Research Article

A Conventional Method for Fermentation and Purification of Recombinant Human Interleukin 24 from *E. coli*

Muhammad Imran Amirzada[1], Xiaohai Gong [2], Yun Chen [2], *Jian Jin[2]

1. Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu 214122, PR China.
2. School of Pharmaceutical Sciences, Jiangnan University, Wuxi, Jiangsu 214122, PR China.

ABSTRACT

Recombinant human interleukin 24 is a member of cytokine family. Recombinant human interleukin 24 is well known as human biological beneficial protein. Recombinant human interleukin 24 production from *E.coli* with a conventional method is a step to produce a low amount of recombinant protein for characterization and biological properties. The expression of eukaryotic proteins in *E. coli* leads to formation of insoluble inclusion bodies (IBs). Inclusion bodies solubilization and refolding is a key challenge for active therapeutic protein production. The recovery of recombinant human interleukin 24 from inclusion bodies is a bottle neck of downstream processing. Protein purification not only increases the final product but also improves the quality of final product. In current research work, we practiced the conventional method for production and purification of recombinant human interleukin 24. The fermentation strategy was based on application of LB media for batch culture. The high pressure homogenizer was used for cell lyses. Traditional approach for IB solubilization and refolding was applied to produce a low sample volume for purification. The anion & cation exchange complex chromatography was applied to remove impurities from the sample and to produce purified product. According to conventional method a negligible recombinant human interleukin 24 was produce with more effort and more time consumption.

Keywords: rhIL24, inclusion bodies, refolding, purification, protein

Received 04 Dec 2013 Received in revised form 15 Dec 2013 Accepted 17 Dec 2013

***Address for correspondence:**
Jian Jin
School of Pharmaceutical Sciences, Jiangnan University, Wuxi, Jiangsu 214122, PR China.
E-mail: jinjian31@126.com, imranamirzada@googlemail.com

INTRODUCTION

In 1995 the melanoma differentiation associated gene-7 (mda-7) was identified [1] and re-designated as interleukin-24 [2], rhIL-24 has a growth suppression and induction of apoptosis in a broad range of cancer cells, but not in normal human cells [3]. The over-expression of recombinant proteins in Escherichia coli (E. coli) is extensively used to produce proteins in large amounts, due to advantages of growth on inexpensive sources, rapid biomass growth and high production [4-7]. Numerous transgenic proteins expressed in E. coli are recovered as insoluble aggregates in the form of inclusion bodies. Inclusion bodies are dense porous particles of aggregated protein. One or a few different proteins are inside the IB, and no ribosomal components or nucleic acids are present and they are held together by non-covalent hydrophobic or ionic bonds [8, 9]. Inclusion bodies are dynamic structures formed by unbalanced equilibrium between aggregated and soluble proteins of E.coli [10] Recovery of recombinant protein from (IBs) is difficult and involves inclusion bodies solubilization in a denaturant and protein refolding [11]. Conversion of the inactive and insoluble protein into soluble and correctly folded product is a major bioprocess challenge [12, 13]. In this study, a conventional approached for production of recombinant human interleukin 24 (rhIL24) was developed which composed of batch culture in LB Medium and a complex time consuming strategy of Anion & Cation Exchange Chromatography.

MATERIALS AND METHODS

Fermentation Media

LB (Luria-Bertani) medium was used for plate cultivation of *E. coli* strain BL21 (DE3) and for preparation of seed culture. Batch cultivations were simultaneously carried out in 2 L bench-top bioreactors with the working volume of 1L [1].

Batch Cultivation

Batch culture was started by adding 100 ml of an overnight-incubated seed culture (OD600= 0.7-1) into the bioreactor containing 900 ml of medium. The pH was controlled at 7±0.05 by the addition of 25% (w/v) NH4OH or 3 M H3PO4. Dissolved oxygen was controlled at 30-40% of air saturation by controlling both the inlet air and agitation rate. Foaming was controlled by adding silicon-antifoaming reagent. In batch culture, cells were induced by the addition of 1mM IPTG. The production phase continued until the growth ceased.

rh-IL24 purification

Cell lysis and IB recovery: The fermented broth was centrifuged at 4°C and 8000 g for 30 min and the obtained pellet was washed twice with 50 mM phosphate buffer pH 7.4. The wet cells (50 g) were suspended in 200 ml of lysis buffer. The lysis buffer composition was 50 mM Tris–HCl containing 1 mM EDTA. The cells were broken by passing the medium through a homogenizer three times at 800 bar. The cell homogenate was centrifuged for 30 min at 6000g at 4°C, the supernatant was discarded and the inclusion bodies recovered.

IBs washing: The IBs pellet obtained in previous step was resuspended in wash buffer (2.5 g l-1 Triton X-100, 50 mM Tris–HCl pH 8.0 containing 5mM EDTA) and incubated 40 min and recovered by centrifugation at 25–28°C for 30 min at 8000 g. In the second washing, the IBs pellet was resuspended in wash buffer (1 M urea) and incubated 40 min and recovered by centrifugation at 25–28°C for 30 min at 8000 g.

IB solubilization and refolding*:* Washed inclusion bodies were dissolved in 30m M Tris–HCl, pH= 8, containing 6 M urea,1 mM EDTA and 100 mM GSH, The solution was incubated at 25–28 °C for 45 min and spun down at 10,000 g for 30 min to get rid of insoluble cell debris and recovered by centrifugation at 25–28 °C for 30 min at 8000 g. and then solubilized inclusion bodies was refolded by refolding buffer that the refolding buffer protocol is 30 mM Tris–HCl (pH =7.5), 2 mM GSSG, 20 mM GSH, 1 mM EDTA, 3M Urea and incubated 12 hours at 4°C. After completion of refolding, the protein pH was adjusted with 2 M citric acid and centrifuged at 10,000 g for 20 min at 4°C.

Anion & Cation Exchange Chromatography*:* The pH of Refolded protein was adjusted to 5-6 by adding 2 M Acetic acid and then loaded in mono Q column. The column temperature and flow rate were maintained at 20 °C and 1 ml/min respectively throughout the process. The column was equilibrated with 3 bed vol. of 25 mM sodium acetate buffer (pH= 4.5). The refolded protein sample was directly loaded on to the column at the same flow rate. The column was extensively washed with 3 beds vol. of the same buffer but with 1 M NaCl. The columns Q-Sepharose eluted peak secondly flow through the SP-Sepharose Fast Flow column and at 5% to 30% elution conditions eluted peaks were collected.

SDS-PAGE

In the reduced SDS-PAGE 15% gel and rh-IL24 and a molecular weight marker were used. Sample buffer [0.5 M Tris pH 6.8, 50% (v/v) glycerol, 100 g l-1 SDS, 20 g l-1 bromophenol blue and 50 g l-1 2-mercapto ethanol (2-ME) was added to samples before boiling for 5 min. The samples were loaded on to the gel and ran at a constant voltage of 120 V for 100 min. Gels were stained with Coomassie brilliant blue R250.

RESULTS AND DISCUSSION

Cell lysis and IB isolation

The cell lysis was experiential at different sonication pulses of 5, 10, 15 and 20 s and at a different homogenizer pressure (600, 800. 1200 bar). The highest IB recovery was observed by passing the medium through a homogenizer three times at 800 bar. IB recovery was measured by the Bradford method.

IB washing

The IBs separated after homogenizer was found to be contaminated. The Impurities interfere with refolding and purification. IBs washed repeatedly to make free from

impurities. Removal of the impurities and obtaining the IBs with high recovery is difficult task and bottle neck for high purity protein production. The optimization of detergent concentration also has effect on the efficiency of IB recovery. Triton X-100 was used to solubilize the bacterial cell wall components and sodium deoxycholate also applied to remove any residual cell debris particles

IB solubilization and refolding

IBs solubilization was achieved to applied minimum amount of denaturant. IBs produced in *E. coli* used high amount of detergent and chaotropic agent, and additional steps had to be in use to eliminate these agents, so we optimize the amount of denaturant use in solubilization. The combination of denaturant/reducing agent has an impact on solubilization and refolding. Hence we use different concentration of urea (3, 6, 8 M) in different pH (6, 8, and 12). The key to refolding is to remove enough denaturant to allow the protein to fold properly, but keep enough denaturant in the refolding buffer to allow proteins to fold/refold several times until attains the proper conformation protein. A single straight forward method which satisfies all protein folding requirements remains the desire object. A novel solubilization process which reduces the propensity of protein aggregation followed by improved refolding will help in the high recovery of recombinant protein from inclusion bodies [14]. As previously reported in our work that the composition of medium and strategy for solulization and refolding not only reduce the cost but also improved the quality and quantity of final product [15].

Anion & Cation Exchange Chromatography

The ion-exchange column was used for the removal of impurities and high purity. In this study we use Q Sepharose Column and Sp Sepharose Column. The purpose for dual purification was to remove impurities and obtained high quality rh IL24.

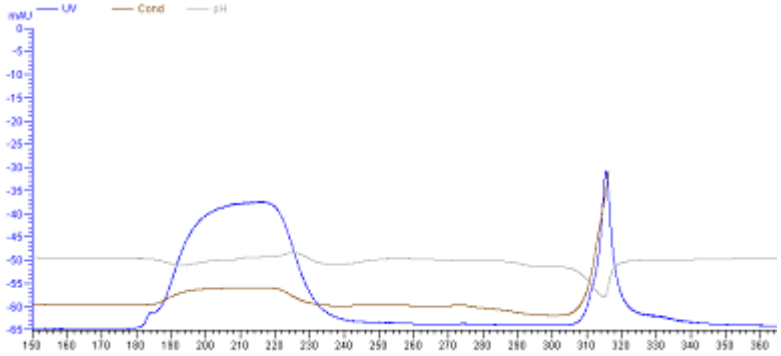

Figure 1: rhIL24 Refolded Sample Flow through the Q Sepharose Column for Initial Purification

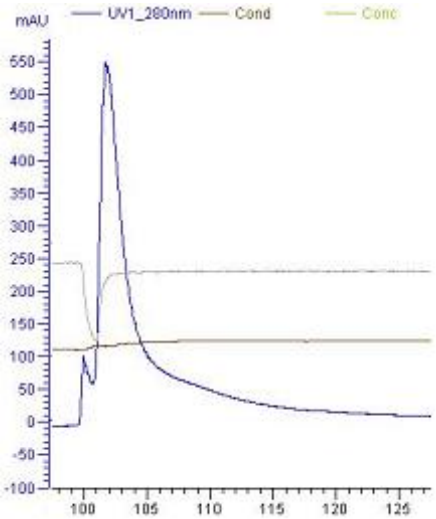

Figure 2: Initially Purified Sample Flow through SP Column for Final Purification

This was not so convenient for large scale production but helpful to obtain low amount with less impurities. The results obtained determine improvement of the method for effective removal of impurities.

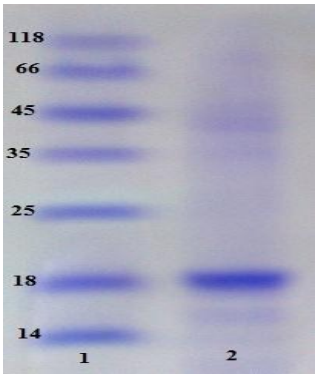

Figure 3: SDS-PAGE of Purified rhIL24. (Lane # 1 is molecular weight marker and Lane #2 is purified recombinant human interleukin 24)

The SDS-PAGE gel electrophoresis shows the purified rh-IL24 by IEC. The final step of the purification showed a single band of 19 kD.

CONCLUSION

In the present study, a conventional method for production of recombinant human interleukin 24 from *E. coli* was developed. The final yield of purified recombinant human interleukin 24 was negligible but although the process established in current research may be functional in the recovery of other proteins expressed in *E. coli.*

REFERENCES

1. Jiang H, Su Z.Z, Lin J.J, Goldstein N.I, Young C.S, Fisher P.B, The melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression, Oncogene (1995) ;11: 2477–2486.
2. Caudell E.G, Mumm J.B, Poindexter N, Ekmekcioglu S, Mhashilkar A.M, Yang X.H, Retter M.W, Hill P, Chada S, Grimm E.A, The protein product of the tumor suppressor gene, melanoma differentiation- associated gene 7, exhibits immunostimulatory activity and is designated IL-24, J. Immunol (2002); 168: 6041–6046.
3. Amirzada M. I, Jin J. Therapeutic applications of interleukin 24, A review. Trop. J. Pharm. Res (2012) ; 12 (6) : 1023 – 1027.
4. Baneyx F, Protein expression technologies: current status and future trends. Wymondham, Norfolk, U.K., Horizon Bio-science (2004).
5. Baneyx F, Recombinant protein expression in Escherichia coli. Curr Opin Biotechnol (1999) 10: 411–421.
6. Baneyx. F, Mujacic, M. Recombinant protein folding and misfolding in Escherichia coli. Nat Biotechnol (2004); 22:1399–1408.
7. Makrides SC, Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev (1996);60:512–538.
8. Menzella HG, Gramajo HC, Ceccarelli EA. High recovery of prochymosin from inclusion bodies using controlled air oxidation. Protein Expr Purif (2002) ; 25(2) :248-255.
9. Parrilli E, Giuliani M, Marino G, Tutino ML. Influence of production process design on inclusion bodies protein: the case of an Antarctic flavohemoglobin. Microb Cell Fact (2010); 9: 19.
10. Villaverde, A. and Carrio, M.M. Protein aggregation in recombinant bacteria. bilogical role of inclusion bodies. Biotechnol. lett. (2003) ;25:1385-1395.
11. Carrio M.M, Villaverde A. Construction and deconstruction of bacterial inclusion bodies, Journal of Biotechnology (2002) ; 96: 3–12.
12. Clark,E.D. Protein refolding for industrial processes.Curr.opin.Biotechnol. (2001) ; 12, 202-207.
13. Panda,A.K. Bioprocessing of therapeutic proteins from the inclusion bodies of Escherichia coli.Adv. Biochem. eng/Biotechnol., (2003) ; 85:43-93
14. Singh,S.M. and Panda, A.K. Review, Solublization and refolding of bacterial inclusion body proteins. J. Biosci. Bioeng., (2005) ;99 (4) : 303-310.
15. Amirzada MI: Yu M: Gong X: Chen Y: Zhu R: Lei J et al: Cost-effective production of recombinant human interleukin 24 by lactose induction and a two-step denaturing and refolding method. (Accepted, in press. DOI: 10.1007/s10295-013-1367-2).