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A RAPID METHOD FOR LARGE SAMPLE NUMBER EXTRACTION OF PROTEINS FROM MAMMALIAN CELLS

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ABSTRACT

Finding the best method of cell lysis and extraction of protein is the key step in detection and identification of proteins in all applications of proteomics. The methods currently used for protein extraction from mammalian cells are usually laborious, expensive or insufficiently reliable. Recently, researchers working with mammalian cells conduct protein extraction widely and frequently. This frequent dealing with a large number of samples becomes tedious work. Even though there are many commercial lysis buffers on the market, they are usually expensive. Therefore, in this study, protein extraction lysis buffer was designed to provide highly efficient total protein extraction from cultured mammalian cells. The simple and unique composition of this lysis buffer containing sodium dodecyl sulphate (SDS) dissolves cell membranes and extracts total cellular protein in only 45 minutes. The novel extraction method and lysis buffer composition was formulated using classical optimization or one factor at a time (OFAT) study. In this method, cell pellet from mammalian cells (CHO-K1) were subjected to different lysis buffer treatment procedure and then incubated at room temperature. The SDS-PAGE was performed to confirm the proteins extracted. The method was extended to bacteria (*Escherichia coli*, BL21-AI), yeast (*Pichia pastoris*) and repeated for mammalian (CHO-K1) for confirmation. It yields complete extraction for bacteria and mammalian cells. As such, this lysis buffer when used for preparing protein extracts from bacteria and mammalian cells offers rapid, reliable and cheap protocol which is applicable to routine laboratory analysis especially for protein estimation and SDS-PAGE.

Introduction

Extraction of proteins is challenging and inconsistent and has long been an issue for scientists [1]. Recently, researchers working with mammalian and bacteria cells frequently conduct protein extraction procedure and this often involves large number of samples and tedious work.

Protein extraction begins with cell lysis or cell disruption. Many techniques including mechanical and chemical methods are available for cell disruption and protein extraction. These techniques have been used by many researchers in their own works for different purposes [2]. Mechanical disruption techniques include sonication, blending or grinding with abrasives, agitation with glass beads, or use of the French press. These techniques have been successfully used with a variety of microorganisms. On the other hand, chemical disruption techniques including autolysis and chemical treatments have proven useful in specific cases. The choice of specific extraction procedures is dependent on the particular microorganism being studied, the quantity of protein required, and the type of analyses to be performed. Mechanical disruption techniques are not always applicable. This is because of the potential biohazard problems associated with

contamination of equipment and generation of aerosols. Therefore, the use of chemical disruption may avoid this problem. However, their addition to a cell extract may increase the complexity of subsequent protein isolations [3].

According to Brown and Audet (2008), there are two types of chemical disruption methods namely detergent lysis and alkaline lysis. Detergent lysis has been well developed for bulk biochemical assays and translates well into single- cell level. Many different detergents are used for this purpose including ionic, non-ionic and zwitterionic moieties. The selection of surfactant is important since it can affect the speed of cell lysis as well as the protein extraction efficiency. Strong ionic detergent such as sodium dodecyl sulphate (SDS) is able to perform cell lysis in seconds but tend to denature proteins from the cells. Milder non-ionic detergents such as Triton X-100 has slower cell lysis but have lower tendency to denature proteins and break up protein complexes; thus are preferable for applications involving protein structure or activity [4]. Zwitterionic detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate can be used for cell lysis as well, and result in no net change in the charge of solubilized protein. However, care must be taken as they can result in the suppression or reversal of electroosmotic flow in downstream electrophoretic separation methodologies [4].

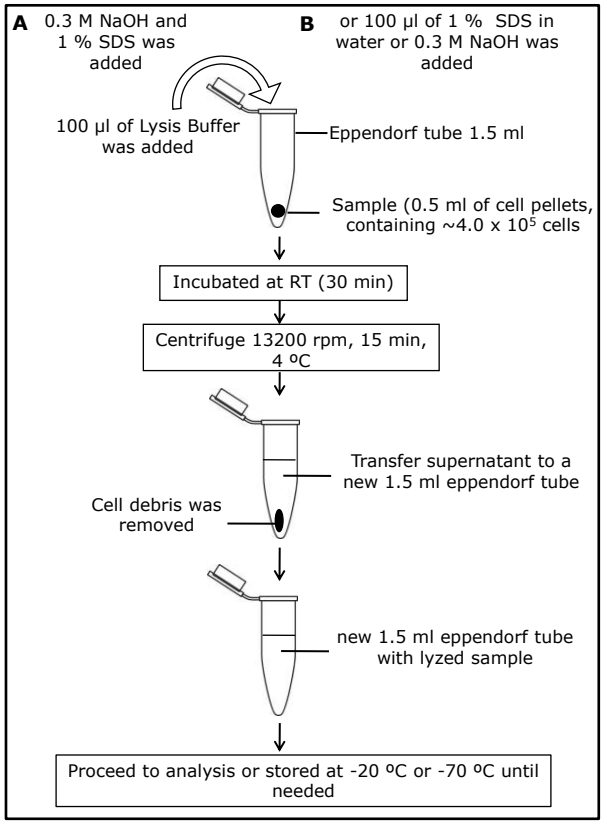

Fig. 1 Procedures for protein extraction adapted from Freshney (2005) with improvement.

Another method for chemical cell lysis relies on the generation of OH^- ions at electrodes to create significant concentrations to drive cell lysis [4]. Most of the protocols are being developed for microorganisms as described in Table 1. Meanwhile, there is limited information regarding protein extraction methods including composition of lysis buffer for mammalian cells. Products from mammalian cell culture are normally excreted into the supernatant thus harvesting of products do not require cell lysis. However, data on total protein is crucial for understanding of the mammalian cell culture system and improving its efficiency in producing desired products. The objective of this study is to design an efficient protein extraction protocol including its lysis buffer for cultured cells. It was found that the protocol was suitable for both mammalian and bacteria cells. The protocol was rapid, cheap, straight forward and reliable thus suit the need of processing large sample number for routine laboratory applications particularly for protein estimation and SDS-PAGE.

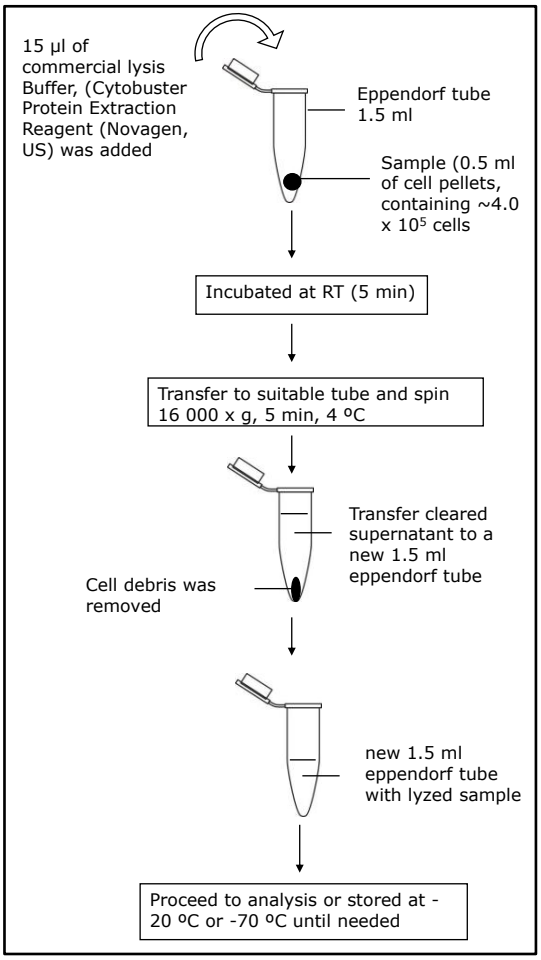

Fig. 2 Procedures for protein extraction using commercial lysis buffer (CytoBuster Protein Extraction Reagent (Novagen, US)).

Table 1 Protein extraction methods (lysis techniques) for bacteria and yeast

Lysis Technique	Application	Cell type	Species/ Strain	References
Mechanical disruption (Sonication, centrifugation and rupture by glass beads; FastPrep)	Proteomics studies	Bacteria	Enterecoccus faecalis V583 Lactococcus lactis NIZO 0900 Pediococcus pentosaceus OZF	Mehmeti *et al.* [1]
Chemical disruption (acetone treatment and 1 % SDS) **Mechanical disruption (sonication and glass bead disruption)**	Protein studies	Pathogenic and nonpathogenic bacteria	Staphylococcus aureus 184 and 196E Escherichia coli 20S0 Bacillus cereus 5065 Clostridium botulinum Type C/AO28	Bhaduri and Demchick [3]
Chemical disruption (modified alkaline extraction method; 0.3 M NaOH) **Mechanical disruption (Glass bead method)**	Western blotting	Yeast	Fission yeast	Matsuo *et al*. [5]
Chemical disruption (post-alkaline extraction; 100 µl 0.2 M NaOH) **Mechanical disruption (Glass beads lysis)**	Electrophoretic analysis	Yeast	S. cerevisiae 5V-H19 H. polymorpha DL-1 (ATCC 26012)	Kushnirov [6]
Chemical disruption (1 ml of NaOH-SDS solubilization; 0.2 M, 2.5 %) **Mechanical disruption (Sonication or French press treatment)**	Cell volume determination	Bacteria	*Clostridium perfringens* NCTC 8798	Guerlava*et al*. [7]
Microbial cells (Bugbuster protein extraction reagent)	Electrophoretic analysis	Bacteria	*Escherichia coli*	De Mey *et al.* [8]

2.0 Materials and Methods

2.1 Bacteria, Yeast and Mammalian Cells

Three types of cells (Bacteria; *Escherichia coli* BL21-AI, Yeast; *Pichia pastoris* and Mammalian; CHO-K1 (Chinese Hamster Ovary) Cells from ATCC, CCL-61) were used in this study. For bacteria and yeast cells, the cells were obtained in pellet form from our research laboratory. Meanwhile, for mammalian cells, the CHO-K1 was maintained in Hams F12 Media (Cellgro, US) supplemented with 10% (v/v) of serum (Gibco, Invitrogen US) at 37˚C/5% CO_2. The passage four cells were used for protein extraction study. Three biological replicates (independent cultures) for each protocol were made and samples (0.5 ml each containing ~4.0 x 10^5 cells) in the late exponential phase were used for analysis. The pellets for each sample were obtained by centrifugation at 1200 rpm for 2 min. Then, the supernatant was removed and 1 ml of phosphate buffer saline (PBS) was added before another

Table 2 Three different sets of extraction procedures investigating different types of lysis buffer, volumes and concentrations used to extract proteins or lyse cells from mammalian cell line (CHO-K1).

SET	Lysis Buffers	Sample	Concentration (Molar, M or %)	Total Volume	Incubation method	Protein Bands	Gel images (Figure 3)
1	% SDS in 0.3 M NaOH	a	2%	500ul	30 min at RT	No Band	A
		b	2%	100ul		No Band	
		c	1%	500ul		No Band	
		d	1%	100ul		No Band	
2	% SDS in distilled water	a	2%	500ul	30 min at RT	No Band	B
		b	2%	100ul		No Band	
		c	1%	500ul		No Band	
		d	1%	100ul		YES	
3	% SDS in 0.3 M NaOH but washed with water	a	2%	500ul	30 min at RT	No Band	C
		b	2%	100ul		No Band	
		c	1%	500ul		No Band	
		d	1%	100ul		No Band	

centrifugation at 2000 rpm for 5 min at room temperature and stored at -80˚C until required.

2.2 Materials and Reagents

All reagents were obtained from Bio-Rad (Bio-Rad, CA). All plastics and nuclease-free plastics tubes were obtained from Orange Scientific (ORANGE SCIENTIFIC, Belgium). The Protein ladder was purchased from Fermentas (FERMENTAS, USA).

2.3 Classical Optimization or One Factor at One Time (OFAT)Analysis

The effect of sodium dodecyl sulphate (SDS) concentration was investigated with the one-factor-at-a-time (OFAT) method to investigate the most suitable parameters for optimal protein extraction.

2.4 Protein Extraction

In this investigation, three different extraction protocols including different types of buffer, volumes and concentrations (Table 2) were used to extract proteins or lyse cells from mammalian cell. The procedure was adapted from Freshney(2005) [9] and being improved. 100 µl of lysis buffers (A and B; see figure 1) were added to CHO-K1 cell pellet before incubating at room temperature (RT). Next, the cell debris was removed by centrifugation at max speed (13 200 rpm) for 15 min at 4°C. The supernatant was transferred to a new tube containing protein extracts for further analysis or stored at - 20 °C until required. The overall procedure is depicted in Figure 1. The efficiency of the successful procedure of cell lysis was then reconfirmed in triplicates for mammalian cells and further tested on bacteria and yeast samples.

The effectiveness of the successful procedure of cell lysis developed from this study was compared with the commercial lysis buffer CytoBuster Protein Extraction Reagent (Novagen, US). The procedure was as per manufacturer's instructions as depicted in Figure 2. Briefly, 15 µl of CytoBuster Protein Extraction Reagent were added to the CHO-K1 cell pellet (10^5 cells) and then incubated at room temperature (RT) for 5 minutes. After 5 min, the samples were transferred to a suitable tube and spun for 5 min at 16 000 x g (16 000 rpm) at 4°C. The supernatant containing protein extracts was

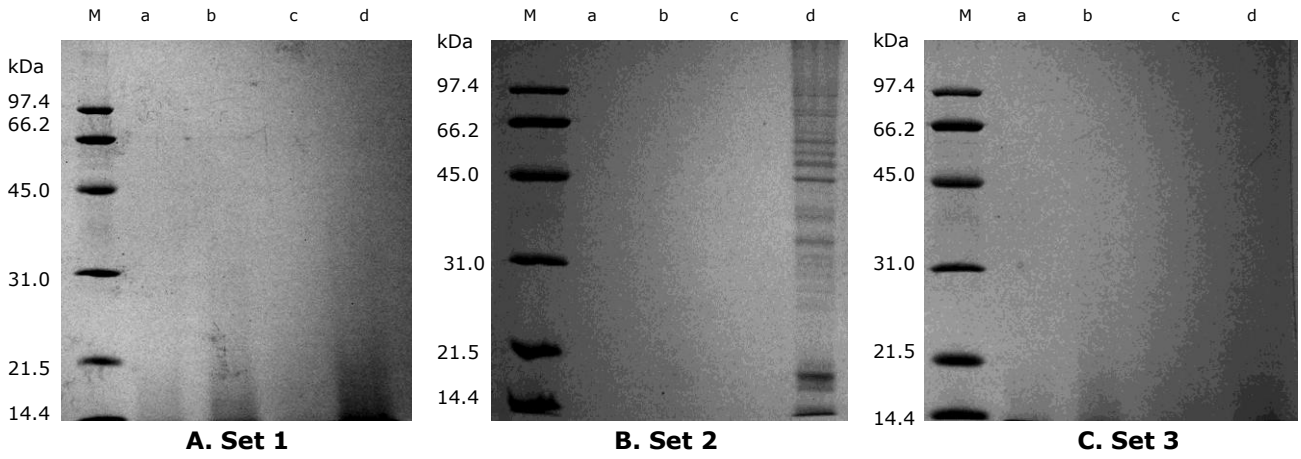

Fig. 3 Gel images of protein bands from CHO-K1 cell lysate obtained from different protein extraction protocols. Set 1 to 3 comprise of combination of different lysis buffer, volumes and concentrations as laid out in Table 2. Only sample d from Set 2 showed protein bands. M, molecular weight marker (Bio-Rad, CA). Samples were run at 12 % resolving and 4 % stacking gels at 250 V for 80 min.

transferred to a new tube for analysis or stored at - 20 °C until needed.

2.5 SDS-PAGE

The SDS-PAGE was performed for all extracted protein samples. Acrylamide separating gel and stacking gel were used for SDS-PAGE. The gels were run at about 200 to 250 V for about one hour. At the end of electrophoresis, gels stained with Coomassie brilliant blue and destained for 2 h in a solution containing 7.5 % methanol and 5 % glacial acetic acid for visualization.

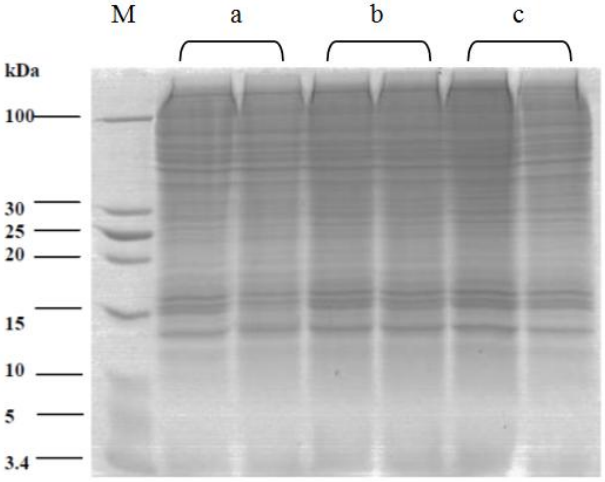

Fig. 4 Lysis buffers were applied to mammalian (CHO-K1) cells (n=3) to reconfirm the efficiency of the protein extraction protocol. M is molecular weight marker ranging from 3.4 to 100 kDa (Fermentas, USA), a is lysis buffer 1, b is lysis buffer 2 and c is lysis buffer 3. Samples were run at 15 % resolving and 4.5 % stacking gels at 200 V for 80 min.

2.6 Total Protein Content Analysis using Bio-Rad Protein Assay

Protein concentration for selected samples (protein samples extracted from successful procedure of lysis buffer developed from this study and the commercial buffer) were determined by Bio-Rad protein assay (Bio-Rad, CA; Catalog no. 1-800-4BIORAD) with bovine serum albumin as the standard, according to manufacturer's instructions. Absorbance was measured using microplate reader, Infinite 200 Pro Series (Tecan, USA) at 595 nm. Mean protein concentrations (µg/ml) between different lysis buffers were compared using SPSS software (t-test). The level of significance was set at $P<0.05$.

3.0 Results and Discussion

3.1 Protein Extraction and SDS-PAGE for Three Different Extraction Protocol

The development of the method began as an attempt to obtain a rapid, efficient and cheaper protein extraction lysis buffer for a large number of mammalian cells (CHO-K1) samples. Three different methods with different types of lysis buffer, volumes and concentrations were investigated as depicted in Table 2. The method was designed based on Freshney (2005) [9] and optimized using classical optimization or one-factor-at-a-time (OFAT) method to observe the possible optimum levels of the parameters. Figure 3 showed the gel images of cell lysate following SDS-PAGE procedure, obtained from protein extraction protocols as laid out in Table 2 respectively. Only protocol from set 2 (sample d, 1% SDS in distilled water, 30 min incubation at room temperature (RT)), showed protein bands suggesting that this protocol was successful. From these results, it can be concluded that the

chemical lysis with detergent (SDS) works well in rupturing the membrane cell. Although the extraction method in this study was adopted from Freshney (2005), only SDS was successfully used to extract proteins from CHO-K1 cells. This may be due to SDS being a strong ionic detergent which is able to lyse cells [4]. According to Anselmetti [10], a typical mammalian cell has a diameter of roughly 10 µm, with a volume of 0.5 pL and a total protein content of 50 pg. By assuming an average molecular mass of 30 kDa, the cell contains about 2 fmol total protein or about one billion copies of protein molecules. To our knowledge, there were limited studies on protein extraction method for mammalian cells. However, in 2008, Brown and Audet [4] have listed the comparative list of single-cell lysis for mammalian cells.

Table 3 Protein concentrations extracted from CHO-K1 cells using lysis buffer from successful procedures and commercial reagent (n= 3, Mean ± SD)

Lysis Buffer	Protein concentrations (µg /ml)	t-test
100 µl of lysis buffer from successful extraction procedure (per ~4 x 10^5 cells)	919.1 ± 693.14	p = 0.096 (p > 0.05)
15 µl of commercial lysis buffer (per ~4 x 10^5 cells)	2388.5 ± 72.83	No significant difference between the two groups

3.2 Confirmation of Successful Protein Extraction Protocol

In order to confirm the protein extraction protocol (Set 2, Sample d), the successful lysis buffer was prepared in triplicates. The lysis buffer was added to the CHO-K1 cell pellet and the extracted protein was subjected to SDS-PAGE. The results showed successful extraction of protein ranging from 3.4 to 100 kDa for all samples (Figure 4).

3.3 Protein Extraction and SDS-PAGE for Bacteria (E.coli BL21-AI), Yeast (Pichia pastoris) and Mammalian Cells (CHO-K1)

The method was tested on bacteria (*E. coli*), yeast (*P. pastoris*) and repeated for mammalian cells (CHO-K1). The proteins were successfully extracted for bacteria and mammalian cells (Figure 5). The use of 1 % SDS in this study to extract proteins from bacteria was similar to Bhaduri and Demchick (1983) where they applied 1 % SDS concentration in acetone-SDS treatment [3]. However, in this study no protein was able to be extracted from yeast using this method suggesting that other method might be used to extract proteins from yeast. Thus, alkaline methods have been reported to be suitable for protein extraction from yeast [5].

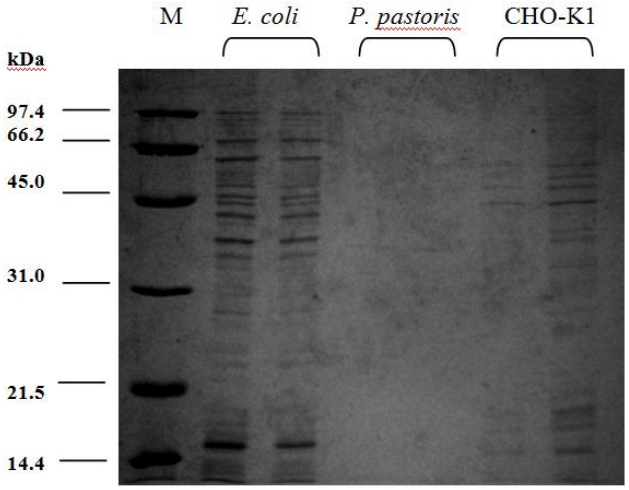

Fig. 5 Optimized lysis buffer was applied to bacteria cells (*E.coli*), yeast cells (*P. pastoris*) and was repeated for mammalian (CHO-K1) cells (n=2). M is molecular weight marker ranging from 14.4 to 97.4 kDa (Bio-Rad, CA). Samples were run at 12 % resolving and 4 % stacking gels at 250 V for 80 min.

3.4 Comparison between Lysis Buffer from Successful Protein Extraction Procedure and Commercial Lysis Buffer using SDS-PAGE and Bio-Rad Protein Analysis

The successful lysis buffer was compared with the detergent based commercial lysis buffer [11]. 20 µl of the samples extracted using both lysis buffers according to protocols were loaded to mini SDS-PAGE. The protein bands were observed for all samples. The protein bands for samples extracted with commercial lysis buffer were denser than the samples extracted by our lysis buffer (Figure 6). The amount of proteins extracted was further measured using Bio-Rad Protein Assay as described in method section. The concentration obtained from commercial reagent was approximately one times higher than the developed buffer (Table 3). However, there is no significant difference between the concentrations of proteins extracted in the method compared. Although the proteins amount was higher when lysed with commercial reagent, the two lysis buffers had given sufficient amount of proteins that can be used for further analysis.

Compared to procedure suggested by Freshney [9] where samples were heated at 100 °C for 30 minutes or alternatively incubated at room temperature for 30 minutes, our protocol is in agreement with the latter suggested procedure. Although this is longer than commercial reagent (5 min), it is suitable for handling large number of samples where cycle time is longer. From cost point of view, SDS is certainly much

cheaper compared to commercial reagent. This may help maintain cost-effectiveness of work involving large sample number. In addition, preparation of the lysis buffer is very straight forward thus improve the efficiency of the whole process.

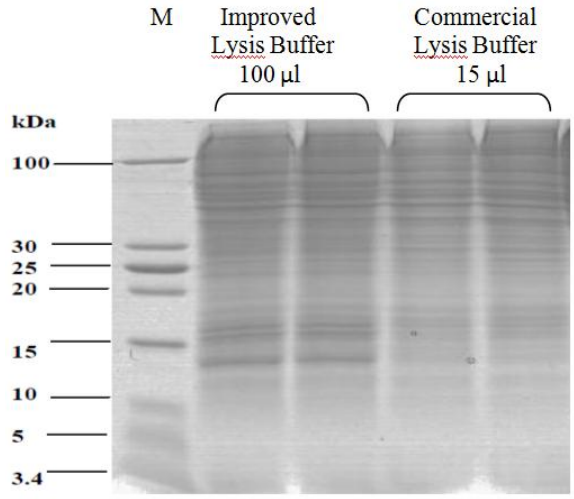

Fig. 6 Lysis buffer from successful procedure (100 µl) and commercial lysis buffer (15 µl) were applied to mammalian (CHO-K1) cells (n=2). M is molecular weight marker ranging from 3.4 to 100 kDa (Fermentas, USA). Samples were run at 15 % resolving and 4.5 % stacking gels at 200 V for 80 min.

4.0 Conclusions

In this study, a simplified optimal method that is both cost-effective and time-saving for obtaining protein extracts from large sample number mammalian cultures has been successfully developed with the use of 100 µl 1 % SDS per 4 x 10^5 cells. This rapid, reliable and cheap method for preparing protein extract for mammalian cells is applicable to routine laboratory analysis such as protein estimation procedure involving Bradford method and for SDS-PAGE. For future improvement, it is recommended that the composition of SDS and Triton may be used to improve cell lysis procedure as well as increase protein yields.

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