Separation of low molecular weight serum proteins using acetonitrile precipitation assessed by one dimensional gel electrophoresis

Ruchika K. Jain, Aruna A. Jawade, Shubhangi K. Pingle*, Rajani G. Tumane and Lucky R. Thakkar

National Institute of Miners' Health, JNARDDC Campus, Opposite Wadi Police Station, Amravati Road, Wadi, Nagpur(M.S.), India
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ABSTRACT

In human serum, low molecular weight (LMW) proteins play an important role in major biological processes which were masked by high molecular weight (HMW) proteins. Separations and identification of LMW proteins of interest from serum is very intricate task. The objective of the present study was to develop in-house acetonitrile (ACN) precipitation method for separation of LMW proteins from high abundance and HMW proteins in serum samples. Blood Samples (n=94) were collected from area of Vidharbha region of Maharashtra, India. Serum precipitation was done by using different dilutions of ACN. After standardization of ACN, 1:1 dilution was used and accordingly serum, supernatant and precipitate was used for one-dimensional sodium dodecyl sulfate/polyacrylamide gel electrophoresis (1D SDS-PAGE) for separation of proteins. Gel image was analyzed by using Gel analyzer 2010 software. It was noted that LMW proteins were present in supernatant in the molecular range of 42 kDa, 32 kDa, 24 kDa, 19 kDa, 5kDa whereas HMW proteins were found in precipitates at the range of 156kDa, 115 kDa, 102 kDa, 95 kDa, 72 kDa, 69 kDa, 58 kDa, 43 kDa. Results showed that HMW proteins present in precipitated and LMW proteins were concentrated in supernatant. In conclusion, a rapid ACN precipitation method has been developed that reproducibly depletes high abundance and HMW proteins from serum prior to mass spectrometry based proteomic approaches.

Keywords: Acetonitrile precipitation, HMW proteins, LMW proteins, One dimensional electrophoresis, Precipitate, Supernatant
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INTRODUCTION

Human Serum contains thousands of proteins varying in concentration, the majority of which are low molecular weight (LMW). The normal laboratory value of serum total protein is around 7 g/dl (1)**.** The analysis of proteins present in human serum is of great importance in the study of both health and diseases. Diagnostic molecules are often LMW proteins secreted into the blood stream as a result of the disease progress (2, 3). However, the diverse range of proteins present in the serum can lead to difficulties when looking for those present at low concentrations (4). In serum the most abundant proteins includes albumin and immunoglobulin G (IgG) which masked the expression of LMW proteins (5). Removal of highly abundant proteins, coupled to powerful protein separation methods are required for facilitating detection and identification of low abundant masked proteins.

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Proteins with molecular weights of <25kDa are involved in major biological processes such as ribosome formation, stress adaption and cell cycle control (6, 7). Despite their importance, the coverage of smaller proteins in standard proteome studies is rather sparse. Several technique like affinity chromatography, ethanol precipitation, specific antibodies or size-exclusion filtration are available for the separation and identification of major serum proteins, but effective methods are yet to be proven successful for LMW molecules (8 - 13). All the above mentioned techniques are costly, time consuming and require tedious procedure. Therefore, there is a need to develop easy and cost effective precipitation method which separate and analyze LMW proteins from serum samples. The use of acetonitrile precipitation satisfies both criteria: large abundant proteins such as albumin are effectively precipitated while peptides and small proteins remained in solution and can be analyzed for further studies. The effect of ACN precipitation on serum proteins was evaluated by 1D SDS-PAGE enabling them to be compared and identified using different mass spectrometry approaches (14, 15).

The objective of the present study is to develop an in-house acetonitrile precipitation method that would allow selective precipitation of large abundant proteins and at the same time dissociates and extracts smaller peptides/proteins. The main rationale was to develop a cost effective method that would allow separation of such proteins, which can be used further for identification and functional studies.

MATERIALS AND METHODS

Study Subjects and Sample Collection:
A total 94 serum samples were collected from the Vidharbha region of Maharashtra state, India out of which n= 46 were males and n= 48 were females. The age of the participant was ranged between 15-78 years. Baseline characteristics and clinical findings of participants were recorded. Blood samples were collected and centrifuged at 5000 rpm for 10 min. After centrifugation serum was transferred to sterilized tube and stored at -40^0C for further analysis.

Acetonitrile Precipitation
Acetonitrile was used for precipitation of serum proteins. Three different dilutions of serum and ACN (1: 0.5, 1:1, and 1:2) were used for the precipitation. The serum and ACN mixture was placed on ice for 1 hour. After cooling it was centrifuged at 5000 rpm for 5 minutes. The supernatant was removed and precipitate was dissolved in distilled water. The serum, precipitate and supernatant were directly applied for 1D electrophoresis after protein estimation.

Estimation of protein concentration:
The protein concentration of the serum sample, precipitate and the supernatant was determined by using the Biuret Biosystem kit (total protein) following the manufacturer's instructions. The protein absorbance was measured at 545nm using a semi auto analyzer.

One Dimensional SDS-PAGE gel electrophoresis
Approximately 40-60 μg of the serum, supernatant and precipitate were loaded into a well of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed with vertical slab gel electrophoresis system by standard Laemmlli's method using 10% running gel and 5% stacking gel. The gel separation was done at 250 Volts. After completion of electrophoresis gels were developed by Coomassie staining. Briefly the gels were fixed with a methanol: acetic acid: water (4:1:5) solution, stained with Coomassie brilliant blue Stain and destained in a solution of 10% methanol and 7% acetic acid.

Densitometric analysis:
After staining, gel images were taken with the help of gel scanner. The densitometric analysis of protein profile of developed gels was then studied using gel analyzer 2010 software.

RESULTS

The result of 1D SDS PAGE of serum samples prior to ACN precipitation method reveal that an albumin band at approximately 66kDa and the IgG bands at approximately 43 kDa and 29kDa corresponding to heavy and light chain of IgG respectively. Result showed that the bands of albumin and IgG heavy and light chain were prominent and both together represent about 90% of the serum proteins. Therefore, the depletion of this high abundance protein is very essential for successful separation of LMW proteins from serum samples.

The total average serum protein concentration observed in the present study was 7.8gm/dl. After ACN precipitation of serum samples the total protein recovery is only 35 to 40% this demonstrating the efficiency of the method for the depletion of HMW proteins. The use of one volume of ACN can efficiently precipitate or depleted large abundant HMW protein such as albumin and IgG while LMW proteins remained in the supernatant.
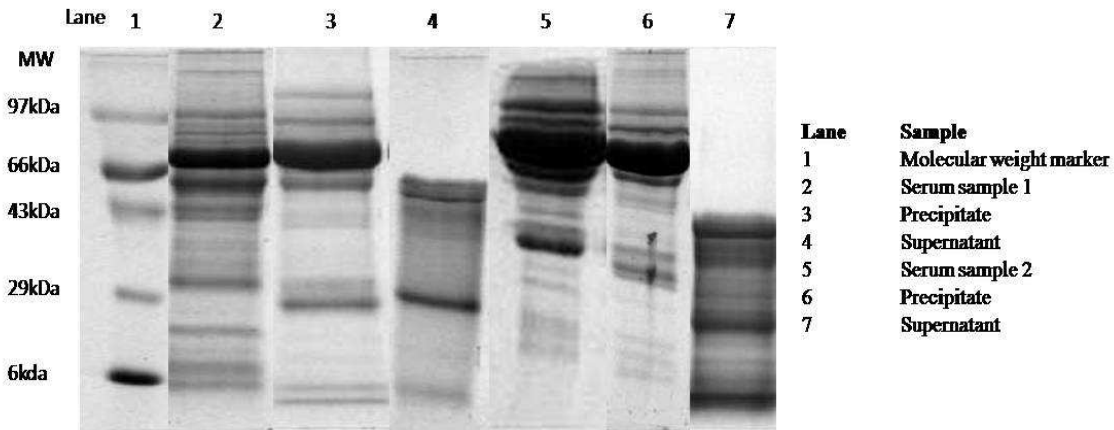
Note: After standardization of ACN (1:1) all serum samples were loaded accordingly

Figure: 1. Show protein profiling of 1D SDS PAGE gel of the serum samples 1 & 2 (lane 2 & 5) and its precipitates (lane 3 & 6) and supernatants (lane 4 & 7) after coomassie staining
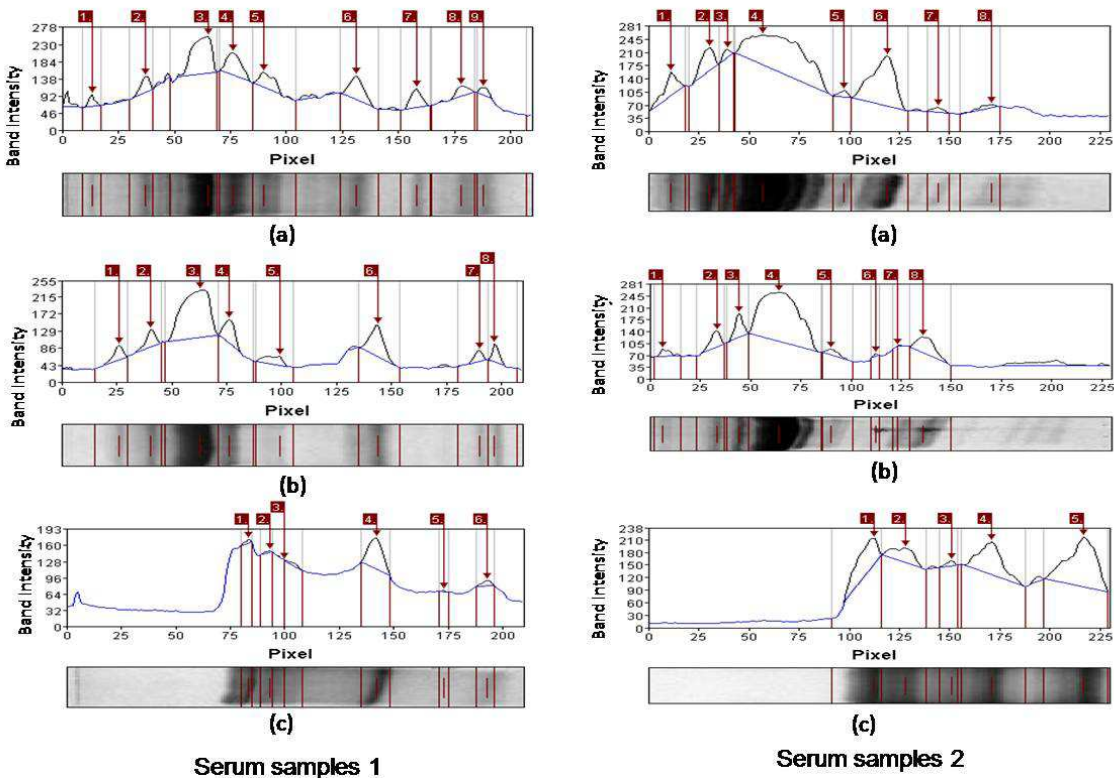

Figure 2: Represents the densitometric analysis of protein bands intensity in serum samples (1a, 2a), its precipitate (1b, 2b) and supernatant (1c, 2c) after 1-Dimensional SDS page profile (as shown in Fig. 1)

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The present study included a standardization of ACN method by using three different dilutions (1:0.5, 1:1 and 1:2 dilutions) of ACN with serum samples. For standardization all the dilutions were loaded separately on to the gel. It was observed that the ratio of serum to acetonitrile i.e. 1: 0.5 is not showed properly for depletion of HMW protein like albumin and heavy and light chain of IgG. However, the ratio of serum to ACN i.e. 1:1 & 1:2 are very effective for the depletion of high molecular weight proteins and the high recovery of the protein in precipitate and supernatant. After centrifugation of the reaction mixture, less soluble heavy proteins should be found in the precipitate, while the more soluble lighter ones remained in the supernatant. The proteins in the supernatant and precipitate were range from approximately 6 – 205kDa. This represents a wide range of proteins with regards to size.

Based on 1D SDS PAGE, proteins were separated by using 1:1 standardized dilution which was differentially expressed in supernatant and precipitates as shown in figure 1.

Depending upon expression of proteins, selected gels were subjected to densitometric analysis which is shown in figure 2. of each serum samples.

Table 1: Depicts the molecular weight (kDa) of expressed protein bands in serum samples, its precipitate and supernatant after densitometric analysis of 1-Dimensional SDS page (as shown in fig.1)

Sample Number	Serum samples Protein Bands Molecular Weight (KDa)	Precipitate Protein Bands Molecular Weight (KDa) (ACN dilution 1:1)	Supernatant Protein Bands Molecular Weight (KDa) (ACN dilution 1:1)
1	138	115	-
	99	95	-
	67	69	-
	58	58	52
	48	43	46, 42
	28	24	24
	20	-	-
	15	14	17, 14
	6, 5	6, 5	6, 5
2	143	156	-
	104	102	-
	93	90	-
	75	72	-
	55	55	-
	44	43	-
	32	38, 33	32
	22	-	26
	14	-	19, 13
	-	-	7, 5

Table. 1 depicts the separated protein bands according to their molecular weight in precipitate and supernatant by ACN precipitation method. In all precipitates protein bands were found in the range of 156kDa, 115 kDa, 102 kDa, 95 kDa, 72 kDa, 69 kDa, 58 kDa, 43 kDa while in all supernatants protein bands were found in the range of 42 kDa, 32 kDa, 24 kDa, 19 kDa, 5kDa.

DISCUSSION

Abundance amount of proteins present in the cells having wide range of proteins which create great complexity while analyzing the whole cell (16). Reducing this complexity by analyzing protein subsets has improved analysis of LMW proteins and contributed information about the localization of various proteins in the cell. Several methods are available for the separation and identification of HMW proteins, but effective methods are yet to be proven successful for LMW proteins (17, 18). The development of new techniques is needed for the separation of LMW proteins which were masked by HMW proteins. The present study focused on the development and standardization of ACN precipitation method for elimination of HMW proteins from serum sample and separate LMW proteins.

The present study was supported by many research papers as separation of LMW proteins from HMW proteins by ACN precipitation is effective method. Ulunna K. Ofurum *et.al.* (19) worked on evaluation of ACN as a method for separating LMW from HMW proteins in cytosol from MCF-7 Breast Cancer Cells . They reported that the bands from the precipitate revealed the same large proteins as the cytosolic fraction. The supernatant was containing mainly LMW proteins. On the basis of their results they concluded that, although ACN precipitation did not result in

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complete protein separation, there is obviously enrichment for LMW proteins in the supernatant. Similar findings were observed in our study, more LMW proteins bands were expressed in supernatant while HMW proteins were found in precipitate after ACN precipitation method.

Wei Sheng Yan Jiu. *et.al.* (20) worked on various separation techniques such as multiple affinity chromatography, ethanol and ACN precipitation method with the aim to determine reliable separation method. On the basis of result they concluded that multiple affinity chromatography method could effectively remove the high abundance proteins and keep a large number of low abundance proteins and ethanol precipitation could eliminate part of high abundance proteins in serum, but low abundance proteins was less harvested. While serum proteins separation by ACN method was able to eliminate the vast majority of high abundance proteins and more proteins of LMW were found in supernatant. Their finding strongly suggested that ACN precipitation is one of the best methods to separate LMW proteins which completely support our findings.

Another study of C. Fernández *et. al.* (21) compared the three methods to diminish the content of most highly abundant proteins in human serum with ACN, dithiothreitol (DTT) and protein equalization with the ProteoMiner (PM) which was assessed by 1-D gel electrophoresis and MS. They suggested that out of these three methods, ACN method allows an effective depletion of the protein fraction above 72 kDa. The ACN-depletion strategy offers a viable alternative to the immunochemistry-based protein-depletion techniques commonly used for removing high abundance proteins from serum prior to MS-based proteomic analysis. Thus an establishing the fact that ACN precipitation is used as a viable alternative method for precipitation and/or separation method for LMW proteins.

In conclusion, ACN precipitation is one of the good methods for enrichment of LMW proteins and 1D SDS PAGE electrophoresis is an immense proteomic method to visualize the separation of proteins according to their MW. In future, 2D electrophoresis will be performed for clear visualization of separation of LMW proteins and identification/characterization will be carried out with the help of LCMS or MALDI-TOF-MS analysis.

CONCLUSION

Combination of ACN depletion and one-dimensional electrophoresis enabled the detection of the low abundant serum proteins which has very low concentration in the serum. 1D SDS-PAGE analysis of the supernatant clearly showed the enrichment of LMW proteins after extraction, showed the efficiency of the ACN method for the depletion of HMW proteins. The ACN-depletion strategy offers a possible alternative to the other immunochemistry-based protein-depletion techniques generally used for removing high abundant proteins from serum prior to MS-based proteomic analyses.

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