CHAPTER 4.

RESULTS & DISCUSSION

(Recombinant Human GM-CSF Purification)
Recombinant Human GM–CSF Purification

The essential strategy for generating scFvs against recombinant GM-CSF was to immunize mice and isolate the enriched mRNA to form \( V_H \) and \( V_L \) gene pools. These would be then linked together by the gene spacer peptide to to get scFv in an expression library followed by screening for the scFv with the desired specificity and affinity. As a first step to this protocol it was necessary to optimize the immunization strategy which would lead to maximum enrichment, this would significantly reduce the number of clones that needed to be screened in the expression library. This in turn required the availability of pure GM-CSF typically in milligram quantities a problem given that expression levels of GM-CSF are known to be extremely poor in \( E. coli \). Fortunately work in being carried out in our lab on resolving the bottle necks in GM-CSF expression and thus GM-CSF has all ready been cloned in a series of expression vectors in \( E. coli \). Also we have developed standard protocols for over expression of a wide range of proteins and these guide lines were helpful in designing strategies for over expression of GM-CSF. Essentially two methods of getting over expression are used. The first is to use signal peptides and fusion tags to secrete the protein in its soluble form to the periplasm where its toxicity towards the host is lower, followed by purification using affinity chromatography. The second strategy is to express the protein cytoplasmically as inclusion bodies, thus sequestering it inside the cell in an inactive form. However this requires additional refolding steps in order to get the active protein. A second requirement of purified GM-CSF was to use it to coat the ELISA plates which would then be used to screen the scFv which bind specifically with GM-CSF. The screening strategy for the scFv was to use a sandwich ELISA technique. For this it was decided to produce single chain antibody library in the expression vector pET22b. Here the scFv gene fragments would be cloned downstream of the pel B signal sequence. Expression through this vector would produce a scFv polypeptide with a pelB signal sequence at the N-terminal and a 6X Histidine tag at its C-terminal end. According to our screening strategy, the antigen protein coated onto the ELISA
wells with GM-CSF would bind to the scFv (primary detection reagent) and the bound scFv would be detected in the ELISA using HRP-linked anti His tag antibodies. Hence a first characteristic requirement of our antigen protein (rhGM-CSF) was, that it should not be expressed with a His tag. Moreover we required 90-95% pure antigen protein to prevent non-specific binding.

It was initially decided to obtain GM-CSF in soluble form using a construct pMAL-GMCSF which expresses the recombinant protein without a His tag (Fig. 4.1).

![Diagram](image)

Fig. 4.1: Schematic representation of recombinant plasmid pMAL-GMCSF construct. pMALp2 is 6.72 Kb. GM-CSF gene is 400bp

pMAL p2 expression vectors provide a well established method for expressing and purifying a protein produced from a cloned gene. The cloned gene is inserted downstream from the malE gene of *E. coli*, which encodes a maltose binding protein (MBP), resulting in the expression of an MBP fusion protein (Guan, et al. 1987; Maina, et al. 1988). The method uses the strong "tac" promoter and the malE translation initiation signals to give high-level expression of the cloned sequences (Amann and Brosius, 1985; Duplay, et al. 1984). This is followed by a one step purification of the fusion protein using MBP's affinity for maltose (Kellerman and Ferenci, 1982). Also the protein is secreted to the periplasmic space which harbors less contaminating proteins as compared to the cytoplasm and contains fewer proteases. Thus BL21 (DE3) competent cells were
transformed with the pMAL-GMCSF and cultured in TB (terrific broth) and induced at an O.D. of 2. For higher periplasmic yields of the fusion protein pMAL-GMCSF cultures are recommended to be induced at 0.3mM to 0.5mM IPTG concentrations. Therefore two flasks were used where the above IPTG concentrations were used. Cells were harvested at the 4th hour post induction and periplasmic preparation was optimized with method I and II (see Materials and Methods) for both (0.3mM and 0.5mM) IPTG concentrations. The optimized periplasmic preparation using the flask induced with 0.3mM IPTG gave optimum fusion protein yields (Fig. 4.3a and b).

![Fig. 4.2a: Periplasmic preparation of MBP-GMCSF fusion protein:](image)

Lane 1: Periplasmic MBP-GMCSF fusion marker
Lane 2: Overnight culture (pMAL-GMCSF, BL-21 cells)
Lane 3: 0.3 mM IPTG / 2nd hour / total cell lysate
Lane 4: 0.3 mM IPTG / 2nd hour / periplasm method I
Lane 5: 0.5 mM IPTG / 2nd hour periplasm method I
Lane 6: 0.3 mM IPTG / 2nd hour periplasm method II
Lane 7: 0.3 mM IPTG / 4th hour total cell lysate
Lane 8: 0.3 mM IPTG / 4th hour periplasm method I
Lane 9: 0.5 mM IPTG / 4th hour periplasm method I
Lane 10: 0.3 mM IPTG / 4th hour periplasm method II
The periplasmic sample was then purified using amylose column purified since the GM-CSF contained the MBP fusion which binds selectively to amylose. For this the periplasmic fraction was loaded on to the column in column buffer (20 mM Tris pH7.4, 200 mM NaCl, 10 mM β ME, 1mM EDTA) and eluted using 10mM maltose containing elusion buffer (20 mM Tris pH 8, 10mM maltose, 200 mM NaCl, 10 mM β ME) (Fig. 4.4).
pMAL p2 expression vectors also contain the sequence coding for the recognition site of a specific protease, Factor Xa which cleaves the MBP fusion tag from the expressed protein (Nagai and Thogersen, 1984 and 1987). Factor Xa cleaves after its four amino acid recognition sequence [Ile-(Glu or Asp)-Gly-Arg] so that no vector-derived residues are attached to the protein of interest.

An experiment was set up with the amylose column purified fusion protein to find the optimum protease concentration and time required to get 90%-95% cleavage of the fusion protein. The optimum enzyme concentration was standardized between 0.5% and 1% Factor Xa (0.5 µg or 1 µg, Factor Xa / 100 µg of fusion protein) for 2hrs, 4hrs, 8hrs and 24hrs at 25°C (Fig. 4.5). The experiment showed that Factor Xa protease when used at a concentration of 1 µg per 100 µg of fusion protein cleaves the fusion protein in 8hrs at 25°C to a considerable extent whereas longer incubations resulted in GMCSF degradation.

Another aspect that became clear in this experiment was that, the yield of the cleaved GM-CSF protein was very low at this step itself, which would have reduced further in the following chromatographic step of getting rid of cleaved
MBP and Factor Xa. Secondly the concentration of Factor Xa used to get cleavage was considerably high, hence too costly to be used for a preparative purpose. We required rhGM-CSF both for mice immunization and for subsequent scFv ELISA based screenings. Therefore it was decided to change our strategy to produce rhGM-CSF directly in a soluble form. Instead it was decided to use the inclusion body route to produce large quantities of rhGM-CSF. For this the clone pET28a-GMCSF (Fig. 4.6) was used which expresses GM-CSF in the cytoplasm in the form of IBs. Though this route involves an additional refolding step, this is more than compensated by the high yields and ease of purification of IBs. In this particular clone the GM-CSF gene has been cloned downstream of the asparagenase signal sequence. Expression of the protein is driven by the strong T7 promoter. BL21 (DE3) cells were transformed with pET28a-GMCSF and cultured in LB and TB media. The expression level was almost double in TB culture, hence cells were grown in TB, induced at an O.D. of 2 and cells were harvested at the 4th hour post induction. The cell lysate run on
15% SDS-PAGE with the molecular wt marker showed a strong band of 17kD (Fig.4.7).

Fig. 4.6: Schematic representation of recombinant plasmid pET28a-GMCSF. pET28a is 5.36kb. GM-CSF gene is 400bp.

Fig. 4.7: SDS PAGE analysis total cell protein and IBs of GM-CSF in pET28GMCSFtrasformed BL21 (DE3) cell culture in TB. Lane 1: Mol. Wt marker: Lane 2 & 3: 4th hour culture post induction; Lane 4 & 5: supernatant fraction after sonication of 4th hour post induction cell culture; Lane 7 & 8: pellet fraction after sonication of 4th hour post induction cell culture;
The formation of inclusion bodies offers several advantages for the production of recombinant proteins. These foreign proteins may be unstable in the cytoplasm of *E. coli* due to proteolysis and also may be toxic to the host cell in the native conformation which is the main reason behind poor GM-CSF expression. Under appropriate conditions, the recombinant protein deposited in the form of inclusion bodies may amount to 50% or more of the total cellular protein. Because IBs have a relatively higher density (Mukhopadhayay, 1997) they can be isolated from the cellular proteins by centrifugation and the purity of the resulting preparation may reach 90% under optimal conditions. A number of human proteins expressed in *E. coli* directly e.g. human growth hormone (hGH) (Ikehara, et al. 1984), IFN-γ (Nishi, et al. 1985), interleukin-2 (IL-2) (Sato, et al. 1987) etc. have been shown to exist as aggregates or inclusion bodies. As is clear from the gel picture we were able to obtain extremely high levels of GM-CSF by the optimized induction protocol. Moreover a simple centrifugation step in getting rid of most of the impurities associated with the recombinant protein. It is noteworthy to point out that the levels of GM-CSF expression obtained are the highest reported so far in literature.

Inclusion bodies can be purified from their associated cellular impurities by washing with detergents e.g. Triton X-100 and deoxycholate (Marston, 1986; Marston and Hartley 1990). To wash GM-CSF inclusion bodies we used detergent deoxycholate (Langley, et al. 1987). Its concentration was standardized over a range that dissolves cellular protein impurities but but does not affect the inclusion bodies. The optimized protocol for isolating pure IBs was as follows: A 1L culture Pellet of OD~4.0 was resuspended in 50ml sonication buffer (50mM Tris-Cl, 5mM EDTA, 1mM PMSF pH 8.0) and sonicated [(30sec burst and 30sec pause at 300W) X 12 cycles]. The cell lysate was centrifuged at 12000xg at 4°C for 30 minutes. The pellet, containing inclusion bodies, was washed with deoxycholic acid by resuspending it in sonication buffer containing 0.5%, 1% and 2% DCA (deoxycholic acid) and incubated at 37°C for 1 hr. followed by centrifugation. The pelleted IBs were again washed to remove residual impurities. The IB pellets were given two washes with sonication buffer.
alone and two washes with DDW. 0.5 % DCA gave the optimum IB wash as higher concentrations of DCA tended to solubilize GM-CSF resulting in protein loss in the washing steps. Although the use of higher than 0.5% DCA concentration resulted in protein loss we still washed GM-CSF IBs with this 1% DCA as this resulted in highly pure IBs. This procedure helped in obtaining purified GM-CSF in IB form, SDS PAGE of the washed sample revealed that the protein was > 85% pure at this point (Fig.4.8). A western blot of the same sample was also done to confirm the result (Fig. 4.9).

![SDS PAGE analysis of DCA wash of GM-CSF IBs](image-url)

Fig. 4.8: SDS PAGE analysis of DCA wash of GM-CSF IBs
Lane 1: Mol. Wt. marker; Lane 2: total cell lysate of 4th h culture of pET28-GMCSF transformed BL21 (DE3) cells; Lane 3 & 4: 1% DCA washed GMCSF IBs; Lane 5 & 6: 1st and 2nd DCA wash supernatants.

Following the preparation of an acceptably pure level of inclusion bodies, their solubilization is generally done in concentrated denaturant (Urea or guanidinium chloride, GdmCl) (Fischer, et al. 1993). Wherever target protein contains disulfide bonds, like GM-CSF, the renaturation buffer is supplemented with a redox
system. Addition of a mixture of the reduced (RS-) and oxidized (RSSR) forms of low molecular thiol reagents such as glutathione, cystine (molar ratios of reduced to oxidized compounds 5:1 to 10:1, respectively) are generally made (Lilie, et al. 1998; Ahmed, et al. 1975; Wetlaufer, et al. 1987). In renaturation buffers L-arginine is also a popular additive (Rudolph and Lilie, 1996; Lilie, et al. 1998). In addition to this, in some cases, non denaturing concentrations of chaotrophic agents such as urea or GdmCl have been found essential for renaturation, e.g. in the case of chymotrypsinogen A (Orsini and Goldberg, 1978). We used urea as the denaturing agent for our work. To find out the minimum concentration of urea required for solubilization of the IB, different concentrations of ureawere used. Along with urea 5M GdmCl was also tested. It fully solubilized the IBs but since it tended to get precipitated its use was dropped. It was observed that the maximum concentration of 8M urea was required for solubilization since at lower concentration the protein was found mostly in the pellet (Fig. 4.10). GM-CSF IBs were therefore solubilized in buffer (50mM Tris Cl, 8M urea, pH 8.0) at a protein concentration of 1mg/ml. After centrifugation to remove the insolubles the denatured protein was subjected to dilution refolding. More than 10 fold dilution was done in refolding buffer [50mM Tris, 2M urea, 10% sucrose, 1mM EDTA, 1mM EDTA, 1mM PMSF, 3mM Glutathion (reduced), 0.5mM (oxidized), 0.4M NaCl, 0.5M Arginine, pH 8.0], and kept for stirring at 4°C over night. The refolded protein solution was dialyzed against the same refolding buffer without glutathions. This refolded protein solution was then subjected to anion exchange chromatography for further
purification. However we were unable to recover any protein from the column. Most probably after getting concentrated inside the column, it again got aggregated. A similar problem was reported with the GM-CSF/IL-6 fusion protein. Whereas the protein precipitated in column when being purified by Q Sepharose (H.P.) ion exchange chromatography after renaturation by dilution (Sun Q.M., et al 2002). They solved the problem by first purifying Inclusion bodies by Q Sepharose H.P. ion exchange in 8 M urea, followed by in situ refolding on the column by Sephacryl S-200. Following this procedure they recovered the renatured fusion protein with more than 95% purity. Following the same strategy we solubilized the IBs as before and subjected them to anion exchange in the column buffer (50mM TrisCl buffer, 8M urea in pH 8.0). Bound protein was eluted with 400mM NaCl in column buffer (50mM TrisCl buffer, 8M urea in pH 8.0) (Fig. 4.11). Mono Q column purified protein sample in denatured state was then subjected to 10 fold dilution renaturation in the presence of glutathione redox system (Lilie, et al. 1998; Ahmed, et al. 1975; Wetlauffer, et al.)
1987) with renaturation buffer [50mM Tris, 2M urea, 10% sucrose, 1mM EDTA, 1mM PMSF, 3mM Glutathione (reduced), 0.5mM (oxidized), 0.4M NaCl, 0.5M Arginine, pH 8.0]. Using pulse dilution the protein concentration was reduced from 1mg/ml to 0.1mg/ml. Renaturation was performed at 4°C, the solution being stirred slowly overnight. Partially renatured GM-CSF protein was then dialyzed against the same renaturation buffer without glutathions first and then urea concentration was decreased to 1M and to 0M in the subsequent buffer changes. As the at this stage protein was in dilute form, it was concentrated by centrifugation using centricon filters (filter membrane was of 3.5 cut off limit). Buffer washes were given with PBS. We recovered nearly 2mg partially renatured and more than 95% pure GM-CSF protein. This protein was then used for mice immunizations and scFv screening experiments.