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Proteomics-based approach for identification and purification of human phosphate binding apolipoprotein from amniotic fluid

M. Alam[1], M. Mahajan[1], M. Raziuddin[2], T.P. Singh[1] and S. Yadav[1]

[1]Department of Biophysics, All India Institute of Medical Sciences, New Delhi, India
[2]Department of Zoology, Vinoba Bhave University, Hazaribag, India

Corresponding author: S. Yadav
E-mail: savita11@gmail.com

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ABSTRACT. Human amniotic fluid is of both maternal and fetal origin; it protects the fetus and provides the environment for growth and development of the fetus. We used a proteomics-based approach for targeting and purifying human phosphate binding protein, a member of the DING family of proteins from amniotic fluid, using Blue Sepharose CL-6B, DEAE-Sephacel and gel filtration chromatography. The protein had earlier been reported to be serendipitously purified along with PON1 (paraoxonase 1). It was identified using electro-spray-ionization-time-of-flight mass spectrometry and was found to be human phosphate binding protein. Human phosphate binding proteins have been reported to play a role as phosphate scavengers and may have a protective function against phosphate-related disorders, such as atherosclerosis, diabetes and kidney stones.

Key words: Amniotic fluid; Human phosphate binding protein; Proteomics

INTRODUCTION

Human amniotic fluid is a protein-rich fluid formed from maternal blood ultrafiltration by the placenta and excretion from the fetus urine (Lotgering and Wallenburg, 1986). Human amniotic fluid has been shown to contain 412 proteins, as found by MALDI-MS and MALDI-MS-MS (Tsangaris et al., 2006), which are either fetal or maternal in origin (Campbell and Lees, 2000). Apart from the physical environment, which protects the growing fetus (Verburg et al., 2007), amniotic fluid also provides constituents essential for fetal growth and well-being (Strong Jr. et al., 1990; Whitfield and Ritche, 1995), in which proteins play an important part (Chevallier et al., 1998; Heizmann, 1999; Tsangaris et al., 2006). Human phosphate binding protein (HPBP) is an apolipoprotein, 38-45-kDa glycosylated protein, found mainly in plasma (Renault et al., 2006) and also in human amniotic fluid (Tsangaris et al., 2006). HPBP was first purified by chance along with PON1. HPBP is a member of the DING family, which suggests its distribution in a wide number of eukaryotes (Morales et al., 2006, 2007). The DING protein family seems to have emerged by consensus for ubiquitous but genetically elusive proteins, usually with a molecular weight of around 40 kDa, of which the N-terminal sequence is DINGGG- (Adams et al., 2002). HPBP plays a key role, being identified as the first transporter to bind phosphate ions in human plasma (Berna et al., 2008), and it helps in the function of PON1 (Rochu et al., 2007a). HPBP is also known to stabilize the PON1 and high-density lipoprotein association (Rochu et al., 2007b) and promotes phosphotriesterase activity (Rochu et al., 2007a). Both HPBP and PON1 associated with high-density lipoprotein (Harel et al., 2004) are governed by plasma calcium and phosphate concentrations (Fokine et al., 2003) and work in tandem to reduce the risk of phosphate-dependent diseases such as athrosclerosis (Schmidt et al., 1998), diabetes (Dursun et al., 2006) and kidney stones (Kumar et al., 2004).

We purified HPBP from human amniotic fluid using a proteomics-based approach to target protein on the basis of molecular weight and pI, as evident on the 2-D gel map. The role of HPBP in amniotic fluid is still not clear, but its role in fetal development and well-being will be interesting none the less.

MATERIAL AND METHODS

Samples of 5 mL amniotic fluid each from 5 documented Rh-sensitized pregnancies at the 24th-36th week of gestation were pooled, which were planned from amniocentesis to evaluate other gestational diseases. The specimens were obtained from the Department of Obstetrics and Gynecology, All India Institute of Medical Sciences, New Delhi, after obtaining written informed consent from the patients. The study was approved by the university Ethics Committee (Ref. No. A-16/25.07.2007).

Method of sample collection

Detailed ultrasound was performed to delineate fetal heart rate, site of placenta, amount of fluid, and any gross fetal malformation. The patients were placed in lithotomic position and their lower abdomen was cleaned with antiseptic solution. A 23-G spinal needle was used to aspirate the amniotic fluid per abdomen under ultrasonic guidance by a medical professional specialized in the field of prenatal diagnostic procedures and fetal medicine. The first

1 mL amniotic fluid was discarded, and then 5 mL amniotic fluid was drawn. Samples were centrifuged at 20,000 *g* for 30 min, and the supernatant was used for different experimentation.

Standardization of samples

Each sample was scanned by UV spectroscopy and a Lilly chart was obtained to establish whether the pregnancy was Rh-isoimmunized. Samples were stored at -20°C until further use.

Albumin removal by Blue Sepharose CL-6B chromatography

Albumin, which comprises more than 50% of the protein content of amniotic fluid, tends to mask the other proteins, which are over-expressed, giving rise to false-negative results. Hence, albumin needs to be removed before loading the sample on the IPG strip. To address this problem, the pooled samples were submitted to albumin depletion using Blue Sepharose CL-6B affinity chromatography. The sample was first centrifuged at 20,000 *g* for 30 min. The supernatant was collected and mixed with equilibrating buffer (0.05 M Tris-HCl, pH 8.0) before loading onto a Blue Sepharose CL-6B (Amersham Biosciences) column (2.6 × 20 cm). Passing of amniotic fluid through a column of Blue Sepharose CL-6B column results in the removal of approximately 60 to 70% of the albumin **(**Travis et al., 1976; Angal and Dean, 1977).

Precipitation of protein for 2-D gel electrophoresis

Proteins from the unbound fraction of Blue Sepharose CL-6B were precipitated using 9 mL 0.05 M Tris-HCl buffer, pH 8.0, and 2% protease inhibitor cocktail solution [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mM EDTA, 130 mM bestatin, 14 µM E-64, 1 mM leupeptin, 0.3 µM aprotinin]. After incubation at -20°C for 2 h, the mixture was vortexed, cooled on ice repeatedly, and then centrifuged at 15,000 *g* for 15 min at 4°C. Cold acetone was added to the supernatant to a final concentration of 80%, v/v, and the mixture was kept at 4°C for 1 h. After centrifugation, the pellets were washed with 80% acetone and harvested again by centrifugation. The precipitate obtained was dried and solubilized in 0.5 mL lysis buffer (6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (w/v) Triton X-100, 40 mM Tris-HCl, 50 mM DTT, 0.2% pH 3-10 Bio-Lyte). The solution was centrifuged at 15, 000 *g* at 4°C for 15 min to obtain the soluble protein in the supernatant (Park et al., 2006). Protein was determined by the Bradford method (Bradford, 1976).

2-D gel electrophoresis

Rehydration of IPG strips

Precast IPG strips with nonlinear immobilized pH 3-10 gradient were rehydrated overnight with a 1000-µg protein sample complemented with 0.0025% (v/v) bromophenol blue and 1% (v/v) IPG buffer (Rabilloud et al., 1994).

Isoelectric focusing

Isoelectric focusing was performed using the IPGphor™ IEF system (Amersham Bio-

sciences): 100 V/h, 300 V/h, 500 V/h, 1000 V/2 h, linear gradient from 1000 to 8000 V, and a final phase at 8000 V for 12 h, resulting in a total of 100,000 V/h (Gorg et al., 1991). Strips were equilibrated for 10 min in 9 M urea, 50 mM Tris-HCl buffer at pH 8.8, 30% (v/v) glycerol, 2% SDS, 0.001% (w/v) bromophenol blue and 65 mM DTT, and finally for 15 min in the same solution except that DTT was replaced by 13.5 mM iodo-acetamide (Gorg et al., 1988, 1997).

SDS-PAGE

Protein was separated on 12.5% SDS-polyacrylamide gels at a constant voltage of 50 V for 2 h followed by 150 V for 6 h at 25°C, using an Iso-DALT electrophoresis unit (Amersham Biosciences). Gels were stained with colloidal Coomassie blue (G-250).

In-gel digestion and ESI-Q-TOF/MS analysis

The stained protein bands were excised and incubated 24 h in water, followed by partial drying and then, washing once with 150 µL 50% acetonitrile (ACN) in 200 mM ammonium carbonate, pH 8.9, for 20 min at 30°C and twice with 100% ACN for 10 min each. The gel was dried in a vacuum drier. Trypsin solution (20 µL; 5 µg/mL in 25 mM ammonium bicarbonate) was added to the dried gel and incubated overnight at 37°C. The resulting peptides were recovered by three extractions with a 100-µL solution of 60% ACN and 0.1% TFA at 30°C. The extracts were combined and concentrated to 20 µL in a Speed Vac. At the time of injecting sample in ESI-Q-TOF/MS, 0.1% formic acid was added to acidify the peptide mixtures, and the ESI-Q-TOF/MS was performed in the positive mode.

Purification and identification

A total of 100 mg protein from the unbound fraction of Blue Sepharose CL-6B (as done for sample preparation of 2-D gel electrophoresis) was loaded onto a DEAE-Sephacel column (2.6 × 16 cm), which was pre-equilibrated with 0.05 M Tris-HCl, pH 8.0. After adequate washing, a linear gradient of 0.0-0.5 M NaCl, in the same buffer was done. The fractions were collected until the absorbance at 280 nm was 0.005. The purification status of proteins was checked at every step using SDS-PAGE. The bound fractions of 0.1 M NaCl, onto a DEAE-Sephacel column, were pooled separately, concentrated and desalted using ultrafiltration with a 3-kDa-cutoff membrane (Millipore). A total of 25 mg lyophilized protein was loaded onto a G-75 Sephadex column (1.6 × 125 cm), pre-equilibrated with 0.05 M Tris-HCl, 0.2 M NaCl, and fractions were eluted at a flow rate of 0.2 mL/min. Absorbance was measured at 280 nm. Peak 1 containing proteins was pooled and concentrated by ultrafiltration using a 3-kDa cutoff membrane. SDS-PAGE was performed on 10% polyacrylamide gels, and proteins were stained with colloidal Coomassie blue (G-250). The single band obtained on SDS-PAGE was excised, trypsin digested and analyzed by ESI-Q-TOF/MS.

RESULTS AND DISCUSSION

All samples of amniotic fluid used for experimentation were obtained for detection of status of Rh-isoimmunized pregnancy. The Lilly chart was obtained using a UV-visible

spectrophotometer at 450 nm, and samples with absorbance more than 0.1 were used for proteomics and purification.

Figure 1 depicts the typical Lilly chart for the amniotic fluid. A volume of 25 mL amniotic fluid pooled from 5 cases of Rh-isoimmunized pregnant mothers was loaded on 40 mL Blue Sepharose CL-6B (2.6 × 20 cm) and eluted with 0-0.5 M NaCl gradient, and the unbound protein was collected in order to remove excess albumin and immunoglobulins masking the gel, shown in Figure 2 depicting their reduction on SDS-PAGE (10%).

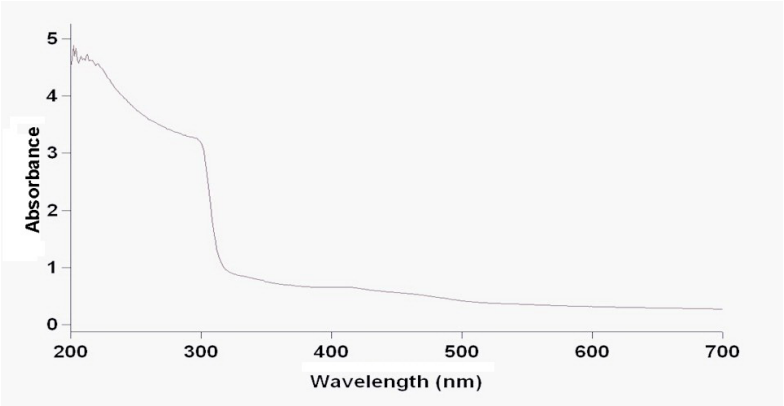


Figure 1. Lilly's chart obtained using UV-visible spectroscopy of amniotic fluid from Rh-isoimmunized pregnancy.

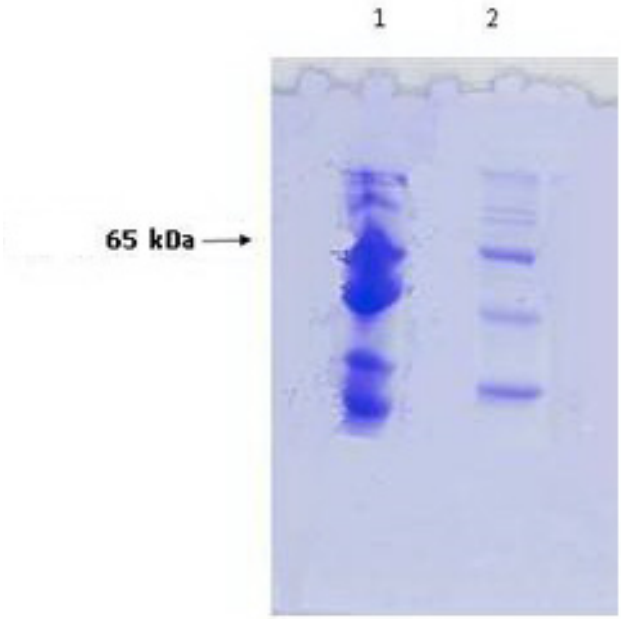


Figure 2. SDS-PAGE (10%) showing removal of albumin by Blue Sepharose CL-6B on colloidal Coomassie blue (G-250) stained gel. *Lane 1* = Crude profile of amniotic fluid. *Lane 2* = Unbound fraction from Blue Sepharose CL-6B with decreased content of albumin.

A total of 1000 µg quantified and precipitated protein from the unbound fraction of Blue Sepharose CL-6B was separated to obtain a 2-D gel electrophoresis map, and 35 proteins were identified using ESI-Q-TOF/MS. Figure 3 shows the 2-D gel electrophoresis map of the amniotic fluid and highlights the presence of HPBP in the amniotic fluid.

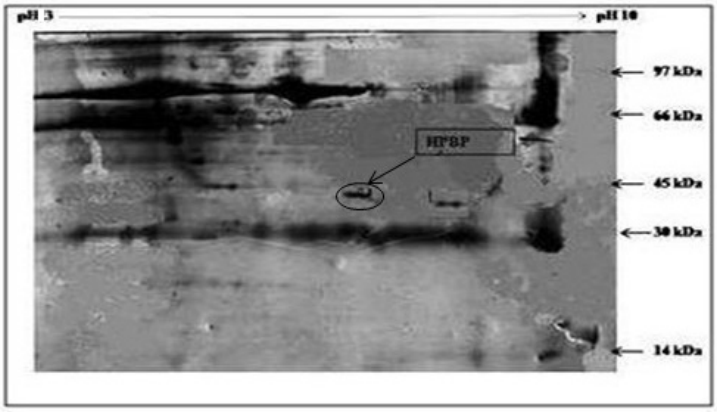


Figure 3. The 2D-gel (12.5% and pH 3-10) map from pooled samples of 5 cases of Rh-isoimmunized pregnancy.

The unbound fraction of the Blue Sepharose CL-6B was then loaded onto a DEAE-Sephacel column, which was pre-equilibrated with 0.05 M Tris-HCl, pH 8.0, and eluted with a 0-0.5 M NaCl gradient. The DEAE-Sephacel bound fraction at 0.1 M NaCl was pooled separately, concentrated and desalted using ultrafiltration. Figure 4 shows the elution profile of DEAE-Sephacel column chromatography.

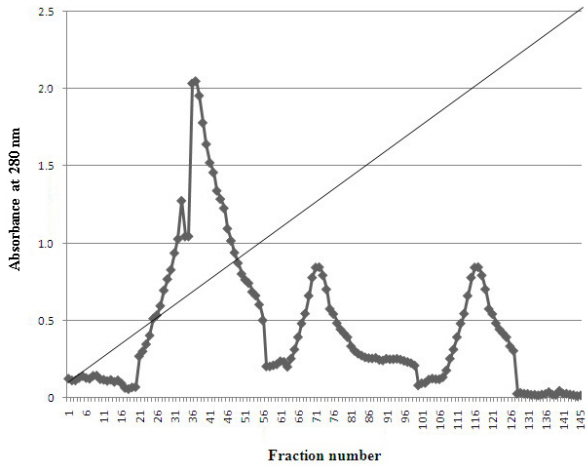


Figure 4. Ion-exchange chromatography of amniotic fluid on a DEAE-Sephacel column (2.6 × 16 cm). The column was pre-equilibrated with 0.05 M Tris-HCl, pH 8.0, at flow rate of 0.2 mL/min and then eluted with a linear gradient of 0-0.5 M NaCl.

An aliquot of 25 mg of the lyophilized protein from the DEAE bound at 0.1 M NaCl was dissolved in 0.05 M Tris-HCl, pH 8.0, and 0.2 M NaCl and loaded onto a Sephadex G-75 (1.6 × 125 cm) column pre-equilibrated in the same buffer. Fractions were collected at a flow rate of 6 mL/h and monitored at 280 nm. Figure 5 shows the elution profile of the Sephadex G-75 chromatography, and SDS-PAGE (10%, inset) shows DEAE-Sephacel fractionated protein at 0.1 M NaCl and purified protein from the Sephadex G-75 column. The single band from the gel was cut, trypsin digested and analyzed by ESI-Q-TOF/MS. Figure 6 shows the MS profile of the purified protein, and the peptides matched with HPBP.

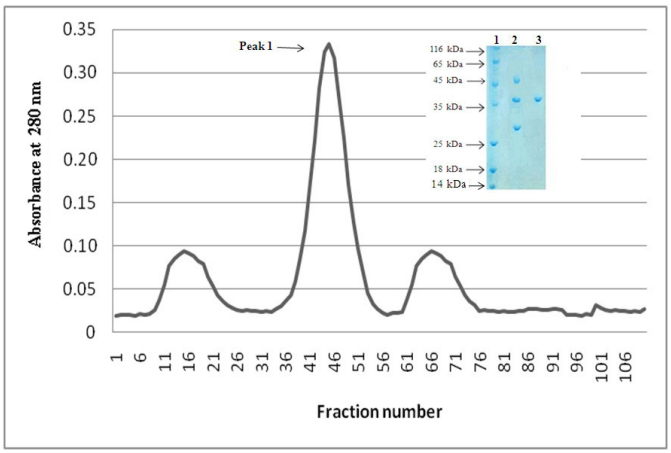
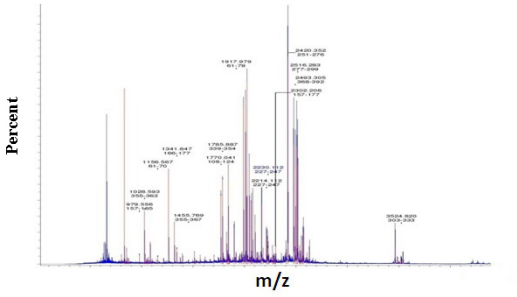


Figure 5. Elution profile of Sephadex G-75 (1.6 × 125 cm) equilibrated in 0.05 M Tris-HCl, pH 8.0, and 0.2 M NaCl, flow rate of 6 mL/h, 2-mL fractions. SDS-PAGE was performed on 10% polyacrylamide gels, and proteins were stained with colloidal Coomassie blue (G-250). *Lane 1* = SDS-PAGE analysis. *Lane 2* = Molecular mass markers. *Lane 3* = DEAE-Sephacel, 0.1 M NaCl. Purified protein from Sephadex G-75.



Human phosphate binding protein (HPBP) Apo-lipoprotein:

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DINGGGATLPQKLYLTPDVLTAGFAPYIGVGSGKGKIAFLENKYNQFGTDTTKNVHWAGS 60
DSKLTATELATYAADKEPGWGKLIQVPSVATSVAIPFRKAGANAVDLSVKELCGVFSGRI 120
ADWSGITGAGRSGPIQVVYRAESSGTTELFTRFLNAKCTTEPGTFAVTTTFANSYSLGLT 180
PLAGAVAATGSDGVMAALNDTTVAEGRITYISPDFAAPTLAGLDDATKVARVGKGVVNGV 240
AVEGKSPAAANVSAAISVVPLPAAADRGNPDVWVPVFGATTGGGVVAYPDSGYPILGFTN 300
LIFSQCYANATQTGQVRDFFTKHYGTSANNDAAIEANAFPLPSNWKAAVRASFLTASNAL 360
SIGNTNVCNGKGRPQ 375
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Figure 6. Mass spectrum of purified protein and peptides matched with human phosphate binding protein.

HPBP is a novel member of the DING family of proteins, which are likely ubiquitous in eukaryotes. In humans, DING protein had been identified from synovial fluid of rheumatoid arthritis (Hain et al., 1996; Blass et al., 1999), breast cells (Belenky et al., 2003) and kidney epithelial cells (Kumar et al., 2004), but the first structure was deduced from the HPBP concomitantly purified with PON1 from human serum (Morales et al., 2006). The structure clearly showed a relationship between HPBP and ABC (ATP binding cassette transmembrane) transporters in prokaryotes. The role of DING protein in humans as an inorganic phosphate scavenger may protect against cardiovascular disease such as atherosclerosis (Dorozhkin and Eppe, 2002; Amann et al., 2003). Interestingly, it has also been shown that this protein prevents kidney stone formation by inhibiting calcium phosphate precipitation (Kumar et al., 2004).

We report here for the first time the use of proteomics in targeting and identifying human phosphate binding apolipoprotein from amniotic fluid. The use of proteomics-based purification and subsequent characterization through ESI-Q/TOF is found to be a reliable, quick and high throughput method of purifying HPBP, a member of the DING protein family.

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