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The Optimized Conditions of Two Dimensional Polyacrylamide Gel Electrophoresis for Serum Proteomics

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Abstract

Serum proteome analysis provides a potential promising approach in disease diagnosis and therapeutic monitoring. However, the large dynamic range of proteins, high-abundant proteins, excess of salt and lipid in serum makes the analysis very challenging. Therefore, it is imperative to improve the dissolution of proteins in two-dimensional gel electrophoresis (2-DE), and enhance the ability to analyze the proteome of serum under a wide variety of physiological conditions. This study examined the effects of various combinations of depletion high-abundant protein, precipitated means, hot SDS treatment and IPG strips with different pH on 2-DE map for mouse serum. Finally the removal of high-abundant proteins in serum by the ProteoExtract™ Albumin Removal column, ethanol precipitation, the heating with 2.5% SDS and 2.3% DTT to denature sample at 95°C for 3 min, and IEF on pH 4-7 IPG strips (17cm) with 100 μg depleted serum proteins are generally recommended for serum proteome analysis on 2-DE by silver staining, which can effectively improve the resolution and intensity of low-abundant proteins. The optimized conditions help to produce a better reference 2-DE gel of serum samples for following identification potential novel disease markers.

Keywords: Serum proteomics; Two-dimensional gel electrophoresis; High-abundant protein; Optimial condition

Introduction

Serum proteins are useful diagnostic tools and alteration of the expression of some serum proteins is an early sign of an altered physiology that may be indicative of disease (Poon and Johnson, 2001), therefore serum is usually a source for biomarker discovery by proteomics analysis. Potential novel disease markers are often present at low concentrations. However, the large dynamic range of proteins in serum makes the analysis very challenging because high-abundant proteins, such as albumin, IgG, antitrypsin, etc, tend to mask those of lower abundance on two-dimensional gel electrophoresis (2-DE) (Ahmed et al., 2003). There have developed several depletion columns to remove of a few high-abundant proteins in serum, which can increase the sample loading capacity and improve the detection sensitivity of low-abundant proteins (Björhall et al., 2005). In addition, many factors including sample preparation method, pH value of IPG stripe, staining approach, etc can affect protein separation and identification on 2-DE. 2-DE has major advantages in discovery of the overall alternations of specific proteins expression from serum sample (Anderson and Anderson, 1977; Omenn, 2004) , and the comparison of protein expression between normal and abnormal states of serum is widely applied to clinical and biomedical research in many reports (Judith et al., 2007; John et al., 2007; Bijon and Jürgen, 2007; Baukjede et al., 2008; Zhao et al., 2007; Feng et al., 2005).

In order to improve the separation of serum samples prior to comparative analysis for disease marker identification, we optimized 2-DE conditions for serum proteomics from sample preparation to deletion of high-abundant proteins and IPG stripes with different pH, etc several factors. The optimized conditions could substantially improve the solubility and resolution of the protein mixture derived from serum on 2-DE, which holds promise to accelerate the discovery of novel serum protein biomarkers.

Materials and Methods

Serum sample collection

Mouse blood serum and human serum(except hemolysis) were respectively collected from the BALB/c mice and healthy offers with their informed consent , and clotted for 2 h at room temperature (Jose et al., 2007). The clotted material was removed by centrifugation at 3000 rpm for 15 min. The concentration of the serum protein was determined using a protein quantitation kit (Bio-Rad, USA), by bovine gamma globulin as the standard. The protein samples were aliquoted and stored at -80°C without any treatment until further assayed.

Protein preparation for IEF

The serum samples were mixed with ice-cold ethanol or acetone in 1:4 v/v ratios and precipitated at -20°C overnight. The precipitated proteins were then centrifuged at 18000 *g* for 10 min, and air-dried pellets were dissolved in 20ul of a buffer containing 2.5% SDS and 2.3% DTT, denatured at 95℃ for 3 min(Joo et al., 2003). The concentration of the processed serum protein was determined using a Protein Quantitation Kit (Bio-Rad, USA). The serum proteins were suspended in sample buffer (7 mol/L urea, 4% CHAPS, 60 mmol/L DTT, 2% BioLyte 3-10, BioRad) for the first dimensional isoelectric focusing (IEF) analysis on 7-cm, pH3-10 IPG strips.

Depletion of high-abundant proteins

In order to remove the main high-abundant proteins from serum, the ProteoExtract™ Albumin Removal column (ProteoExtract™ Albumin Removal Kit; Calbiochem, San Diego, CA) was used to treat mouse serum. The orginal 40ul serum sample was processed with one removal column for each time, and the depletion procedure was performed at room temperature according to manufacturer's instructions. The depleted serum samples were precipitated with 4 times of ice-cold ethanol as above, and same amount of original and depleted serum was performed 2-DE in parallel.

IEF on IPG strips with different pH value

In the first dimension, proteins were separated by IEF with precast IPG strips (nonlinear gradient pH3-10 or pH 4–7, 17 cm, BioRad). Proteins were diluted in the lysis buffer (7 mol/L urea, 4% CHAPS, 60 mmol/L DTT, 2% BioLyte 3-10 or 4–7; BioRad) to obtain a total volume of 350ul per strip. Focused IPG strips were rehydration at 50 V for 12 h. IEF was performed at 20°C with a constant power (50*u*A/ IPG-strip) at 250 V for 30 min, linear; 1000 V for 1 h, rapid; linear ramping to 10000 V for 5 h; and finally 10000 V for 6 h.

As for the IEF on 7-cm IPG strip, the depleted serum proteins were diluted in the lysis buffer (7 mol/L urea, 4% CHAPS, 60 mmol/L DTT, 2% BioLyte 3-10 or 4–7; BioRad) to obtain a total volume of 125ul per strip. Focused IPG strips were rehydration at 50 V for 12 h. IEF was performed at 20°C with a constant power (50*u*A/ IPG-strip) at 250 V for 30 min, linear; 1000 V for 1 h, rapid; linear ramping to 4000 V for 3 h; and finally 4000 V for 4 h.

SDS-PAGE

After IEF, IPG strips were equilibrated in 50mM Tris/HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS and 10 mM DTT for 15 min, washed with 50 mM Tris/HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, and 200 mM iodoacetamide for another 15 min. Then the IPG strip was transferred to the top of 12% SDS-PAGE gel, and finally embedded in low melting agarose subjected to SDS-PAGE electrophoresis at constant current (20mA for initial 30min, then 30 mA/ IPG-strip for the rest of time for 17-cm IPG strip; 80V for initial 30min, then 120V/ IPG-strip for the rest of time for 7-cm IPG strip). According to the previous reported gel staining approaches of SDS-PAGE for serum sample (Joo et al., 2003; Zhang et al., 2004) and our experiments in advance (data not shown), the proteins were stained with silver staining , which shows more sensitive for detection the low-abundant proteins than staining with colloidal Coomassie brilliant blue. Therefore, proteins were stained with silver staining after separation on SDS-PAGE.

Silver staining

The silver staining for SDS-PAGE gels was performed as following. Gels were fixed in 45% ethanol and 5% acetic

acid for 2 h, washed with deionized water for 20 min twice, and then incubated in the sensitization solution containing 0.02% sodium thiosulfate for 2 min. After washing with water for 5 min for 3 times, gels were stained in a solution containing 0.1 % silver nitrate for 30 min at 4°C. Protein spots were developed in a solution with 2% (w/v) sodium carbonate and 0.04% formaldehyde until spots were clearly visible. Then 1% acetic acid was applied to halt development and stained gels underwent three 5-min washes in water.

Image analysis

The images were scanned using the Bio-Rad GS-800 scanner under white lights (400-750nm). Gel image was processed and features were detected with PDQuest software (Version 6.1, Bio-Rad).The computer program PDQuest identified protein spots from the digitalized images of the gel, and the 2-D PAGE resolved proteins were located and quantified automatically. Each serum sample was repeated three times and variability between the experiments was assessed on three different gels. The data was analyzed by comparision the reproducibility of the position and quantity of protein spots between the two repeated 2-DE gels for one same sample (Pánek and Vohradský, 1999), by which the 2-DE map was determined to be reproducible when more than 80% matching was achieved among gels (Blomberg et al., 1995).

Results

Hot SDS treatment can improve protein separation

In order to desalt and remove lipid in the serum, the hot SDS method was used to enhance the solubilization of serum as following: the air-dried of serum by ethanol precipitation were dissolved in 20ul of a buffer containing 2.5% SDS and 2.3% DTT, denatured at 95°C for 3 min. And finally 200ug total proteins was diluted to 125ul with a sample rehydration buffer to run IEF. As a result, we can obviously observe that more spots were detected on the 2-DE (7-cm, pH3-10 nonliear IPG) via hot SDS treatment than that not using this method. However, the concertration of the SDS in the protein samples should be limited under 0.25%, w/v ratio, due to negative charges of SDS may affect the PI of sample.

Precipitation optimization

Because the salts, nucleic acids and lipids in serum could interfere with the 2-DE, precipitation is generally employed to concentrate and selectively separate proteins in the sample from the contaminating species. We compared the effects of two different precipitation solvent (Jiang et al., 2004), respectively ethanol and acetone. Serum was mixed with ice-cold ethanol or acetone at 1:4 v/v ratios and precipitated at -20°C for overnight .The precipitated samples were then centrifuged at 18000 *g* for 30 min, the supernatant was removed and the pellet was air dried. The pellet was suspended in 500*ul* of sample buffer to run 2-DE. As a result, more protein dots with middle and low molecular weights appeared on the 2-DE map by ethanol precipitation than that by acetone precipitation (Figure 2).

Effect of the Removal of High-Abundant Proteins

Serum albumin can constitute 50-70% of the total serum proteins and IgG is up to10-25%, the presence of these high-abundant proteins can mask other low-abundant proteins and cause loss of resolution in 2-DE. More less-abundant proteins from the depleted serum samples were separated and visible on 2-DE, and the aggregation of high-abundant proteins was greatly reduced , which resulted in the appearance of new protein spots compared to the crude serum smples (Figure 3).Therefore it is important to deplete high-abundant proteins, which is about 80% of total serum proteins, by using ProteoExtract™ Albumin /IgG remove column, as an essential first step in the characterization of serum so that 3-4 times more enriched sample can be loaded on 2-DE.

Efficiency of IPG strips with different pH range

In order to enhance resolution of serum low-abundant proteins, we compared with two kinds of IPG strips, respectively pH 3-10 (NL) and pH4-7. The protein spots mainly appeared on the acid part of 2-DE gel with pH 3-10 (NL) IPG strip, in contrast, the whole protein pattern was relatively uniform and more protein spots were separated on 2-DE gel with pH 4-7 IPG strip (Figure 4). This was due to the majority of the serum molecular and PI gathered between 45-80kD and 4.5-6, therefore, using narrow range immobilized pH gradients with a large sample loading volume allows an efficient resolubilization of polypeptides after the first dimension (Bjellqvist et al., 1993).

The optimized conditions of 2-DE for serum sample

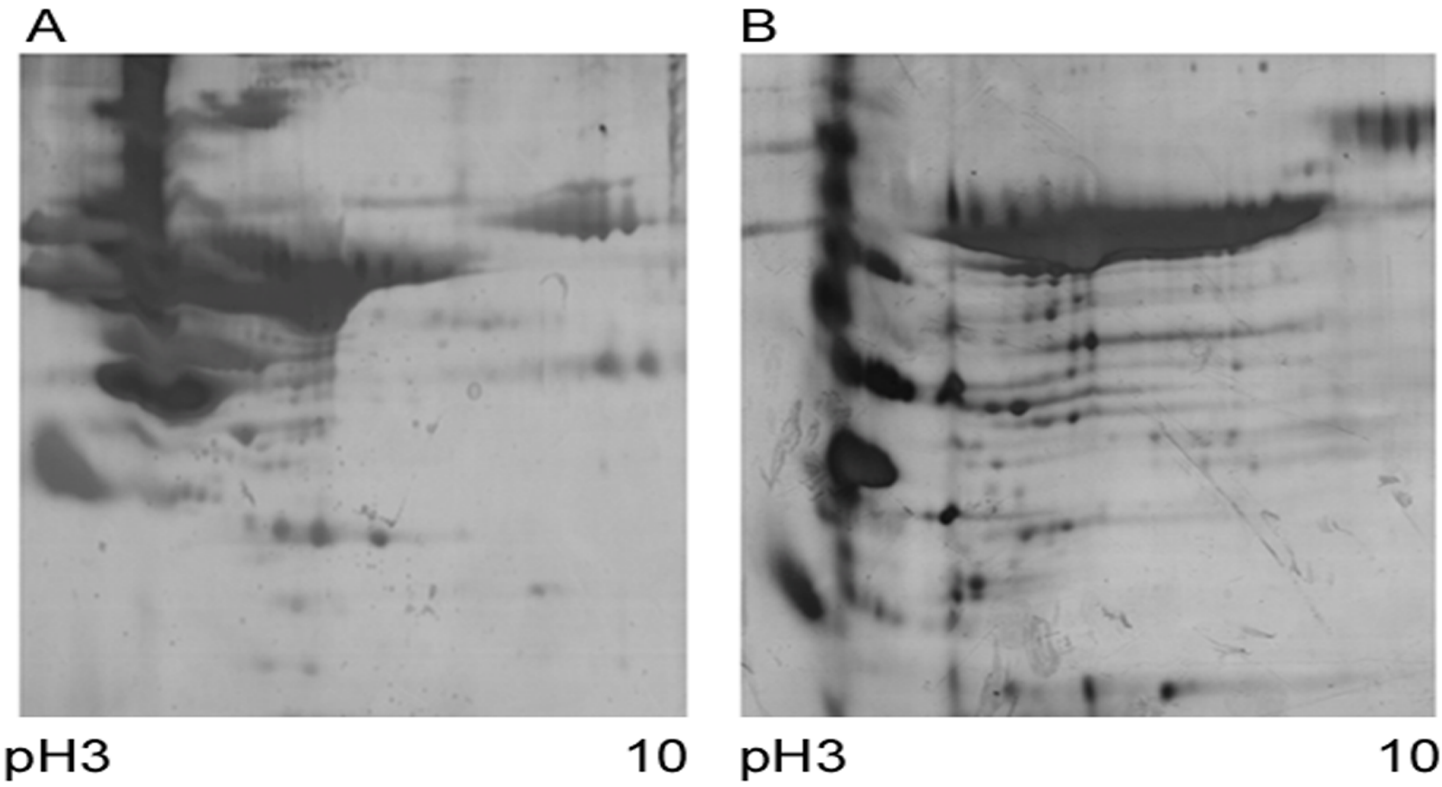

Figure 1: The 2-DE image of serum proteins prepared by hot SDS method.(A) Unprocessed by hot SDS method, and (B) processed by hot SDS method. The IEF was performed on 7-cm IPG strips (3-10 NL), loading with 200*u*g of total mouse serum proteins.

Based on our above experiment results, the conditions including the removal of high-abundant proteins using ProteoExtract™ Albumin /IgG remove kit, then depleted serum sample precipitated by ethanol, following hot SDS treatment and denatured at 95°C for 3 min, and finally IEF manipulation on pH 4-7 IPG strips, were the optimized parameters to run 2-DE for serum samples. Under these conditons, 100 µg depleted mouse serum samples was loaded on the 17-cm IPG strip with pH4-7 and sufficiently separated on 2-DE (Figure 5A). Similarly, the 2-DE map was clearly with 40µg depleted serum proteins loading on a 7-cm IPG strip(pH4-7) (Figure 5B).

Each experiment was performed triplicate to ensure the accuracy of analyses. The maps were analyzed by PDQuest software Version 6.1 (Bio-Rad). The 2-DE gel pattern was similar to each other, and almost 90% matching on spot position and quantity was achieved. As for the 3 repeated 17-cm gels with pH4-7 for mouse depleted serum analysis, a subset of 316 spots was matched in all gels out of an average of 342 resolved proteins. In other words, the spots matching percentage was about 92±3%. By using only the spots that matched across all gels, the mean standard deviation of 20.6% was calculated among the three gel replicates. An average deviation of 0.72mm (*x*-position) and 0.45 mm (*y*-position) were obtained for all three gels. Similarly, we applied this optimized conditions to human serum and obtained a good 2-DE map repeatedly (Figure 5C). These comprehensive optimized 2-DE conditions for serum proteomic analysis provided a better foundation later to find and identify more candidate changed proteins between two serum samples.

Discussion

Appropriate sample preparation is essential for obtaining good results in 2-DE. The removal of high-abundant proteins from serum is a necessary step in biomarker discovery studies. There have several commercially available depletion columns for removal of high-abundant protein in serum, which have different depletion efficiency and binding specificity (Ahmed et al., 2003). In addition, Valerie et al. (2005) used the Gradiflow BF400 as a fractionation tool to deplete highly abundant albumin from human plasma (Valerie

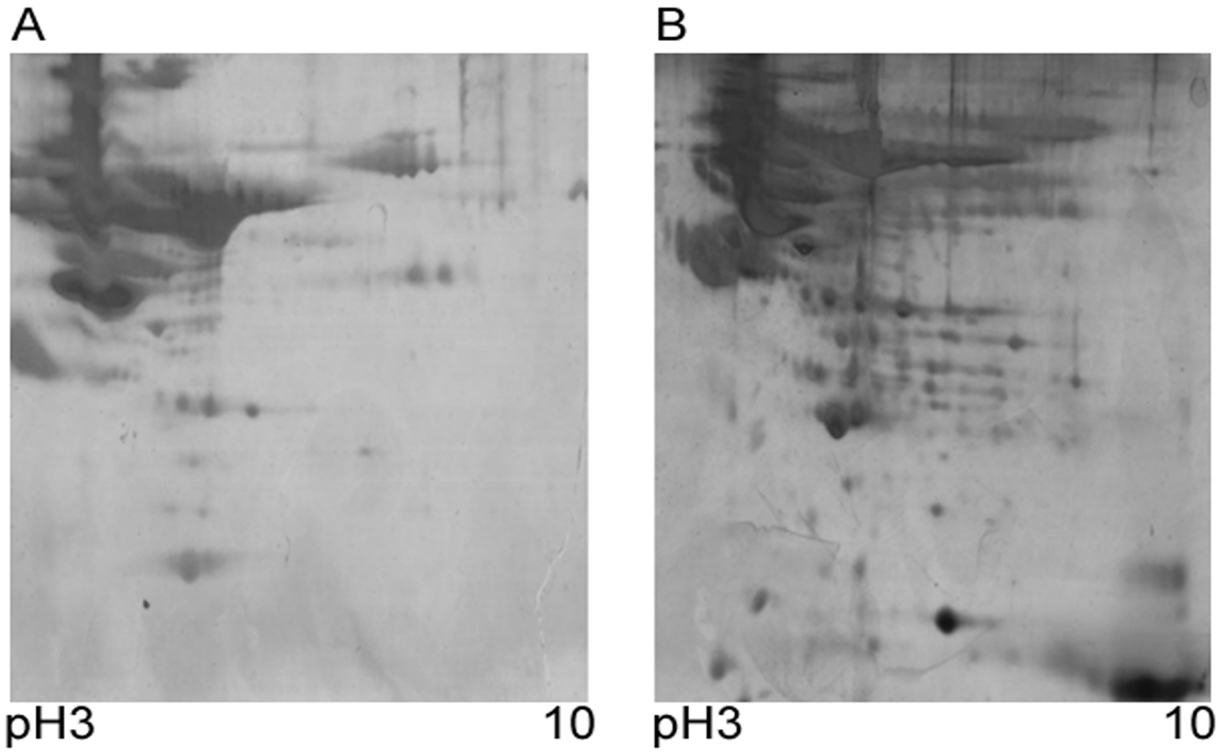

Figure 2: The 2-DE images of serum proteins respectively prepared by precipitation with acetone (A) and ethanol (B). The IEF was performed on 7-cm IPG strips (3-10 NL), loading with 200*u*g of total mouse serum proteins.

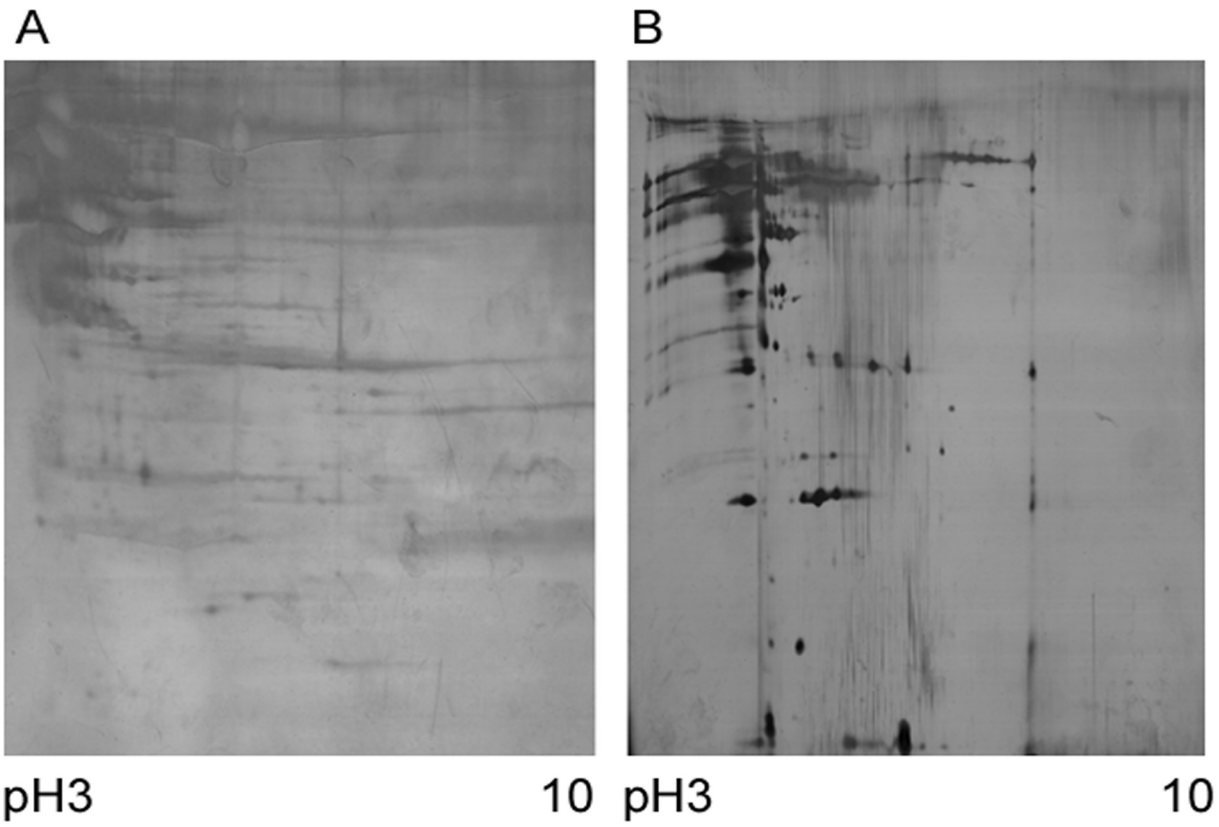

Figure 3: The serum 2-DE patterns with depletion of high-abundant proteins. (A) Unprocessed mouse serum sample, and (B) the mouse serum after removal of high-abundant proteins. The IEF was performed on 17-cm IPG strips (pH3-10 NL), loading with 100*u*g of total serum proteins.

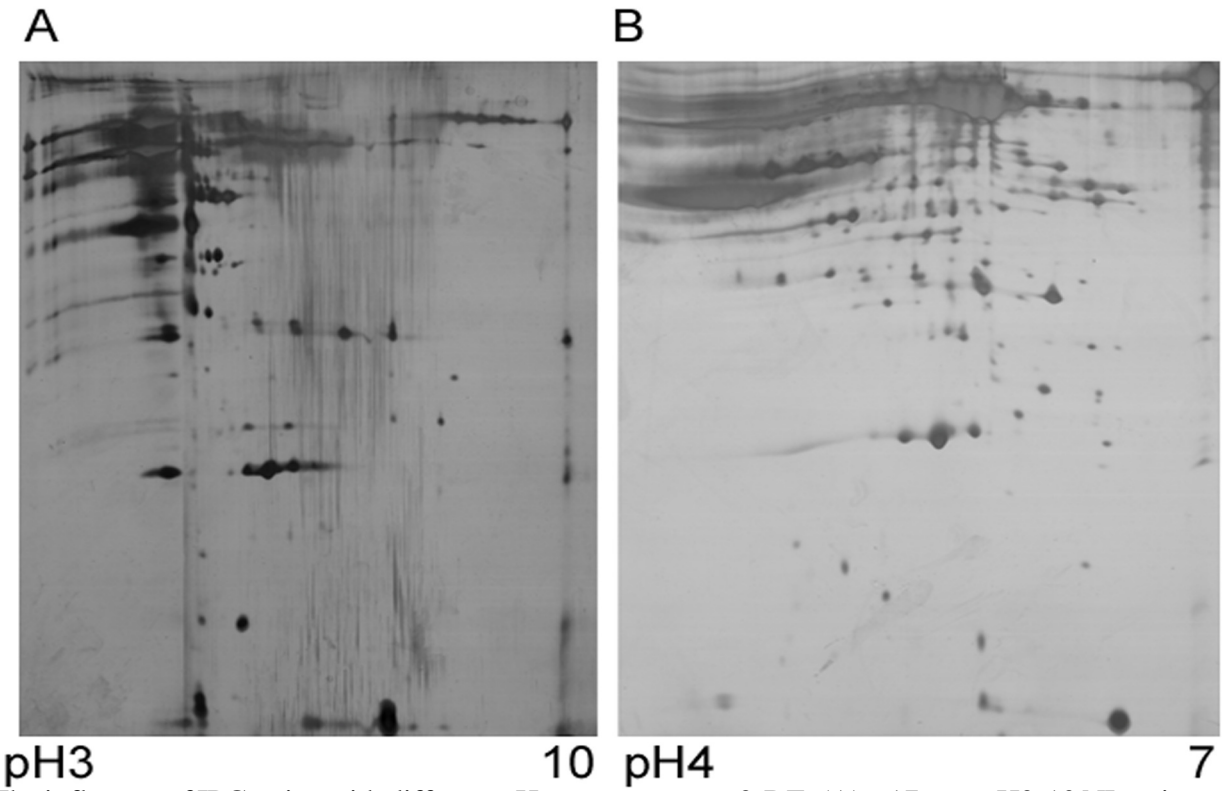

Figure 4: The influence of IPG strips with different pH range on serum 2-DE. (A) a 17-cm, pH3-10 NL strip and (B) a 17-cm, pH 4-7 IPG strips, loading with 100*u* g of depleted mouse serum proteins respectively

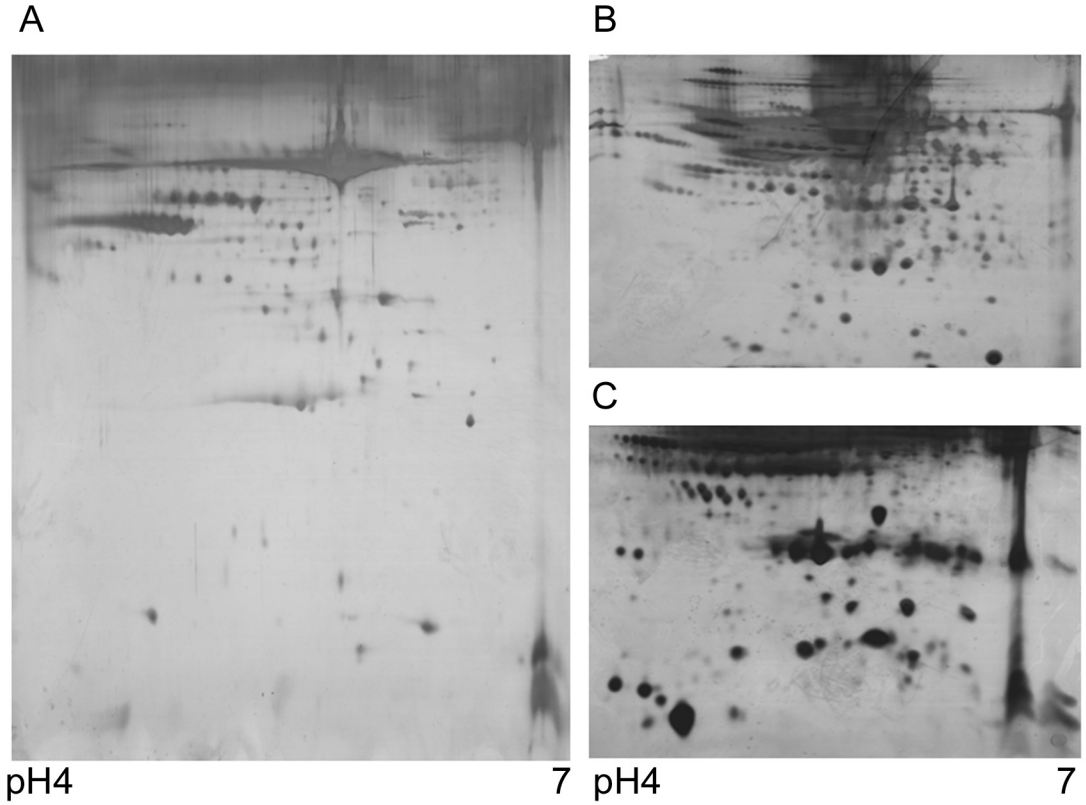

Figure 5: The best optimized 2-DE conditions for serum samples. (A) a 17-cm, pH 4-7 strip loading with 100*u*g of depleted mouse serum proteins; and (B) a 7-cm, pH 4-7 strip loading with 40*u*g of depleted mouse serum proteins; (C)a 7-cm, pH 4-7 strip loading with 40*u*g of depleted human serum proteins.

et al., 2005). However, pre-separation for serum samples is required more advanced methods. Our goal was to find the most efficient method of sample analysis for serum proteomics.

Because serum albumin is a predominant protein in serum, we used an albumin depletion strategy prior to serum sample proteomic analysis. The albumin depletion approach we used is based on disposable (single-use) prepacked albumin-specific binding resin columns (ProteoExtract™ Albumin Removal Kit; Calbiochem, San Diego, CA), which was validated to be the best albumin/IgG removal kit with good efficiency, specificity and reproducibility (Ahmed et al., 2003; Björhall et al., 2005). According to the datasheet provided by the user protocol of ProteoExtract™ Albumin Removal Kit, more than 80% and 70% of serum albumin and IgG can be removed respectively from human and mouse plasma, which was almost verified by our serum sample manipulation (data not shown). Furthermore, this kit was simple to manipulate. However, it was disposable for single use and only small amounts of depleted proteins was available each time. Our results showed that more less-abundant proteins from the depleted mouse serum samples were separated and detected on 2-DE. Similarly, other prefractionation techniques including serum precipitation, hot SDS treatment and IPG strips with pH4-7 can effectively increase the resolution of low-abundant serum proteins. These modified approachs are also applied for other serum samples from other species including human beings, because the main components of human serum are similar to that of mouse serum. Therefore, the removal of high-abundant proteins in serum, ethanol precipitation, the heating with 2.5% SDS and 2.3% DTT to denature sample at 95°C for 3 min, and IEF on pH 4-7 IPG strips are generally recommended for serum proteome analysis on 2-DE. Overall, our study provides comprehensive optimized conditions, and assists in the following discovery and detection of low-abundant proteins in serum by mass spectrometry that may prove to be informative biomarkers.

Acknowledgement

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