

Isolation of human surfactant protein A from amniotic fluid

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Abstract

Surfactant protein A (SP-A) is one of the four known surfactant-associated proteins found in human lungs. It plays a major role in determining regulation of surfactant uptake and resecretion. Qualitative and quantitative deficiencies of SP-A may contribute to neonatal respiratory distress syndrome. The measurement of its level in amniotic fluid or neonatal tracheal aspirate may be useful in the assessment of replacement therapy using natural or synthetic surfactants. In order to develop an in-house immunoassay to detect the level of SP-A, we used a discontinuous sucrose density gradient to isolate SP-A from amniotic fluid. Polyacrylamide gel electrophoresis was carried out on the isolates with low molecular weight markers. We successfully isolated SP-A from 12 out of 31 samples of amniotic fluid. The isolates were found to be relatively pure and have a molecular weight of about 35 kD. The isolated SP-A were used as immunogens to raise antibodies in rabbits for the immunoassay.

Key words: SP-A, respiratory distress syndrome, polyacrylamide gel electrophoresis, sucrose density gradient.

INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids, proteins and carbohydrates which is synthesized in type II pneumocytes.^{1,2} Among the surfactant associated proteins found in human lungs, surfactant protein A (SP-A) remains the major component and it plays an important role in the regulation of surfactant uptake and resecretion. It also acts synergistically with the mixture of phospholipids and helps in determining the regulation of phospholipid metabolism.^{3,4}

Research done by Avery and Mead about 30 years ago had successfully shown that the mechanical problem faced in lungs of neonates with respiratory distress syndrome (RDS) is mainly due to the lack of surfactant and thus high tension occurs in the air water layer of the lining of the alveoli.^{5,6}

Until recently clinical trials done in neonatal units have not demonstrated substantial improvement in the mortality and morbidity rate of RDS neonates^{7,8} with the use of natural and synthetic surfactant therapy. Nowadays RDS can be classified into 2 groups: surfactant responsive and surfactant non-responsive.⁹ Qualitative and quantitative deficiencies of SP-A may contribute to this syndrome. The measurement of its level in amniotic fluid or

neonatal tracheal aspirate may be useful in the assessment of replacement therapy for this syndrome. In order to develop an in-house ELISA for quantitation of SP-A, a relatively pure source of SP-A needs to be isolated. We attempted to isolate this protein from amniotic fluids of uncomplicated deliveries.

MATERIALS AND METHODS

Isolation of SP-A from amniotic fluid

All samples were obtained from Ward 1, 6 and 7, Kuala Lumpur Maternity Hospital from cases of uncomplicated delivery. Samples were collected using sterile techniques from normal

pregnancies during artificial rupture of the membrane and kept in ice-packed containers from delivery and during transport to the laboratory. Amniotic fluid samples that were grossly contaminated with blood or meconium

were discarded. Surfactants were isolated from individual amniotic fluid samples at room temperature within hours of collection according to the method of Hallman *et al.*¹⁰

The samples were centrifuged at 1,000 g for 10 min to separate out debris and blood cells. The supernatant was recovered and recentrifuged at 7,500 g for 10 min at 4°C using a Beckman ultracentrifuge and rotor SW 28 (Beckman, USA).

The resultant pellet was suspended in phosphate buffered saline (Sigma Diagnostic, USA) and layered carefully over a discontinuous system of sucrose density gradient which consisted of (a) 8 ml 0.55 M sucrose, 20 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4); (b) 8 ml 0.27 M sucrose, 20 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4) (c) 8 ml 0.15 M NaCl. Each centrifuge tube contained material from 100 ml of amniotic fluid.

After centrifugation at 10,000g for 90 min the interphase between the two sucrose layers was recovered and resuspended in phosphate buffered saline. The surfactant was then sedimented by centrifugation at 7,500g for 120 min. The final pellet was then resuspended in phosphate buffered saline and kept at -20°C for further analysis.

Determination of SP-A purity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis system used was a discontinuous gel system adapted from Laemmli, 1970." All chemicals used as stock solution as described were electrophoresis reagents from Sigma Co., USA.

Acrylamide bis-acrylamide: 30.0% acrylamide (w/v), 1.0% N,N'-bis methylene acrylamide. The solution was kept at 4°C.

0.75 M Trizma base (pH 8.8): 9.075% trizma base (w/v), pH adjusted by using concentrated HCl.

1.0 M Trizma base (pH 6.8): 12.1% trizma base (w/v), pH adjusted by using concentrated HCl.

Lauryl sulphate, sodium salt (SDS): 10.0% SDS (w/v).

Ammonium persulphate: 1.0% ammonium persulphate (w/v). The solution was prepared fresh just before use.

Sample buffer: 20.0% 1.0 M Tris pH 6.8 (v/v), 10.0% SDS (v/v) 48.0% urea (v/v), 0.005% bromophenol blue (w/v), 6.0% DL-dithiothreitol (w/v).

10x electrophoresis buffer: 3.0% trizma base (w/v), 14.4% glycine (w/v).

Top buffer reservoir: 10.0% 10x electrophoresis buffer (v/v), 1.0% SDS (v/v).

Staining solution: Coomassie brilliant blue R-250 (w/v), 4.0% ethanol (v/v), 10.0% acetic acid (v/v).

Destaining solution: 30.0% ethanol (v/v), 5.0% acetic acid (v/v).

Components of polyacrylamide gel

Components of 7.5% running gel and 3.0% stacking gel were prepared from the following stock solution:-

7.5% running gel: 25.0% acrylamide bis-acrylamide (v/v), 50.0% 0.75 M tris pH 8.8 (v/v), 1.0% SDS (v/v), 10.0% ammonium persulphate (v/v) 0.6% TEMED (v/v).

5.5% stacking gel: 17.7% acrylamide-bis acrylamide (v/v), 12.0% 1 M tris pH 6.8 (v/v), 0.95% SDS (v/v), 9.5% ammonium persulphate (v/v), 0.6% TEMED (v/v).

Sample preparation

Low molecular weight markers obtained from Pharmacia Biotech, USA were used as protein standards. 100 µl of sample buffer was added to each vial of low molecular weight markers and mixed. 15 µl of this mixture was applied to the gel for electrophoresis after boiling for 7 min at 100°C. Sample buffer was added to each sample in the ratio of 1:1 followed by 0.05% sucrose (w/v). 15 µl of sample was applied to the gel for electrophoresis after boiling under the same condition.

Electrophoresis condition

Electrophoresis was carried out using cylindrical rod at room temperature for 10 min under a constant current of 10 mAmp and 5.5% stacking gel followed by 4 hr under a constant current of 14 mAmp and 7.5% running gel. The electrophoresis system used was obtained from Bio-Rad, USA.

Staining and destaining of gel

Gel was soaked in staining solution for 30 min. Then it was washed with water and destained in destaining solution overnight.

After washing with water, the gel was preserved under 10.0% acetic acid. R, for both the protein standard and samples were calculated with the following formula:

$$R = \frac{\text{distance protein migration from the origin}}{\text{distance of bromophenol blue migration from the origin}}$$

Determination of SP-A concentration

SP-A concentration was determined by a uv method based on absorption in ultra-violet spectrum under wavelength of 215-225 nm.¹² Quartz cuvetts (Starna Ltd, UK) was used for the optical density (OD) measurement. Each sample

TABLE 1: Protein concentrations of samples where SP-A isolation was successful or unsuccessful

SP-A successfully isolated		SP-A unsuccessfully isolated	
Sample	Protein concentration (mg/dl)	Sample	Protein concentration (mg/dl)
1	380	1	123
2	570	2	141
3	950	3	42
4	681	4	20
5	260	5	0
6	530	6	20
7	244	7	50
8	384	8	31
9	683	9	111
10	490	10	123
11	537	11	196
12	827	12	196
		13	172
		14	162
		15	152
		16	152
		17	172
		18	190
		19	223
Mean	544.7	Mean	119.8

was diluted 1:1000 in triplicate with phosphate buffered saline and measurement was done by using phosphate buffered saline as blank. Mean protein concentration of each sample was calculated with reference to the standard curve generated using bovine serum albumin (Sigma Co., USA) as standard protein solution.

RESULTS

The concentrations of the protein in amniotic fluid samples are shown in Table 1.

SP-A was successfully isolated from 12 out of 31 amniotic fluid samples collected. SP-A isolated were found to be relatively pure with a molecular weight of about 35 kD (Fig. 1) as demonstrated by PAGE.

DISCUSSION

SP-A can be isolated by various methods: sucrose density gradient,¹⁰ extraction with butanol,¹³ isoelectric focussing¹⁴ and modification of sucrose gradient.¹⁵ We have experimented with

these approaches described in the literature, and found that the method based on discontinuous sucrose density gradient gave successful yield in some samples of amniotic fluid. We described in this paper the method modified from Hallman *et al.*¹⁰

Only 12 out of 31 samples seemed to show SP-A band by PAGE. This could be due to the low content of SP-A in the amniotic fluid samples. Previous studies showed that the SP-A level in amniotic fluid obtained from normal pregnancy was about $(9.91 \pm 0.34) \mu\text{g/ml}$ ¹⁶ whereas the sensitivity of protein detection with Coomassie Brilliant Blue staining was about 0.5 μg per band¹¹. The mean protein concentration determined by using ultra-violet method¹² of successfully and unsuccessfully isolated samples were 544.7 mg/dl and 119.8 mg/dl respectively (Table 1). Not all these proteins are SP-A. Besides, the isolation procedure entails loss of SP-A during the process. This may explain why we could not demonstrate SP-A from low protein samples even with a

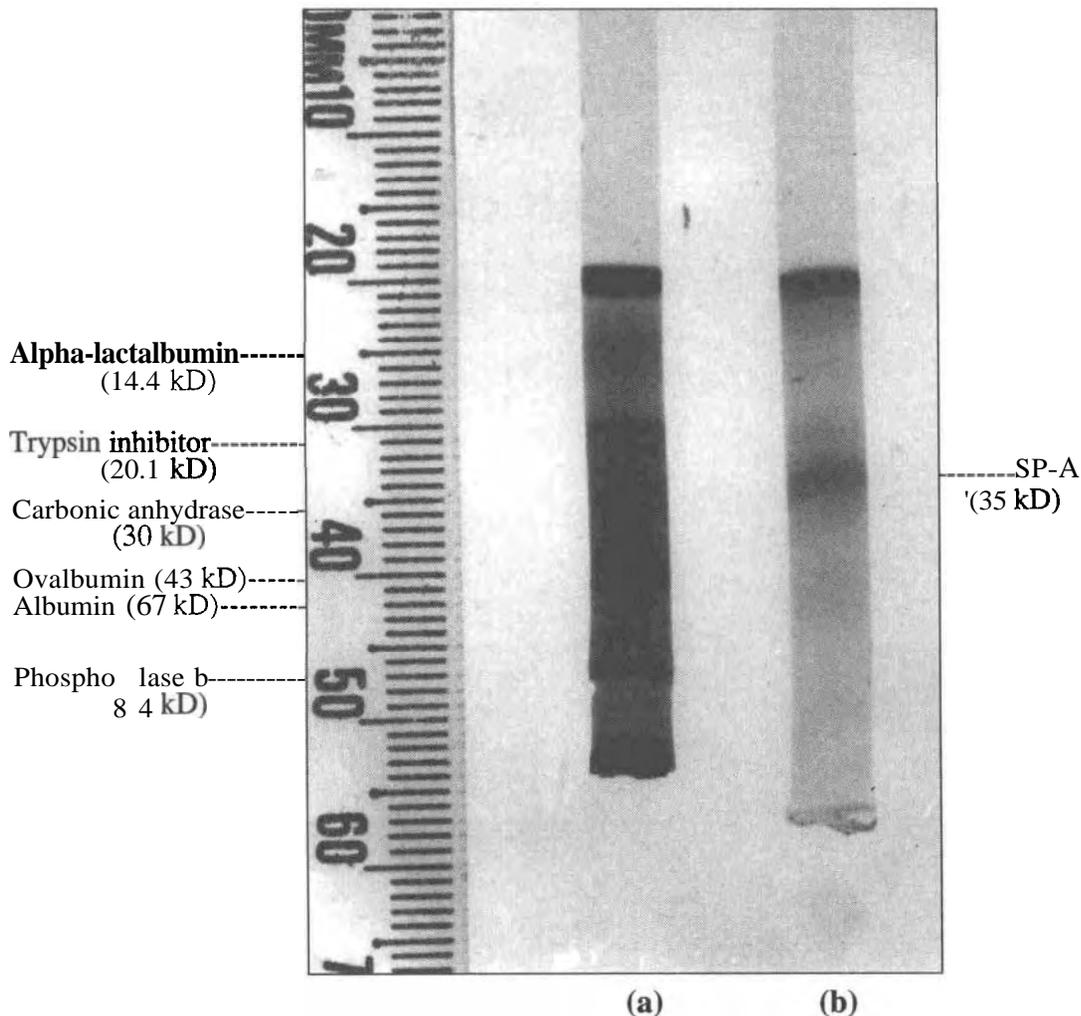


FIG. 1: Protein band in (a) low molecular weight markers and (b) SP-A after SDS-PAGE.

sensitive protein stain such as Coomassie Brilliant Blue.

Samples demonstrated to have SP-A bands with a molecular weight of 35 kD were pooled for production of antibodies in rabbits and antisera were subsequently used to establish an in-house ELISA for quantitation of SP-A in body fluids such as amniotic fluid, gastric aspirate and tracheal aspirate to investigate their clinical usefulness in neonatal respiratory distress syndrome.

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