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Full Length Research Paper

Enrichment methods for revealing lower molecular weight biomarkers in human breast plasma proteome

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Breast cancer is the most common malignancy in women, comprising about 18% of all female cancers. The most important tools in screening and early detection are imaging techniques: mammography, ultrasonography and magnetic resonance imaging. However, up to 20% of new breast cancer incidents cannot be detected by these methods. Up to now, poor diagnosis of breast cancer is due to a lack of specific biomarkers. Therefore, there is much interest in identifying useful pathological markers that can help in detection and treatment of this disease. Up to now, detecting minor proteins in human plasma is a difficult strategy due to the presence of several highly abundant proteins such as albumin and haptoglobins. The objective of this study was to develop a reproducible method that depletes high-abundant proteins and improves detection of minor ones. For this purpose, we evaluated the impact of using Affi-gel blue affinity columns and organic solvent (acetonitrile) precipitation on the removal of high abundant proteins. Fractionated plasma protein extracts were subsequently analyzed by SDS-PAGE and two dimensional gel electrophoresis. Our findings confirm that Affi-gel blue can enhance detection of minor plasma proteins and could thus be a useful strategy in the discovery of new breast cancer markers.

Key words: Affigel blue, albumin, acetonitrile, plasma, proteomic separation, breast cancer.

INTRODUCTION

Breast cancer is the most frequent malignancy among women (Hondermarck, 2003). Current methods used to detect breast tumors are based on mammography, ultrasonography and magnetic resonance imaging. However, since a tumor should be at least a few millimeters in size, it is already late when breast cancer is detected. So, there is a considerable need for the identification of useful pathological markers that can help not only in early detection but also for treatment (Vercoutter-Edouart, 2001). Proteomics approaches have brought with it the hope of discovering novel biomarkers that could be used to diagnose diseases (Hondermarck, 2003; Vercoutter-Edouart, 2001).

Human plasma has an important clinical value for identification and detection of biomarkers. It is considered as the most diverse proteome among body fluids. In addition, plasma is very interesting from the medical point of view as most cells communicate with it and many cells release at least part of their content into it upon damage or death (Anderson, 2002; Hulmes, 2004; Jacobs, 2005). Actually, plasma proteomics has become an important research strategy for biomarker screening, diagnosis and therapy. However, proteomic analysis of plasma is analytically challenging due to the high dynamic concentration range of some of its constituents.

In blood, the most abundant proteins include albumin,

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Abbreviations: 2DE, Two-dimensional dlectrophoresis; **SDS-PAGE,** sodium dodecylsulfate-polyacrylamide gel electrophoresis; **IEF,** isoelectric focusing; **ACN,** acetonitrile; **pI,** isoelectric point; **MW,** molecular weight.

haptoglobins and immunoglobulins. They represent approximately 90% of the bulk mass of proteins but they are less than 0.1% of total proteins. These high abundant proteins, in particular albumin, interfere with the detection of minor protein components. In this way, many strategies have been developed for the selective removal of albumin and other high-abundance proteins like, immunoaffinity columns, isoelectric trapping, dye-ligand chromatography, and peptide affinity chromatography (Fountoulakis, 2003; Govorukhina, 2003).

Actually, it has been estimated that over 10000 different proteins are commonly present in the plasma, most of which are at very low relative abundances (Adkins, 2002). The majority of potential disease biomarkers may be present at extremely low concentrations at low ng/ml to pg/ml levels, and thus might be masked by the presence of more abundant proteins (Anderson, 2002; Jacobs, 2005).

As an example, Chen (2005) performed a stepwise immunoglobulin G (IgG) and albumin protein depletion by affinity chromatography, followed by ultra high efficiency capillary liquid chromatography interfaced with ion trap-tandem mass spectrometry. This extensive fractionation led to the identification of approximately 2400 peptides and proteins in human plasma. Also, Moritz (2005) successfully used, 2D-free-flow electrophoresis, RP-HPLC to remove high-abundance proteins and to fractionate the plasma prior to proteome analysis. In addition, Tang (2005) used a four-dimensional strategy combining protein and peptide separation to increase the detection of low-abundant proteins in plasma and serum proteomes.

To detect minor abundant proteins in plasma at least two points must be settled: (1) Efficient throughput depletion strategy of high-abundance proteins and (2) postdepletion fractionation. To find the optimal conditions for 2-DE analysis of minor plasma proteins in breast cancer we therefore tested in the current study two different depletion methods (acetonitrile and Affi-gel blue affinity columns) and explored the impact of these procedures on the removal of high abundant proteins, as well as, on the amount of minor proteins that may remain after depletion.

MATERIALS AND METHODS

Plasma collection, preparation and storage

Breast cancer patients (n=6) were selected from the same population living in the middle coast of Tunisia. Tumors classified as infiltrating ductal carcinomas (IDCA) were pathologically staged according to the tumor node-metastasis classification system. Histological grade was assessed according to the system of Elston and Ellis. All patients were divided according to their disease stage. Stage grouping was based on TNM formula (Tumor, Node, Metastasis) of the patient, depending on tumor size, presence of regional metastatic lymph nodes and presence of distant metastases. Plasma was obtained at the time of diagnosis prior to any therapy from patients with histologically diagnosed breast cancer at the department of gynecology at Sousse Hospital after informed consent was given. The method for the collection of the plasma samples is of great important, as the composition and concentration of plasma proteins varies with the collection method. To prepare plasma, EDTA (ethylenediamine tetraacetic acid), was added to the blood samples which were then centrifuged at 1500 g for 10 min /4°C to avoid hemolysis, decanted and transferred into Eppendorf tubes as aliquots and frozen at -80°C until use.

Acetone precipitation

Four volumes of ice-cold acetone (400 µl) were added to 100 µl of plasma in order to precipitate total plasma proteins. Samples were allowed to stand for 30 min at -20°C. The sample solution was centrifuged at 12 000 rpm for 10 min at 4°C and supernatant was removed. The pellet washed with cold acetone (80%) then dried under vacuum was suspended in 100 µl of adequate solubilization buffer prior to SDS-PAGE or 2-D gel electrophoresis analyses.

Depletion of high-abundance plasma proteins

Acetonitrile precipitation

Acetonitrile (ACN) precipitation method involved adding 1 or 2 volumes of HPLC grade ACN (200 µL, 400 µL) containing 0,1% trifluoroacetic acid (TFA) to 200 µL of plasma, issued after acetone precipitation followed by vigorous vortexing for 5 s, and allowing it to stand at room temperature for 30 min. Samples were then spun for 10 min at 12000 rpm in a centrifuge at room temperature. Protein concentration in the supernatant was determined according to the procedure described by Bradford (1976) and modified by Ramagli and Rodriguez (1985).

Chromatography on mimetic blue (Affi-gel blue) and protein A/G

Because albumin and immunoglobulin IgG collectively account for 75 90% of the total plasma protein content, we selectively removed these proteins to enrich for proteins of minor abundance. Prior to SDS-PAGE and 2-DE analyses, immunoglobulins (Igs) were depleted by using protein A/G beads (Pierce) according to the manufacturer's instructions. Albumin was removed using Affi-Gel Blue (Bio-Rad, Hercules, CA, USA), a cross linked agarose bead with covalently attached Cibacron Blue F3GA that has previously been used successfully to visualize proteins of low abundance (Ahmed, 2003; Moutaouakkil, 2011). The attached dye functions as ionic, hydrophobic, aromatic, or sterically active binding sites for protein. Affi-Gel Blue has a high affinity for albumin and is suited for albumin removal. Briefly, breast patient's plasma samples (100 µl) were equilibrated in 20 mM phosphate buffer pH 7.1 (buffer A) by rapid column desalting on an Econo-Pac 10 DG column. Equilibrated plasma samples were applied to the Affi-Gel Blue column and washed with 2 bed volumes of buffer A. The effluent from this step contains the albumin-depleted fraction. The albumin enriched fraction was collected using 1.4 M NaCl in 20 mM phosphate buffer pH 7.1 and desalted as described previously before SDS-PAGE and 2-DE experiments. The albumin/IgG depleted plasma samples were stored at -80°C.

First dimension separation: Isoelectrofocalization (IEF)

Analytical 2-DE was carried out in a Bio-Rad system (Miniprotean II) as described previously (Hamrita, 2008). Equal amounts of proteins (80 µg) issued from breast plasma samples were applied

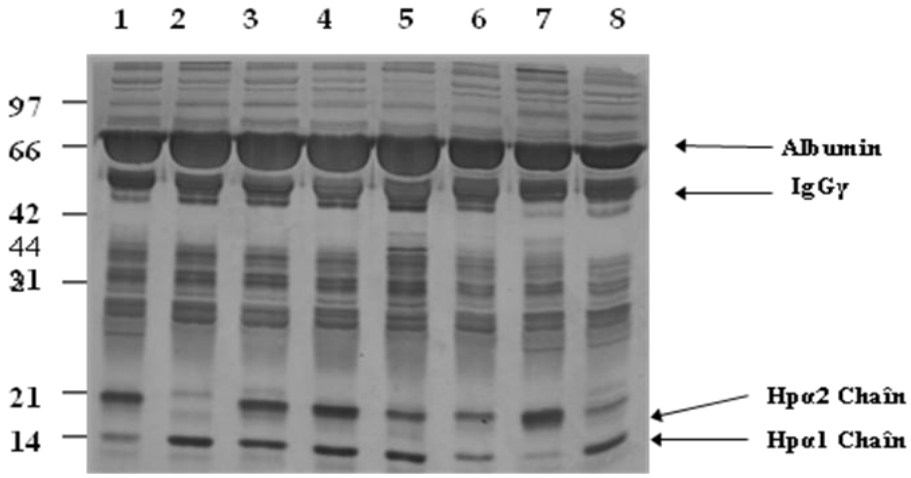

Figure 1. SDS-PAGE analysis of human plasma proteins before treatment. Four volumes of ice-cold acetone were added to plasma samples and proteins were. suspended in solubilisation buffer prior to SDS-PAGE. Proteins were stained with Coomassie blue (Lanes 2, 4, 6 and 8: plasma from breast cancer patients; Lanes 1,3,5 and 7: plasma from healthy controls).

to the first dimension and at least three IEF gels were run for each sample. IEF was performed on 7 cm IEF rod gels [pH 4.0–8.0] at 200 V for 15 min, 300 V for 15 min and 400 V for 18 h. Focused strips were equilibrated in SDS equilibration buffer (125 mM Tris–HCl pH 6.8, 2.5% (w/v) SDS, 10% (w/v) glycerol, 0.025% (w/v) bromophenol blue) and were then loaded onto 12% SDS gel slabs for separation in the second dimension. For each experiment, IEF and SDS-PAGE were carried under similar conditions. Electrophoresis was performed in triplicate to ensure reproducibility between runnings.

Second dimension separation : SDS-PAGE analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out with the Tris/glycine buffer system. 10 µg of plasma proteins were separated under reducing conditions on 12% SDS-PAGE mini gels (10 x 10.5 cm) at 140 V, and constant current for 1 h and visualized by colloidal Comassie Blue G-250 or silver staining according to standard protocols. For 2-DE analyses IEF gel strips were equilibrated in SDS equilibration buffer and were then loaded onto 12% SDS gel slabs for separation in the second dimension.

Protein visualization and image analysis

After separation, the proteins were visualized by a sensitive colloidal Coomassie G250 stain or by silver staining. Briefly, the dye solution containing 17% (w/v) ammonium sulfate, 3% (v/v) phosphoric acid, 0.1% (w/v) Coomassie G250 and 34% (v/v) methanol was changed once after 12 h staining and the gel slabs subjected to a 24 h cycle for increasing dye deposition on low abundance proteins. The detection was then increased by placing the gel into 1% v/v acetic acid for producing a better contrast between spots and gel. Coomassie stained gels were scanned and spot detection and quantification were carried out using PDQuest 7.1 software (Bio-Rad). In the silver staining method, the gels were fixed in 50% v/v methanol, 12% v/v acetic acid and 0.05% v/v formaldehyde for at least 2 h. The fixed gels were rinsed with 50% v/v ethanol three times for 20 min each, then again sensitized with 0.02% w/v sodium thiosulfate followed by three washings with milli-Q water each for 20 s. The gels were immersed in 0.1% w/v silver nitrate and 0.075% v/v formaldehyde for 20 min then rinsed with milli-Q water twice for 20 s each and developed in 0.05% v/v formaldehyde. Finally, the reaction was stopped with a solution consisting of 50% v/v methanol and 12% v/v acetic acid (Yan, 2000).

RESULTS AND DISCUSSION

The goal of the present study was to improve the detection of low-abundance proteins in human plasma by using two different experimental procedures including Affi-gel blue affinity chromatography and acetonitrile precipitation.

In the current study, prior to 2-DE analyses, we have used in the one hand, the acetonitrile precipitation (ACN) method and in the second hand, the Affi-Gel Blue, which is a cibacron-based dye, that has previously been used successfully to visualize proteins of low abundance (Ahmed, 2003; Moutaouakkil, 2011). For this purpose, plasma samples obtained at the time of diagnosis from 6 breast cancer patients were collected and processed as described previously.

Prior to acetonitrile precipitation and chromatographic analyses, acetone extracted plasma was analyzed by SDS-PAGE after staining proteins with the coomassie brillant blue method (CBB G-250; Figure 1). The procedure was repeated three times to control the reproducibility and to establish standard operation procedures. By using the SDS-PAGE analysis, we were able to reveal the albumin protein band, at approximately 64 kDa and the IgG bands, at approximately 50 and 25

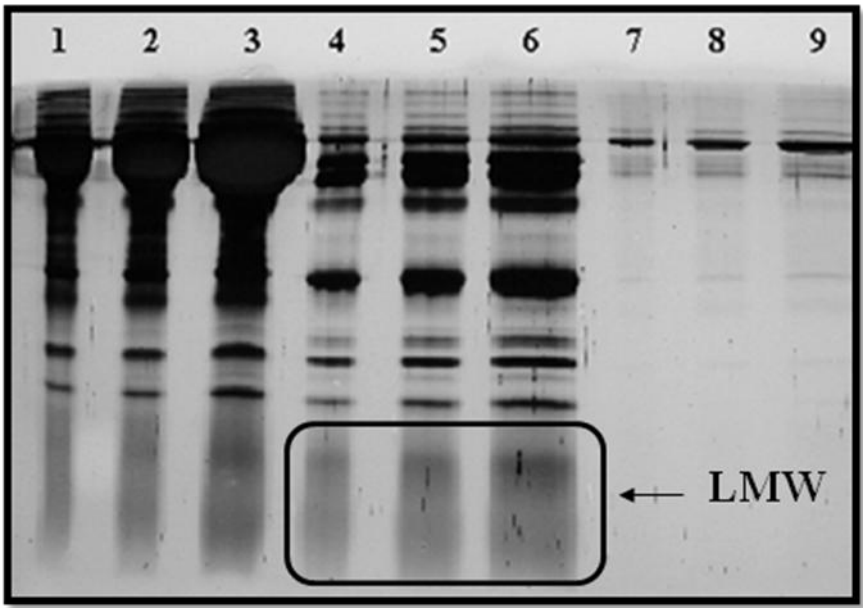

Figure 2. SDS-PAGE obtained after protein precipitation with acetonitrile. Lanes 1, 2 and 3: initial plasma protein sample before depletion (10 µg). Lanes 4, 5 and 6: plasma protein profile (2µg) after precipitation with acetonitrile [1 v / 1v]. Lanes 7, 8 and 9: plasma protein profile (1µg) after precipitation with acetonitrile [1v plasma / 2v acetonitrile]. LMW: Low Molecular Weight.

kDa corresponding to heavy and light chains of IgG, respectively (Figure 1). Figure 1 shows that albumin was the predominant spot in parallel with a small number of minor proteins of low molecular weight. Results demonstrate that the most abundant proteins are albumin and the immunoglobulin heavy and light chains, representing together about 60% of the plasma proteins. Therefore, depletion of at least these two high abundance proteins is a prerequisite of a successful search for biomarkers in plasma.

In the current investigation the effect of ACN precipitation on plasma proteins was evaluated by 1D and 2-DE analyses (Figures 2, 3 (A and B). Based on previous studies, protein precipitation with acetonitrile has been suggested for the removal of protease activity and biological contaminants and enrichment of proteins (Jiang, 2004). However, precipitation is prone to protein losses due to poor precipitation or incomplete resolubilisation (Jiang, 2004). From our findings, ACN precipitation resulted in a loss of several proteins as assessed by 1-D SDS-PAGE (Figure 2). A significant decrease in albumin levels and high molecular weight proteins was observed. Figure 3 (A and B) shows the 2D electrophoresis pattern of ACN-treated plasma samples. No significant difference was observed between untreated plasma (Figure 3-A) and ACN treated samples (Figure 3-B). As shown in Figure 3-B, ACN can efficiently remove Apolipoprotein-A1, whereas albumin is still present in the ACN depleted fraction. These findings are not in agreement with a previous study showing that the use of two volumes of acetonitrile in the presence of TFA effectively precipitate large abundant proteins such as albumin while peptide and small proteins remained in solution and could be explored (Chertov et al., 2004).

The enriching ability of the Affi-Gel Blue method was also investigated in the current study. Affi-Gel Blue is a polycyclic anionic ligand that interacts with proteins via ionic and hydrophobic interactions. Affi-Gel Blue has long been used as an affinity reagent for protein purifications, including albumin (Hamrita, 2009; Fredolini, 2010; Altintaş, 2011). This method also has several advantages, including high-loading capacity and low cost. Due to their relatively low cost, they are disposable, which prevents any cross-contamination between different samples. Successful application of Cibacron Blue in biomarker discovery was also reported (Hamrita, 2009; Fredolini, 2010). Affi-Gel Blue ligand together with protein A/G kits were also developed and commercialized as affinity-depletion methods (Moutaouakkil, 2011; Altintaş, 2011). At the early stage of a proteomic sample preparation, this latter method was widely used for albumin and IgG depletion from human plasma samples.

In the current study, more than 45% of high molecular weight proteins including albumin were removed after Affi-Gel Blue treatment. We found that protein A/G beads depleted a major fraction of immunoglobulins from plasma as assessed by SDS-polyacrylamide electrophoresis (Figure 3(C, D), Figure 4).

Figure 4, shows SDS-PAGE analysis of eluates and retained fractions from Affi-Gel Blue columns. After

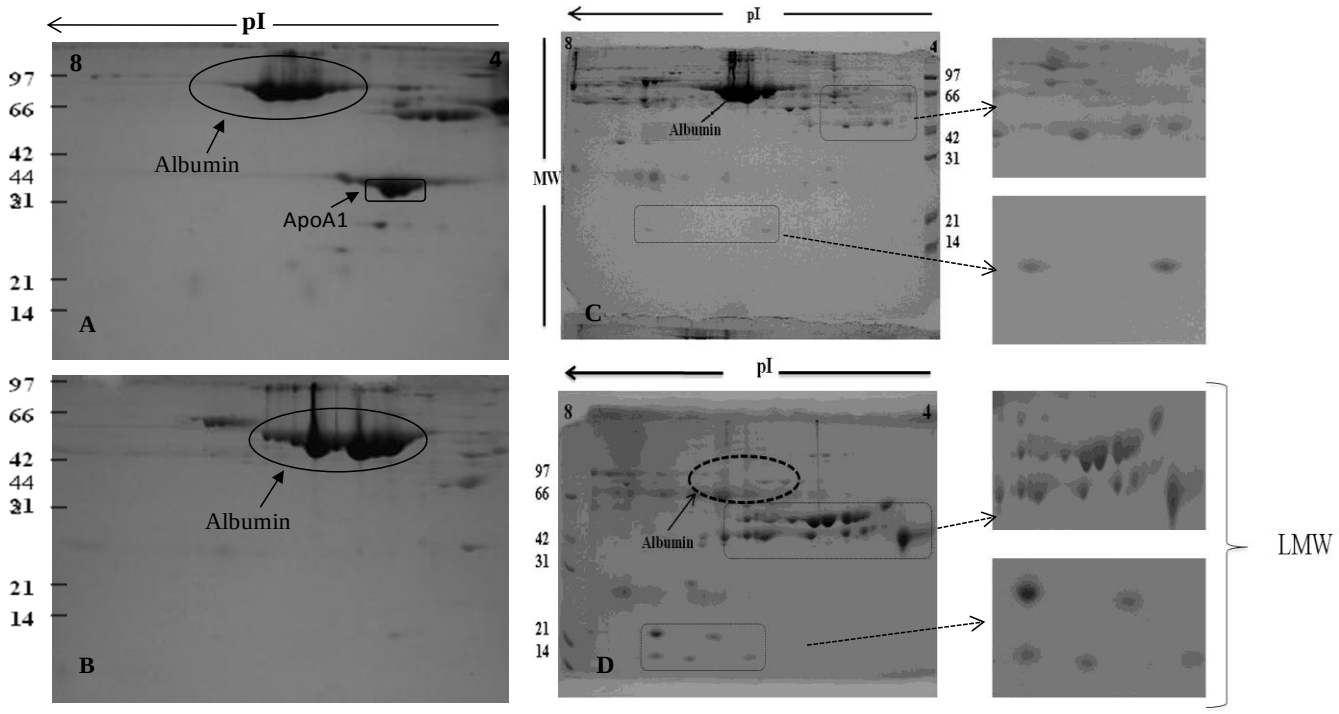

Figure 3. Two-dimensional maps of the human plasma proteins. Figures A and B: Protein precipitation with acetonitrile [A: 1v plasma is added to 0.5 v acetonitrile]. [B: 1v plasma is added to 1v acetonitrile]. Figures C and D: Proteins fractionated with the Affi-Gel Blue column. Left panels: No significant differences were observed between untreated plasma (Figure A) and treated samples (Figure B). Albumin is still present after precipitation with acetonitrile. As shown in Figure B, ACN can efficiently deplete only minor high-abundance proteins, like Apolipoprotein-A1(ApoA1). Middle panels: The proteins were separated as stated under materials and methods. The gel was stained with Coomassie Colloidal Blue. Albumin is absent in the Affi-Gel blue depleted fraction. The Affi-Gel Blue method enhances the intensity of minor proteins by several-folds (Figure D). Right panels: close-up sections of coomassie blue stained 2-DE after removal of high abundant proteins including albumin. The two close-up sections of Figure D, showing a much cleaner and more even distribution of low-abundance proteins in comparison with the image in Figures B and C.

albumin depletion and 2D-analyses, a more efficient detection of weaker spots became possible (Figure 3 D). Passage through the Protein A/G column resulted in an efficient removal of approximately 75% of the immunoglobulins and an enrichment of weaker protein spots (Figure 3 D). As shown in figure 3, the presence of immunoglobulins may mask the detection of proteins with similar molecular weights and pI's and also limit the protein loading capacity on 2-DE gels.

Figure 3 D displays a representative 2-DE image after IgG depletion, showing a much cleaner and more even distribution of low-abundance and low molecular weight proteins in comparison with data obtained after acetonitrile precipitation (Figure 3B).

According to the literature removal of highly abundant proteins using an albumin and IgG removal kit results in 4- to 6- fold increase in medium- and low- abundance proteins (Fredolini, 2010). From our preliminary findings, compared with ACN precipitation, the major advantage of the Affi-Gel Blue method could be related to its ability to remove high abundant proteins such as albumin and to enrich plasma samples in low molecular weight forms (Altintaş, 2011).

In summary, we describe herein a sensitive method for the discovery of new disease biomarkers among low molecular weight plasma proteins. From our findings, the Affi-Gel Blue method combined with protein A/G beads is more effective for isolation of the low molecular weight fraction and albumin removal than acetonitrile depletion. Further studies on additional breast tumor samples can improve disease classification, early detection and intervention, assessment of disease progression, and possibly long-term outcomes.

Conclusion

To improve breast cancer management and survival, there is a great need to develop screening programs, early diagnosis and potential therapeutic targets. In the current study, our goal was to explore two different strategies in an attempt to improve separation and identification of low-abundance proteins in the human plasma. We confirm, that Affi-Gel Blue beads can enhance detection of low-molecular-weight species which may aid in the discovery of new breast cancer markers

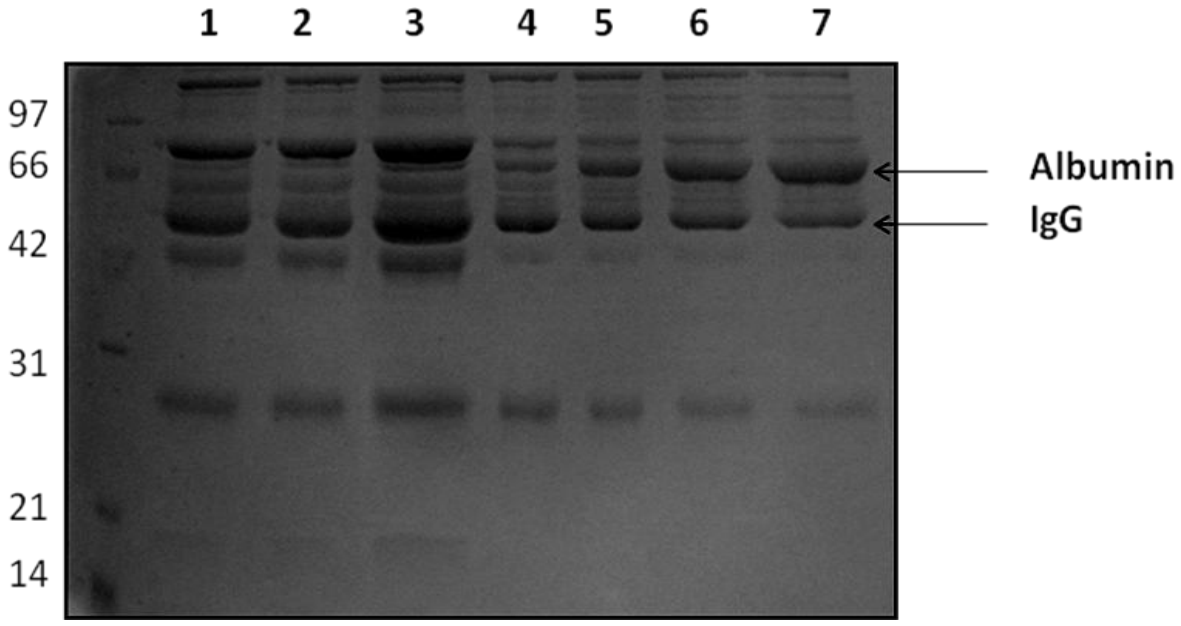

Figure 4. SDS-PAGE obtained after protein depletion with Affi-Gel Blue. Lanes 1, 2 and 3: Fractions not retained on the column of Affi-Gel Blue. Lanes 4, 5, 6 and 7: Fractions retained on the column of Affi-Gel Blue. As shown by SDS-PAGE, we note that albumin and immunoglobulins, are visibly depleted by this method.

that could be useful in early diagnosis, as well as to monitor disease progression.

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