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Short Research Communication

EFFECT OF LIPID ON GEL BASED ELECTROPHORETIC PROTEOMIC EXPERIMENTS

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Abstract: Gel based clinical proteomic experiment requires an optimum quality of proteins extracted from the human tissues for good resolution of the spots on the gel. Lipid is one of the common contaminants of tissue samples in clinical proteomics. To determine the effects of lipid in gel based proteomic experiments, constant amounts of protein with varying concentrations of phosphotidyl choline was subjected to single and two dimensional electrophoretic separations. While a lipid concentration of 5µg distorts the resolution and visualization of the protein bands on SDS PAGE, higher amounts of lipid in the order of 200µg have a very minimal effect on the resolution of the spots in two-dimensional gel. The biophysical principles causing the varying effects of the lipid on the two types of gel based proteomic experiments is highlighted.

Keywords: lipid; proteomics; two-dimensional gel electrophoresis.

Introduction

Proteomics pertains to the study of a specific proteome of a tissue or an organelle, including their modifications, abundances and variations along with their interacting partners and networks, in order to understand cellular processes. Clinical proteomics is a sub-discipline of proteomics that involves the application of proteomic technologies on clinical specimens such as human body fluids and tissues to understand better the various physiological and pathological conditions for biomarker and drug target identification (Hariprasad *et al.*, 2013). Proteome analysis is a technically challenging task accentuated by the fact that under a given condition and time, thousands of proteins are expressed in cells. Two dimensional electrophoresis has been successfully applied for such complex proteomic analysis because it separates proteins on the basis of charge and molecular weight and helps to quantify the relative abundance (Rukmangadachar et al., 2011). With the wide spectrum of diseases and the variety of tissues, efficient sample preparation is extremely important for considerable resolution of the proteins in electrophoretic separation (Kataria *et al.,* 2011). This makes it essential to ascertain the quantity of protein to be loaded and minimize the presence of interfering non-protein contaminants.

The possible contaminants of clinical proteomic samples include: salt, DNA and polysaccharides. These contaminants have a tendency to interfere with resolution and visualization of protein spots. Understanding the effect of these contaminants and importance of removing them has been shown to save labor, cost and most importantly for efficient use of precious

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tissue samples (Hariprasad *et al.*, 2011; Berkelman *et al.*, 2008). Though there have been a number of studies discussing the role of ionic impurities and nuclear molecules, there has been little done to understand the role of lipids on elecrophoretic proteomic experiments.

Some of the important aspects pertaining to lipids and proteomic experiments are: (i) increased use of lipid-rich tissues such as brain, spinal cord, adipose tissue and oil-plant tissues in proteomic experiments (Manral *et al.*, 2012; Kaur *et al.*, 2012). (ii) failure to eliminate lipids and hydrophobic molecules after acetone precipitation; (iii) presence of high amounts of lipid bound protein resistant to solubilization by conventional detergent based buffers and (iv) increased focus of research on hydrophobic rich lipid-membrane proteins (Saravanan *et al.*, 2007). Lipid interference is therefore a dimension that may affect the outcome in terms of visualization of proteins, their resolution and reproducibility of the gels in clinical proteomics.

Materials and Methods

Ethics, patient selection criteria and consent

The study was approved by the Ethics committee of All India Institute of Medical Sciences (Ref. No. IEC/NP-23/2013) and procedures followed were in accordance with the ethical standards formulated in the Helsinki declaration. Written informed consent was taken from the patients before enrolling them into the study. Cyto-reductive debulking surgery was done for patients with advanced stage ovarian cancer at the Department of Obstetrics and Gynecology. Tissue samples were taken from the dissected ovarian cancer, washed gently with 1x PBS and stored in -70 °C.

Sample Preparation

Snap frozen ovarian cancer tissue samples were homogenized, resuspended in lysis solution (8M Urea, 2M thiourea and 4% CHAPS) and centrifuged at 5000rpm for 15minutes at 4°C. Debris was discarded and the supernatant was transferred onto a fresh Eppendorf tube. The protein concentration was estimated using Bradford method and lipid concentration was estimated using vanillin-phosphoric acid method (Cheng *et al.*, 2011). A gradation type lipid-protein mixture were prepared by adding varying amounts of phospholtidyl choline to constant amounts of the protein extracted from the ovarian cancer tissue samples.

SDS PAGE

0µg, 2.5µg, 5µg, 10µg, 25µg and 50µg of phospholtidyl choline were added to 100µg protein. The SDS loading dyes was added to the lipid-protein mixture samples and loaded for electrophoresis on a 10% acrylamide gel. The electrophoresis was conducted at a voltage of 20V during the stacking phase and increased to 40V during the separating phase of the gel. The electrophoresis was run for till the tracking dye was seen to be running out of the gel. The gel was stained with coomassie brilliant blue-250.

2D Electrophoresis

Rehydration

0µg, 25µg, 50µg and 75µg of phospholtidyl choline were added to 300µg protein. The lipid-protein mixtures were taken for 2D electrophoresis. 1.25µl of IPG-buffer (pH 3-10NL) (Amersham Biosciences, USA), 0.002% bromophenol blue and 0.75mg of DTT was added to the each sample. Final volume was adjusted to 250µl lysis buffer. After mixing, the tube containing samples were centrifuged at 16,000xg for 2min and loaded on a rehydration tray (Amersham Biosciences, USA). IPG-strip of pH range 3-10, 13cm was used for IEF. Plastic cover on the strip was carefully removed and the strip was placed over the sample for overnight (14-16h).

Iso-electric Focusing

Rehydrated IPG strip was subjected for iso-electric focusing in an IPGphor 3 (Amersham Biosciences, USA) as per the following program: 1. Step mode, 50V for 30min; 2. Step mode, 100V for 30min; 3. Step mode, 200V for 2h; 4. Step mode, 500V for 1; 5. Gradient mode, 500-1000V for 1h; 6. Step mode, 1000V for 2h; 7. Gradient mode, 1000-6000V for 2h; 8. Step mode, 6000V for 3.30h or till total volt-hours of 29,000 was achieved.

Strips were covered with mineral oil and temperature was set at 20 °C.

Second dimension separation

At the end of iso-electric focusing, the strips were equilibrated in SDS-equilibration buffer containing 50mM Tris-HCl, pH 8.8, 6M urea, 30% glycerol, 2% SDS, and 0.02% bromophenol blue. Strip was first equilibrated for 15min with 0.05% DTT prepared in 5ml of SDS equilibration buffer at RT. The solution was decanted carefully and replaced with 1.25% iodoacetamide solution, prepared in SDS equilibration buffer, for 15min at RT. Meanwhile a 12% polyacrylamide gel was cast by on a Ruby gel apparatus (Amersham Biosciences, USA). Strip was carefully loaded on the PAGE and sealed with 0.5% agarose containing 0.002% bromophenol blue prepared in SDS-electrophoresis buffer (25mM Tris base, 192mM glycines 0.1% SDS). Gel was run at 15mA for 30min and then at 30mA till the bromophenol blue came out of the gel. The gels were stained by colloidal coomassie blue and destained by 1% acetic acid in water.

Results and Discussion

Tissue is composed of a variety of lipids like cholesteryl esters, triglycerides, free fatty acid, monoglycerides, diglycerides and phospholipids. In this study, the concentration of the lipid in the ovarian cancer tissue was 3.2µg/100µg of protein. Phospholipids form more than 62% of the total tissue lipids. Phosphatidylcholine is the most abundant phospholipid in the human body amounting to 50% of the total and is the main constituent of membrane bilayers. Also, phosphatidylcholine is the principal phospholipid circulating in plasma as an integral component of the lipoproteins. Therefore, investigation of lipid interference in gel electrophoresis was performed using phospahidylcholine.

Lipid-protein samples were run on the SDS PAGE. It is observed that the resolutions of the bands are distorted with increase in concentration of lipid (Figure 1). The explanation lies in the interference of phospahidylcholine with SDS-protein interactions. The proteins migrate as SDS-protein micelles in SDS PAGE. Phospahidylcholine interferes in the formation of

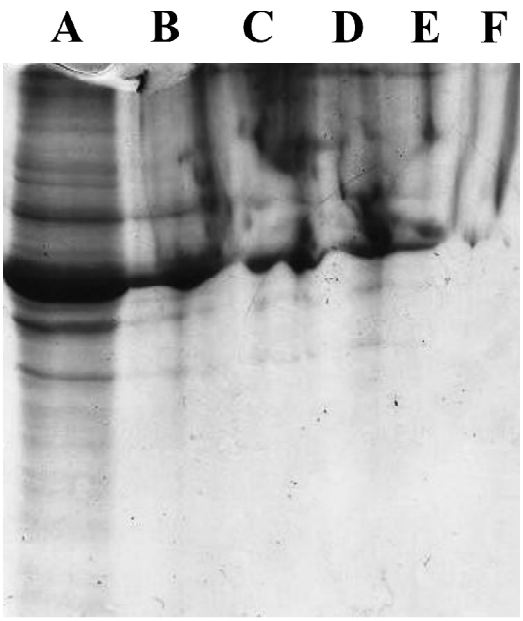

Figure 1: **SDS PAGE of 100µg protein from ovarian cancer tissue with (A) 0µg, (B) 2.5µg, (C) 5µg, (D) 10µg, (E) 25µg and (F) 50µg of lipid. The lanes B, C, D, E and F show distortion of the bands due to the presence of lipid**

SDS-protein micelles by interacting individually with proteins and SDS. This results in phospahidylcholine-protein micelles and phospahidylcholine-SDS micelles. The phospahidylcholine micelles are formed with a CMC ranging from nM to mM depending on the substitution at C1 and C2 of glycerol. This leads to more heterogeneity of the sample with respect to the distribution of lipid-protein complex of varying sizes. In addition, choline in phospahidylcholine imparts a positive charge to the complex thereby negating the negative charge bearing SDS-protein complexes further adding to the distortion. These factors affect the resolution of the bands seen on SDS PAGE.

However, the varying concentration of lipid on the 2D electrophoresis showed interesting results. The two most evident findings from the 2D gels are: (1) complete absence of spots on the acidic side of the gel, and (2) good resolution of the spots across the rest of the gel area (Figure 2). Phospholtidylcholine is a neutral lipid, but has an electric dipole moment of 10D (Mashaghi *et al.*, 2012). This results in the protonation of the negatively charged phosphate groups at acidic end of the strip to give the lipid a net positive charge. This increase in the ionic concentrations

at the acidic end of strip has resulted in burning and a subsequent loss of focusing of proteins at this end. The burning is clearly demarcated by the thick vertical streaking at the left part of the gels and the length of the strip lost is seen to be proportional to the amount of lipid loaded. Streaking seen in the first gel (Figure 2A) is due to inherent lipid in the ovarian cancer tissue. However, the resolution of the rest of the proteins is not affected and is evident by the presence of the spots across the remaining width of the gel. Considering the fact that there are less than 2% proteins with a pI less than 4, the extent of spots that are not visualized because of lipid may not be very significant in 2D gel based tissue proteomic experiments.

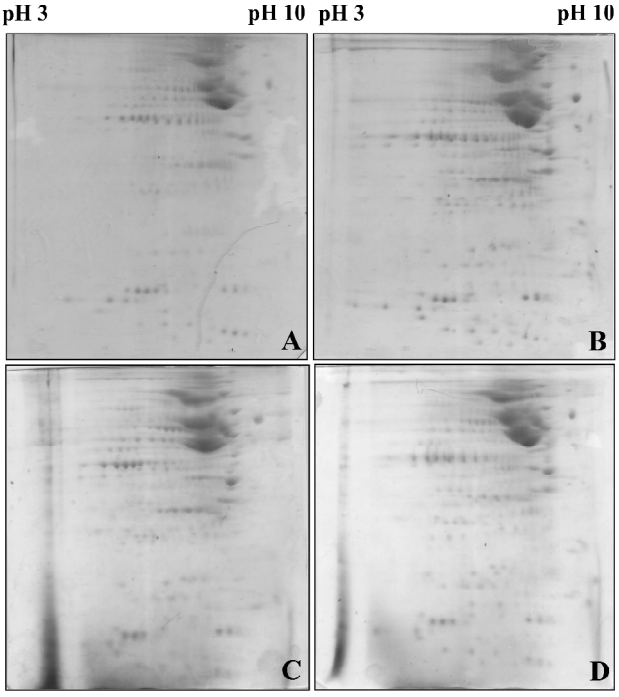

Figure 2: **2D electrophoresis of 300µg of ovarian cancer tissue proteins with (A) 0µg, (B) 75µg, (C) 150µg and (D) 225µg of lipid. Protein spots are absent on the extreme left part of the gels. Vertical streaking is seen on the acidic side of the gel.**

Conclusions

Lipid adversely affects resolution of protein bands in SDS PAGE experiments while it has less influence on the resolution of spots in 2D elecrophoretic experiments. It is therefore concluded that lipid removal is essential for SDS-PAGE but not mandatory for 2D gel elecrophoretic proteomic experiments with tissue extracts.

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Abbreviations

CMC, Critical Micellar Concentration; SDS PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; DTT, Dithiothreitol; 2D, Two-dimensional.

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