THE INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLEDGE

Extraction and Detection of Bovine Lung Surfactants, Comparison with Human Surfactants and Their Antibacterial Role

B. Vijay Kumar
Associate Professor, Department of Biotechnology, PG Studies & Research Centre, Karnatak College, Karnatak, India
N. Sandeep
Research Scholar, Department of Biotechnology, PG Studies & Research Centre, Karnatak College, Karnatak, India
S. Najminara
Research Scholar, Department of Biotechnology, PG Studies & Research Centre, Karnatak College, Karnatak, India
Rosemary
Research Scholar, Department of Biotechnology, PG Studies & Research Centre, Karnatak College, Karnatak, India

Abstract:
Bovine (Deoni cattle) lung extract containing surfactants have significant role in prevention of various respiratory disorders are studied in comparision with human lung surfactants. Molecular weights of the surfactants of Bovine lungs ranged between 40 & 58 kDa which is very close to the values of human lung surfactants. Further, the surfactants SP-A & SP-D of cattle matching with surfactants of human. Cattle lung extract has shown significant inhibitory effect on growth of lung infecting pathogens such as Pseudomonas aeruginosa & Staphylococcus aureus in vitro.

Keywords: *Bovine (cattle), Surfactants, Lungs, Staphylococcus aureus, Pseudomonas aeruginosa*

1. Introduction

Deoni cattle is a native breed of India. They originated in the talukas of Basvakalyan, Bidar and Bhalki of Bidar district in Karnataka and adjointing Latur district of the State of Maharashtra. Known to be both good milk yielders and draft animals. These animals are quite popular in the Karnataka and Maharashtra. The body is moderately developed. Cows of this breed are moderately good milkers, yielding about 1135 kg in a lactation period of 300 days. Well-bred herds in farms yield on an average up to 1580 kg during the same period. (Sastry & Salunke, 2005). The percent of the live animal weight that becomes carcass weight, for fed beef is usually around 62-64%. The expected yield of retail cuts from beef carcasses ranges from approximately 55% to 75%, depending on the fatness and muscling of the animal, and the type of cuts produced. However cattle lung is considered as poor quality meat by local people hence, generally considered as waste and thrown.Therefore lung is selected for the study to get its valuable products like surfactants which would be predicted as curable agents in human lung disorders. The lung is a uniquely vulnerable organ. Human lung infection is caused by a variety of different elements. The most common of these are pneumonia, bronchitis; asthma and tuberculosis arise from the presence of microorganisms such as bacteria, fungi, or viruses. More common bacterial lung infection is usually *Staphylococcus aureus* and *pseudomonas aeruginosa*.This causes swelling in addition to the production of mucus and increased blood flow.Hence present study is made whether cattle lung extract (surfactants) ha s any cruical role to check the multiplication of these bacteria invitro.

2. Materials

2.1 Biuret reagent: Dissolve 1.5 grams of CuSO4 and 4.5 grams of Na-k tartate in 250ml 0.2N NaOH solution to this solution is added 2.5 grams of KI and volume made upto 500ml with 0.2N NaOH.
2.2 0.2N NaOH:8gms of NaOH dissolved in 1000ml of Distilled water to get 0.2N NaOH solution.
2.3 Acrylamide stock (44:0.8): 44.0gm of Acrylamide, 0.8gm of Bis acrylamide were dissolved in 100ml of distilled water
2.4 Acrylamide stock (30:0.8): 30.0gm of Acrylamide, 0.8gm of Bis acrylamide dissolved in 100ml distilled water.
2.5 Seperating gel buffer:18.15gm of Tris (1.5M)dissolved in 50ml of distilled water, adjusted PH to 8.8 with concentrated HCl&made to final volume of 100ml with distilled water.
2.6 Stacking gel buffer: 6.5gm of Tris (0.5M) dissolved in 50ml of distilled water, adjusted PH to 6.8 with concentrated HCL, made to final volume of 100ml with distilled water.
2.7 Ammoniun per sulphate (APS):
2.7.1 APS(10%): 0.5gm of APS dissolved in 5 ml distilled water
2.7.2 APS (1%): 0.5gm of APS dissolved in 50ml distilled water. Then (Both 2.7.1 & 2.7.2 made every time fresh solution).

2.8 Seperating gel solution: 7.50ml of Acrylamide : Bis acrylamide (44:0.8), 7.50ml ofseparating gel buffer, 0.60ml of 10% SDS, 13.60ml ofdistilled water, 0.76ml of 1% APS, 0.06ml of TEMED are mixed and madetotal volume of 30.02ml.

2.9 Stacking gel solution: 2.00ml of Acrylamide: Bis acrylamide (30:0.8), 3.75ml of separating gel buffer, 0,15ml of 10% SDS,8.90ml of distilled water, 0.15ml of 10% APS, and 0.15ml of TEMED aremixed and made total volume 15.10ml.

2.10 Solubilizing buffer (2x): 0.75 gm of Tris (0.125M),1.00gm of SDS(2%), 7.50ml of Glycerol(15%), and 0.005mgBromophenol blue are mixed, dissolved in distilled water &made finalvolume 25ml, adjusted to pH 6.8. Before electrophoresis, the above solution was added with 5% P-mercaptoethanol. Then, it was boiled in a water bath for 5 – 10 mins.

2.11 Running buffer:6.00gm ofTris(1.5M), 1.00gm of SDS and 28.00gm of Gylcine dissolved in 1000 ml of distilled water.

2.12 Coomassie blue stain (for acrylamide gels): 1.25gmCoomassie blue, 255mlmethanol, 255ml distilled water, and 45.0mlacetic acid are mixed & filtered.

2.13 Destaining solution:

2.13.1 Destain-I: 30.0ml, methanol, 7.50ml of acetic acid and 100ml distilled water are mixed together.

2.13.2 Destain-II:50ml of methanol, 7.50ml ofacetic acid and 100ml of distilled water are mixed together.

3. Methods

3.1. Extraction of Proteins from Lungs

About 250grams of fresh cattle lung is brought to laboratory in ice furnace from slaughter house, Bidar. Lung was thoroughly washed using distilled water to remove the excess blood and impurities.Lung was dissected in fine pieces with the help of sterilized surgical blade and again washed with water.Lung pieces were added a sufficient amount of liquid Nitrogen for hardening and ground with help of mortar pestle.1gram of ground tissue was transferred into 1.0ml centrifuge tuba and sufficient volume of cool RIPA lysis buffer (for 0.1gram tissue 1ml of buffer is used) was added and mixed.It was the incubated on ice for 40mins (vortex for 4-6 time during incubation).Above mixture was transferred to another centrifuge tube and centrifuge at 27000rpm for 20mins at 4ºC.Supernatant was collected, stored at 4ºC and bottom cell pellets were rejected.

3.2. Qualitative Estimation of Protein

An equal amount of NaOH and lung extract was mixed & few drops of 1% CuSO4 were added & mixed well. Blue colour changed to deep violet. It is positive result of presence of proteins in lung extract.

3.3. Quantitative Analysis of Protein

The known standard protein solution was taken into series of test tubes 0.0 to 1.0ml with the interval of .02ml of protein solution and made up the total volume of 4ml by the addition of Dist. Water.The blank test solution was prepared by adding only 4ml of dist. water. I another test tube, 4ml of unknown protein extract of lung was taken.Then 4ml of biuret reagent added to each tube and mixed well.The tubes were kept at 37ºC for 10mins during which the purple color was developed.The O.D of each tube was measured at 520nm using a reagent blank.The graph was drawn with O.D v/s concentration of known protein to detect unknown protein concentration in lung extract.

3.4. Sodium Dodecyl Sulphate Polyacrylamide Slab Gel Electrophoresis

The KLAMPHOR slab gel unit was thoroughly cleaned and dried.1 mm thick spacer was fixed to the bottom and two sides of the plates were coated with silicon high vaccums.The other plate was kept on it and both the plates were held by the clips and then fixed in the slab gel unit, Wing nuts were tightened.Separating gel buffer was prepared and separating solution was poured up to the level such that 1 cm gap was allowed for stacking gel solution A 0.1% SDS solution in water was layered to form even layer on the separating gel. It was allowed for 30 mins for polymerization, when the SDS solution level dropped due to run off along the sides, fresh SDS solution was added. Then the entire assembly was covered with plastic bag and left over night at 4 ° C for aging. Stacking gel solution was prepared and carefully layered over the previously placed comb on the separating gel. After 30 mins the comb was carefully removed.For loading the wells, lung surfactant protein samples were prepared by adding 50 micro liters of 2X solubilizing buffer to 50 µl of the sample. Similarly a mixture of protein markers (50 micro liters) in 2X solubilizing buffer (50 µl) were used the following molecular weights. and boiled in the water bath for 5 mins.

About 50 µls of sample was loaded into each well. Running buffer was added to the anode and cathode chambers till the buffer touched the gel.Power supply was connected and 60 V was applied until the marked dye entered the separating gel. Afterwards, the voltage was increased to 120 V and the power supply was maintained until the marker dye reached the bottom of the separating gel. Then the lids were disconnected and the slab gel setup was removed.After removing the glass plates, the gel was placed in coomassie blue stain for 1 hour. Later the gel was destained in Destain-I with frequent changes until clear backgroung was obtained. The gels were stored in Destain-II. (Markers used: Egg albumin - 43.0kDa, Bovine serum albumin - 68.0kDa)

3.4.1. Drying of Gels

The gel was placed in 50% methanol for 30 mins and later it was replaced with fresh absolute methanol and shaking was continued for about 15 mins.The gel was removed and was placed pressed in a polythene bag kept at 45º angle to facilitate the draining of left over solution and drying. Afterwards the gel was rendered flat by drying stored in polythene. At the time of gel scanning, the gel was immersed in 7% acetic acid for about 50mins to get the gel of original nature.

3.5. Detection of Pathogens from Sputum (Gram's Staining Method)

Sample from mass culture was smeared on a clean glass slide, allowed it to air dried and fixed it by flaming.The smear was covered added with crystal violet stain and allowed it to act for 60sec.Excess stain was oozed & then added with Gram's iodine to form complex molecules with crystal violet stain for 1min.in the bacterial cell wall.Excess complex molecules are first washed with distilled water & then with absolute alcohol or acetone for 30secs, again washed with water.The saffranin stain was added on the above washed slide for counter staining, excess stain was oozed out, and then allowed to air dry.The pathogens were observed under magnification of100x using oil immersion in microscope to find out their population density & differentiate gram positive from gram negative bacteria.

3.6. Detection of Role of Surfactants on Pathogens

Two Nutrient Agar plates were prepared aseptically and numbered them as 1 and 2.In the 1st plate, 1ml of Asthma patient's sputum culture was added. In the 2nd plate, 1ml of sputum culture & 1ml of cattle lung extract was added.Both the plates were kept inverted state into the incubator at 37ºC for 24-48hrs for growth.

3.7. Comparative Study of Pathogens of Asthma Patient with Pure Culture

The pure cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were collected from Department of Microbiology, Veterinary University Bidar and brought to the laboratory in nutrient broth.Four Nutrient Agar plates were prepared aseptically and numbered them as 1, 2, 3& 4.In the 1st Nutrient agar plate, 1ml of pure culture of *pseudomonas aeruginosa* pour plated (used as control), in the 2nd Nutrient agar plate, 1ml of pure culture of *Pseudomonas aeruginosa* (as test plate) and 1ml of lung extract was poured, In the 3rd Nutrient agar plate, 1ml of pure culture of *Staphylococcus aureus* was poured (as control) & in the 4th Nutrient agar plate, 1ml of pure culture of *Staphylococcus aureus* and 1ml of lung extract was poured (as test plate)All the 4 plates were kept in the incubator at 37ºC for 24-48hrs for growth.

4. Results & Discussion

The qualitative Biuret test of lung extract shown deep purple colored complex which indicates the cattle lung extract has high amount of protein including surfactants. Similar results obtained with human lung.To determine the total amount of protein (including surfactants) present in lung extract, the extract was subjected to the quantitative analysis by biuret method. It was observed that the lung extract has shown 210 mg/kg (Fig. 1). The reports of others for human lung surfactants showed 100mg/kg in adults & 4-5mg/kg in infants. In this experiment, the excess amount of proteins observed may be due to the presence surfactants, and other lung structural & functional proteins of lungs.

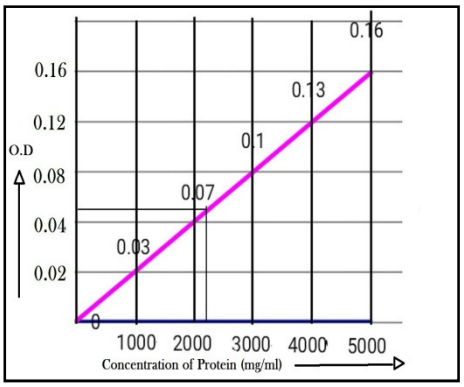

Figure 1: Quantitative analysis for proteins of lung extract

Further, the extracts of cattle lung proteins were subjected to SDS-PAGE electrophoresis to determine molecular weights of lung surfactants for their conformation. In our study, from the cattle lung extract, isolated proteins have the molecular weight ranges from 40-58kDa (Figure 2) which is nearly as same as the Egg albumin and the Bovine serum Albumin which was ranging rear the values of human lung surfactants such as SP-A 28-36kDa & SP-D 42-43kDa as reported by Griese et al. (2013) & Pérez-Gil and Keough (1998). Further, cattle lung surfactants can be modified by adding certain additives to make clinical drug for the treatment of human surfactant deficient respiratory disorders as it was reported by Taeusch et. al.(2002) that "Survanta is a modified natural surfactant prepared by Lipid extraction of mincd bovine lung by addition of DPPC, palmatic acid & tripalmatin which is similar to surfactant TA that is first surfactant used clinically for the treatment of Human Respiratory Disorders in Japan. It is a cow lung minced extracted made by Tokyo and Tanabe company". Rochester (2011) reported that, the first generation of therapeutic surfactant preparations, that are currecntly used in clinical practice, consists either of lipid extracts of natural, nonhuman surfactants containing the lipid components, SP-B and SP-C of whole surfactant(calf lung surfactant extract) or bovine lipid extracted surfactant, or of synthetic, completely protein-free mixtures of phosphatidylcholine, tyloxapol and hexadecanol (Exosurf). The next generation of surfactants will be composed of defined lipids and hydrophobic proteins or peptides.Pulmonary surfactant reduces the surface tension at the air–liquid interface throughout the lung by forming a lining layer between the aqueous airway liquid and the inspired air.

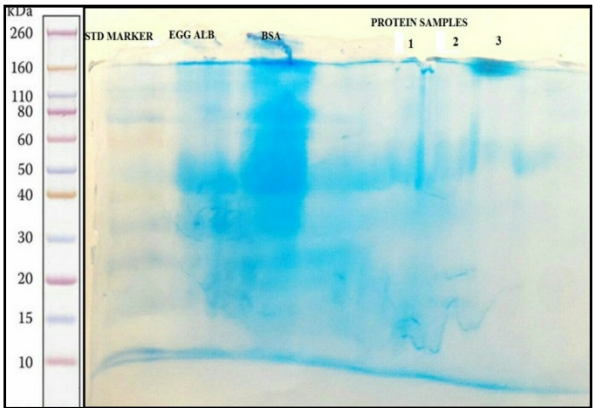

Figure 2:Matching of Std. protein bands of SDS-slab gel electophoresis with known protein bands for detecting molecular weights of surfactants

Further, the cattle lung extract having surfactants were used for knowing its effects on pathogens (*S. aureus& P. aureginosa*) of lungs of asthma patient. The growth of these microorganisms was more in the controls when compared to the growth in test plates. It was merely indicates that, the growth of both pathogens present in sputum was inhibited due to the surfactants of cattle dung (Figure 3). Hence, the cattle surfactants with slight modifications by adding necessary additives can be clinically used for the treatment of asthma that is caued by these pathogens.

Figure 3: Effect of bovine lung extract on sputum pathogens of asthma patients

Further, the effect of cattle lung surfactants on these pathogens was conformed to the pure cultures of these bacteria (Figure 4).It was observed that, the antibacterial activity of lung extract suppressed the growth of these micro-organisms significanty. Hogo (1991) and Russell (1992) have reported that the surfactant interact with various cellular components, in particular proteins and lipids, and consequently cause deleterious effects on the growth and viability of microbial cells. It was also reported that the antimicrobial activity of surfactants is influenced not only by the chemical properties of surfactants but also by how a target micro organism has grown before and during the surfactant treatment. Further, it was re-poured (Basawaraj,2003)that, cationic surfactants such as quaternary ammonium compounds and, although less active zwitter ionic surfactants possess antimicrobial activity at relatively low concentration.

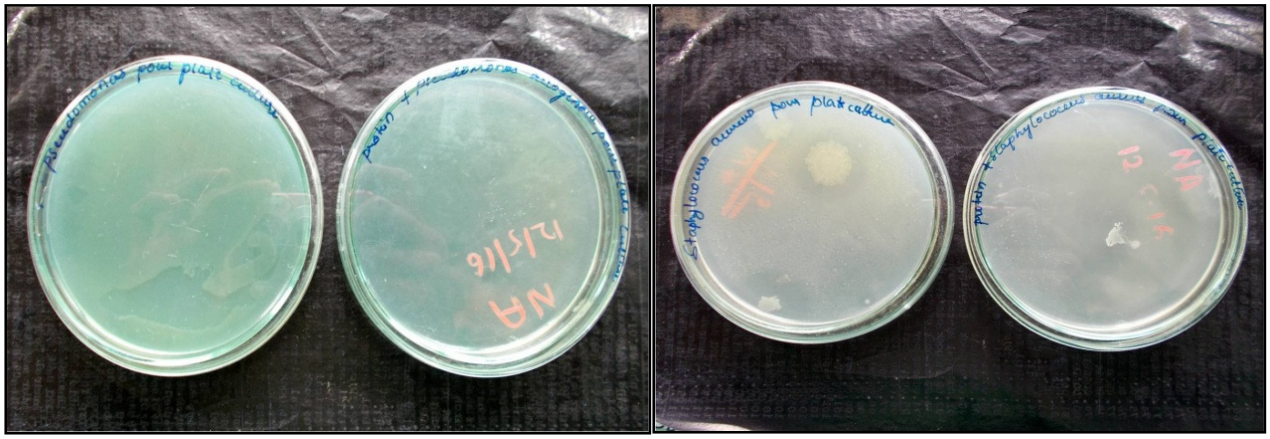

Figure 4: Effect of lung extract sample on p. aeruginosa and s. aureus

5. Conclusion

Cattle lung has valuable surfactant which ranges between 40-58kDa that is nearing the values of human lung surfactants. Cattle lung extract containing the surfactant are closely matching with SP-A and SP-D surfactant of human lungs. Hence, used as drug with slight modification.Human lung disorders can be treated by cattle lung extract since their most of molecular weights is ranging within 40-58kDa.Cattle lung extract has shown significant effect on growth of human lung pathogens such as *pseudomonas aeruginosa* and Staphylococcus aureus.

6. References

i. Basawaraj A. A thesis enitled "Isolation of proteins from varieties of sheeps, 245-250.2003.
ii. Sastry and Dr. Vijaya Salunke."Animal Resources". India: Human Environment. Geography Standard (Grade) IX. Pune: Secretary Maharastra State Board of Secondary and Higher Secondary Education. P. 31.2005.
iii. Jesus Perez-Gil. Biochemical et Biophysics Acta (BBA) - Biomembranes "Structure of pulmonary surfactant membranes and films: The role of proteins and lipid-protein interactions. The interplay of lungs. 2008.
iv. Griese M. Respiratory syncytial virus and pulmonary surfactant. "viral immunol" vol. 15, pp. 357-363.2002.
v. Taeusch H, Lu K, Goerke J and clements J . Nonionic polymers reverse inactivation of surfactant by meconium and other substances. Am J Respir Crit Care Med; 159; 1397-5.1999.
vi. Taeusch HW and Keough KMW. Inactivation of pulmonary surfactant and the treatment of acute lung injuries. Pediatr Pathol Mol med; 20; 519-36.2001.
vii. Hogo WB and Russell AD. Types of antimicrobial agents. In principle and practice of disinfection, preservation and sterilization, 2nd edn. Russell AD, Hugo WB and Ayliffe GAJ. Pp. 34-38. Oxford: Blackwell Science Publications. 1992.
viii. Rochester NY. "Recent advanes in ocular drug delivery system": The first generation of therapeutic surfactant preparations, that are currently used in clinical practice, consists either of lipid extracts of natural, nonhuman surfactants containing the lipid components, SP-B and SP-C of whole surfactant (Survanta). 2011.