the second dimension electrophoresis. Protein spots were visualized by silver staining as mentioned earlier.

Pattern profiles were obtained by sample in gel and sample in cup loading methods is shown in Figures 1 and 2. The obtained results after analysis of protein profile using Image master 2D platinum 6.0 indicated that the number of spots detected by the sample in gel method is higher than the number obtained by sample in cup loading method (Figure 3). As the results indicate, 211 and 719 stained spots are detected in gels loaded by sample in-cup and by sample in-gel application methods, respectively. Also, there was no protein aggregation and spot streaking in the 2-DE gel image with sample in gel application. Therefore, the sample in gel is more effective technique than sample in cup for the 2-DE of serum
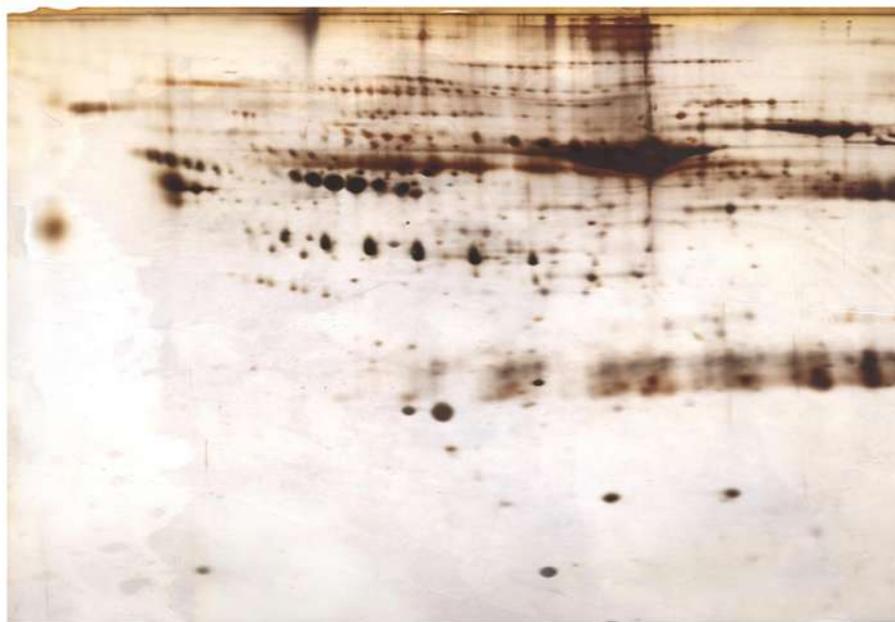
Figure 2. Two-dimensional pattern of human serum proteins in IPG (pH 4-7 and 18 cm) via sample in gel application, stained with silver.

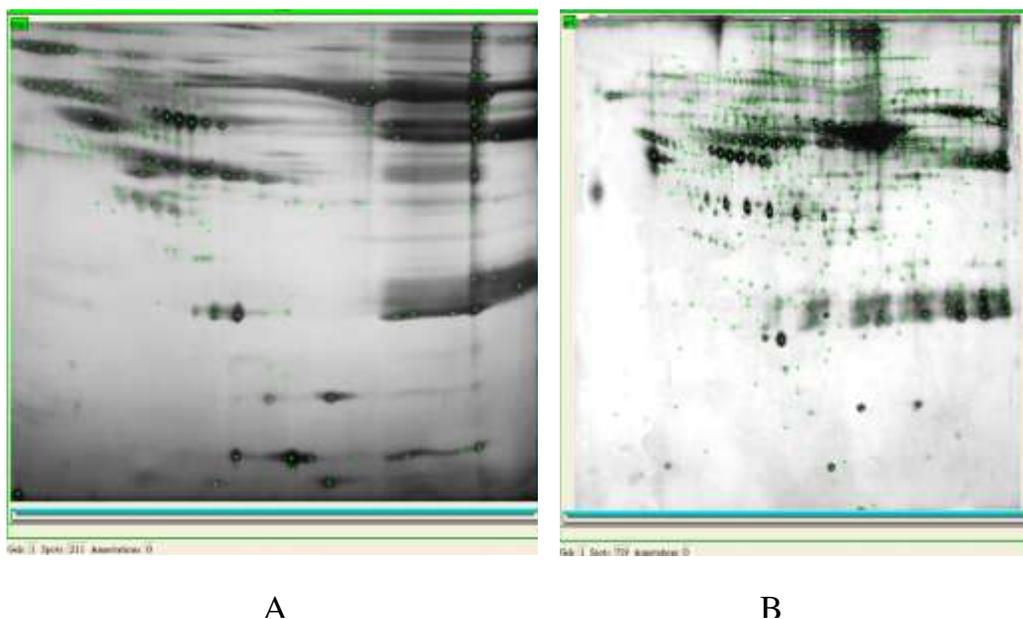A   B

Figure 3. Comparison of 2- DE gel images of human serum proteins in IPG (pH 4-7 and 18 cm) via sample in-cup (A) and sample in-gel (B) application and analyzed with Image master 2D platinum 6.0.

samples. In general, rehydration loading or sample in-gel application have higher capacity (more than 100 µl), technically is simpler and eliminates the aggregation of proteins at the application area.

In the present study, sample contained 220 µg of protein was used for sample loading application method.

However, increase in the amount of protein from 100 to 220 µg, showed significant and positive correlation between sample loading amount and spot numbers, but further increase in sample loading amount resulted in a high decrease in resolution of 2-DE gel (data not shown). This result was in accordance with the results of Sheng-

Bing et al. (2003).

Sample application has been suggested as an important step affecting the quality of 2-DE technology (Rabilloud et al., 1994) and some researchers successfully evaluated sample in gel and sample in cup application for two-dimensional electrophoresis with immobilized pH gradient (Panek et al., 1999; Joo et al., 2003; Barry et al., 2003; Jiang et al., 2004; Long et al., 2006; Luque-Garcia et al., 2006). Result of our study is in accordance with the results of Barry et al. (2003), however, in this report, quantitative evaluation of sample application was employed. Long et al. (2006) have reported a novel droplet-tap sample-loading method for 2-DE, and in this study, resolution compared with cup loading was improved.

Conclusion

We compared the in-gel and in-cup sample application for 2-DE and this method was optimized for plasma proteins analysis. In addition, we introduced modified 2-DE with immobilize pH gradient for serum proteome analysis. Resolution of pattern profiles of this study is better than many other results that resolved proteins of serums sample using 2-DE. These data can be considered as improved set of conditions and basis for future investigation on resolution and analysis of serum proteome.

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