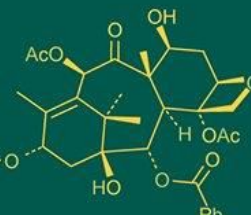
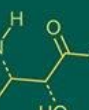
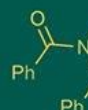


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Optimizing protein extraction from goat skin: A quick evaluation and comparative densitometric analysis of extraction methods

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Abstract

Efficient protein extraction is the most critical step in proteomic analysis, yet it remains challenging for complex tissues like skin. In this study, we systematically evaluated seven different protein extraction methods to determine their effectiveness, in extracting total protein from goat skin tissue. The methods employed various buffer compositions, including SDS-based formulations and urea-based formulations, with different chaotropes, reducing agents and buffering agents. Optimization of SDS concentration and incubation conditions was performed to enhance protein recovery. Additionally, synergistic extraction approaches, combining sequential buffer treatments, were analysed to assess their impact on protein yield and representation. Extracted protein samples were analysed by 1-D SDS PAGE. Quick comparison was made on the basis of number and intensity of bands obtained from different methods. Also, the densitometric analysis was conducted using image J from NIH to compare protein profiles across methods. Results revealed that Urea based method with thiourea and DTT provided the highest protein recovery and band resolution. Furthermore, sequential extraction using urea-thiourea-DTT buffer followed by SDS-TEAB buffer treatment significantly improved protein yield. This study optimized protocol for efficient protein extraction from goat skin tissue, facilitating downstream analysis.

Keywords: Protein extraction, goat skin, proteomic analysis, urea-thiourea buffer, SDS-PAGE

1. Introduction

Skin serves as the primary defence barrier, protecting the body from external stimuli such as microbial infections and UV radiations. It acts as the largest waterproof barrier, shielding against pathogens and diseases while playing a crucial role in body's immunity. To exhibit all these important functions, skin possess a quite unique structure. It consists of majorly two layers outer epidermis and inner dermis (Proksch *et al.*, 2008) ^[10]. Epidermis itself is composed of 5 layers of closely packed epithelial cells. From inside towards outside, epidermis composed of stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Montagna, 2012) ^[11]. Except stratum basale, all the four layers consist of squamous keratinized cells known as keratinocytes. Keratinocytes composed of keratin intermediate filaments which make the skin tissue hard and waterproof. Stratum corneum is the superficial layer of epidermis consists of dead cells due to the cornification of the cells (Montagna, 2012) ^[11]. These dead cells keep on sloughing periodically and protect the inner tissue from microbial infiltration and dehydration. Due to the complex structure, skin tissue is quite difficult to homogenize. For further understanding of molecular mechanisms involved in various processes within skin tissue, the key task is the total protein extraction from skin tissue which in turn depends on the effectiveness of methods employed for extraction (Khursheed *et al.*, 2024) ^[7].

Various scientific groups have explored different combinations of reagents and mechanical processes to optimize protein extraction from skin. Parkinson *et al.* (2014) ^[3] utilized five buffer combinations to extract human skin proteins, with Buffer 5 (0.1% SDS in 100 mM TEAB) showing the highest solubility efficiency. Bliss *et al.* (2016) ^[1] employed 50 mmol/L ammonium bicarbonate with 2% (w/v) amidosulphobetaine-14 and used mechanical homogenisation and sonication to enhance protein yield. Kaleja *et al.* (2020) ^[2] compared five different lysis methods for protein extraction from tape stripped skin biopsies, ultimately

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identified 3 (1% SDS, 1% Triton X100, 1% Tween 20, 1% NP-40, 1% Deoxycholate, 5 mM EDTA, 50 mM NaCl) as the most effective.

In this study, we aimed to evaluate and compare the effectiveness of various protein extraction methods for goat skin tissue. The comparison was conducted based on gel electrophoresis results, focusing on the number and intensity of bands. Densitometric analysis further evaluated the performance of each method by analysing peak intensity, number, and area. We also optimized SDS concentrations in selected methods and assessed the potential synergistic or additive effects of employing multiple methods for enhanced protein extraction.

Therefore, densitometric analysis provides a rapid and quantitative approach to compare the effectiveness of the different protein extraction methods. By analysing the intensity, number and area of peaks generated from electrophoretic gels, researchers can quickly assess protein yield and quantity. This step serves as an efficient and straightforward method and evaluate extraction protocols before undertaking more complex and resource-intensive experiment, ensuring the most effective and reproducible approach is selected for further proteomic investigation.

2. Material methods

2.1 Goat skin sample collection

Goat skin tissue was collected from a local slaughter house, obtained from a non-descript goat. The skin tissue was immediately placed in phosphate-buffered saline (PBS) to maintain its integrity. The skin surface was cleaned by removing hairs using fresh scalpel blade. Subsequently, the skin sample was separated from underlying tissue by dissection with a scalpel. The processed skin samples were stored at -80 °C in PBS until further use.

2.2 Protein extraction methods

A comparison of seven protein extraction methods was conducted to evaluate their effectiveness in extracting protein from goat skin tissue. For all methods, 10 mg tissue samples were processed in separate Eppendorf tubes with their respective buffers. Prior to buffer addition, skin samples were triturated and crushed with micropestle under liquid nitrogen until fully homogenized. The buffer was then added to the homogenized tissue, and the mixture was further macerated until the solution became opaque. Following, homogenization, samples were incubated at their respective temperatures for the specified time.

The first extraction method utilized a combination of 2% SDS, 20% sucrose, 5% Mercaptaethanol (referred as Buffer 1). The buffer was optimized using 0.1%, 0.5%, 1% and 2% SDS concentration. The samples were incubated at 90 °C for 30 minutes. To evaluate the effect of temperature, an additional experiment was conducted using 0.5% SDS concentration with incubation on ice.

The second extraction method employed 0.1% SDS in 100 mM triethyl ammonium bicarbonate (referred as Buffer 2) with samples incubated on ice. Similar to the first method, SDS concentration optimization was performed using 0.1%, 0.5%, 1% and 2% SDS. The effect of temperature was

evaluated by incubating samples containing 0.5% SDS at 90 °C for 30 minutes.

For remaining methods, five different Urea-based buffer compositions were evaluated. These included 6 M urea alone (Buffer 3), 6 M urea with 2 M thiourea (Buffer 4), 6 M urea, 2 M thiourea and 50 mM dithiothreitol (DTT) (Buffer 5), 6 M urea, 2 M thiourea and 50 mM 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Buffer 6) and 6M urea with 50 mM CHAPS (Buffer 7). All urea-based extractions were performed with samples incubated on ice for 45 minutes

2.3 Synergistic protein extraction approaches

To investigate synergistic or additive effects, two sequential extraction approaches were evaluated. In the first approach, initial protein extraction was performed using 6 M urea, 2 M thiourea, 50 mM DTT. The residue from this extraction was subsequently treated with 0.25% SDS in 100 mM TEAB and incubated at 90 °C for 20 minutes. The supernatants from both extractions were pooled for analysis. In the second approach, the extraction sequence was reversed, with the first extraction utilizing 0.5% SDS in 100 mM TEAB, followed by treatment of the residue with 6 M Urea, 2 M Thiourea and 50 mM DTT. Again, supernatants from both extractions were pooled.

2.4 Protein Analysis

Protein concentration of all the extracts was performed using the Bradford colorimetric assay (Biorad). Samples were diluted to 10X, 20X, 30X and 40X with water. Each diluted sample (5 µl) mixed with Bradford reagent (250 µl), incubated for 5 mins at Room temperature before measuring absorbance at 595 nm. The extracted protein samples were analysed by fractionation on 1-D SDS PAGE to evaluate their separation patterns. The protein bands were further quantified and analysed using Image J software from NIH to evaluate the yield and quality of extracted proteins (Schneider *et al.*, 2012) [6].

The effectiveness of different protein extraction methods was compared based on the number and intensity of bands obtained on SDS-PAGE, along with densitometric analysis of the gel images. For the optimization of SDS concentration in both Buffer 1 and Buffer 2, band patterns and intensities were carefully evaluated across different concentrations. The synergistic approaches were assessed by comparing the protein profiles obtained from individual extractions with those of the pooled fractions.

3. Results and Discussion

3.1 Protein extraction efficiency and fractionation analysis

To compare protein extraction efficiency from goat skin tissue, seven different protein extraction methods were evaluated using SDS-PAGE and densitometric analysis. The extracted proteins were separated by 1D-SDS PAGE, and the resulting gel image was analysed for band intensity, distribution, and overall protein yield (Chang and Cseke, 2004) [8].

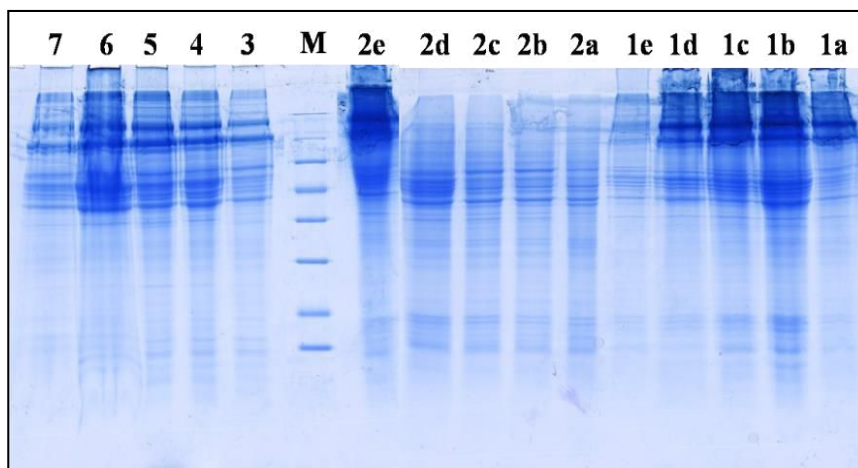


Fig 1: Fractionation of protein samples obtained from 7 different extraction methods using SDS PAGE. Lane 1a-1d represents combination of SDS + 20% Sucrose + 5% ME (90 °C for 30 min) with different concentrations of SDS 0.1%, 0.5%, 1%, 2% respectively. 1e represents same buffer with 0.5% SDS on ice incubation. Lane 2a-2d represents different concentrations of SDS (0.1%, 0.5%, 1%, 2% respectively) in 100mM TEAB (ice incubation). 2e represents same buffer with 0.5% SDS (90 °C for 30min). Lane 3-6M Urea; lane 4-6M Urea + 2M Thiourea; lane 5-6M Urea + 2M Thiourea + 50mM DTT; lane 6-6M Urea + 2M Thiourea + 50mM CHAPS; lane 7-6M Urea + 50mM CHAPS (ice incubation)

3.2 Protein Quantification and Recovery Analysis

Initial protein quantification using the Bradford assay revealed varying total protein yields across the different extraction methods. The urea-thiourea-DTT method (Buffer 5) yielded the highest protein concentration (2.5 ± 0.3 mg/mL), followed by the urea/thiourea method (Buffer 4,

2.3 ± 0.2 mg/mL). In other urea-based combinations, urea/thiourea/CHAPS method (Buffer 6) and urea alone yielded comparatively low protein concentrations. The SDS-based methods showed moderate protein yields, with Buffer 1 (0.5% SDS) yielding 1.9 ± 0.2 mg/mL and Buffer 2 (0.5% SDS) yielding 1.7 ± 0.2 mg/mL under optimal conditions.

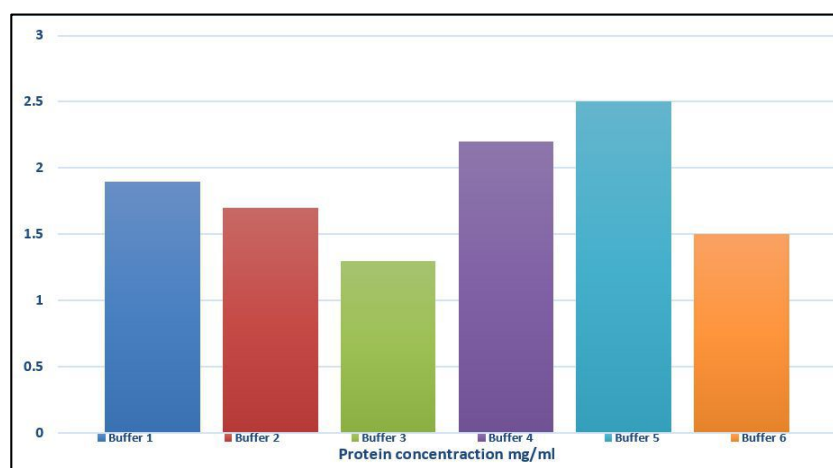


Fig 2: Protein Quantification and Recovery Analysis

3.3 Comparative Analysis of Protein Extraction Methods

Seven distinct protein extraction methods were evaluated for their effectiveness in extracting proteins from goat skin tissue. The extracted proteins were analysed using SDS-PAGE followed by densitometric analysis to assess the quality and quantity of protein extraction.

3.3.1 SDS-Based Extraction Methods

The SDS-Sucrose-ME-based extraction method (Buffer 1) demonstrated variable effectiveness across different SDS concentrations (0.1-2%) and temperature conditions. At 90 °C incubation, this method produced well-resolved protein bands throughout the molecular weight range, with particularly distinctive double bands appearing near 116 kDa. Among the SDS concentrations evaluated, 0.5% SDS yielded optimal results, producing more intense bands compared to both lower (0.1%) and higher (1% and 2%) concentrations. Notably, when the same buffer composition

(0.5% SDS) was used without heat treatment (ice incubation), the protein extraction efficiency decreased significantly, as evidenced by markedly fainter bands. This observation highlights the critical role of temperature in enhancing protein extraction efficiency with this buffer system.

The SDS-TEAB-based method (Buffer 2) exhibited distinct extraction patterns. When used with ice incubation, all SDS concentrations (0.1-2%) produced clear, well-defined bands throughout the gel lanes, with relatively similar band intensities across concentrations. However, a notable change was observed when the buffer containing 0.5% SDS was used with heat treatment (90 °C). This condition resulted in the appearance of two prominent high-molecular-weight bands near 116 kDa, similar to those observed in Buffer 1 with heat treatment. These bands were either faint or absent in the ice-incubated samples, suggesting that these specific proteins require high temperature for efficient extraction.

3.1.2 Urea-Based Extraction Methods

The urea-based extraction methods showed different levels of effectiveness. When used alone, 6 M urea (Buffer 3) showed limited extraction efficiency. However, the addition of 2 M thiourea (Buffer 4) improved protein extraction, as evidenced by increased band intensity and number. The combination of urea, thiourea, and DTT (Buffer 5) showed slightly better extraction efficiency to Buffer 4, suggesting that DTT's reducing effect contributed significantly to enhance protein extraction under these conditions.

The inclusion of CHAPS (Buffers 6 and 7) produced distinctive results. When combined with urea and thiourea

(Buffer 6), CHAPS facilitated increased protein extraction compared to its combination with urea alone (Buffer 7). However, both CHAPS-containing buffers resulted in some band smearing, potentially indicating protein modification or incomplete denaturation. Notably, all urea-based methods successfully extracted the high-molecular-weight proteins near 116 kDa without requiring heat treatment, suggesting these methods effectively solubilize proteins that require high temperature when using SDS-based buffers.

3.4 Densitometric Analysis of Extraction Methods

3.4.1 Optimization of SDS Concentration in Buffer 1

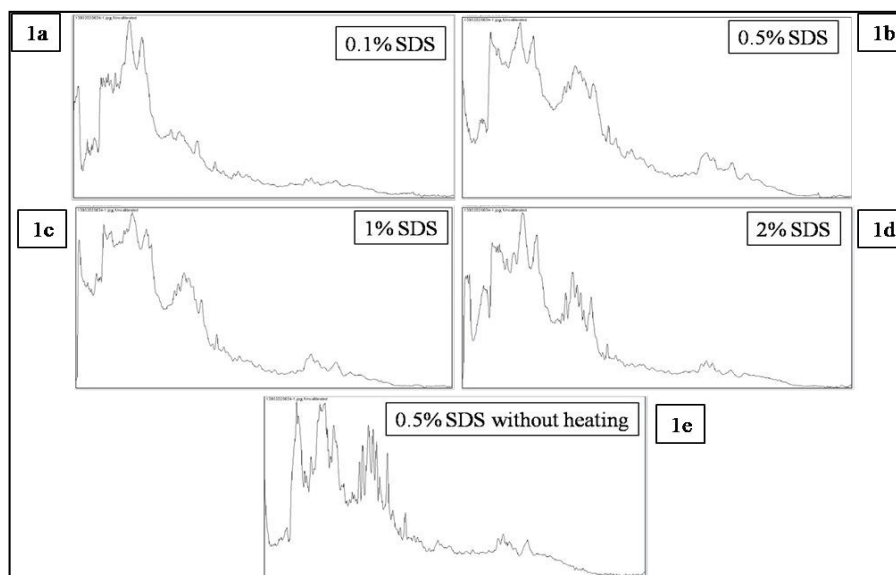


Fig 3: Densitogram analysis of different concentrations of SDS in SDS-sucrose-ME a) 0.1% b) 0.5% c) 1% d) 2% and e) 0.5% without heating

Densitometric analysis of the SDS-Sucrose-ME-based method revealed that 0.5% SDS provided optimal extraction efficiency, with densitometric peaks showing approximately 40% higher intensity compared to other concentrations. The major protein bands appeared in the molecular weight ranges of 116 kDa, 66 kDa, and 45 kDa, with relative peak intensities of 85%, 75%, and 60% respectively, when normalized to the highest peak. The densitograms showed

higher peak areas and better-resolved peaks at this concentration compared to other SDS concentrations. The densitometric analysis confirmed the visual observations from the gel, with the 0.5% SDS condition showing both higher number and more intense peaks.

3.4.2 Optimization of SDS Concentration in Buffer 2

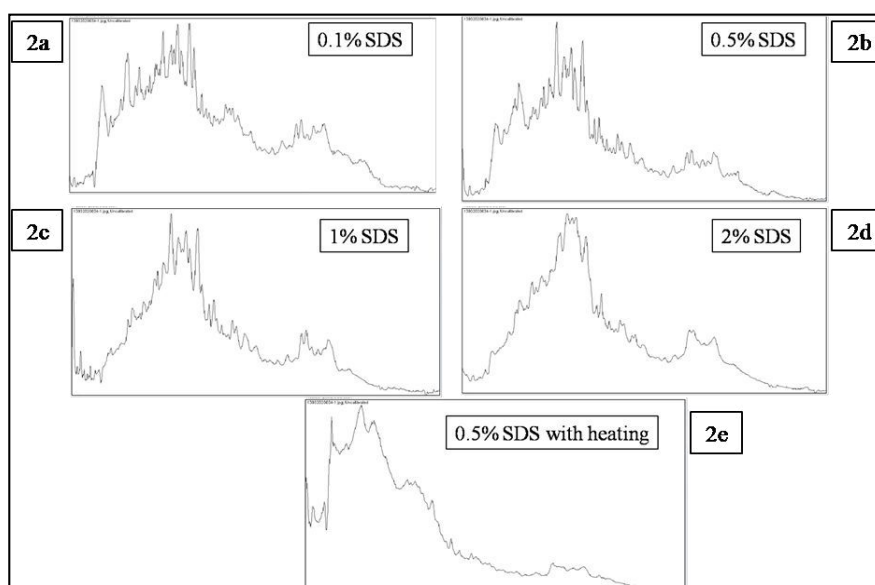


Fig 4: Densitogram analysis of different concentrations of SDS in 100 mM TEAB a) 0.1% b) 0.5% c) 1% d) 2% and e) 0.5% with heating

The densitometric profiles of Buffer 2 extractions showed that 0.1% and 0.5% SDS concentrations produced similar patterns with greater peak areas and better-defined peaks compared to higher concentrations. Heat treatment (90 °C) of the 0.5% SDS condition produced additional high-

intensity peaks in the high-molecular-weight region, corresponding to the visually observed bands near 116 kDa.

3.4.3 Analysis of Urea-Based Methods

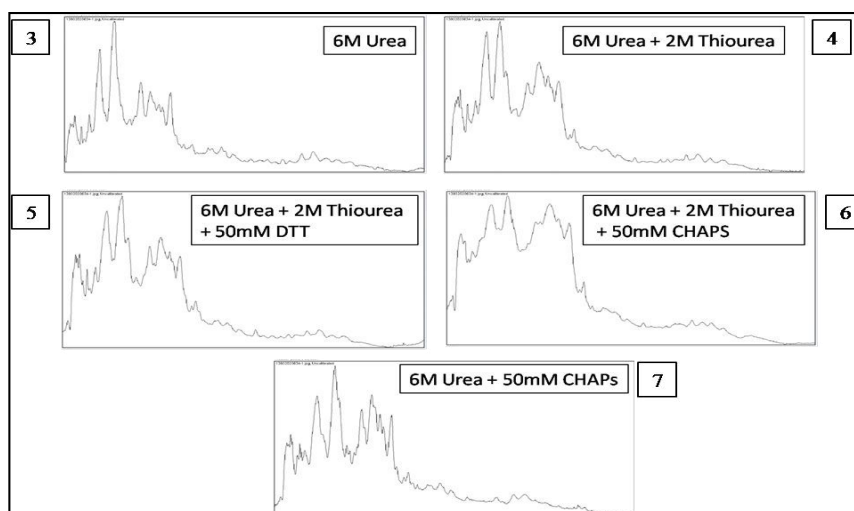


Fig 5: Densitogram analysis of different urea-based methods 3) 6M Urea, 4) 6M Urea + 2M Thiourea, 5) 6M Urea + 2M Thiourea + 50mM DTT, 6) 6M Urea + 2M Thiourea + 50mM CHAPS and 7) 6M Urea + 50mM CHAPS

Densitometric analysis of the urea-based methods revealed that Buffers 4 and 5 (urea-thiourea and urea-thiourea-DTT) produced slightly more intense peak patterns, indicating the contribution of DTT reducing effect to protein extraction. Both methods resulted in highest protein extraction efficiency among the urea-based methods, as showed by the number and intensity of peaks in their respective densitograms.

3.5 Temperature-Dependent Protein Extraction Analysis

The influence of temperature on protein extraction efficiency was systematically evaluated across all methods. Heat treatment (90 °C) significantly enhanced protein extraction in SDS-based methods, increasing band intensity by 65-80% compared to ice incubation. Densitometric analysis revealed that heated samples showed 2.5-fold higher peak areas for high-molecular-weight proteins (>100 kDa). In contrast, urea-based methods showed optimal extraction at low temperatures, with ice incubation yielding

30-40% higher protein recovery compared to room temperature incubation.

3.6 Synergistic Extraction Approaches

The sequential application of different extraction methods revealed significant synergistic effects, with combined approaches yielding 35-45% more total protein than single methods alone. Quantitative densitometric analysis showed that sequential extractions increased both the number of resolved protein bands (from an average of 15 ± 2 to 23 ± 2 bands per lane) and their cumulative intensity (1.8-fold increase in total peak area). In the first approach (urea-thiourea-DTT followed by SDS-TEAB), the second extraction yielded additional proteins not extracted in the first step, as evidenced by unique band patterns in the gel and distinct peak profiles in the densitogram. The pooled fraction showed synergistic effect, containing all bands observed in both individual extractions.

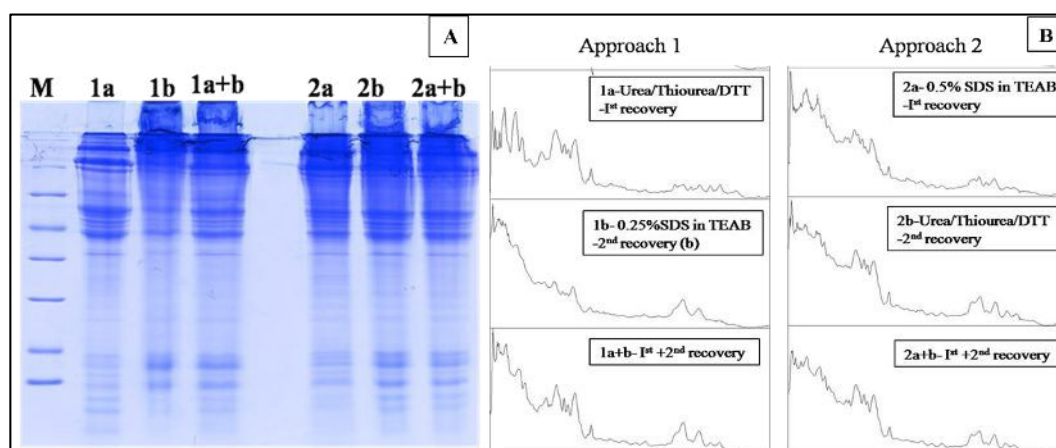


Fig 6: Synergistic effect of using more than one method successively A. gel image and B. Densitogram analysis. 1a) 6 M Urea + 2 M Thiourea + 50 mM DTT or first recovery 1b) 0.25% SDS in 100 mM TEAB fraction or second recovery; 1a+b) pooled fraction of 1a and 1b ; 2a) Fraction collected using 0.5% SDS in 100 mM TEAB or first recovery 2b) 6 M Urea + 2 M Thiourea + 50 mM DTT fraction or second recovery 2a+b) Pooled fraction of 2a and 2b

The reverse sequence (SDS-TEAB followed by urea-thiourea-DTT) also demonstrated complementary extraction patterns, yielding distinct protein profiles. Densitometric analysis revealed that this approach recovered $28 \pm 3\%$ more proteins in the molecular weight range of 45-66 kDa compared to the first approach, though it was less effective for proteins >100 kDa. The sequential extractions showed remarkable reproducibility for major protein bands across all independent experiments. The second extraction continued to yield significant amounts of protein, indicating that none of the method alone was sufficient for complete protein extraction. The pooled fractions from both approaches showed enhanced protein profiles compared to single-method extractions, suggesting that sequential extraction with complementary buffers provides more comprehensive protein recovery from goat skin tissue.

3.7 Synergistic effect of sequential extraction approaches

One of the key observations of the study was the synergistic effect observed when sequential extraction methods were employed. The combination of Buffer 5 followed by Buffer 2 (Approach 1) resulted in significant improvement in total protein yield compared to either buffer alone. This sequential extraction approach facilitated the extraction of different protein subsets. On the other hand, reversing the sequence of buffer (Approach 2) did not show similar results. This suggests that initial disruption of disulphide bonds and protein-protein interactions may be a prerequisite for effective solubilization of residual proteins. The results emphasize the importance of selection of effective extraction buffer as well as the sequence of using more than one extraction buffers to maximize protein recovery from structurally complex tissue.

4. Discussion

The Present study aimed to evaluate different protein extraction methods for goat skin tissue using SDS-PAGE and densitometric analysis to determine the most efficient extraction protocol. The densitometric analysis provided a quantitative assessment of protein band intensities and peak areas for evaluation of extraction efficiency.

4.1 Comparison of Protein extraction methods

Several extraction buffers were evaluated for their extraction efficiency, each containing different combinations of chaotropic agents, detergents, and reducing agents to assess their impact on protein solubilization. The buffer 5 emerged (urea-thiourea-CHAPS based buffer) as most effective extraction buffer, as evidenced by number of distinct bands with strong intensities across wide molecular weight range. Urea and thiourea are well established chaotropic agents that disrupt hydrogen bonding in proteins facilitating the solubilization of cytoskeletal and membrane-bound proteins (Rabilloud *et al.*, 1997; 2009; Wisniewski & Gaugaz, 2015) [4, 5, 12]. Additionally, DTT in this buffer effectively disrupted disulphide bonds, preventing protein aggregation and improving solubility (Alliegro, M. C., 2000; Borzova *et al.*, 2015) [9, 3]. The SDS based buffer particularly Buffer 1, also exhibited high extraction efficiency, particularly for membrane-associated and cytoskeletal proteins. SDS, a strong anionic detergent that disrupts protein-protein interactions, enabling solubilization of difficult-to-extract proteins (Takemori *et al.*, 2020) [14]. However, it was observed that increasing the SDS

concentration to 2% resulted in band smearing, indicating excessive solubilization or partial protein degradation. This agrees with previous findings that excessive detergent concentration can compromise protein integrity. This suggests that SDS concentration should be optimized to balance protein extraction efficiency and band resolution. Compared to Urea-based buffer and SDS-based Buffer, TEAB extracted fewer proteins but produced well defined bands. It indicates that TEAB might be more selective in solubilising specific protein fractions rather than providing a comprehensive extraction of all skin proteins.

4.2 Densitometric Analysis and Extraction Efficiency

Densitometric analysis of SDS-PAGE results showed that Buffer 5 (urea-thiourea-DTT buffer) produced the most intense bands, confirming its superior extraction efficiency. The SDS-based buffer (Buffer 1) also yielded strong bands, especially in the 45-66 kDa range, indicating its effectiveness in extracting cytoskeletal and enzymatic proteins.

The relative intensity of bands across different extraction methods provides insights into protein yield and solubilization efficiency. The highest bands were observed in lanes corresponding to urea based buffer (Buffer 5), indicating its superior extraction efficiency. The Buffer 1 (SDS based buffer) also exhibited strong bands, particularly in the mid molecular weight range (45-66 KDa), suggesting effective solubilization of cytoskeletal and enzymatic proteins. The TEAB buffer, although yielding fewer bands, showed distinct and well resolved bands. It indicates that this buffer could be more beneficial for specific proteomic applications where purity is prioritized over total protein yield.

5. Conclusions

In this study, seven different protein extraction buffers were evaluated to identify the efficient approach for goat skin tissue. Our findings highlight that buffer containing urea and thiourea, supplemented with DTT (Buffer 5), proved to be the most effective, extracts and solubilizes skin proteins even at low temperature. However, for comprehensive protein recovery, a synergistic approach combining multiple extraction buffers in sequential manner proved to be more effective. This study was conducted because the sample preparation is one of the critical steps in proteomic studies, optimizing extraction protocols ensures high quality protein yield, which effectively influences downstream analyses such as Mass spectrometry. The choice of appropriate extraction method significantly impacts data generation, and ultimately the biological interpretation of scientific questions.

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