SOLUBILIZATION, PURIFICATION AND NUCLEASE ACTIVITY OF RECOMBINANT CROSS-REACTIVE MATERIAL (rCRM197) EXPRESSED AS INCLUSION BODIES IN *E. coli*

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Abstract:

Background: In the current research, recombinant Cross-Reactive Material (rCRM 197) designed to form inclusion bodies in recombinant *E. coli* cells. High expression of recombinant proteins forms as aggregates in recombinant *E. coli* with misfolded and acts as inactive proteins. Regularly fermentation process is carried out at 37 °C to produce rCRM197 Inclusion bodies in recombinant *E. coli*, which forms very strong aggregates. As per researcher, reduction of temperature leads to less aggregates and more active refolded proteins can be formed. Inclusion bodies formed at low temperature called as non- classical Inclusion bodies.

Method: The fermentation process of rCRM197 in *E. coli* was carried out at 37 °C before the OD reaches to 55 OD, same time IPTG was inducted into the fermented and further fermentation process was carried out at 25 °C Temperature. After fermentation cells were harvested. Expressed proteins were then treated with 2M, 4M, 6M and 8M urea plus tris buffer at 8 pH. Expressed proteins formed as inclusion bodies in *E.coli* cells were lysed with high pressure homogenizer; lysed cells were centrifuged. Formed cell mass was divided into 2 fractions were washed and 1st fraction was treated with different concentrations of mild solubilizing agents like sodium lauryl Sulphate plus Triton X-100 and 2nd fraction was treated with strong denaturing agents Urea plus tris buffer solution. Purification was carried out with Q Sepharose resin. Purified samples were performed nuclease activity.

Results: Harvested samples were treated with 2M, 4M, 6M and 8 M urea without cell lysis were performed 10% SDA PAGE, expression of protein was negligible. 1st fraction samples were analyzed with 10 % SDS PAGE, results show that expression of protein concentration as compared with standard was less. 2nd fraction samples which were performed gel electrophoresis shown high protein expression and concentration, In 2nd fraction sample 3 was found as best and for the sample purification and nuclease activity was carried out.

Conclusion: Based on the results, 2nd fraction sample, which was solubilized in 8 M urea plus tris buffer was shown high concentration of protein, effective nuclease activity.

Keywords: Cross reactive material, Fermentation; polysaccharide vaccine; recombinant *E. coli.*

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INTRODUCTION

Haemophilus influenzae, *streptococcus pneumoniae*, *Neisseria meningitis*, *salmonella typhi* are polysaccharide capsules surrounds bacteria. This capsule is a virulence factor and it protects the bacteria from phagocytosis. Purified capsular polysaccharides elicit T-independent antibody responses without a memory function, capsulated polysaccharide bacteria like *Pneumococcal, Hib, Typhoid* are restricted to infants and compulsory immunizations at earliest to infants. The absence of efficacy of this vaccine in infants triggered development of conjugate vaccines which are so effective that there is now no room for plain polysaccharide *Hib* vaccines. The variance between polysaccharide antigen and protein antigen is that only polysaccharide vaccine provides T-independent antigens whereas the protein conjugated vaccines provide T-dependent immunity [1-2]. The *Hib* vaccines, *Pneumococcal* conjugate vaccine contain the capsular polysaccharide chemically conjugated to a carrier protein. This makes the non-T-dependent polysaccharide vaccines become T-dependent immune response vaccines for early age infants. One of the most widely used and highly effective carrier protein is Cross-Reactive-Material-197 (CRM197), the carrier protein is covalently linked to poorly immunogenic and T-cell-in-dependent capsular polysaccharides. rCRM197 (G52E mutation) is a mutant of Diphtheria toxin (DT) having 58kDa molecular weight, with less toxicity or non-toxic nature, favorable for the conjugation of most polysaccharide capsule containing *Haemophilus influenza, Streptococcus pneumonia*e of the polysaccharide vaccines [3-9]. As so many Carrier proteins are available to conjugate the polysaccharide vaccines, but the most preferable one is CRM197 because of its nontoxic nature. CRM197 known as a non-catalytic residue, and the crystal structures of DT showed that residue G52 is not directly involved in either NAD binding [10-12]. Production of CRM197 from native organism *Corynebacterium Diphtheria* having limitations like Endotoxin, Low protein recovery, protein folding issues, may require protein specification thereby limiting conjugation efficacy. Due to these limitations maximum researchers selected for expression of CRM197 in *Escherichia Coli (E. coli*) is a Rod–Shaped gram-negative, nonpathogenic bacterium and favorable host for large scale production of recombinant proteins typical strain will double in number every 20 min. Because of its multiplication capacity it's a choice for maximum recombinant protein production. It can grow in minimal medium, proteins under normal laboratory conditions. Bacterial strains used in recombinant DNA work are derivatives of *E. coli* Strain k12 [13, 14].

MATERIALS AND METHODS

Reagents and Chemicals: The E. coli strain BL21 (DE3) pET23 vector- GENSCRIPT, Luria broth medium (Yeast- Himedia, Tryptone- Himedia, Peptone-Himedia, NaCl-Merck), Zinc Sulphate, Copper Sulphate, IPTG, Tris buffer, Urea, Guanethidine- Manufacture by Merck. L-arginine, Triton X-100, Sodium lauryl Sulfate, Tris Buffer from Finar limited, Q Sepharose Resin, DEAE Resin, Column chromatography – GE health Care.

Equipment: Refrigerator Centrifuge– Electro lab, Centrifuge, Eppendorf, Probe Sonicator -PCI analytics, SDS PAGE gel–Bio-Rad, Agarose Gel Electrophoresis – Cleaver (Future bioscience), Mini Orbital Incubator, Rotary shaker - WAIOMETRA, UV spectrophotometer – Lab India. 10 L fermenter- WP-Winpact. Micropipettes -Bio-Rad

METHODS

Selection and optimization of media in shake-flask

Seed preparation: From working cell bank of E. coli (CRM197) which was stored at -70 °C was thawed and was cultured on a streak plate containing antibiotic kanamycin (52 mg /L) and was incubated at 37 °C for 24 hr. to obtain single colonies. Well grown colonies were used for the flask studies

Preparation of LB Media &Amplification of recombinant *E. coli* (CRM197) and Lysis: BL21 (DE3)/pET cells were cultured at 37 °C in 200 ml LB medium (1% Tryptone, 0.5% yeast extract, and 0.5% sodium chloride) supplemented with 52 mg/ml Kanamycin as antibiotic with shaking (240 rpm) in an Erlenmeyer flask (1 L) until they entered a log phase (OD600 = 1). The culture is then transferred into 10 L fermenter, which contains sterilized media adjusted to pH 7.0, temperature 37 °C before culture transfer antibiotic kanamycin was transferred through the sample porters to the sterilized medium. After OD >5.0, feed was given. After attaining the OD 55, isopropyl β-D-1- thiogalactopyranoside (IPTG) was added at the concentration of 0.5 mM to exponentially grown E. coli culture and cells were further cultured at 25 °C for 4 h to facilitate the formation of non-classical inclusion bodies. 100 ml of harvested sample was kept aside and the

remaining harvested sample was centrifuged using Refrigerated Centrifuge at temperature 4º C, 6000 G for 15 minutes. After the centrifugation cycle was completed, the soup was collected in a beaker. The cell pellet was collected in LDPE bags and both were kept at 4º C. Protein expression was done by using 10% SDS PAGE Gel electrophoresis [15].

Recovery of IBS without lysis of Cells: From 100 ml of Harvested sample, 8 Eppondrops were placed 2 ml sample in each tube. of Eppendorf tubes were centrifuged with table centrifuge. Soup was collected in empty Eppendorf and for cell pellet which was in few mg was solubilized with different concentrations of urea to know the inclusion bodies solubilization without cell disruption.

Isolation recovery of IBs: From Harvested cell pellet 400 mg of 4 samples were collected in 4 tubes (Each tube contains 100 mg of cell pellet) and suspended in lysis buffer (20 mM Tris buffer pH 8, with 10 ml buffer per gram wet weight cell pellet), and cell disruption was carried out by using probe Sonicator. The OD values decreased in each cycle. At 4th cycle there was over 95% lysis was completed which was confirmed by OD at 600 nm. The recovery of IBs after lysis optimization was accomplished by centrifugation. The cell lysate was centrifuged at 6000 rpm, 4 °C for 15 min. after centrifugation soup was collected and labelled as 1st wash, for same insoluble pellet lysis buffer was added repeated the centrifugation, collected the soup and labeled as 2nd wash, for same sample third wash done with tris buffer and collected the soup and cell pellet.

Solubilization (Inclusion Bodies -IB): After 3rd wash samples were divided into 2 fractions. These 2 fractions which contains inclusion bodies were treated with different denaturing agents (Low to high concentrated). The 1st fraction with 100 mg inclusion bodies pellet was dissolved with different concentrations of sodium lauryl Sulphate plus Triton X 100. The second fraction with 100 mg inclusion bodies dissolved with different molarities of Urea + tris buffer solution. Solubilization of all samples was analyzed by 10 %SDS PAGE electrophoresis. Based on expression study on SDS PAGE best expressed samples further preceded for purification and refolding.

Purification (Q-Sepharose, Ion Exchange Chromatography):

The Acrylic column was packed with Q-Sepharose and connected to Bio-Rad Chromatography System. The Resin was packed based on the column height and width. The load sample capacity was calculated based on the protein concentration. The resin was equilibrated with Equilibration Buffer (20 mM tris buffer). Pass the Sample solution (Load solution) at 2 ml/min. and collect the sample flow through. Then passed Wash Buffer (20 mM tris buffer), then Elution Buffer (100 to 1 M NaCl and 20 mM tris), collect the fractions according to the absorbance. The protein was analyzed by using SDS PAGE Analysis and under UV-Spectroscopy in OD280 nm [16-18].

Refolding buffer

Refolding buffer contains 15% Glycerol, 0.5 M Glucose, 20Mm of Tris HCl, 1mMEDTA, 0.1MNaCl and 0.4ML-Arginine. Samples which were solubilized in 0.2% Sodium lauryl Sulphate and Triton X-100,8M urea and tris solubilized samples dissolved in 50 ml of Refolding buffer containing L- arginine solution separately and adjusted to pH 8.0.

SDS-PAGE (10%) ANALYSIS

SDS/PAGE electrophoresis was performed to know the expression of protein as well as purified sample analysis. CRM along with the reference CRM 197 was loaded on to 10% SDS-PAGE. Gels were run at 90-150 V different voltage until blue dye reached the bottom of the gel. SDS/PAGE molecular weight markers (Bio labs) were used for molecular weight. After Coomassie Brilliant Blue staining, distain the same gel the image was observed visually.

Nuclease activity of rcrm197 by AGAROSE GEL ELECTROPHORESIS [19-21]

Nuclease activity was analyzed by using agarose gel electrophoresis (1.5 % Agarose gel) and image was captured under UV Trans illuminator. 300 mg of agarose is weighed and mixed with 25 ml of TEA (1 X) and Et-Br is added (1µl). Kept in oven for some time and remove, pour into agarose glass plates. Then do electrophoresis.

Table 1. Nuclease activity Assay: Buffers preparation:

Chemicals	Concentration	Volume	Weight (Gm)
Tris HCl	10Mm	30 ml	0.047g
$CaCl_2$	2.5 mM	30 ml	0.008 g
$MgCl_2$	2.5mM	30ml	0.007 g
Adjusted the pH – 7.6			

Nuclease activity assay for diluted and refolded samples:

Assay I: 1:2 dilutions of solubilizes protein with refolding buffer (20 mM tris + 0.4 ml L-arginine pH-10) was taken and mix with assay buffer incubated at Room temp for 4.5 h along with 3 µl of plasmid DNA.

10 µl of 1:2 dilution of refolded protein + 150 µl of assay buffer (10 mM tris –HCl+ 2.5 mM $CaCl_2$ +2.5 mM $MgCl_2$) + 3 µl of plasmid DNA.

Assay –II: 150 µl of 1:4 dilution of refolded protein + 150 µl of assay buffer + 3 µl of plasmid DNA.

Assay-III: 150 µl of 1:4 dilution of 3M urea containing protein +150 µl of assay buffer +3 µl of plasmid DNA.

RESULTS AND DISCUSSION

rCRM197 working cell bank was thawed and inoculated on agar plate after growing better single colony was transferred to 200 ml media containing flask, after reaching the OD at 600 nm 1, the culture transferred to the 10 L fermenter which was previously sterilized. After 16 hours of fermentation, the OD was reached 55. Then the final hr. harvested sample was collected and analyzed the expression of desired protein with 10 % SDS PGAE gel electrophoresis. After getting confirmation from SDS-PAGE the harvested sample processed further. 10 % SDS PAGE protein expression of 4thhr, harvested sample soup and cell pellet results shown in Figure 1.

From figure 1 observed that rCRM197 was not expressed in supernatant or soup of the harvested sample. Hence the soup or supernatant of the harvested centrifuged sample was discarded.

As the fermentation process was carried out at 25 ℃ after induction of protein expression agent IPTG, the final harvested sample 100 ml collected was decided to perform the solubilization with different molarities of urea and to reduce the tedious process like cell lysis, and cost of the process.

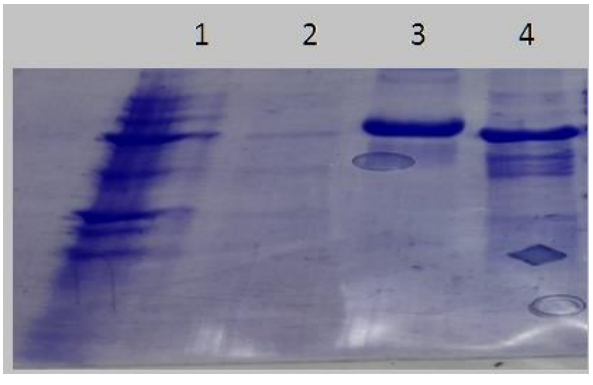

Figure 1. Expression of CRM197 protein samples collected from harvested cells.

Lane 1, 2, 3, 4 are 4th hr. harvested sample, soup of harvested sample after centrifugation, standard sample of rCRM197, cell pellet after centrifugation of harvested sample respectively.

From the 100 ml harvested sample 16 ml of sample was placed in 2 ml volume of 8Eppendorftubes which were then treated with 2M, 4M, 6M and 8M and 10M urea. The samples were loaded in gel and expression results shown in figure 2. Gel was loaded with 4th hr. harvested sample with 15µl, 2M, 4M, 6M and 8 M urea solubilized samples 15 µl along with the Standard, BSA and Protein marker.

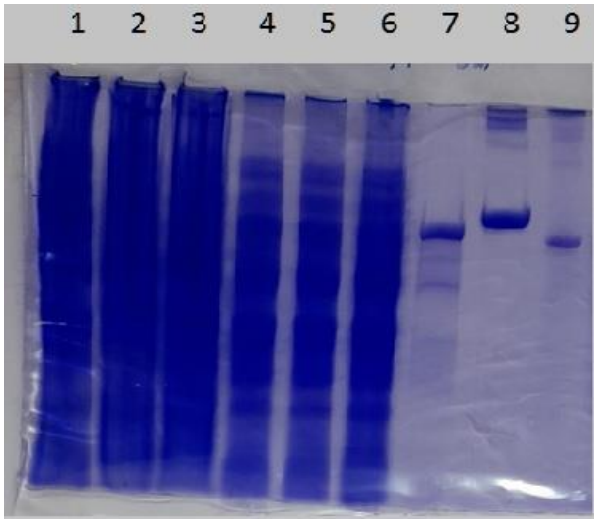

Figure 2. Different Molar concentration of Urea on the solubilization of rCRM197 inclusion bodies without cell lysis.

Lane 1 is 4th hr. harvested sample as load; Lane 2, 3, 4, 5 and 6 are 2M, 4M, 6M, 8M, 10M urea's respectively; Lane 7, 8, 9 are standard rCRM197; BSA (Bovine serum Albumin), protein marker respectively.

From figure 2, 10 % SDS PAGE results it was observed that negligible amount of the sample was expressed which was not matching the intensity of the any standard used in Gel.

Hence further cell lysis was carried out for obtained cell pellet. Isolated inclusion bodies were centrifuged and washed for 3 times to remove the cell debris and impurities. Divide the pellet in to 2 fractions. Few mgs of first fraction sample were collected in 15 ml falcon tube then solubilized the inclusion bodies with 1M to 8 M SLS with triton X-100.And obtained samples were loaded in 10% SDS PAGE, protein expression Results shown in Figure 3.

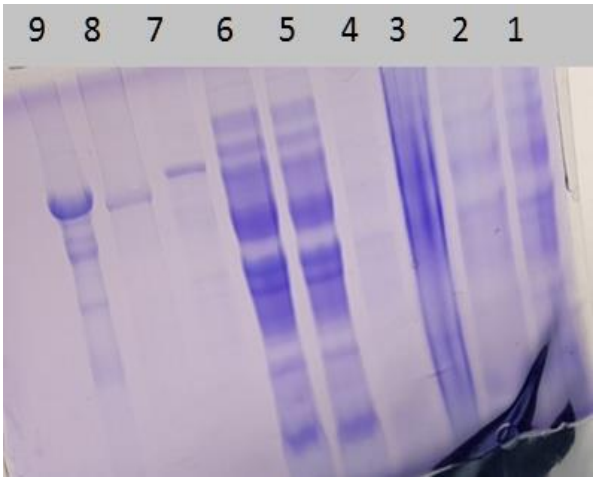

Figure3. Solubilization (SLS and Triton X -100) study expression in 10 % SDS PAGE Gel electrophoresis.

Lane 1, 2, 3, 4, 5, 6, 7 and 8 - 1M, 2M, 3M, 4M, 5M, 6M, 7M and 8M SLS and with Triton X-100 respectively; Lane 9 - Standard rCRM197
As shown in figure 3, inclusion bodies very slightly solubilized in SLS and Triton X-100 detergent solution.

Figure 4: SDS PAGE 10%, expression of rCRM197 with 1M to 8 M urea plus tris buffer pH 8.

Lane 1, 2, 3, 4, 5, 6, 7 and 8 are solubilization of pellet with 1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M urea respectively. Lane 9 - Standard sample; Lane 10 – BSA.

Observations from Figure 4, the expression of protein increased as the urea concentration was increased and the impurity pattern also visible. From 1 M to 8 M the high molecular weight impurities were cleared with urea plus tris solution. The intensity of the protein expression was very high in 8 M urea plus Tris buffer with 8 pH (figure 4) clearly visible that low molecular weight impurities were not cleared from the rCRM197 solubilized samples. Only 8 M urea solubilized samples were purified with Q-Sepharose, ion-change resin with NAC chromatography and samples were loaded in the 10 % SDS Gel electrophoresis.
Purified sample was used for the refolding of rCRM197. By nature, CRM197 has nuclease activity, CRM197 is nontoxic but it has nuclease activity toxic to cells [22]. rCRM197 protein proper folding and activity was confirmed with Agarose gel Electrophoresis, results were shown in figure 6. Refolding activity was Solubilization of IB protein using mild conditions without disturbing the native structure was a key to high nuclease activity. Overnight refolding was carried out in the ratio of 1:20 dilution with 800 ml of refolding buffer.

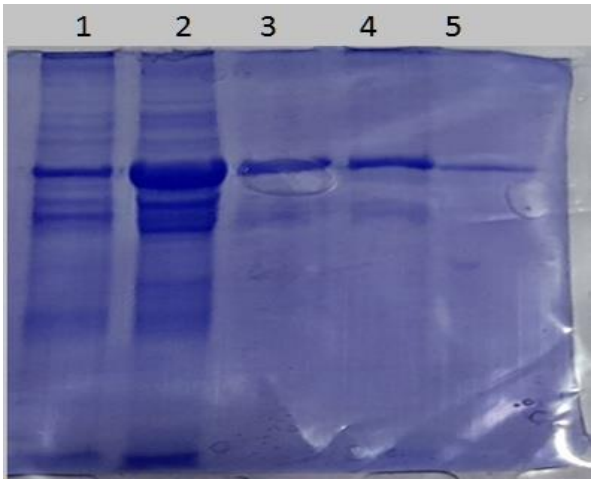

Figure 5. Purification sample (Q-Sepharose) 10 % SDS PAGE, sample solubilized in 8 M urea and tris buffer pH 8.

Lane 1, 2, 3, 4, 5, are sample flow through, solubilized sample, elution 1, elusion 2, elution 3 (Purity high no impurities observed) respectively

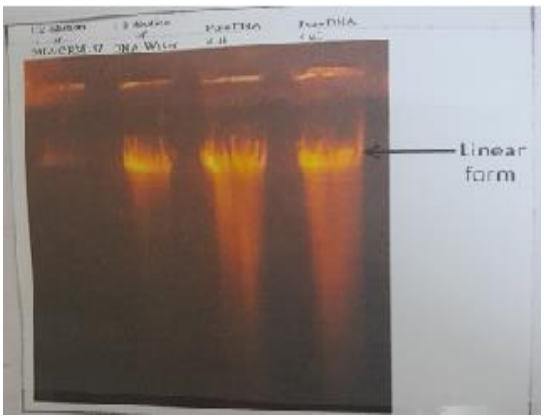

Figure 6. Nuclease activity of sample solubilized in refolding buffer and solubilized in 8M urea and tris buffer at pH8 purified sample.

Lane 1- Assay set 150 µl of refolded protein (1:4 dilution) + 150ul of 20mM tris, 0.4 M arginine containing buffer (pH-10) with rCRm197 along with DNA the clearance of the super coiled form of DNA is more from 1 to 5 hr. incubation of sample; Lane 2- Plasmid DNA with distilled water dilution; Lane 3- Plasmid DNA

CONCLUSION

In this study, fermentation process after IPTG induction was carried out at 25 ℃ and formed inclusion bodies of rCRM197 solubilized in the mild detergents (SLS and Triton X 100) and denaturing agents like Urea plus tris buffer. As per literature fermentation process which is carried out at 25 ℃ after IPTG induction, the formed inclusion bodies were less aggregated and mild misfolded in nature. To solubilize the non-classical inclusion bodies very mild solubilizing agents are required. In this study more misfolded or inactive proteins were observed. The obtained proteins were solubilized in mild solubilizing agents and strong denaturing agents (Urea plus tris buffer) with different concentrations. Samples shown more protein concentration which are dissolved in 8M urea plus tris buffer (pH 8). Purification was carried out with Q-Sepharose resin. The purified proteins of rCRM197 refolded with arginine were shown effective nuclease activity.

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