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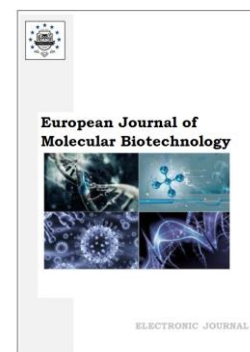

Complex Extraction of Surfactant Proteins from the Farm Animal Lungs Using a Non-Ionic Detergent Tween 20

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Abstract

The article presents the pilot extraction technology of surfactant proteins, represented by hydrophilic (SP-A, SP-D) and hydrophobic (SP-B, SP-C) fractions from bovine and porcine lungs. Borate buffer, tris buffer, and phosphate buffer saline solution were the basis for extraction solutions. Twin 20 was applied in various concentrations as a mild detergent capable of preserving the spatial structure of the protein. To identify and give a semi-quantify estimation of surfactant protein presence in extracts we used one-dimensional electrophoresis and mass spectrometry technique. A semi-quantitative assessment of the protein concentration using integrated density on electrophoregrams showed that the borate buffer allows us to isolate the largest amount of SP-A, and the tris buffer SP-D without adding Tween 20 proved analogue effect if we perform extraction from the bovine lungs. Phosphate buffered saline solution + 1 % Tween 20 demonstrated the best efficiency of extraction of SP-A and SP-D from porcine lungs. The extract solution of Tris-buffer + Tween 20 content demonstrated the highest efficiency, and Phosphate-buffered saline + 1 % Tween 20 was the least effective, failing to isolate SP-C. The detergent addition was critical to the degree of surfactant proteins extraction. The development of a comprehensive technology for the extraction of surfactant proteins will reduce the cost and laboriousness of their production. This technology will make it possible to reduce the cost of surfactant-based drugs and make them more accessible to the population.

Keywords: protein extraction, surfactant proteins, nonionic detergents, Tween 20, lungs, farm animals.

1. Introduction

Today, there is an increasing need in creating technologies aimed at obtaining a larger number of proteins and other functional compounds from various objects of plant and animal origin, reducing the prime and labor costs of the final product (Faustino et al., 2019).

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Currently, of particular interest is the development of a surfactant protein (SP) complex extraction. SPs play an important role in medicine, as being used for the treatment of pulmonary diseases such as respiratory distress syndrome, acute lung injury syndrome, and oncology (Casals et al., 2012, Lopez-Rodriguez et al., 2014, Bayat et al., 2015, Fedorchenko et al., 2016, Cheung et al., 2017). Interest in SPs has increased due to the pandemic caused by SARS-CoV-2. Some hypotheses say that SARS-CoV-2 may cause an intense loss of surfactant proteins (Takano, 2020, Avdeev et al., 2021) and that the exogenous surfactant proteins may assist in the recovery of damaged alveoli and prevent severe acute respiratory failure (Ghati et al., 2021).

Pulmonary surfactant is a lipid: protein complex containing four proteins dipalmitoyl-phosphatidylcholine as the major component. The pulmonary SPs family includes four types of proteins. Hydrophilic SP-A a is of 5.3 % and SP-D is of 0.6 % of all SPs, hydrophobic SP-B is of 0.7 % and SP-C is of 0.4 % of ones. All SPs are synthesized by alveolar type II cells (Chroneos et al., 2010). The lipid complex includes phosphatidylcholine as the predominant species (70-80 %) and neutral lipids as cholesterol (5-8 %). Among these, the saturated dipalmitoyl-phosphatidylcholine accounts for 40 % in average whereas unsaturated phosphatidylcholine and anionic phospholipids as phosphatidylglycerol (8 %) and phosphatidylinositol (PI) are also important components (Cañadas et al., 2020).

Such surfactant-based drugs as synthetic Exosurf (Glaxo-Wellcome, USA-UK) and ALEK (Britannica, UK), semi-synthetic Surfactant-TA (TokyoTanabe, Tokyo, Japan) and Survanta (AbbVie Inc, Chicago, USA), natural Curosurf, (Chiesi Farmaceutici, Farma, Italy), Infasurf (Forrest Labs, St. Louis, USA), CLSE (Rochester, NY, USA), and Surfactant-HL and Surfactant-BL, (Russia) are known to be the most popular in clinical practice. Synthetic surfactants demonstrate less effective, although they are certainly more available (Patel, 2018). At the same time, these drugs, unlike natural ones, do not contain SPs. This fact is extremely important for their properties, which are crucial for surfactant phospholipid ability to reduce surface tension at the phase boundary (alveolar surface – air), while synthetic drugs, including Exosurf, do not contain these proteins.

Currently, existing technologies allow us to distinguish all four isoforms of SPs, but such techniques are suitable only for research tasks. There is currently no way to isolate all SPs for further purification and use as medicines. Most of the existing technologies are based on the Bligh and Dyer method including Russian developments (Beers et al., 1992, Strong et al., 1998, Rozenberg et al., 2019). They are very laborious and expensive because it is necessary to carry out additional stages of lung homogenate purification. Nonionic detergents are the best choice for membrane protein isolation. Tween 20 (TW20), one of them, is highly soluble in water. With the hydrophilic-lipophilic balance of 16.7, TW20 gently destroys lipid bilayer membranes, preventing protein denaturation, and has the lowest critical micelle concentration of 0.06 compared to other detergents from this group (Seddon et al., 2004, Johnson, 2013).

This study aims to develop a pilot technology for the complex extraction of all 4 surfactant protein isoforms from bovine and porcine lungs using the non-ionic detergent TW 20.

2. Materials and methods

We used bovine and porcine lungs as raw materials.

The lungs were immediately frozen at -20°C after the removal, then transported to the laboratory and prepared for the extraction of SPs and further mass spectrometric studies (Cox et al., 2006).

2.1. Surfactant protein extraction

The protocol included lung washing in cold PBS for 1 hour in a reciprocating shaker. The lung samples were homogenized in 15 ml of extraction solution (Table 1) per gram of tissue using an Ultra-Turrax Tube Drive homogenizer (IKA, Germany) and glass beads in a BMT-20 S/G tube for 5 minutes at 5000 rpm.

Next, we centrifuged the samples at 700g for 5 min in a ScanSpeed Mini centrifuge (Labogene, Denmark). The protein concentration in the supernatant was determined by the Lowry method. The obtained samples were divided into two parts: the first part was used for SDS-PAGE, the second part was placed in cryovials and frozen at -80°C in an MDF-C8V1 freezer (Sanyo, Japan) for subsequent mass spectrometry analysis.

Table 1. Extraction solutions

Base	Concentration	pH	Detergent
Tris-buffer	10 mM	7.4	None or 0.1 % TW20 or 1 % TW20
Borate buffer	10 mM	9.18	
PBS (Phosphate buffer+0,9% NaCl)	-	7.4	

2.2. SDS-PAGE

We performed one-dimensional SDS-polyacrylamide gel electrophoresis to separate and identify extracted proteins in a Mini-PROTEAN Tetra Cell (BIO-RAD, USA) under denaturing conditions using SDS according to the Laemmli method and 10-250 kDa markers, Precision Plus Protein™ Unstained Protein Standards (BIO-RAD, USA). The obtained protein fractions were mixed with sample buffer (SB-buffer) in a ratio of 1:10 and thermostated at 95°C for 5 minutes. The stacking (5 %) and resolving (12 %) gel solutions were prepared for electrophoresis. After electrophoresis, the gels were placed in a gel-fixing solution for 15 minutes, stained with Coomassie Brilliant Blue G-250 (Russia, Dia-M) for an hour, and then placed in a gel-washing solution overnight. Then the gels were soaked for 15-60 minutes in ddH_2O, and the ChemiDoc™ Touch Imaging System (BIO-RAD, USA) used to captured and make digital images (Ruano et al., 1998). A semi-quantitative assessment of the surfactant protein concentration in the gels was carried out using ImageJ 1.53K software (NIH, USA) by measuring the integrated density. SP-A detected in 28-36 kDa range (Kankavi et al., 2004), SP-D had mass of 43 kDa (Crouch et al., 1994), SP-B had mass 8 kDa as monomeric form and of 16 kDa as dimeric form (Simonato et al., 2011), and SP-C one detected in 3.7-21 kDa range (Beers et al., 2017).

2.3. Mass spectrometry

2.3.1. Sample preparation

The preliminarily purification of sample hydrolysates from urea concluded in hydrophobic chromatography using ZipTip microcolumns (Millipore, Germany) with C_{18} resin. After being applied to the microcolumn, the samples were washed with 4 % acetonitrile solution (Merck, Germany) and 0.1 % trifluoroacetic acid (PanReac AppliChem, Germany) in deionized water. The definitive washing of peptides was conducted on the column with 80 % acetonitrile solution and 0.1 % trifluoroacetic acid and drying on a vacuum concentrator.

2.3.2. Mass spectrometry protocol

The first stage of analysis concluded in applying the hydrolysate of analyzed samples on a reversed-phase column with subsequent peptide separation in an acetonitrile gradient. The peptides eluted from the column entered the ionization chamber, where the ions were analyzed by tandem mass spectrometry on an Orbitrap Elite ETD high-resolution mass spectrometer (Thermo Scientific, Germany). Ion fragmentation was carried out by two independent methods HCD and CID.

2.3.3. Data analysis

During mass spectrometry we obtained sets of peptide and ion fragment masses and processed them using a commercial program PeakStudio 7.5. (Bioinformatics Solutions Inc, Canada). Then we identified peptides using the UniProt database. Protein identification was considered reliable if the value of the identification confidence level -10 lgP was ≥ 20.

2.4. Statistical Processing

We processed quantitative data in the Statistica 12.0 (StatSoft Inc., USA) with indicator calculations used to characterize nonparametric samples in biomedical studies: the normality of the distribution, the median [1st quartile, 3rd quartile] and assessed the significance of sample differences. We also used the Mann-Whitney test with a p-value less than 0.05 to analyze the differences between two independent samples.

3. Results

The eluting solutions showed different results in the dependence of animal species, buffer kind, and TW20 concentration. Figure 1 demonstrates that the complex extraction of SPs let to the highest protein output from porcine lungs in cases of PBS with 0.1 % TW20 and 1 % TW20

application. If we took bovine lung samples the using borate buffer with 0,1 % TW20 provided the best result. The addition of Tris-buffer in 1 % concentration let to worse protein outcome, 3-4 times less compared to other buffers.

Figure 2 presents the results of electrophoresis application to identify the concrete SPs in obtained samples from bovine lungs (2A) and porcine lungs (2B). We can see small peaks on the electropherogram of bovine lung extracts at the 37 kD marker level corresponding to the mass of an SP-A monomer, in case of borate buffer, borate buffer + 0.1 % TW20, and tris-buffer without detergent revealed at first, second, and fourth tracks, accordingly.

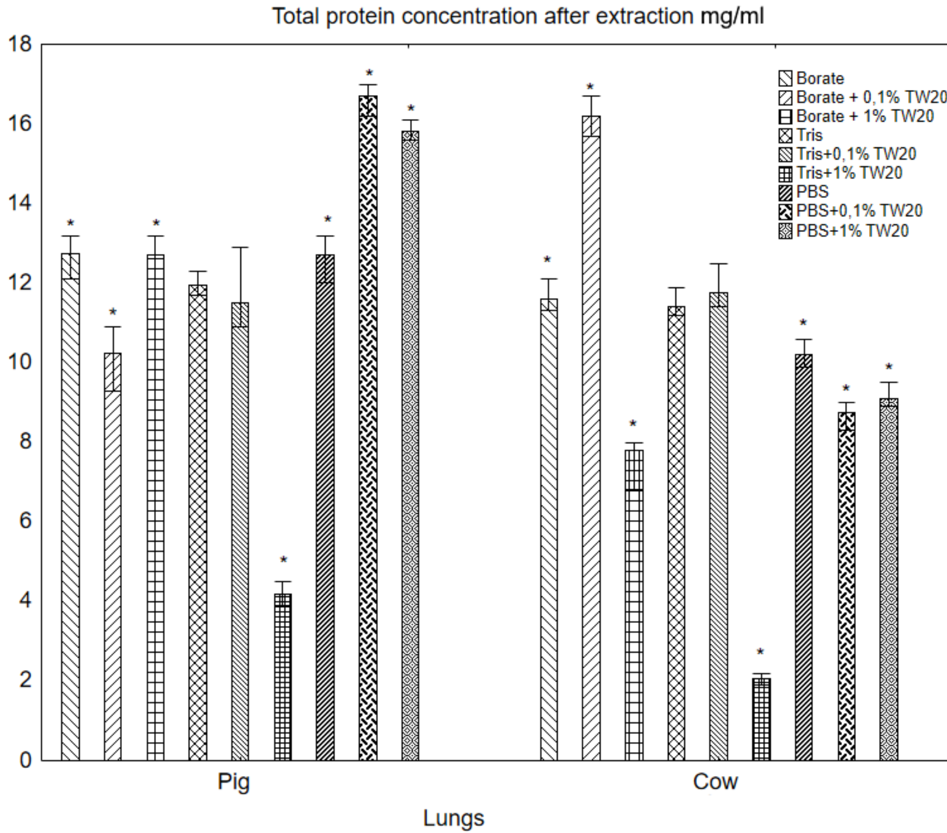

Fig. 1. Total protein concentration in the samples obtained by different extraction solutions.
* – statistically significant differences (nonparametric Mann-Whitney test, $p < 0.05$)

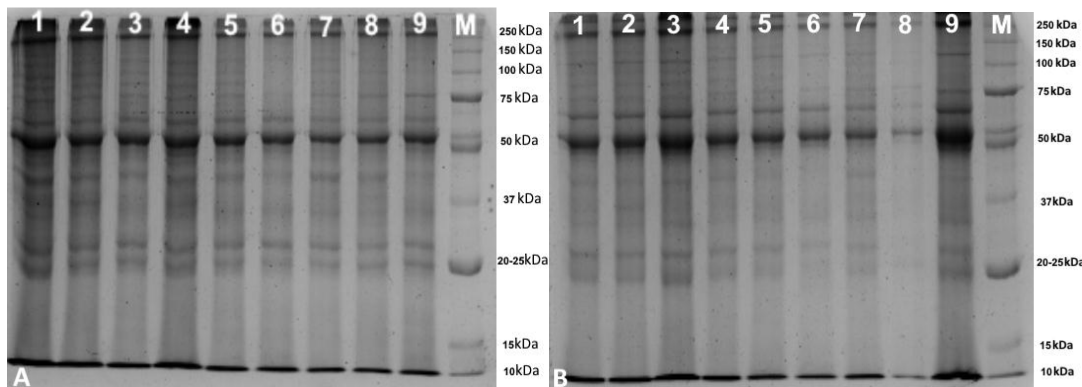

Fig. 2. SDS-PAGE electropherogram of bovine lung (A) and porcine lung (B) extracts. Coomassie Brilliant Blue G-250 staining, 1:10 dilution. Protein bands: 1 – Borate, 2 – Borate + 0.1 % TW20, 3 – Borate + 1 % TW20, 4 – Tris, 5 – Tris + 0,1 % TW20, 6 – Tris + 1 % TW20, 7 – PBS, 8 – PBS + 0,1 % TW20, 9 –PBS + 1 % TW20, 10 – BIO-RAD protein markers.

In all tracks we see an electrophoretic strip between the 37 kDa and 50 kDa markers, presumably appropriating SP-D. No peaks corresponding to the dimeric form of SP-B were found, but it is possible that the monomeric form is on a peak at the 10 kDa marker. All sample tracks have a blurred strip at the 20-25 kDa marker corresponding to different SP-C.

On the electropherogram of porcine lung extracts we can see three blurry peaks at the 37 kD marker level corresponding to the mass of an SP-A, in a case of borate buffer + 0.1 % TW20, borate buffer + 1 % TW20, and PBS + 1 % TW20 revealed at second, thirdly and ninth tracks, accordingly. In all tracks except for PBS + 0,1 % TW20, we see an electrophoretic strip between the 37 kDa and 50 kDa markers, presumably appropriating SP-D. The electrophoretic strip corresponding to the dimeric form of SP-B is not revealed on electropherogram porcine lung extracts. Probably SP-C is present in an electrophoretic strip corresponding to the 10 kDa marker, but all sample tracks, except for PBS + 0.1 % TW20, have a strip at the 20-25 kDa marker corresponding to SP-C preform.

Table 2 presents an estimate of the integrated density of electrophoretic strips obtained by extraction with different eluents. This indicator made it possible to select six samples for mass spectrometric analysis and protein identification.

These samples were represented by extract SPs, obtained in PBS + 1 % TW20, borate buffer + 0.1 % TW20, and tris-buffer of bovine lungs and porcine lungs. The flattening electrophoretic strip did not allow the evaluation of integrated density for the hydrophobic fraction of SPs.

Mass spectrometry allowed identifying all SPs in experimental samples. The results of mass spectrometry are consistent with electropherograms. They prove the presence of all hydrophobic and hydrophilic SPs in the electrophoretic strip corresponding to molecular markers. The extraction solution containing PBS + 1% TW20 made it possible to isolate only three SPs from the lung extracts of bovine and porcine. Mass spectrometry revealed SPs in porcine lung and bovine lungs extracts, but also to other closely related mammalian species.

Table 2. SDS-PAGE analysis of SPs for each solvent-detergent extraction

Solvent	Concentration TW 20	Protein	Integrated Density	
			Bovine lungs samples	Porcine lungs samples
PBS	0 %	SP-A SP-D	0,60 0,73	0,79 0,78
	0,1 %	SP-A SP-D	0,57 0,71	0,82 0,83
	1 %	SP-A SP-D	0,60 0,70	0,96 0,95
Borate buffer	0 %	SP-A SP-D	0,96 0,85	0,87 0,89
	0,1 %	SP-A SP-D	0,78 0,89	0,94 0,91
	1 %	SP-A SP-D	0,66 0,77	0,67 0,68
Tris-buffer	**0 %**	SP-A SP-D	0,77 0,94	0,82 0,86
	0,1 %	SP-A SP-D	0,74 0,73	0,78 0,80
	1 %	SP-A SP-D	0,58 0,66	0,72 0,69

This result may be associated with a low degree coverage of amino acid sequences from 1 % to 8 %, and a low significant value. The SP-A extracted with tris-buffer without TW20 from porcine lung sample had highly identified (-10 lgP 36.62) with an amino acid sequence coverage of 10 %. The use of borate buffer + 0.1 % TW20 allowed the isolation of SP-A from bovine lungs to have a high degree identity (-10 lgP 118.31) and an amino acid sequence coverage of 21 %. Figure 2 presents the electrophoretic strips corresponding to molecular markers 21 kDa and therefore SP-C. It should be noted for separation of the hydrophilic SPs to be more successful compared to ones.

The results of mass spectrometry and electrophoresis confirm the fact, that all SPs fractions were isolated during extraction by various eluting solutions.

4. Discussion

We choose the TW20 detergent because it has a gentle action on cell lipids. It destructs lipid-lipid and lipid-protein interactions and preserves native protein structure and function. In addition, TW20 has the best physicochemical properties compared to other detergents from the nonionic group, like Triton-x100. On the other hand, it is necessary to find a way to purify the obtained extracts from TW20 (Seddon et al., 2004) before using them in developing drugs based on SPs.

Porcine lung fat percentage is much higher than in bovine lungs. These differences affect the quality of extraction and the effectiveness of the detergent, resulting in extraction degree. Ionic strength and pH may also be effective (Ruano et al., 1998). An alkalization can lead to the destruction of lipids (Spilling et al., 2013), which we can observe in protein extraction from the porcine lung. The dimeric form of SP-B was absent, and this fact indicates the breaking hydrophobic bond between the molecules after the detergent addition, and the presence of hydrophobic proteins was confirmed by the mass spectrometry.

Since mass spectrometry analysis involved the use of a complete database of all protein sequences of the studied organisms, commonly we see in the samples major plasma proteins such hemoglobin, albumins, immunoglobulins, etc. Particularly, its presence reduced the quality of the analysis. In future, the purification from the major proteins of blood plasma over 60 kDa may improve the quality of SPs identification in extracts.

5. Conclusion

The use of extraction solutions based on borate buffer and PBS with 0.1 % and 1 % TW20 makes it possible to isolate more proteins from the bovine and porcine lung extracts. However, SP-C and/or SP-D may be absent in some samples if non-effective eluting formula was used. Tris-based extraction buffer with and without TW20 seems to be most effective for isolating all fractions of SPs, which confirmed by the results of mass spectrometry. To sum up, we can state that it is necessary to use tris-buffer as the main solvent for further modification and optimization of the complex SPs extraction.

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