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COMPARISON OF METHODS OF EXTRACTION AND SEPARATION OF PROTEINS FROM URINE

COMPARACIÓN DE MÉTODOS PARA LA EXTRACCIÓN Y SEPARACIÓN DE PROTEÍNAS DE LA ORINA

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Resumen: las proteínas son excelentes biomarcadores pues proporcionan una indicación directa del estado biológico, y pueden ser analizadas en los fluidos corporales. La orina ha despertado un gran interés en los últimos años como fuente de biomarcadores, ya que es una ultrafiltración del plasma, su colección es mínimamente invasiva y las muestras son fáciles de manejar. Sin embargo, ciertas características de la muestra, tales como la dilución de las proteínas y el alto contenido de sal, presentan desafíos para el análisis de proteínas. En este artículo corto se comparan cuatro métodos para la extracción de proteínas de la orina, concluyendo que la precipitación con acetona produce los mejores resultados en cuanto a concentración de proteínas y resolución en electroforesis 2D. Este método de extracción se puede realizar fácilmente en cualquier laboratorio sin la necesidad de kits comerciales.

Palabras clave: electroforesis, extracción, orina, proteínas

Abstract: proteins are valuable biomarkers because they provide a direct indication of a biological state or condition, and they can be assayed in body fluids. Urine has roused great interest in recent years as a source of biomarkers, as it is an ultrafiltration of plasma, its collection is minimally invasive and samples are easy to handle. However, certain features of the sample, such as the diluted protein concentration and high salt content, present their own unique challenges for protein analysis. In this brief communication we compare four methods of protein extraction from urine, and conclude that acetone precipitation yields the highest protein concentration, as well as the best resolution of protein spots in 2D electrophoresis. This method of extraction can be easily performed in any lab without the need for commercial kits.

Keywords: electrophoresis, extraction, protein, urine.

1. INTRODUCTION

Proteomics, which studies the proteins expressed by a system (called proteome), is an area that has been widely researched in the past decade. Proteins are directly responsible for carrying out most tasks within the cell; therefore a change in protein expression levels is an indication of a pathological process (Xiao et al., 2005). In the last ten years very Sensitive techniques have been developed for proteome analysis from biological samples such as cells, tissues and body fluids (Fang & Zhang, 2008; Patterson & Aebersold, 2003). This has led to the discovery of biomarkers, which are molecules whose expression levels or changes in the body are indicative of a biological state (Goo & Goodlett, 2010), and which can be used to assist in the diagnosis of a disease, to evaluate prognosis and to monitor response to treatment.

There are different types of biomarkers; these can be genetic (DNA and RNA), proteins, secondary metabolites produced by signaling cascades activated in pathological processes, or other organic molecules. Although DNA and RNA biomarkers have been widely studied, protein biomarkers have gained recognition due to their close association with the cell's biological activity.

As mentioned earlier, biomarkers can be detected in various biological samples, but blood has been the most widely studied of these as it comes into contact with the whole body. However, proteomic analysis of this sample has certain challenges such as the activation of proteases during sample collection, which generates a range of proteolytic products and introduces sample variability (Omenn et al., 2005). Moreover, in blood there are 20 high abundance proteins that make up approximately 99% of the proteins in the sample (Caubet et al., 2010; Veenstra et al., 2005), and which mask other less abundant, but potentially interesting, proteins. For these reasons urine has roused great interest in recent years. Urine is an ultrafiltration of plasma and is very stable compared to blood, it is easy to handle and large volumen can be obtained in a minimally invasive manner (Gonzalez-Buitrago et al., 2007). However, certain features of the sample, such as the diluted protein concentration and high salt content, present their own unique challenge for protein analysis. Therefore, one of the priorities in the field of urinary proteomics in recent years has been to optimize methods of protein extraction from urine. In this brief communication we compare four methods of protein extraction that can be performed in any research lab without the need of expensive commercial kits.

2. MATERIALS AND METHODS

2.1 Sample Collection

Second morning urine samples were collected from three non-diabetic healthy males, between 30 and 40 years of age. Volunteers with hypertension, prostate disorders, urinary tract infections or those taking medication were excluded.

2.2 Urine sample processing

The collected samples were centrifuged at 2000 rpm for 10 minutes in order to remove cells, cellular debris and mucus present in the sample. Subsequently, the supernatant was filtered with 0.2

Membrane filters (Millipore0). Finally the samples were pooled and stored at -20°C until protein analysis.

2.3 Protein extraction and quantification

To extract the proteins present in the pooled urine sample four precipitation methods using acetone, ethanol, methanol and acetonitrile were tested. The sample was mixed with the solvent in a 1:4 volume ratio and was incubated overnight at -20°C. The following day the sample was centrifuged at 11,000 g for 30 min at 4°C, and the pellet washed twice and resuspended in ultrapure water. The protein yield of each extraction method was quantified using the bicinchoninic acid method according to the manufacturer's specifications (BCA-kit, Bicinchoninic Acid Kit, Sigma-Aldrich®). Each extraction method was performed twice using different samples.

2.4 Two-dimensional electrophoresis (21))

To confirm protein integrity the pooled samples were first analyzed by one-dimensional electrophoresis run on 12% polyacrylamide gels (SDS-PAGE). Following this, two-dimensional electrophoresis was performed on the protein samples from the two most effective extraction methods.

For the first dimension (isoelectric point, pI), 50 ug of protein were resuspended in hydration buffer (7M/2M urea/tiourea, 4% CHAPS, 100 mM DTT and 1% carrier ampholytes pH 3-10) at 4°C. The IPG strips (range pH 3-10, 7 cm, Invitrogen ®) were rehydrated with 190 µL of the protein sample (49 ug total protein) and incubated overnight at room temperature. The next day the first dimension was run for 1 hour and 40 minutes, with a progressive increase in voltage as follows: 200V - 450V - 600V - 750V - 950V for 5 minutes , 1200V - 1400V - 1600V for 10 minutes each and finally 2000V for 45 minutes (ZOOM IPG RunnerTM , Invitrogen ®). After this, the IPG strips were equilibrated with running buffer (Invitrogen® LDS buffer) plus DTT for 15 minutes at room temperature. For the second dimension (separation by molecular weight), the strip were placed in the pre- formed gels (4-12 % polyacrylamide Bis-Tris, NuPAGE-gel, Invitrogen®) and the gels were run at 200 volts (XCell Sure LockTM , Invitrogen O) for 45 minutes with NuPAGE MOPS SDS Running Buffer 1X (Invitrogen ®).

2.5 Gel staining

The protein bands on the SDS-PAGE gel were visualized using Coomassie blue, whereas for the 2D gels silver staining was performed according to the manufacturer's recommendations (SilverQuest TM Staining Kit, Invitrogen O).

3. RESULTS AND DISCUSSION

To determine the best method for extracting protein from urine samples we compared four methods of extraction using different organic solvents. The best protein yield was obtained with acetone precipitation (1.49 ug/ul), followed by ethanol (1.04 µg/µl); while protein precipitation using methanol and acetonitrile gave the lowest yields, 0.17 µg/µl and 0.60 respectively, as can be seen in Fig. 1.

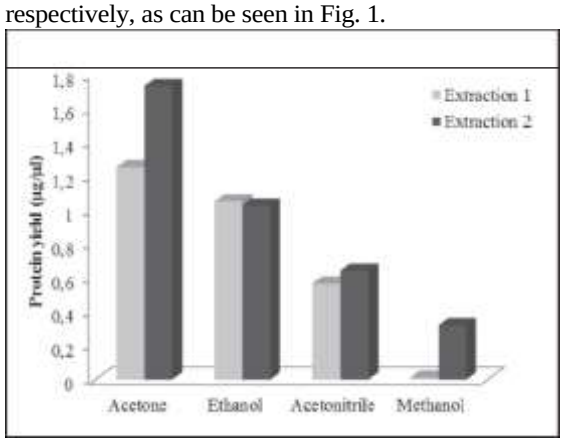

Fig. 1. Comparison of four methods of protein extraction from urine.

The proteins extracted with each method were subjected to separation by SDS-PAGE and stained with Coomassie blue to evaluate protein integrity. As can be seen in Fig. 2, protein bands can be observed across the whole range of molecular weights, with the biggest bands at approximately 70 kDa and 25kDa. It is likely that these bands correspond to albumin and 1-microglobulin, the two most abundant proteins in urine, although it is difficult to identify a band simply on the basis of its molecular weight.

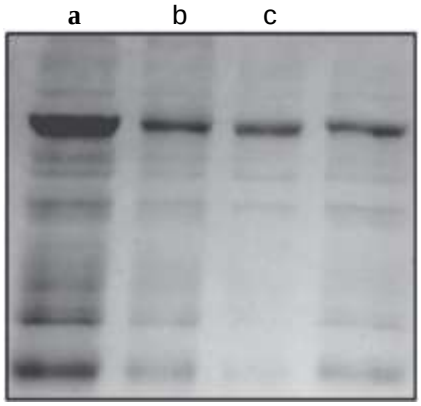

Fig. 2. Protein separation by SDS-PAGE. a) acetone, b) ethanol, c) methanol, d) acetonitrile.

Proteins extracted from the two most successful methods, acetone and ethanol precipitation, were subjected to separation by 2D electrophoresis and stained with silver nitrate. The highest number of protein spots was obtained with acetone precipitation, as can be seen in Fig. 3. As mentioned previously, the high salt content in urine can affect the subsequent analysis of the sample. Therefore, we recommend dialysis of the protein extracts before 2D electrophoresis to improve the resolution of protein spots.

In general, the threshold of glomerular permeability in the kidney is around 68 kDa, therefore urine contains many low molecular weight proteins, as can be seen below.

Acetone Ethanol

Fig. 3. Comparison of the 2D urinary protein profile acetone and ethanol precipitation.

4. CONCLUSIONS

In this brief communication we have shown that acetone precipitation of proteins in urine yields the highest protein concentration, as well as the best resolution of protein spots in 2D electrophoresis. This method of extraction can be easily performed in any lab without the need for expensive commercial kits. Future work will focus on testing desalting methods to increase resolution of protein spots in 2D electrophoresis.

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