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RESEARCH ARTICLE

Residual protein analysis by SDS–PAGE in clinically manufactured BM-MSC products

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Abstract

Residual substances that are considered hazardous to the recipient must be removed from final cellular therapeutic products manufactured for clinical purposes. In doing so, quality rules determined by competent authorities (CAs) for the clinical use of tissue- and cell-based products can be met. In our study, we carried out residual substance analyses, and purity determination studies of trypsin and trypsin inhibitor in clinically manufactured bone marrow-derived mesenchymal stromal/stem cell products, using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) method. Despite being a semiquantitative method, SDS–PAGE has several benefits over other methods for protein analysis, such as simplicity, convenience of use, and affordability. Due to its convenience and adaptability, SDS–PAGE is still a commonly used method in many laboratories, despite its limits in dynamic range and quantitative precision. Our goal in this work was to show that SDS–PAGE may be used effectively for protein measurement, especially where practicality and affordability are the major factors. The results of our study suggest a validated method to guide tissue and cell manufacturing sites for making use of an agreeable, accessible, and cost-effective method for residual substance analyses in clinically manufactured cellular therapies.

KEYWORDS
advanced therapy medicinal products, bone marrow-derived mesenchymal stromal/stem cells (BM-MSCs), mesenchymal stromal/stem cells (MSCs), residual protein analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Abbreviations: TI, trypsin inhibitor; TRYP, trypsin; W0, supernatants; W1, first step wash sample; W2, second step wash sample; W3, third step wash sample.

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1 | INTRODUCTION

All medicinal products manufactured at Ankara University Stem Cell Institute Tissue and Cell Manufacturing Center—hereon forward referred to as the Manufacturing Center—are human cellular therapy medicinal products manufactured for clinical use within the scope of the manufacturing license no. 2014/02 granted to the Manufacturing Center by the Turkish Ministry of Health Turkish Medicines and Medical Devices Agency (TİTCK) on 19 December 2014. In the manufacturing steps of cellular therapy medicinal products, there may be a few intermediary substances such as, but not limited to, enzymes, growth supplements, stimulating factors, and antibiotic cocktails to induce safety, growth promotion, and further proliferation stimulation to obtain the required number of cells.

In the scope of the manufacturing processes of bone marrow-derived mesenchymal stromal/stem cell (BM-MSC) products at the Manufacturing Center, the most critical intermediary agents are the enzymes and enzyme inhibitors used while passaging cells in a culture dish. In the manufacturing process of most adherent cellular therapeutic products, nonhuman-derived enzymes or different methods are used during the passaging step [1]. Passaging is mainly done for the propagation process to produce a larger cell density from preexisting cells [2]. While passaging, trypsinization is required to dissociate cells from the bottom of the cell culture dish in which they are proliferating. This is traditionally done by a proteolytic enzyme, a serine protease, trypsin (TRYP), which breaks down proteins to be able to dissociate adherent cells [2, 3]. The activity of TRYP is then chemically inactivated using a commercially available trypsin inhibitor (TI), a serine protease-inhibiting protein.

Any reagent used in the manufacturing process that is not intended to be a part of the final product must be documented in the investigational new drug application and accounted for in the final product, following the current good manufacturing practices (cGMP). This is because the activity of proteolytic enzymes can lead to cell lysis, which is a significant parameter for characterizing bioprocesses and impacts both upstream and downstream performance [4]. Therefore, the quantification of residual process components or impurities holds immense importance [5, 6]. The presence of residual enzymes in therapeutic products is a critical aspect of ensuring their safety, and it is necessary to eliminate these enzymes from cell therapy products during the final stages of manufacturing [7]. Contaminant residues found in the manufacturing process of the finished product are referred to as impurities. Contaminants could be leftover solvents, antibiotics, or animal products that were added to tissue culture media, however, not meant to be in the finished product. These could cause allergic reactions or other unfavorable outcomes. Competent authorities (CAs) either look for proof that such contaminants were removed from the finished product, or that the finished product was tested to make sure that the residual levels are within permitted bounds. In the case of clinically manufactured human medicinal products, risky situations or complications that may threaten human health should be prevented, and at the same time, the quality of the final product should not be compromised. Therefore, during the culturing phases of clinically manufactured BM-MSC products, TRYP and TI should be reduced, and the final products obtained should be free of undesirable residual substances, however, with the desired efficacy.

Characteristics specific to the amino acid composition of each protein (i.e., size, net charge, isoelectric point, and heat stability) can be used in the design of the purification strategy [8–14]. Proteins can be purified of foreign protein content in a few ways where wash step with basic buffers such as the phosphate-buffered saline (PBS) [15] is one of the most easily accessible among all. General methods for isolation, protein sample purity assessment, protein expression evaluation, immunochemical protein identification and quantification, and protein purification are used to separate a few proteins—if not one only protein—from a complex biological structure [16–19], such as cell culture supernatants as in the case of our study. Methods of assessing protein purity can go from general quantification methods such as the Bradford assay to analytical and size analysis methods such as high-performance liquid chromatography and size exclusion chromatography, respectively, to as far as native/denaturing electrophoresis methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) chromatography, first described by Laemmli [20] and which is the most preferred identification and quantification method for proteins, is a separation technique based on the difference in the molecular weight (MW) of proteins and other macromolecules [16, 18, 19]. This technique is used to isolate one or more components, determine the MW of a compound, and remove nonprotein contaminants and protein aggregates.

In SDS–PAGE, proteins are initially denatured with the anionic detergent, SDS, and then migrate in response to an electric field through pores in a polyacrylamide gel matrix. The system consists of a double gel layer, a separation gel in which the proteins separate because of their MW, and a stacking gel in which the proteins are concentrated before the protein mixture enters the gel. The compositions and pH of the stacking gel, the separating gel, and the electrophoresis buffer produce a system that can precisely separate proteins relative to their specific MW. The

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combination of pore size and the charge, size, and shape of the protein determines the rate of migration [18, 20]. SDS–PAGE is used to identify molecules in terms of MW and to identify and quantify impurities. Determination of MW by SDS–PAGE is a reliable, cost-effective, and relatively easy method [21] that can precisely separate proteins and, therefore, is widely preferred.

The main aim of our study was to use the SDS–PAGE method as a simple and cost-effective technique to determine the TRYP and TI content as residual proteins in BM-MSC culture supernatants. In doing so, we optimized the SDS–PAGE method for general applications of protein sample purity assessment and immunochemical protein identification and quantification and established the risk/benefit balance of TRYP and TI in the step-by-step PBS-washed supernatants obtained from BM-MSC cultures. We then performed residual substance analyses and purity determinations, and by the interpretation of the study outputs, we determined to what scale TRYP and TI should be removed from the clinically produced BM-MSC products at the Manufacturing Center. Though a few studies address analytical methods for quantification exclusive to TRYP [22–25], none such studies have used the conventional, easily accessible, and applicable cost-effective SDS–PAGE method. To date, literature has not yet included an SDS–PAGE method validation for TRYP/TI quantification in clinical BM-MSC products. Accordingly, the novelty of our study is that we have performed a comprehensive set of experiments using a common method to identify and quantify residual substances in clinical BM-MSC products to local and international regulatory quality standards for advanced therapy medicinal products.

2 | MATERIALS AND METHODS

The manufacture of BM-MSC products for clinical use was performed from donor tissues at the Manufacturing Center, with the approval of the CA, with approval number 2014/2 for the manufacture of human medicinal products.

2.1 | Donor consent

Study samples were collected and processed from one donor tissue. The research was conducted ethically by the World Medical Association Declaration of Helsinki. Written informed consent was collected from the donor. The donor had agreed to extend the use of their tissue samples for further experiments.

2.2 | Sample collection and storage

Supernatants (W0) from cells centrifuged by adding soybean-derived TI after incubation with recombinant TRYP used at the cell dissociation stage, the first step wash sample (W1) obtained from cells resuspended in PBS after trypsinization, and the second step wash sample (W2) obtained from resuspending the pellet already obtained in step W1 with PBS were collected.

Wash samples (i.e., W0, W1, and W2) were collected from the finished products, aliquoted, and stored at −20°C, according to the length of the storage period until the execution of the study.

2.3 | SDS–PAGE method

Optimization of the SDS–PAGE method and protein identification and quantification processes were performed in aiming to understand to what extent the residual substances were removed with PBS at each wash step [15]. The study was executed mainly based on Laemmli's SDS–PAGE protocol [20]. In our study, 8%–16% polyacrylamide gradient gels were used. Separate solutions of 8% and 16% gels were prepared according to the formulation found in Table S1. Subsequently, 2.3 mL of each gel solution was drawn into a serological pipette, and finally, an air bubble was drawn into the pipette to create a gradient within the serological pipette. Then, the gel mixture was poured into the gel casting apparatus. Following the polymerization of separating gel, stacking gel was prepared as specified in Table S2. During electrophoresis, SDS–PAGE running buffer (1×) and Laemmli sample buffer (3×) were used, prepared according to Tables S3 and S4, respectively. Color prestained protein standard (10–250 kDa; broad range) (New England Biolabs) was used as an MW marker.

Throughout the study, the reference standards (bovine serum albumin [BSA], TRYP, and TI) and wash samples were prepared with the Laemmli SDS sample buffer and boiled in a 90°C water bath for 15 min to reduce the protein contents.

Electrophoresis experiments were conducted following the procedure outlined by Jan et al. [26]. Following the electrophoresis, the stacking gel was excised and disposed of, whereas the separating gel segment underwent incubation in fixation solution for 30 min and then stained with colloidal Coomassie dye. Excess dye was removed by shaking the gels in a destaining solution overnight. The fixative solution, the colloidal Coomassie blue staining solution, and the destaining solution were prepared as given in Tables S5–S7, respectively. The VersaDoc imaging system was used to visualize the gels.

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Optimization was carried out at the first stage of the study. Different amounts of protein standards BSA, TRYP, and TI were applied to the SDS–PAGE gel to establish the most effective sample loading quantity for detection (data not shown). In the second step, the optimal quantities of BSA, TRYP, and TI, as determined during the optimization phase, were applied to a single SDS-PAGE gel, and the protein segments of these standards were identified. Subsequently, culture supernatant samples and protein standards were analyzed in the same gel, and SDS–PAGE gel images were recorded.

2.4 | Data analysis

Gel images were analyzed through the GelAnalyzer 2010a software (Lazar Software, Debrecen, Hungary) to identify and quantify the remaining TRYP and TI protein segments in the culture supernatant samples.

In the analysis of each gel, the lanes were marked, and the background noise signal was removed by the "rolling ball radius" method. The area under the curve (AUC) was estimated after eliminating the background signal for the lane profiles from each region. The AUC values obtained for the quantification standards were used to construct the linear regression curve for known protein amounts. This curve equation was then used to calculate protein concentrations for the regions defined above in other lane profiles.

3 | RESULTS

To determine the protein amounts required to visualize reference proteins using the SDS–PAGE method, varying concentrations of BSA, TRYP, and TI samples were included in the gel, and the ideal range for each was defined (data not presented).

Following the optimization stage, protein standards BSA, TRYP, and TI were all introduced into a single gel, and the protein bands for each standard were successfully identified (Figure 1).

Utilizing the GelAnalyzer2010a software, quantitative analyses of SDS–PAGE gels, which contained both the wash samples and protein standards, were carried out, as shown in Figures 2 and 3.

After subtracting the background signal for the lane profiles taken from each region (Figures 2B and 3B), the AUC was calculated (Figures 2C and 3C). AUCs from the quantitation standards were utilized to construct a linear regression curve for the known protein quantities of BSA standards. Calibration curves for both gels were constructed using profile data for BSA standards at 10, 17, and 23.5 ng, with the exclusion of the data for 30 ng BSA.

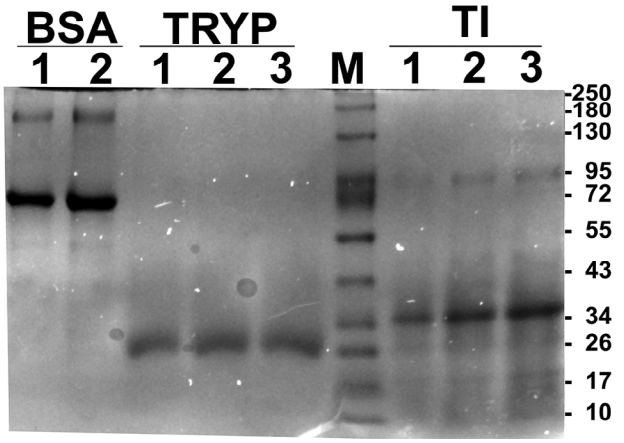


FIGURE 1 Visualization of standard proteins bovine serum albumin (BSA), trypsin (TRYP), and trypsin inhibitor (TI) at varying concentrations on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Lanes represent BSA 1 (20 ng BSA), BSA 2 (30 ng BSA), TRYP 1 (3% TRYP), TRYP 2 (3.5% TRYP), TRYP 3 (4% TRYP), M (protein molecular weight marker), TI 1 (8.35 μg TI), TI 2 (13.35 μg TI), and TI 3 (20 μg TI), respectively.

Examining the bands of TRYP and TI protein standards in Figure 2A,B provided the identification of protein bands in the wash samples. Consequently, it was determined that bands within the 24–25, 34–40, and 30–34 kDa ranges corresponded to the remaining segments of TRYP, TI 1, and TI 2, respectively (Figures 2A and 3A).

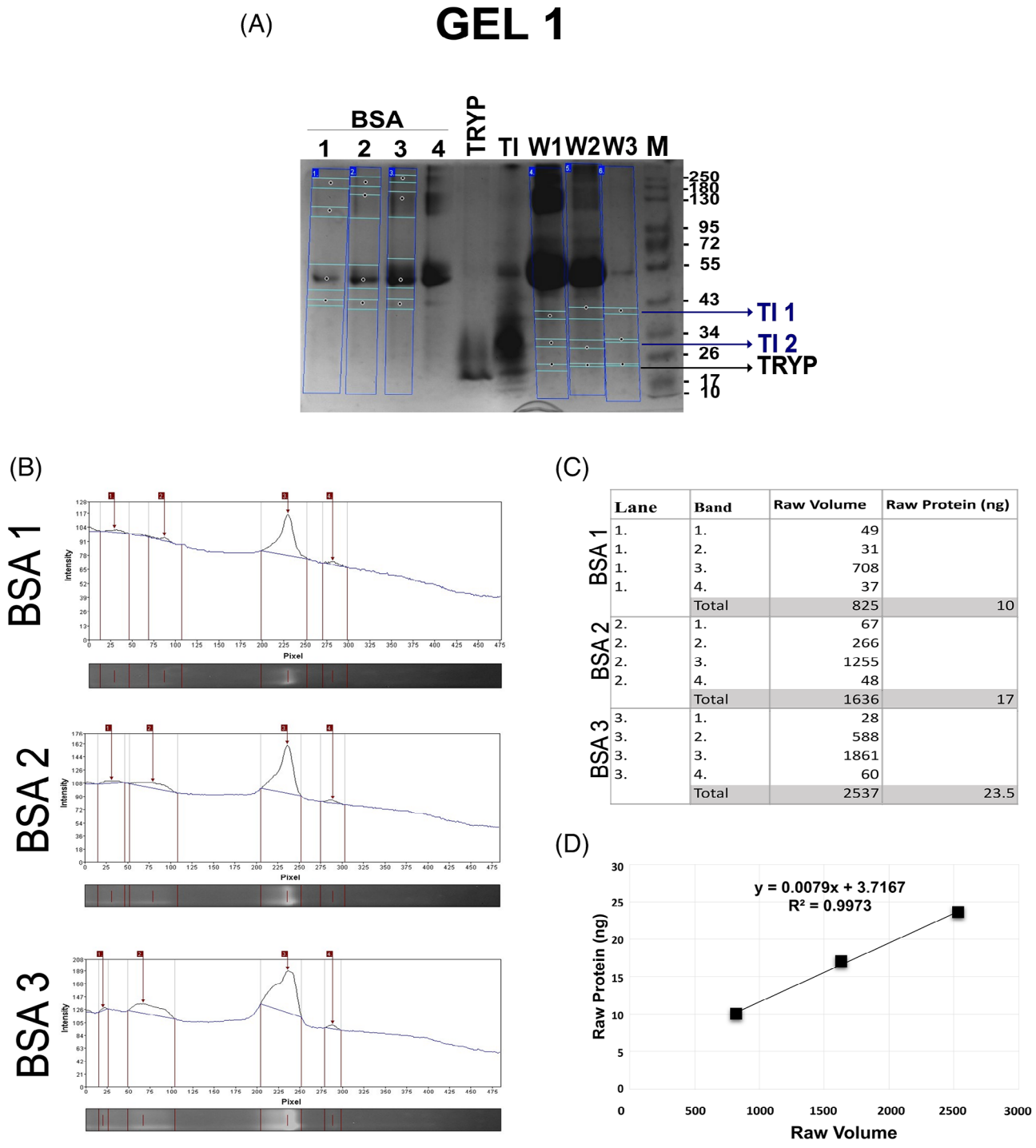
Protein concentrations for TRYP, TI 1, and TI 2 segments calculated based on the calibration curves of both gels are presented in Table 1.

Various concentrations of a quantitation standard were loaded and analyzed in different lanes to ensure result accuracy. The study suggested that the total free protein concentration was consistent in W0 and W1, however, significantly divergent in W2. Going on to W3, an increase in total protein indicated excessive intervention, likely from lysed cells. This would substantially affect the quality of BM-MSC products for clinical use, leading to the recommendation to avoid using W3 in removal procedures at the Manufacturing Center. Consequently, the decision was made to optimally implement W2 procedures before releasing the BM-MSC products to the clinic.

4 | DISCUSSION

Cellular therapeutics are pioneers of what is referred to today as "living medicinal products" as they contain cells, tissues, and/or genetic constituents as active ingredients. In the broadest sense, such medicinal products can be

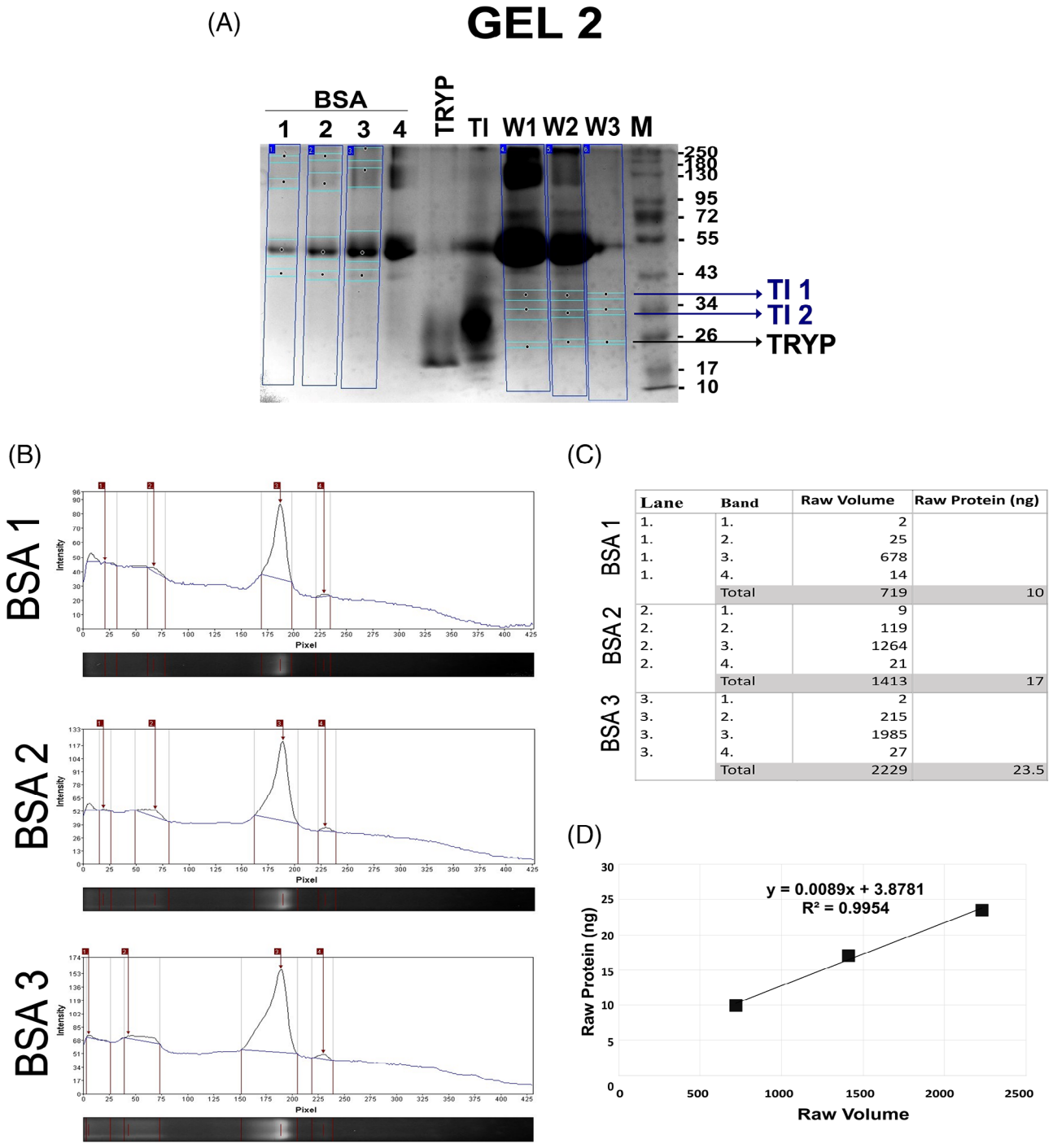
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	Lane	Band	Raw Volume	Raw Protein (ng)
BSA 1	1.	1.	49	
	1.	2.	31	
	1.	3.	708	
	1.	4.	37	
		Total	825	10
BSA 2	2.	1.	67	
	2.	2.	266	
	2.	3.	1255	
	2.	4.	48	
		Total	1636	17
BSA 3	3.	1.	28	
	3.	2.	588	
	3.	3.	1861	
	3.	4.	60	
		Total	2537	23.5

FIGURE 2 1D gel lane profile analysis for Gel 1: (A) marking lanes and segments in each lane on Gel 1. The lanes in the gel represent bovine serum albumin (BSA) 1 (10 ng BSA), BSA 2 (17 ng BSA), BSA 3 (23.5 ng BSA), BSA 4 (40 ng BSA) standards, TRYP (4% trypsin), TI (20 μg trypsin inhibitor), W1 (first step wash sample), W2 (second step wash sample), W3 (third step wash sample), and M (protein molecular weight marker), respectively; (B) profiles of BSA 1 (10 ng BSA), BSA 2 (17 ng BSA), and BSA 3 (23.5 ng BSA) standards segments; (C) raw volume and raw protein values of segments of BSA 1, BSA 2, and BSA 3 protein standards; (D) creation of quantitation curves from lane profiles of quantitation standards.

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	Lane	Band	Raw Volume	Raw Protein (ng)
BSA 1	1.	1.	2	
	1.	2.	25	
	1.	3.	678	
	1.	4.	14	
		Total	719	10
BSA 2	2.	1.	9	
	2.	2.	119	
	2.	3.	1264	
	2.	4.	21	
		Total	1413	17
BSA 3	3.	1.	2	
	3.	2.	215	
	3.	3.	1985	
	3.	4.	27	
		Total	2229	23.5

FIGURE 3 1D gel lane profile analysis for Gel 2: (A) marking lanes and segments in each lane on Gel 1. The lanes in the gel represent bovine serum albumin (BSA) 1 (10 ng BSA), BSA 2 (17 ng BSA), BSA 3 (23.5 ng BSA), BSA 4 (40 ng BSA) standards, TRYP (4% trypsin), TI (20 μg trypsin inhibitor), W1 (first step wash sample), W2 (second step wash sample), W3 (third step wash sample), M (protein molecular weight marker), respectively; (B) profiles of BSA 1 (10 ng BSA), BSA 2 (17 ng BSA), and BSA 3 (23.5 ng BSA) standards segments; (C) raw volume and raw protein values of segments of BSA 1, BSA 2, and BSA 3 protein standards; (D) creation of quantitation curves from lane profiles of quantitation standards.

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TABLE 1 Protein concentrations of trypsin (TRYP), TI 1, and TI 2 segments were calculated based on the quantitation (W1: Wash 1, W2: Wash 2, W3: Wash 3).

GEL 1				GEL 2			
Lane	**Band**	**Raw volume**	**Raw protein (ng)**	**Lane**	**Band**	**Raw volume**	**Raw protein (ng)**
W1	TI 1	39	6.7977	**W1**	TI 1	40	4.2341
W1	TI 2	10	4.5067	**W1**	TI 2	2	3.8959
W1	TRYP	4	4.0327	**W1**	TRYP	4	3.9137
W2	TI 1	6	4.1907	**W2**	TI 1	25	4.1006
W2	TI 2	4	4.0327	**W2**	TI 2	7	3.9404
W2	TRYP	4	4.0327	**W2**	TRYP	4	3.9137
W3	TI 1	15	4.9017	**W3**	TI 1	3	3.9048
W3	TI 2	1	3.7957	**W3**	TI 2	2	3.8959
W3	TRYP	2	3.8747	**W3**	TRYP	1	3.887

defined as pharmaceutical formulations containing biological, synthetic, or semisynthetic active ingredients that can treat or prevent diseases by posing physiological activity on the organism via their pharmacologic, metabolic, or immunologic effects to renew, repair, correcting, replacing, or altering physiological functions such as damage to tissues or cells. Biological activity per unit is a common way to represent the relative purity of a biological product, and it varies greatly depending on the technique. As a result, a variety of analytical techniques are used to thoroughly assess product purity. The operator must investigate any residual substances and/or contaminants in addition to checking the purity of the intended ingredient associated with the product. These impurities may be well-known chemicals, compounds with incomplete characterization, or unexplained substances, and they may result from the manufacturing process or the product itself. Wherever feasible, impurities that can be produced in sufficient quantities should be thoroughly identified, and their biological activities should be assessed. Cell substrates, such as host cell proteins or DNA, cell culture components such as inducers, antibiotics, medium components, or downstream processing procedures can all contribute contaminants to the process. These impurities might appear at different phases of the manufacturing process [6]. There has been considerable interest in developing a safe manufacturing process for clinical use that involves the utilization of stem cells in diverse cell therapy applications. During the manufacturing process, determining the absolute and relative purity of a biological product presents substantial analytical challenges and depends on both the technique used and the findings attained. In cellular therapies, cells isolated from donors (allogeneic) or patients (autologous) are cultured in plastic cell culture vessels for use with various strategies. Enzymes used to passage the cells during culture are inhibited, and the cells are subsequently removed through centrifugation or filtration [20].

In cell culture, recombinant TRYP is used to digest proteins at the point of cell attachment. By digesting these proteins, TRYP induces cell detachment from the culture vessels [27, 28]. Other methods for dissociating adherent cells include scraping, microjet, sonication, and the use of synthetic peptides [29–33]. Some of the methods result in decreased cell viability due to damage to the cell membrane, whereas others are difficult to locate or are costly to implement [33]. Hence, the TRYP-mediated technique continues to be the most popular and practical way to separate adherent cells from culture containers. The breakdown of these surface proteins and subsequent cell detachment affects cell morphology and metabolic pathways [34, 35].

The trypsinization process leads to the formation of round-shaped cells with modified physiological activity [34]. TI is used to neutralize TRYP after the cells have been detached, inhibiting its ability to operate as a protease. TRYP is used in large-scale adherent cell cultures. To avoid unintended immunological responses or pathogen contamination, animal-derived proteins should not be used in such cultures [36]. The recombinant TRYP used in our study is of soybean, nonanimal origin. Regardless of the source of TRYP and TI, trypsinization can downregulate and meanwhile upregulate growth-related and apoptosis-related protein expressions, respectively [2]. Although trypsinization may be open to debate as a step in need of elimination from clinical application, trypsin inhibition does not appear to be a potential hazard when clinically administered [37]. The effective removal of TRYP and TI [38, 39] from cell therapy medicinal products while maintaining the quality properties of the cellular content is potentially efficient in developing finished products that will have less apoptotic risks and therefore higher and safer therapeutic benefits for recipients.

In commercial solutions, the concentration of porcine TRYP, an animal-derived source, is typically between 500

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and 2500 μg/mL [22], whereas the TRYP concentration in normal human serum is below 460 ng/mL or below ~0.02 μmol/L [24]. Even though most of the enzyme is typically eliminated during the final stages of the manufacturing process of cell therapy products, it is necessary to assess the residual amount of TRYP to confirm its complete absence [7].

Wu et al. [24] analyzed human sera for TRYP content using protamine-mediated fluorescent enhancement of DNA-templated gold nanoclusters. Here, the limit of detection (LoD) for TRYP was estimated as 1.5 ng/mL. Song et al. [23] reached an even higher sensitivity with a LoD value of 0.5 ng/mL using a fluorescent microsphere probe. Zhou et al. [25] found a LoD of 2.8 ng/mL in their study by sensitive colorimetric detection of TRYP in human urine samples. We found a baseline LoD TRYP value of 4.5 ng/mL. Our LoD value seems to be two-, three-, and ninefold higher than those of previous studies using various other techniques for TRYP analysis, suggesting a possible variation between the detection of TRYP in supernatant samples used in our study versus whole tissue samples used in the others.

Braatz et al. [22] analyzed a recombinant, GMP-grade porcine TRYP in cellular therapy products to find a value of 2.9 ± 0.4 μg/mL residual TRYP resulting from hollow fiber tangential flow filtration. The resulting TRYP concentrations in Wu et al. [24]'s study ranged from 5 to 100 ng/mL. Zhou et al. [25], on the other hand, found TRYP levels of 52.4–142.5 ng/mL. In our study, we interestingly found TRYP and TI concentrations ranging from ~2.9 to 4.7 ng/mL in both W0 and W1 samples. Resulting in similar concentrations to the Braatz et al. [22] study from cell culture, we reduced TRYP and TI content by 100% in the W2 samples. Our results correlate more with those of Braatz et al. [22] in that both studies analyzed TRYP content from direct cell culture rather than whole tissue samples as in the studies of Wu et al. [24] and Zhou et al. [25].

The most common method for determining and displaying the presence of residual protein contaminants in medicinal products is the enzyme-linked immunosorbent assay (ELISA) [40]. However, in cellular therapies, it is preferable to detect the presence and concentration of TRYP residuals used in the manufacturing process using SDS–PAGE instead of relatively expensive methods like ELISA [21]. Already, intermediary steps used in the clinical manufacture of cellular therapies are quite costly. It is therefore preferable to make use of less expensive yet reliable and easily validated methodologies for the quality control of finished products. SDS–PAGE is easy to apply and does not involve light-sensitive or time-dependent steps, making it cost-effective. Therefore, SDS–PAGE can be applied to large experimental contents and provides advantages over methods like ELISA [4].

SDS–PAGE is a cornerstone of quality control methods due to its simplicity and reliability, wherein proteins are dissolved and separated based on their MW [41]. This chromatography method is used to separate and remove molecules (especially proteins) based on differences in size. This method is often preferred for the removal of protein aggregates [20]. The quantity of protein samples loaded onto the SDS–PAGE gel is a crucial factor in obtaining accurate protein resolution. Overloading the gel with protein samples can result in distorted band patterns, as the electrophoretic pattern of separated proteins is influenced by the amount of loaded protein. Therefore, it is important to avoid overloading the gel to prevent distortions in adjacent lanes. Conversely, using an insufficient amount of protein sample on the gel can reduce the detection of trace proteins in the mixture [42]. Consequently, the quantity of loaded protein samples directly affects the sharpness and intensity of a protein band [43]. The limit of quantification baseline value of 15 ng/mL in our study presents the minimum quantity of protein sample loading for the generation of an accurate protein band.

A variety of staining methods can be used to visualize protein bands in SDS–PAGE analyses. Coomassie blue, silver, and Oriole staining are the most used visualization methods [44]. Coomassie blue staining is the most widely used and accepted, although less sensitive than silver staining—the gold standard—and Oriole staining, as it is compatible with mass spectrometry (MS) analysis. It is fast and easy to apply and gives good results at high protein concentrations, and protein bands can be visualized over a wide range [45, 46]. In this study, 1 ng of protein per well could be detected by staining with the widely used colloidal Coomassie blue, as previously reported in the literature [46–49]. Thus, we excelled in yielding results close to Oriole fluorescent staining [50]. Though silver staining is known to have the highest sensitivity [51], we corroborated previous findings that staining with colloidal Coomassie blue is an agreeable alternative method with its easy application.

There are several operator-dependent limitations when setting up an SDS–PAGE experiment protocol. The SDS–PAGE method has remained substantially the same since Laemmli's first discovery in 1970 [20, 52]. The percentages of acrylamide gel to be used need to be considered. The higher the gel concentration, the more difficult for the proteins to migrate. A feasible solution for protein content to be loaded evenly would be the use of gradient percentages of the separating gel. By this, there is a chance to observe and evaluate all detail in depth within a sample.

It is also quite ideal to use an approximately 1 mm-thick gel to aid in the migration of the proteins [53]. The 1 mm gel is extremely fragile. One careless minor snatch at either end or corner of it will lead to it breaking hence

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manifesting poor gel images at the end of the day. Proteins have a high chance of denaturing even before being boiled in preparation for electrophoresis. The operator in the process might need to act at a swift pace to avoid excess loss of protein content in the study samples.

Band discrepancies in general may be attributed to several paradigms during the analytical protocol—that is, sample preparation, electrophoresis conditions, gel staining, and protein denaturation [19]. In our study, any risks of unequal protein concentrations or insufficient sample volume were ruled out during our extensive protocol optimization. Parameters such as buffer composition, voltage, run time, and running conditions were justified with optimization experiments run against BSA. Loading volumes for each protein sample were also optimized against the standard proteins. β-Mercaptoethanol was added to the sample buffer as a reducing agent to successfully disrupt protein structure. Any compromise to the integrity of the gel structure was omitted from the study. Throughout our optimization study, all gels were cast on the same day, and the length of gel casting was consistent across all samples. Likewise, after the optimization phase, Gel 1 and Gel 2 were cast on the same day, under identical conditions, and with identical durations for all phases in the gel casting process. Furthermore, runs for Gel 1 and Gel 2 were performed on the same day, within the same SDS–PAGE tank, to reduce any changes in migration time and band profile between runs. If any, a step that might have affected band consistency could only relate to the protein aggregation or degradation that might have occurred in the samples after being collected from the Manufacturing Center [54]. However, each sample was stored at the same temperature and time interval as we have stated in our manuscript. It may be then regarded as a minor error due to the nature of the samples, which could be observed during any experimental phase.

Despite being a semiquantitative method, SDS–PAGE has several benefits over other methods for protein analysis [55]. ELISA is a widely used method for detecting and quantifying residual proteins in cellular products. It offers high sensitivity and specificity for detecting residual proteins and is often used for routine quality control in biopharmaceutical manufacture [56]. Western blotting, also known as immunoblotting, is another common method for residual protein analysis [57]. This method allows for the detection of specific proteins and can be used to assess protein size, abundance, and posttranslational modifications in cellular products [58]. MS is another analytical technique used for identifying and quantifying proteins in cellular products. MS offers high sensitivity and specificity and can provide detailed information about the identity and structure of residual proteins present in cellular products [56].

The reason why SDS–PAGE has been nominated as the most popular method is mainly due to its ease of use, reproducibility, relative affordability, efficiency, and length of loading [14, 21]. This cost-effective and conventional method is widely used to analyze proteins in complex extracts [52]. Gel electrophoresis and immunoblotting that follow this process respectively are predominant procedures for the isolation of proteins on nano-/microscale applications [53]. Nevertheless, if a more precise MW determination is required, the MW of an unknown protein should always be obtained by MS [59]. Although SDS–PAGE can lack sensitivity, specificity, and quantification capability in comparison with other analytical methods, its moderate throughput and ease of use is a preference for preliminary screening and qualitative analysis of residual proteins in cellular products, which when combined with its semiquantitative capabilities. SDS–PAGE is less sensitive compared to ELISA, Western blotting, and MS, typically detecting residual proteins in the microgram range. It offers moderate specificity, as protein bands can sometimes be difficult to distinguish based solely on size. However, for minimal sample sets such as samples of cellular products, SDS–PAGE has adequate throughput [60–62]. Compared to the other methods, SDS–PAGE is also relatively simple and cost-effective, requiring basic laboratory equipment and reagents, which makes it an agreeable alternative for researchers.

Experimental setup and optimization studies in SDS–PAGE analyses are crucial, especially in terms of quantification, reproducibility, and reliability of the method. To validate the method and analyze samples against standards, optimization studies with the use of standard agents versus commercially available protein standards and samples versus standard agents/commercially available protein standards need to be carried out until statistically significant accurate linear results are obtained each time.

Optimizing a method for undesired protein content identification and quantification will aid in future studies to be conducted for the removal of potentially more dangerous and harmful intermediary agents such as penicillin, which, when administered to a patient in a finished product, is well-renowned for its potential lead to morbid and mortal consequences such as allergic reactions and even further on, anaphylaxis [63–67].

5 | CONCLUDING REMARKS

We used BM-MSC product samples from a single donor in our study and hence suggest that future research should include a broader range of donor tissues and sample sizes to analyze potentially hazardous substances for more accurate results. Despite the common use of SDS–PAGE, the

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surprising lack of optimization and validation studies for identifying and quantifying advanced therapy medicinal products in the literature was noted. Our study emphasizes the need for further SDS–PAGE method validation to ensure the quality of protein content used in manufacturing these products. The limited number of existing studies has made it challenging to compare and discuss the results on a broader scale, underscoring the importance of additional research in this area.

AUTHOR CONTRIBUTIONS

Pelin Kilic designed, conducted and supervised all steps of the study. All authors contributed to the study conception and implementation. Additionally, material preparation, data collection, reporting and interpretation of the study outcomes were performed by Pelin Kilic, Sema Karabudak and Begum Cosar. All authors commented on the previous versions and agreed to the final draft of the manuscript, and Pelin Kilic approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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