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Research Article

OPTIMIZATION OF REFOLDING PROCESS FOR PRODUCTION OF RECOMBINANT CRM_{197} PROTEIN FROM *E.COLI*

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ABSTRACT

E. coli is most widely, well studied and highly characterized microorganism available for production of recombinant protein. $rCRM_{197}$ (Recombinant Cross Reacting Material) is a genetically modified recombinant protein which is widely used as a carrier protein. When a recombinant protein is over expressed in heterologous expression system of *E. coli*, it gets misfolded and aggregated in the form of inclusion bodies. Recovery of active recombinant protein from the inclusion bodies is a cumbersome task. In the current study, $rCRM_{197}$ was over expressed in *E. coli* based host system using optimized semi defined media. The purified inclusion bodies were isolated by optimization of Cell lysis step. Solubilization was performed using high concentration of Chaotropes at alkaline pH. A refolded and functional form of native CRM_{197} protein was recovered by dilution of solubilizing agent using Cystine/Cysteine redox agents in Refolding buffer. The optimized process resulted in generating high quality cost effective CRM_{197} with drastic reduction in the production time.

Keywords: *E.coli*, Fermentation, Inclusion bodies, Solubilization, Refolding, $rCRM_{197}$.

1. INTRODUCTION

Availability of cost effective vaccines for all has been the point of concern in the scientific community and Biological industry since long. The cost of production varies from product to product and the choice of the adopted process to produce the vaccine. For production of the targeted protein or polysaccharide, various microorganisms are utilized including Bacteria, Virus, Yeast or animal cells including human cell lines. While a lot of time gets consumed in research of a suitable host for production of a recombinant protein, *Escherichia coli* (*E. coli*) offers advantage over the other microorganisms. *E.coli* is most widely, well studied and highly characterized microorganism available for production of recombinant protein [1].

$rCRM_{197}$ (Recombinant Cross Reacting Material) is a recombinant protein which is a nontoxic form of diphtheria toxin which is a genetically modified [2]. $CRM_{197}$is used as a carrier protein for conjugating a polysaccharide antigen which itself is unable to trigger a T cell mediated memory cell immune response [3]. Advantage of using CRM_{197} as a carrier protein is that it is a genetically detoxified and antigenically indistinguishable from diphtheria toxin and has more lysyl side chain for conjugation reaction. Currently, CRM_{197} is used in various licensed vaccines as a carrier protein. The maximum achievable yield of CRM_{197} with the traditional methods *i.e.* by using *Corynebacterium diphtheriae* C7 strain is very low and their production requires a Biosafety level II facility which further adds up in the cost of the vaccine [4].

When a recombinant protein is over expressed in heterologous expression system of *E. coli*, it gets aggregated in the form of inclusion bodies (IBs) [5, 6]. For long, IBs were considered as non useable product. The proteins in IBs get miss-folded and recovery of a biologically active protein from IBs is a cumbersome task [7]. The expression of $rCRM_{197}$ in *E. coli* is also dependent on temperature of fermentation culture [8, 9]. When the temperature of fermentation culture is reduced post induction, the protein is expressed in soluble form, whereas at higher temperature, the protein is expressed in in-soluble form and aggregates to form IBs. Purification of recombinant protein from its soluble form has low yield and does not sustain the commercial scale production [10]. IBs consist of pure, miss-folded, stable and bioactive aggregates of expressed protein formed because of protein-protein interactions [11]. Recovery of bioactive protein from IBs involves Cell lysis, Solubilization, Refolding and

Purification steps. Lysis of *E. coli* cell can be achieved by mechanical, chemical, enzymatic or a combination of either method. Homogenization is considered as the most efficient method of choice [12]. Solubilization can be achieved using Chaotropes (Urea, Guanidine hydrochloride (GdnHCl) etc.) or non-denaturing buffers (Tris-HCl) without solubilizing agent [13]. Refolding of the solubilized protein is achieved by removal of the solubilization agent which in turn is achieved by dilution of the solubilized IBs with Refolding buffer. The buffer containing optimized redox agent concentration is an important factor to be considered during Refolding step [14].

In the current study, fermentation was carried out using optimized media for production of $rCRM_{197}$ using *E. coli* BL21 (DE3) cells. The harvested cells were solubilized and Refolding was performed with optimized procedure for recovery of bioactive $rCRM_{197}$ protein. The study provides an opportunity to recover $rCRM_{197}$ protein in an efficient and industrially sustainable procedure. With the availability of cost effective $rCRM_{197}$ carrier protein, the overall production cost of conjugate vaccine can be reduced and the conjugate vaccines can be available for all.

2. MATERIAL AND METHODS

2.1. Strain used

The recombinant cells consist of pTWIN1-CRM_{197} Kanamycin resistant expression vector. The sequence for CRM_{197} gene was synthesized and cloned in pTWIN 1 vector by New England Biolabs, UK (United Kingdom).

2.2. Fermentation

The cells were streaked on LB agar media and incubated for overnight at 37°C. For Pre-Seed flask preparation, 2 to 5 colonies from the streaked plate were inoculated in flask containing 200 mL of Luria Bertani (LB) media. The inoculated flask was incubated at 37°C and 250 RPM (Rotation per Minute) for 4 to 20 hours. After completion of incubation period for Pre-Seed culture, the culture was checked for its purity by gram staining and OD_{600} was checked. For Seed culture preparation, when the OD_{600} (Optical Density at 600 nanometer) of Pre-Seed culture was ≥1, 20 mL culture was inoculated in flask containing 600 mL of LB media and incubated overnight at 37°C and 250 RPM. When the OD_{600} of the culture reached ≥1, 4L of Seed culture was inoculated into fermenter containing optimized media for fermentation. The semi defined fermentation media consists of K_2HPO_4 (11.43g/L), $(NH_4)_2SO_4$ (5.71 g/L), Citric Acid (2.43 g/L), $MgSO_4$ $7H_2O$ (0.57 g/L), $FeCl_3$ $6H_2O$ (142.86 mg/L), $MnSO_4$(20 mg/L), $ZnSO_4.7H_2O$ (17.4 mg/L), EDTA (12 mg/L), H_3BO_3(4.29 mg/L), $Na_2MoO_4 2H_2O$ (3.43mg/L), $CoCl_2$ $6H_2O$ (3.43mg/L), $CuCl_2 4H_2O$ (2.14 mg/L), Glucose (15 g/L), Kanamycin (50 mg/L), Antifoam-PPG (25%) and Yeast extract (7.14 g/L).

Fermentation was carried out at 80L scale in Fed batch mode at temperature: 30±0.2°C, airflow: 80 to 150 SLPM, agitation: 200 to 900 RPM, back pressure: 0.3 bars, pH: 6.8±0.2 and DO (Dissolved Oxygen): ≥ 30%. Oxygen was maintained in cascade mode. Glucose solution containing trace elements and Yeast extract solution was used as feed. Fermentation culture was induced with 0.5 mM IPTG (Isopropyl ß-D-1-thiogalactopyranoside) solution at OD_{600} between 40 and 50. Fermentation was continued till the culture reached stationary phase i.e. the difference between two consecutive OD_{600} was less than 1. Samples were withdrawn at regular interval for OD_{600}, Glucose concentration and Protein expression analysis by SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis) and Western Blot. The fermentation broth was harvested and centrifuged for 30 minutes at 8000 RPM. The pellet was stored at -70°C.

2.2.1. SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis)

Reducing and Non-Reducing SDS PAGE technique was performed for separation of the expressed protein and analysis as per standard protocol [15]. 12% Tris-glycine gel was used. 80V voltage was applied for initial 10 minutes and there after 200V till completion of gel run. The gel was loaded with the prepared samples (20µL), Molecular weight marker (11, 17, 22, 25, 32, 46, 58, 80, 100, 135, 190 and 235 kDa; New England Biolabs), rCRM standard (10µL, 58 kDa, GSK). The gel was stained with Coomassie Brilliant Blue R250 dye and image captured on Trans-illuminator (Bio-Rad Chemidoc XRS+ system).

2.2.2. Western Blotting

For Western blotting, the SDS PAGE gel was transferred to Nitrocellulose membrane (Bio-Rad, Cat# 1620115) using standard protocol [16]. The western blot was incubated for 60 minutes and probed with anti diphtheria polyclonal antibody (Abcam, Cat#ab 151222). The probed membrane was incubated with secondary antibody (Rabbit, Make: Santa Cruz

Biotechnology, Cat#SC2054) conjugated with HRP (Horseradish peroxidase) enzyme. The bands were observed with 3, 3'-diaminobenzidine (DAB) substrate. Further the image was captured on Trans-illuminator.

2.2.3. Protein content (BCA-Bicinchoninic acid Method)

Protein content was estimated using standard Pierce BCA Protein Assay Kit (Thermo, cat#23225) at 562 nm (nanometer) wavelength [17].

2.2.4. HPLC-SEC (Size Exclusion High Pressure Liquid Chromatography)

SEC HPLC was used to monitor the refolding efficiency [18]. Following are the method details for HPLC: Column: Shodex Protein® 5 µm KW-803, LC Column 300 x 8 mm, Mobile Phase: 10mM Phosphate, 100mM NaCl, and pH 7.2, Flow rate: 1mL/min, Run Time: 30min, Detection: UV 280 nm.

2.3. Preparation of Inclusion bodies

2.3.1. Optimization of Cell Lysis step

From 80L scale fermentation lot (Lot 1); approximately 12 Kg of cell pellet was obtained. For optimization of Cell Lysis step, 4 lots of 2 Kg cell pellet each were taken. Cell pellet was suspended in Lysis buffer (20 mM Tris, 0.25M NaCl and pH 8±0.2) in 1:10 ratio. The cell pellet in Lysis buffer was initially homogenized in cold condition (on ice) at 800 bars in homogenizer (Microfluidics) [12]. 3 to 6 passes were performed to check the lysis of the cells.

Based on the analysis of the results, the homogenization step was further optimized by increasing the homogenization pressure to 1200 bar. At both the homogenization pressure, samples were withdrawn for analysis of $OD_{600}$to check the percentage of ell lysis. After Homogenization, the cell lysate was centrifuged at 8000 RPM for 60 minutes between 2 to 8°C (Beckman Coulter, Avanti j-265XP). Pellet was collected and supernatant was discarded.

2.3.2. Washing of Pellet

Washing was carried out in three stages (Wash 1, 2 and 3) with three different buffers [19]. The pellet obtained from Cell Lysis step was re-suspended in Wash buffer 1 (20 mM Tris, 0.25 M NaCl and pH: 8±0.2) in 1:10 w/v ratio. At each washing step, the suspension was thoroughly mixed for 30 minutes at 300 RPM (Cole Parmer, IKA mixer). After mixing, the suspension was subjected for centrifugation at 8000 RPM for 60 minutes between 2 to 8°C. The collected pellet was used for the next step of washing i.e. Wash 2 (1% Triton X 100, 20 mM Tris, 0.25 M NaCl, 2M Urea and pH: 8±0.2) and Wash 3 (20 mM Tris and pH: 8±0.2). All the 3 wash buffers were chilled before use. Sampling was done after completion of washing step. Purified Inclusion bodies were analyzed for Protein content, Western blot and SDS-PAGE.

2.4. Solubilization of Inclusion bodies

Solubilization of IBs was performed using high concentration of Chaotropic agent. 500 g of purified IBs obtained from washing step was suspended in Solubilization buffer (20 mM Tris, 8M Urea and pH: 8±0.2) in 1:20 w/v ratio [6, 19]. The suspension was mixed for 90 to 120 minutes at 300 RPM. After mixing, the suspension was subjected for centrifugation at 8000 RPM for 60 minutes between 2 to 8°C. Pellet was discarded and supernatant was clarified using 0.8/0.45 µm filter. The filtrate was used in Refolding step. Sample was withdrawn for analysis of Protein content.

2.5. Optimization of Refolding Process

Refolding of $rCRM_{197}$ protein was performed by dilution method. Different concentration of cysteine and cysteine redox agents was used [14]. Arginine was used as an aggregate suppressor [20, 21]. 20L of Solubilized IBs were mixed into Refolding buffer in 1:20 ratio v/v (50mM Tris, 2mM Cysteine, 1mM Cystine, 250 mM Arginine and pH 8.0) with a flow rate of 40 mL/min at 2 to 8°C. Samples were withdrawn for % refolding by SEC HPLC and Protein content analysis by BCA method.

3. RESULTS AND DISCUSSION

3.1. Fermentation

To optimize the Refolding process for production of $rCRM_{197}$ protein and to get the starting material *i.e.* Inclusion bodies, an 80L scale fermentation lot was produced using semi-defined media. Fermentation was carried out at specified parameters and the culture was induced with 0.5M IPTG solution. Harvest volume of 90L was collected at the end of fermentation with final OD_{600} of the culture equal to 142. During fermentation, samples were withdrawn for growth profile and Glucose concentration profile analysis (YSI analyzer). Post induction samples were withdrawn for analysis of protein expression by SDS PAGE and Western blot. Fig. 1 describes OD_{600} profileand Glucose profile, Fig.

2: SDS PAGE and Fig.3: Western blot of the fermentation lot.

Fig. 1 shows that the fermentation lot followed typical Sigmoidal growth curve and as the fermentation progresses, the glucose concentration reduced in the culture leading to stationary phase.

It is evident from Fig. 2 and 3 that the post induction samples for 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs and final harvest sample shows clear band with respect to rCRM standard. The western blot confirms the presence of $rCRM_{197}$ protein as it is recognized by anti CRM antibody probed on the Western blot.

Although, chemically defined media and complex media has been used by many researchers for production of high cell density culture for production of recombinant protein [22, 23], our study has shown that a chemically defined media or complex media alone is not suitable for a consistent and robust fermentation process for production of $rCRM_{197}$ protein.Rather use of Semi defined media *i.e.* inclusion of Yeast extract in fermentation media as well as feed solution resulted in high density cell culture with maximum achieved OD_{600} of 140.

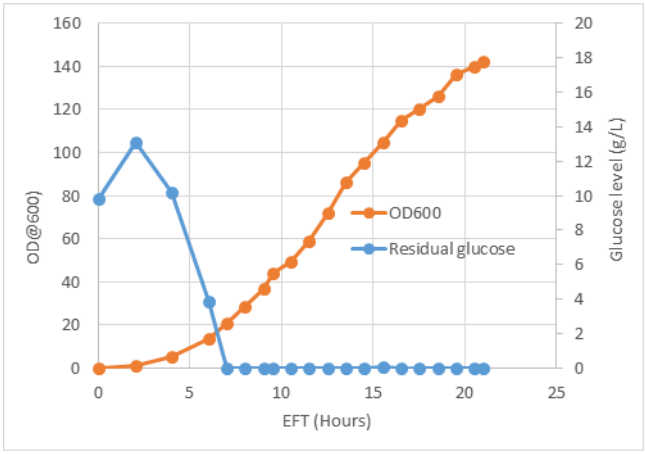

Fig. 1: A-OD_{600} profile and Glucose profileof fermentation lot

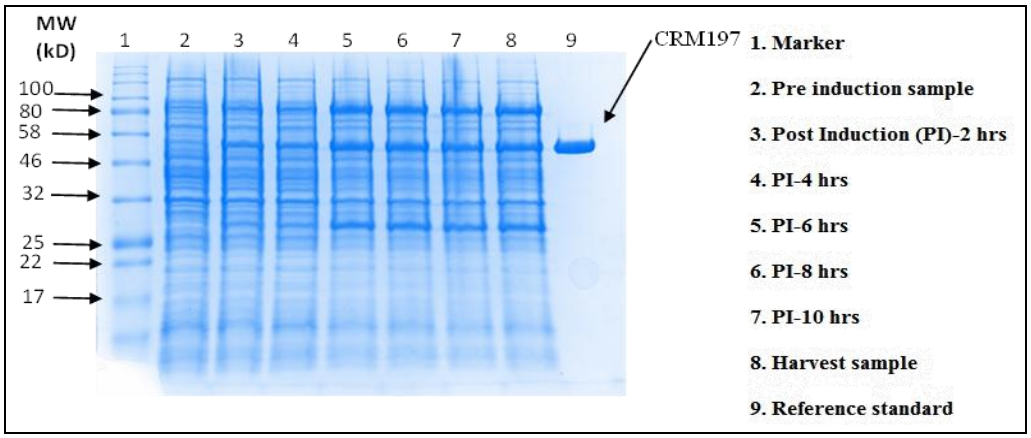

Fig. 2: SDS PAGE (Reducing) analysis of Pre induction and post induction samples from fermentation lot

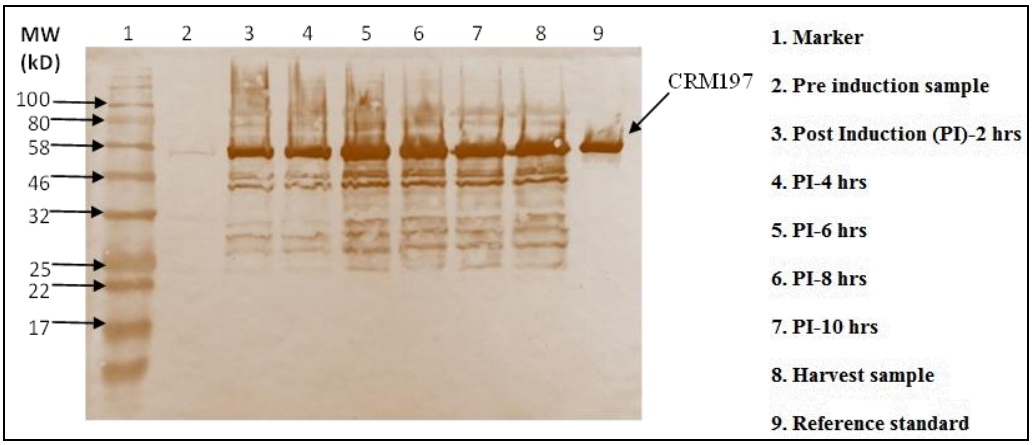

Fig. 3: Western blot analysis of the Pre induction and post induction samples from fermentation lot

3.2. Inclusion bodies (IBs)

3.2.1. Cell Lysis and Washing of Pellet

The cell pellet obtained after centrifugation of fermentation broth was mixed in Lysis buffer and homogenized initially at 800 bar and then at 1200 bar. Samples from 4 IBs lots were analyzed for reduction in OD_{600}after each pass. Fig.2 indicates the reduction in OD_{600}(A) at 800 barand (B) at 1200 bar.

It is evident from the data that in all the 4 lots, at 1200 bar reduction in OD_{600} was greater as compared to reduction of OD_{600} at 800 bar. It was also observed that after 3rd pass, there was no significant reduction in OD_{600}. In all the 4 lots, at 1200 bar, the % reduction in OD_{600} was ≥75%. Hence the homogenization parameter was finalized at 1200 bar, for 3 passes and reduction in OD_{600} should be ≥ 75%.

After completion of 3 washing steps with 3 different Wash buffers, the samples were analyzed for the Protein content by BCA method (Table 1)and Protein Expression by SDS PAGE (Figs. 5 and 6) and Western Blot (Figure 7 and 8). Protein expression analysis was performed in Reducing as well as Non Reducing states.

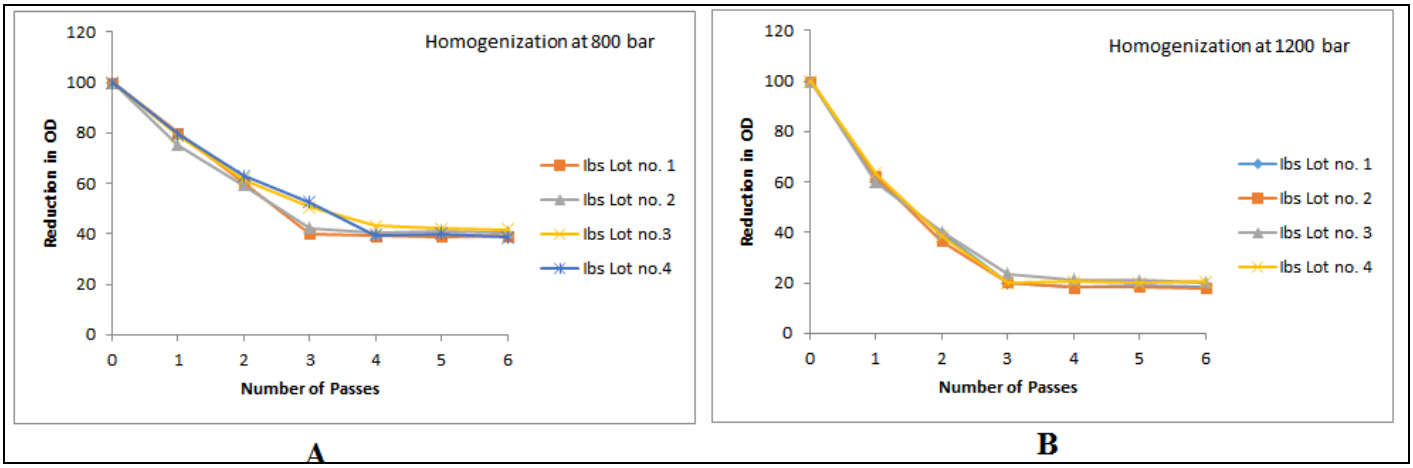

Fig. 4: Reduction in OD_{600} at (A) 800 bar and (B) 1200 bar for IBs Lot no.1, IBs Lot no.2, IBs Lot no.3 and IBs Lot no.4.

Table 1: Comparison of 4 IBs lot (IBs Lot no.1, IBs Lot no.2, IBs Lot no.3 and IBs Lot no.4)

Stage	Description	IBs Lot no.1	IBs Lot no.2	IBs Lot no.3	IBs Lot no.4
Purified Inclusion Bodies	IBs Wet Weight (g)	535	450	514	558
	Protein Content (mg/mL)	7.8	10.3	9.6	10.3
	SDS-PAGE	+Ve	+Ve	+Ve	+Ve
	Western Blot	+Ve	+Ve	+Ve	+Ve

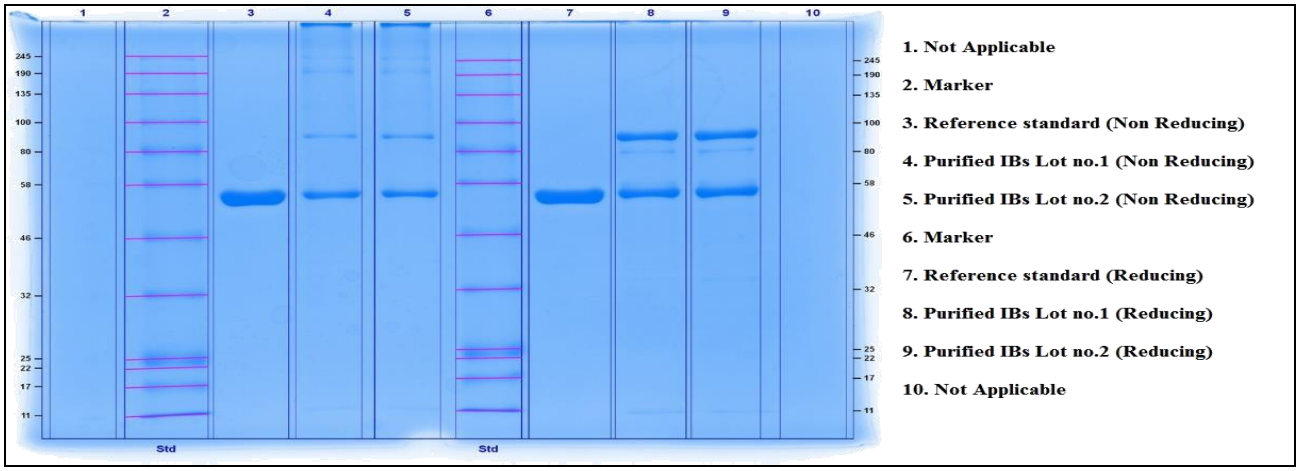

Fig. 5: SDS PAGE analysis (Reducing and Non-Reducing) of Purified IBs Lot no.1 and Lot no.2

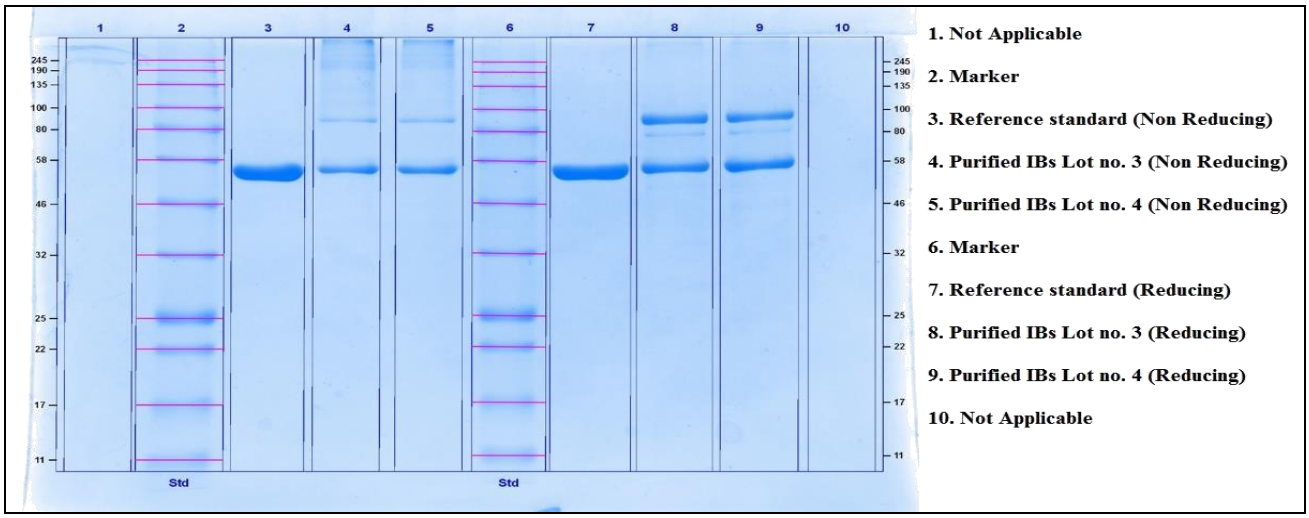

Fig.6: SDS PAGE analysis (Reducing and Non-Reducing) of Purified IBs lot no.3 and Lot no.4

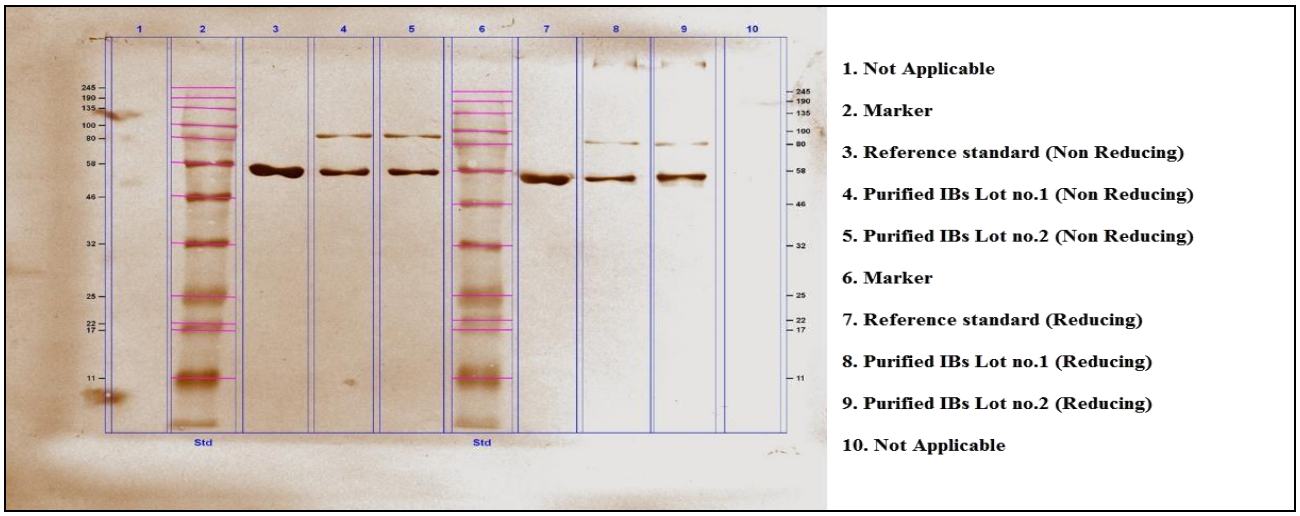

Fig. 7:Western blot analysis (Reducing and Non-Reducing) of Purified IBs lot no.1 and Lot no.2

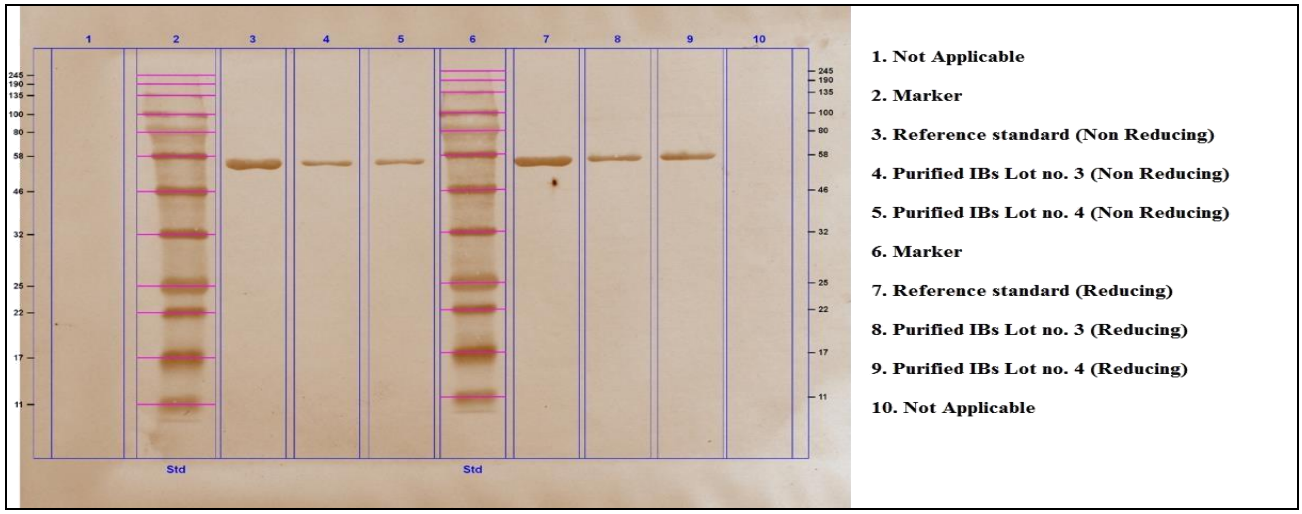

Fig. 8: Western blot analysis (Reducing and Non-Reducing) of Purified IBs lot no.3 and Lot no.4.

It is evident from figs. 5-8 that in all the 4 lots, the purified IBs sample shows clear band with respect to rCRM standard. The western blot confirms the presence of $rCRM_{197}$ protein as it is recognized by anti CRM antibody probed on the Western blot. SDS PAGE and Western blot profiles indicates consistent quality for purified inclusion bodies.

Earlier reported studies [10] have shown the use of 20 mM Tris-HCl buffer (pH 7.5) with protease inhibitor and Ultra sonicator for lysis of harvested cells. In the current study, no protease inhibitor was used; instead Tris buffer with 0.25M NaCl was used for lysis of cells on homogenizer. Approximately 500g of IBs were isolated from 2000g of cell pellet *i.e.* 25% of recovery was achieved after optimizing the homogenization pressure.

3.3. Solubilization of IBs

The purified IBs were used in the further purification step for solubilization. IBs were solubilized using high concentration of Chaotropic agent i.e. 8M Urea. 4 different lots were taken from the purified IBs i.e. SIB Lot no.1 to SIB Lot no.4. In all the 4 lots, supernatant sample of Solubilized IBs (SIB) was withdrawn for Protein content analysis by BCA method.

Table 2: Protein content of 4 Solubilized IBs lots (SIB Lot no.1, SIB Lot no.2, SIB Lot no.3 and SIB Lot no.4)

Stage	Description	SIB Lot no.1	SIB Lot no.2	SIB Lot no.3	SIB Lot no.4
Solubilization	Protein content (mg/mL)	9.7	8.5	7.0	10.1

Use of 2 different solubilizing agents in combination along with reducing agents like Dithiothreitol (DTT) and β-mercaptoethanol has been reported by Esmaili, *et al*. Different concentration (2-6M) of Urea and guanidine hydrochloride (GdnHCl) was used for isolation of Reteplase inclusion bodies [19]. Key for efficient solubilization lies in pH and composition of solubilizing agent and based on our results, 8M urea with 20 mM Tris and pH: 8±0.2was found to be the best solubilizing agent for $rCRM_{197}$ protein.

3.4. Refolding Optimization

The balance between flexibility/solubility of the intermediates (or the unfolded structure) and structure collapse affects the refolding course. Misfolding or aggregation competes with Folding into native structure. Optimal refolding is often achieved by forcing denatured protein to collapse and simultaneously maintaining solubility and flexibility of the molecule. Such balances are often achieved by two different means. First is reducing the concentration of denaturant and second is how fast the denaturant concentration is reduced. Several parameters are known to affect the refolding of proteins. Most common are buffer composition and pH, protein concentration, presence of excipients and redox potentials, temperature and time of refolding. For proteins containing free cysteine and disulphide bonds, Refolding buffers containing redox agents are considered critical factor [14]. Most common redox agents used for the purpose are oxidized and reduced glutathione. However due to substantial cost of glutathione for large scale applications, alternative pairs of Cysteine-Cystine was used in the current optimization study.

Dialysis and Dilution of solubilized proteins in refolding buffer are two widely used methods [6]. In the current study, dilution based refolding was done for $rCRM_{197}$. Different parameters evaluated for refolding are Redox potential, Temperature of refolding process, Time of refolding reaction, Protein concentration and Aggregate suppressor.

3.4.1. Redox Potential

The process of refolding can be controlled to large extent by using appropriate redox system in the Refolding buffer [14]. The redox system controls the process of refolding by reducing aggregate formation and forming correct refolding of protein.

Refolding of $rCRM_{197}$ in presence of various concentration of redox system (Cystine/Cysteine) was monitored at various time intervals by SEC HPLC. The chromatogram presented in fig. 9 shows that Cystine/ Cysteine in the concentration of 2mM and 1mM respectively is optimum for the refolding.

Other concentrations of redox systems have shown higher aggregates as compared to target protein as depicted in Figure 9.

3.4.2. Effect of Temperature of refolding process

Temperature plays vital role in refolding process. Literature reports that refolding process follows first order kinetics whereas aggregation follows the second

order kinetics because of which aggregation predominates over the aggregation at higher temperature [24]. In the current study it was observed that cold condition yields in higher percentage of correct refolded form with minimal aggregates (Fig. 10).

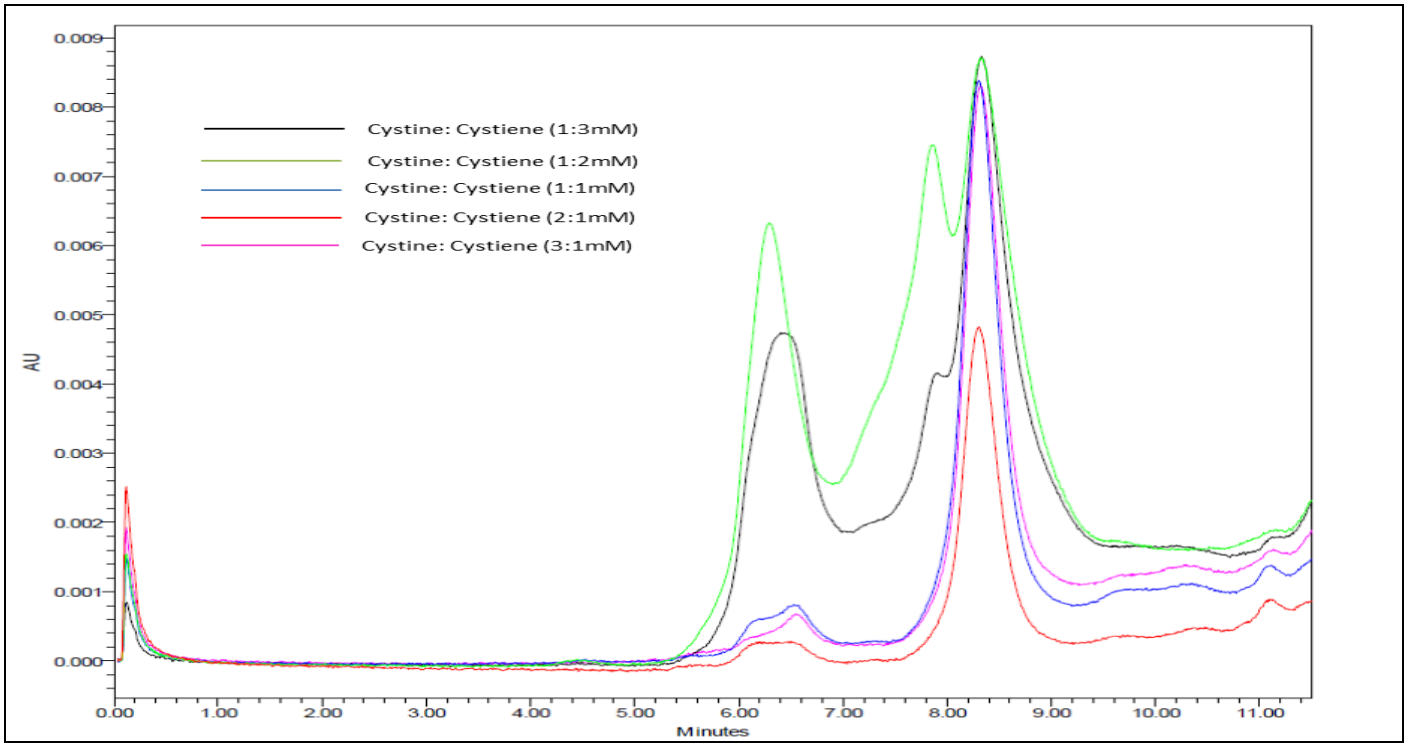

Fig.9: SEC HPLC profiles for variable redox potentials

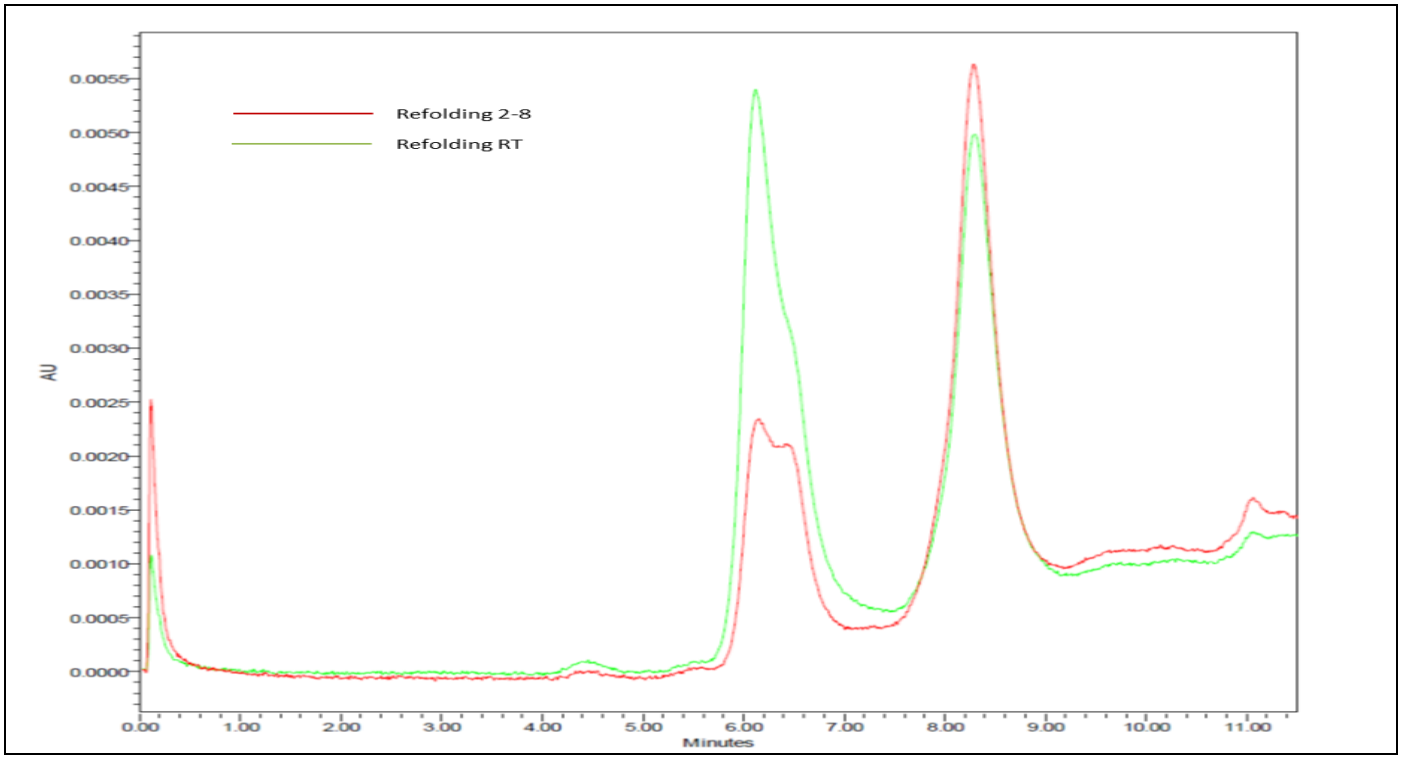

Fig. 10: SEC HPLC for refolding at different temperature

3.4.3. *Effect of Time of refolding reaction*

Refolding reaction was monitored for various time intervals using SEC HPLC. It was observed that refolding occurs spontaneously as solubilized IB is diluted with optimum Refolding buffer. There was no significant change in the SEC profile over the time (Fig. 11).

3.4.4. *Effect of Protein concentration*

Protein aggregation is higher order reaction while refolding is first order reaction. At higher initial protein concentration, the rate of aggregation is higher than rate of refolding. Thus with the increase in initial protein concentration the yields of correct refolded protein decreases [24]. In the current studies good yields of correct refolded proteins were obtained for the protein concentrations in the range of 0.1-0.3 mg/mL. As the protein concentration was increased beyond 0.5 mg/mL rate of aggregation was higher (Fig. 12).

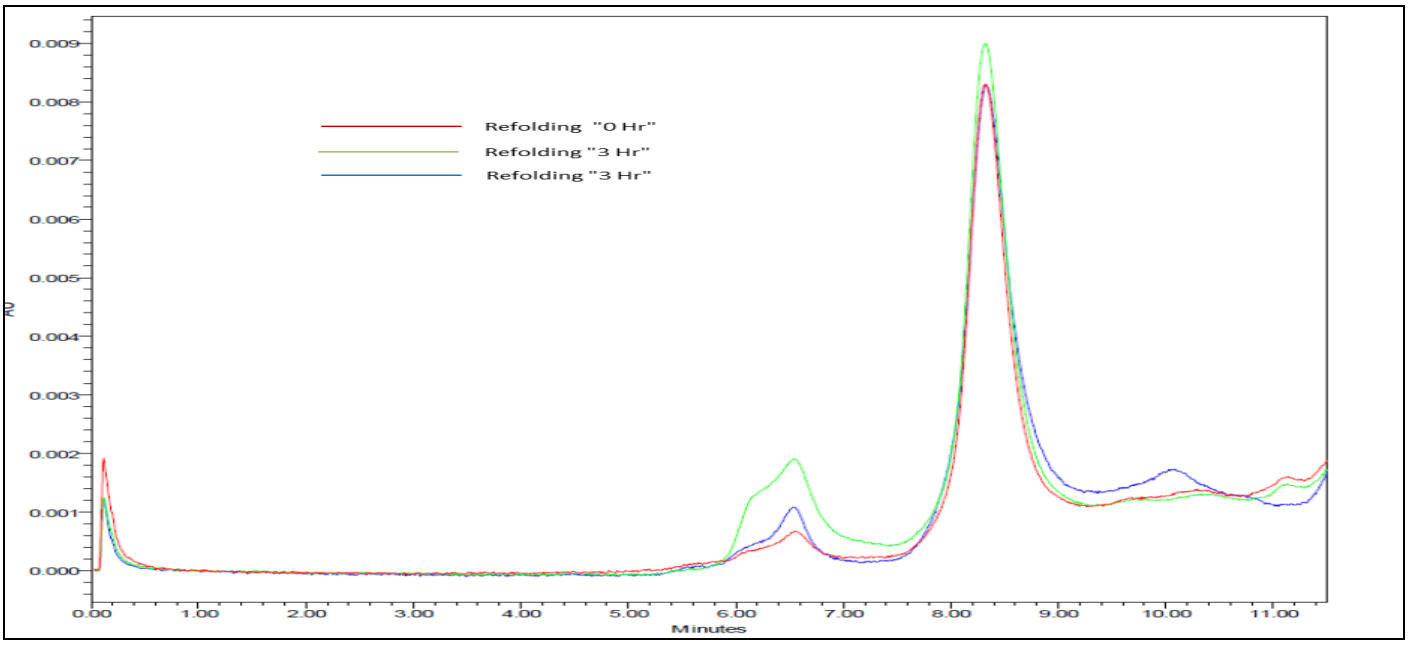

Fig. 11: SEC HPLC profiles for refolding samples at different time points

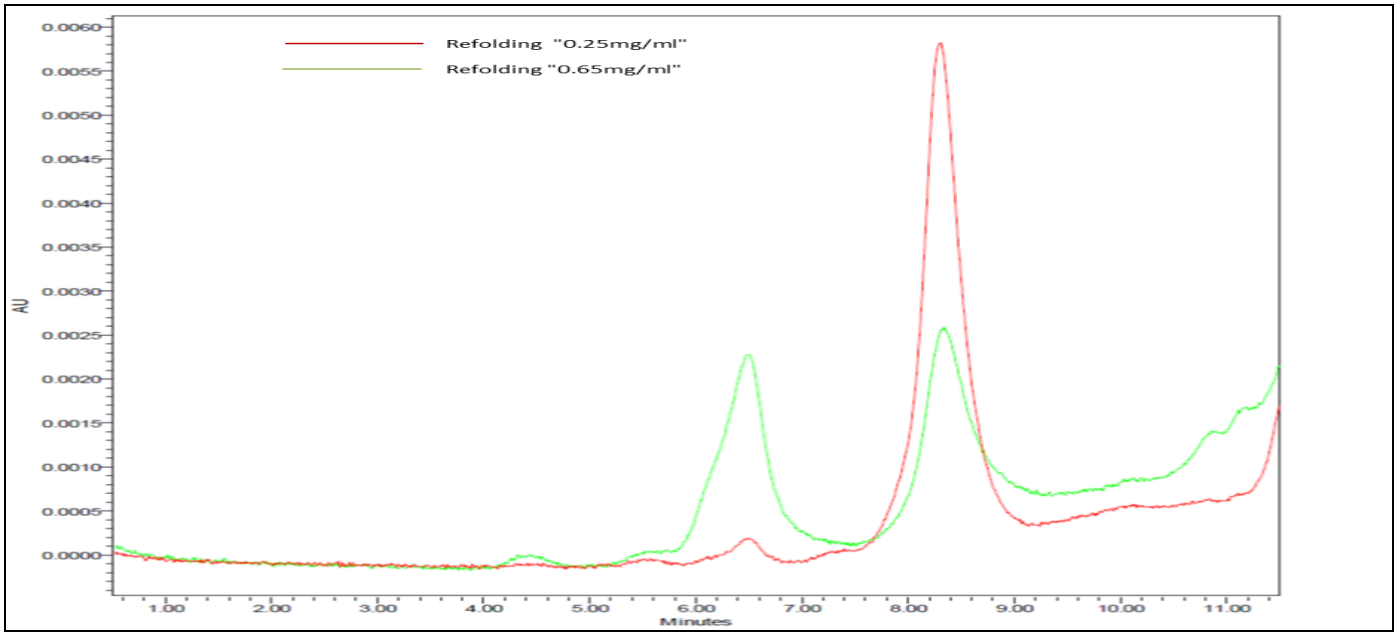

Fig.12: SEC HPLC Profiles for refolding with varying protein concentration

3.4.5. *Effect of Aggregate suppressor*

During refolding, proteins assume a partially folded state for a prolonged period instead of getting converted to the native structure. Thus, either protein structure must be stabilized or the structures that are not in the native state must be kept soluble. There are numerous solution additives that have been proved useful for this goal. They are mainly grouped into two classes, protein stabilizer and denaturants [24]. Arginine is used as an aggregation suppressor during refolding [21]. In the current study different concentration of Arginine (Arg) has been evaluated and it was observed that arginine at the concentration of 250mM gives best yield for correct refolded protein with minimum aggregate (Fig. 13).

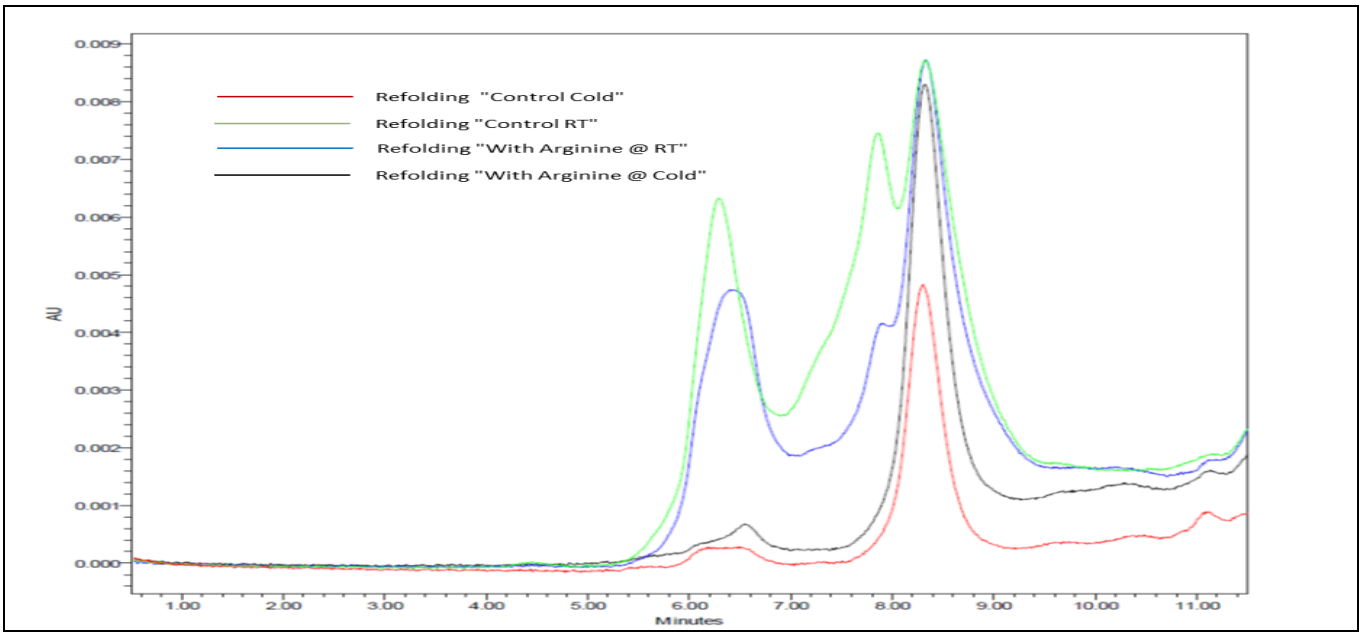

Fig. 13: SEC HPLC Profiles refolding with arginine at variable temperature

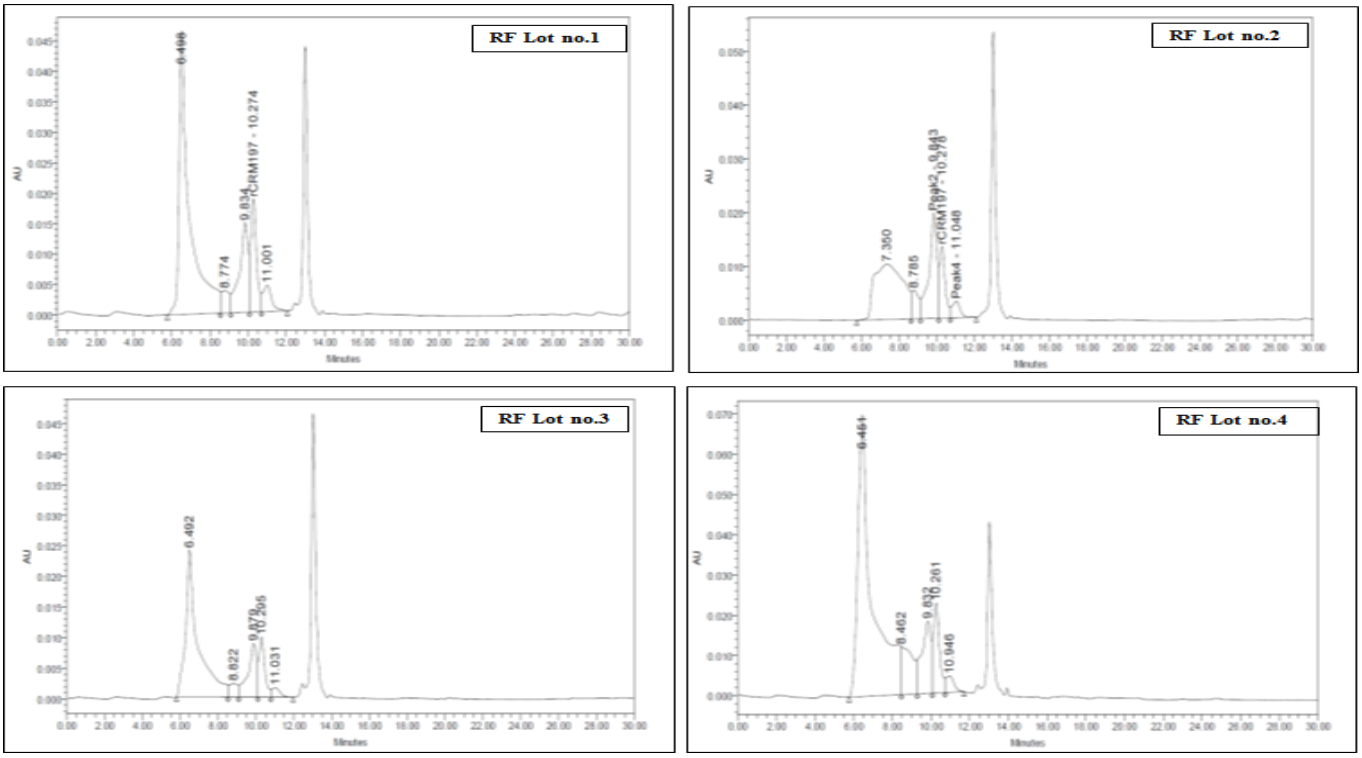

Fig. 14: SEC HPLC Profile of all the 4 lots of Refolding

Based on the above optimization study, all the 4 lots of solubilized IBs were processed further for Refolding batches (RF Lot no.1, RF Lot no.2, RF Lot no.3 and RF Lot no.4). After completion of refolding step, samples were withdrawn for Protein content analysis and SEC HPLC.

From the SEC HPLC profile of all the 4 lots, it can be observed that the Retention time for $rCRM_{197}$ protein is at 10.2 minutes. The first peak corresponds to higher molecular weight aggregates and the last peak corresponds to buffer.

From the above table, it is evident that in all the 4 lots, % Refolding is above 10% and Protein content is above 0.3 mg/mL. In all the 4 lots a consistent refolding profile was observed and a robust Refolding process was established for $rCRM_{197}$ protein.

Table 3: Comparison of all 4 lots of Refolding

Stage	Description	Lot no.1	Lot no.2	Lot no.3	Lot no.4
Refolding	HPLC-SEC CRM peak(% Refolding)	13.9	12.5	12.2	11.2
	Protein content (mg/mL)	0.64	0.30	0.38	0.60

4. CONCLUSION

E. coli offers a fast growth rate with high product yield. In the current study, $rCRM_{197}$ protein was expressed the *E. coli* BL21 (DE3) recombinant cells. Fermentation was performed using semi-defined optimized media for achieving high cell density culture. Over expressed proteins in the form of Inclusion bodies were recovered in refolded and functional form of native $CRM_{197}$by optimization of Cell lysis, solubilization and Refolding process. A robust & scalable process was developed which is safer to handle, compared to other traditional approaches. The optimized process resulted in generating high quality cost effective CRM_{197} with drastic reduction in the production time.

5. ACKNOWLEDGEMENTS

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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