3. Isolation, purification and characterization of inclusion bodies

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3. Isolation, purification and characterization of inclusion bodies

3.1 Introduction

Recombinant protein expression in *Escherichia coli* often results in formation of insoluble aggregates of expressed proteins defined as inclusion bodies (IBs) (1-4). The inclusion body formation inside host cells depends on various parameters; intrinsic as well as extrinsic factors associated with the host cells besides nature of protein itself. Most effective intrinsic factors which affect inclusion body formation are- genetic constitution of host cell, vector, promoter strength and plasmid copy number while extrinsic factors include growth media compositions, pH, temperature and inducer concentration (5-12). Most of the time, final outcome of high level expression in *E. coli* is the formation of protein aggregates. Inclusion body aggregates chiefly consist of expressed protein in denatured or misfolded states. The major possible reason for aggregation has been the association of partially denatured or misfolded proteins molecules through strong hydrophobic interactions (13). Beside higher percentage of expressed protein; inclusion bodies also consist of other proteins, nucleic acids, lipids and carbohydrates in significant proportion as impurities. Protein aggregates in inclusion bodies have been reported to be specific in nature (14). Thus, it would be appropriate if they are separated and purified to homogeneity before solubilization and refolding (15-16). As contaminating proteins affect the refolding yield (17), it is preferable to purify the inclusion body aggregates to homogeneity before solubilization and refolding.

Optimization of expression conditions, inclusion body isolation and purification protocols are thus essential for achieving high throughput protein recovery. Four inclusion body forming recombinant proteins (*E. coli* L-asparaginase II, *Mycobacterium tuberculosis* enolase, human growth hormone and human Cu-Zn superoxide dismutase) were used in this study to analyze the characteristics of protein aggregates. These proteins were chosen on the basis of their physical properties like molecular nature, molecular weight, isoelectric point and hydrophobicity to develop better insight about underlying forces responsible for aggregation during recombinant protein production. Description about the
physical properties, expression vector and host for all the four proteins is given in table 3.1.

In this chapter, expression studies of these four proteins were carried out in detail. These four proteins were expressed as inclusion body in *E. coli*. Effect of induction time on quality and quantity of protein expressed as inclusion body was evaluated. Sucrose density gradient ultra-centrifugation and detergent washing methods were used to isolate pure inclusion bodies from *E. coli* cells. Inclusion bodies of all the four proteins were purified to homogeneity. *In vitro* aggregates of proteins were compared with *in vivo* aggregates (inclusion bodies). They were characterized in terms of size, surface charge and aggregation behavior.

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Table 3.1 Details about recombinant *E. coli* clones and properties of proteins (L-asparaginase, enolase, hGH and Cu-Zn SOD).
3.2 Materials and methods

3.2.1 Chemicals and reagents

Culture media ingredients, tryptone and yeast extract were from Difco Laboratories, India. Tris buffer, Glycine, IPTG, sodium dodecyl sulphate, PMSF, and deoxy cholic acid were from Amresco, USA. Ammonium persulphate, acrylamide and bis-acrylamide from Sigma chemicals, USA. TEMED, EDTA, Bromophenol blue from BIO-RAD, USA. Coomassie brilliant blue R-250 and ampicillin and kanamycin were from USB Corporation, Cleveland, Ohio. SDS-PAGE low molecular weight marker was purchased from GE Healthcare, USA. Micro BCA assay kit from Pierce, USA. Glucose, NaCl, reagents and other chemicals were from Qualigen, India.

3.2.2 Strains and plasmids

*Escherichia coli* BL21 (DE3) and M 15 strain were from Novagen, USA. Strain DH5α was obtained from Invitrogen, USA. Vectors were obtained from Novagen, USA.

3.2.3 Cloning of L-asparaginase II, enolase, hGH and Cu-Zn SOD

The detail of vectors used for cloning these genes has been given in table 3.1. Briefly, these genes were amplified using specific primers and then inserted into their respective vectors by ligation methods. After successful ligation, these recombinant vectors were transferred into expression hosts and expression level of genes were analyzed at protein level by SDS-PAGE. L-asparaginase II gene was amplified from the genomic DNA of *E. coli* K-12 strain (JM109) using primers (forward) 5’ GTGCAGCACATATGTTACCAATATCACC 3’ and (reverse) 5’ GGCGGGATCCTAGTACTGATTGAAGA 3’. *Ndel* and *BamHI* restriction sites were incorporated in the primers to facilitate cloning of the structural asparaginase gene (without its native signal sequence) in the *E. coli* expression vector pET14b in fusion with a six histidine tag at the N-terminus. The resultant recombinant plasmid pET14b-Asp was sequenced to confirm the asparaginase gene insert. *E. coli* BL21 (DE3) cells were transformed with recombinant pET14b plasmid vector to get the expression strain. *Mycobacterium* enolase gene was amplified from genomic DNA of *Mycobacterium tuberculosis*, H37Rv strain using
forward primer 5'GGAACATATGCCGATATCGGCAGG3' and reverse primer 5'CCAAGCTTTTTCGTCTCGCACGCGAACC3'. Cloning was carried out in expression vector pET22b at Nde I and Hind III restriction sites and transferred to E. coli BL21 (DE3). Similarly, hGH and Cu-Zn SOD were cloned in pQE 60 vector and transferred in expression host E. coli M15 strain for protein expression (17). Recombinant clones of enolase, hGH and Cu-Zn SOD were obtained from Dr. L. C. Garg, NII, New Delhi and of L-asparaginase was from Prof. K. J. Mukherjee, School of Biotechnology, Jawaharlal Nehru University, New Delhi.

3.2.4 Preparation of glycerol stocks of recombinant clones

E. coli cells expressing above mentioned genes were grown overnight in Luria-Bertani (LB) media (containing ampicillin or/and kanamycin depending on the nature of vector and host cells as described in table 3.1) at 37°C and 200 rpm. Overnight grown primary inoculums (1ml) were transferred to fresh LB medium (100 ml) and grown up to 0.8 optical density (OD) at 600 nm. Cells were harvested and re-suspended in 50 ml LB medium consisting of 20 % glycerol. Aliquots (1 ml) were prepared and transferred to -20°C for 24 hrs. Finally glycerol stocks of various clones were stored at -70°C. These glycerol stocks were used for preparing culture for protein expression and purification.

3.2.5 Growth and expression kinetics of recombinant clones

E. coli strains expressing asparaginase, enolase, hGH and SOD were inoculated from glycerol stock to 20 ml sterilized LB medium containing 100 μg/ml ampicillin or/and 25 μg/ml kanamycin depending on the nature of clones. E. coli cells were grown overnight at 37°C and at 200 rpm in an orbital shaker. Overnight grown E. coli cells (20 ml) were inoculated into 2 litre LB medium containing 100 μg/ml ampicillin or/and 25 μg/ml kanamycin. Cells were allowed to grow until the optical density (OD) of the culture at 600 nm reaches 0.6-0.8. At this time point, E. coli cells were induced with 1 mM isopropyl β-D-thio-galactopyranoside (IPTG) and were grown for another 3.5 hrs. E.coli cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C. Cell pellets were re-suspended in Tris-HCl buffer and centrifuged again at 12,000 rpm for 20 min at 4°C. The cell pellet obtained was used for inclusion body extraction or stored at -20°C for further processing. The harvested cells were checked for expression of induced and un-induced
recombinant clones by SDS-PAGE. For growth kinetic analyses, OD of culture at 600 nm was recorded at fixed time intervals (~30 minutes). Uninduced culture was used as control. Expression of protein was checked by SDS-PAGE (12 %).

To evaluate the effect of induction time, E. coli cells were induced at early log phase, log phase, late log phase and stationary phase of the growth. The cells were grown for 3 hours after induction. Equal biomasses of cells were processed and loaded on SDS-PAGE to check the yield of expressed protein. Inclusion body yield per unit biomass of cells were estimated by micro BCA method.

3.2.6 Inclusion body isolation and purification

All four proteins resulted in the formation of inclusion body aggregates during expression. Cells expressing recombinant proteins as inclusion bodies were re-suspended in appropriate buffer solution and lysed by sonication (10 cycle of 1 minute each with one minute gap, 60 % duty cycle, power output 25 watts) method. The lysed cells were centrifuged at 12,000 rpm and at 4°C to obtain the inclusion body aggregates. Further purification of inclusion bodies was achieved through detergent washing method (19). Most common detergents used during inclusion bodies washing steps are basically anionic in nature. These detergents behave as mild denaturants and good surfactants. They are Triton X-100, Deoxycholic acid (DOC). Generally, 1 to 2 % solution of Triton X-100 or DOC is used during sonication and washing steps for inclusion body isolation and purification. They form micelle around amphipathic membranous debris and other cellular bodies and solubilize them. Thus these detergents help in removing the attached membranous impurities from inclusion bodies surface. However, their use also depends on the nature of inclusion body aggregates. Sodium salt of Deoxycholic acid (DOC) is most widely used during cell lysis to purify inclusion bodies. The detail protocol used during isolation and purification of inclusion bodies of asparaginase, enolase, hGH and Cu-Zn SOD are given below in flow charts 3.1 to 3.4.
3.2.6.1 Isolation and purification of L-asparaginase II inclusion bodies

Induce *E. coli* cells (2 L culture volume) expressing asparaginase with 1 mM IPTG at an OD of 0.7 at 600 nm and grow for 3.5 hrs. Harvest cells by centrifugation (6000 rpm for 10 minutes at 4°C).

Re-suspend and homogenize the cells pellet in 20 ml of 50 mM Tris-HCl, 5 mM EDTA and 1 mM PMSF at pH 8.5 and sonicate (10 cycles, 60 % duty, power 25 watt of 1 minute each with 1 minute gap on ice). Centrifuge the lysed cells at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend and homogenize the pellet in 20 ml of 50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF and 0.1 % deoxycholate (DOC) at pH 8.5 and sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF at pH 8.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl and 5 mM EDTA at pH 8.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of MQ water. Homogenize (5000 rpm, 2 minutes) it and centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend the pure inclusion body pellet in two ml MQ water.

Flow chart 3.1: Isolation and purification of L-asparaginase inclusion bodies.
3.2.6.2 Isolation and purification of mycobacterium enolase inclusion bodies

Induce *E. coli* cells (2 L culture volume) expressing r-enolase with 1 mM IPTG at an OD of 0.7 at 600 nm and grow for 3.5 hrs. Harvest cells by centrifugation (6000 rpm for 10 minutes at 4°C).

Re-suspend and homogenize the cells pellet in 20 ml of 50 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl₂ and 1 mM PMSF at pH 7.5 and sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge the lysed cells at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend and homogenize the IB pellet in 20 ml of 50 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl₂ and 1 mM PMSF at pH 7.5 and sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl, 1mM EDTA, 5 mM MgCl₂ and 1 mM PMSF at pH 7.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl and 5 mM MgCl₂ at pH 7.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of MQ water. Homogenize (5000 rpm, 2 minutes) it and centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend the pure inclusion body pellet in two ml MQ water.

Flow chart 3.2: Isolation and purification of enolase inclusion bodies.
3.2.6.3 Isolation of and purification of hGH inclusion bodies

Induce *E. coli* cells (2 L culture volume) expressing hGH with 1 mM IPTG at an OD of 0.7 at 600 nm and grow for 3.5 hrs. Harvest cells by centrifugation (6000 rpm for 10 minutes at 4°C).

Re-suspend and homogenize the cells pellet in 20 ml of 50 mM Tris-HCl, 5 mM EDTA, 1 mg/ml lysozyme and 1 mM PMSF at pH 8.5 and leave for 2 hours and then sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge the lysed cells at 12,000 rpm for 20 min at 4°C and discard supernatant.

Re-suspend and homogenize the inclusion body pellet in 20 ml of 50 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF and 2 % deoxycholate (DOC) at pH 8.5 and sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl, 5 mM EDTA, 2% DOC and 1 mM PMSF at pH 8.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl and 5 mM EDTA at pH 8.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of MQ water. Homogenize (5000 rpm, 2 minutes) it and centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend the pure inclusion body pellet in two ml MQ water.

Flow chart 3.3: Isolation and purification of hGH inclusion bodies.
3.2.6.4 Isolation and purification of human Cu-Zn SOD inclusion bodies

Induce *E. coli* cells (2 L culture volume) expressing SOD with 1 mM IPTG at an OD of 0.7 at 600 nm and grow for 3.5 hrs. Harvest cells by centrifugation (6000 rpm for 10 minutes at 4°C).

Re-suspend and homogenize the cells pellet in 20 ml of 50 mM Tris-HCl, 1 mM EDTA, 1 mM CuSO4, 1 mM ZnSO4 and 1 mM PMSF at pH 7.5 and sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge the lysed cells at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend and homogenize the inclusion body pellet in 20 ml of 50 mM Tris-HCl, 1 mM EDTA, 1 mM CuSO4, 1 mM ZnSO4 and 1 mM PMSF at pH 7.5 and sonicate (10 cycles, 60 % duty, power 25 watts of 1 minute each with 1 minute gap on ice). Centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl, 1 mM CuSO4, 1 mM ZnSO4 and 1 mM PMSF at pH 7.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend inclusion body pellet in 50 ml of 50 mM Tris-HCl at pH 7.5. Leave it for one hour and then centrifuge it at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend IBs pellet in 50 ml of MQ water. Homogenize (5000 rpm, 2 minutes) it and centrifuge at 12,000 rpm for 20 min at 4°C and discard the supernatant.

Re-suspend the pure inclusion body pellet in two ml MQ water.

Flow chart 3.4: Isolation and purification of Cu-Zn SOD inclusion bodies.
3.2.7 Purification of inclusion bodies by sucrose density gradient ultracentrifugation

In spite of detergent washing, inclusion body aggregates always consist of detectable amount of impurities in form of membranes debris and other proteins in aggregated form. As inclusion bodies are denser than aggregated impurities, their separation can be carried out by density gradient ultra centrifugation methods (20). This method is very useful and results in good yields of pure inclusion bodies which can be further used for their characterization, solubilization and refolding to obtain bioactive proteins. This method does not require any detergent treatment. Basic steps involve in sucrose gradient method are as follow:

1. *E. coli* cells expressing recombinant proteins (1 litre LB culture) were re-suspended in 10 ml lysis buffer as described in IB isolation flow chart.
2. Cells were sonicated (10 cycles, 1 min each and 1 min gap, 50 % duty cycle, and output control 4, on ice) for cell lysis. The cell lysate was centrifuged at 12,000 rpm for 20 min at 4°C to obtain IB pellets.
3. Inclusion body pellet was re-suspended in 2 ml Tris-EDTA buffer.
4. Sucrose step gradient was prepared in ultracentrifuge rotor tubes by drop wise addition of sucrose solution. 1 ml of each of 80 %, followed by 72 %, 70 %, 68 %, 66 %, 64 %, 62 %, and 60 % (w/v) of sucrose solution was added from bottom to top of the tube to prepare the sucrose gradient.
5. 1 ml inclusion body suspension was added on top of the 60 % sucrose layer in the tube, and centrifuged at 1,20,000g for 6 hrs in a swinging rotor at 4°C.
6. Inclusion bodies were seen in the sucrose gradient as a dense layer below impurities. The dense layer was carefully removed by pipetting without disturbing the other layers and washed with M.Q. Purity of the inclusion bodies was checked by SDS-PAGE analysis.
3.2.8 Gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to method of Laemmli on a slab gel containing 12 % running gel and 5 % stacking gel (21). Vertical mini-gel apparatus (GE Healthcare, USA) was used for electrophoretic separation of proteins. Inclusion body samples were prepared by dissolving them in 10 % SDS solution and then adding appropriate amount of sample buffer. Protein bands were stained with 0.1% Coomassie Brilliant Blue R-250 in 10 % acetic acid and 40 % methanol solution for 20 minutes. Gels were de-stained with 10 % acetic acid and 40 % methanol solution.

3.2.9 Estimation of protein concentration

Protein concentration was determined by micro BCA assay kit (Pierce, USA) using Bovine serum albumin (BSA) as standard. Inclusion bodies were dissolved in 2 % SDS solution for 2 hours. It was then centrifuged at 13,000 rpm for 20 minutes to remove particulate matter and supernatants were used for protein estimation. BSA standards (5 to 70 µg/ml) were prepared in 2 % SDS solution.

3.2.10 Preparation of in vitro aggregates

In vitro aggregates of recombinant hGH and asparaginase were prepared by high pH denaturation and rapid lowering of pH of the solubilized proteins. Human growth hormone and asparaginase IBs were solubilized in 2 M urea, 50 mM Tris-HCl, 100 mM β-mercaptoethanol, pH 12.5 buffer for two hours at room temperature. After this, denatured inclusion bodies were centrifuged at 13,000 rpm for 30 minutes to remove un-dissolved aggregates. The pH of denatured IB proteins was brought to pH 4 by adding 5 N HCl solution. Lowering of pH resulted in formation of large aggregates. Similarly, mixed aggregates of hGH and asparaginase inclusion bodies were prepared by denaturation of proteins in same solubilization buffer. Densities of these aggregates were determined by sucrose density gradient method as described earlier. Morphologies of in vitro aggregates were analyzed by transmission electron microscopy (TEM). Samples for TEM were prepared as described in 3.2.11.
3.2.11 Transmission electron microscopy of inclusion bodies and in vitro aggregates

Ultra pure inclusion bodies obtained by sucrose density gradient ultra centrifugation methods were homogenized and diluted to 1 mg/ml. 10 µl of the diluted samples of IBs were placed on carbon-coated copper grids and dried in a desiccator for 30 minutes. Then grids were washed with distilled water and stained with 2 % (w/v) uranyl acetate for 5 minutes. Similar procedures were used for preparing the carbon grids for in vitro aggregate samples. Morphology and refractive properties of inclusion bodies and in vitro aggregates were analyzed under transmission electron microscope (Jeol JSM 35CF, Japan).

3.2.12 Determination of surface charge of inclusion bodies

Surface charge distribution has important role in solubility and stability of purified inclusion body aggregates. It also reflects the ionization state of surface molecule decorating it. The most commonly used method for determination of charge of aggregate molecule is conductivity method. Zeta potential of inclusion body surface was determined using Malvern Nano-Zeta sizer (Malvern Inc., USA). Before measurement, IBs were homogenized and passed through narrow gauze syringe to make homogeneous solution. This homogeneous solution was diluted in 50 mM Tris-HCl buffer at pH 7.5 to the turbidity of one optical density at 350 nm and used for the measurements.

3.3 Results and discussion

3.3.1 Growth kinetics and expression of recombinant proteins

Bacterial growth in LB medium showed the typical sigmoid type of growth pattern. E. coli growth curve consists of three distinct phases characterized by different growth rates. These were lag phase, log or exponential phase and stationary phase. During lag phase, cells spent most of the time in adapting themselves to the medium and external conditions. Generally it was of one to one and half hour duration for the most of recombinant clones, when they were grown in LB medium in shaker flask culture. Reasons for this long duration of lag phase are differences in media composition of primary inoculum and fresh media (importantly pH, nutrients composition and toxic substances) and significant
difference in cell density in inoculum and secondary culture. These differences create stress responses in primary inoculum cells. After adapting to these stresses, cells enter into active growth phase characterized by rapid cell division and short doubling time (~25 to 30 minutes). This phase is defined as exponential growth phase owing to logarithmic increase in cell numbers on linear time scale. This active growth phase persists for 3.5 to 4 hours in shaker flask culture. When all the media gets completely utilized by growing cells they enter to the stationary phase. It also results due to accumulation of toxic materials and alteration of pH of media.

Growth curves of *E. coli* cells expressing asparaginase, enolase, hGH and Cu-Zn SOD are shown in figures 3.1a, 3.2a, 3.3a and 3.4a respectively. It was evident from the growth curves that induction of *E. coli* cells during log phase of growth resulted in cell growth reduction. It also yielded low biomass in comparison to un-induced culture. This could happen due to diversion of host anabolic processes toward synthesis of recombinant proteins. Thus engagement of cell synthetic machinery in recombinant protein expression creates scarcity of proteins and other cellular molecules inside host cells which are required of growth. Beside this, it also generates heat shock responses in host cells that ultimately lowered the cell growth rate.

Expression of asparaginase in *E. coli* led to formation of inclusion bodies (Figure 3.1a and 3.1b). The lag phase for asparaginase expressing *E. coli* was of 1 hour duration and log phase of approximately 5 hours duration. The optimal concentration of inducer (IPTG) for maximum expression of asparaginase was 1 mM. There was a small but significant decrease in cell growth after induction of culture with 1 mM IPTG (Figure 3.1a). Expression level of asparaginase at different time points after induction was analyzed by SDS-PAGE (Figure 3.1b). Equal amount biomasses were used to check the expression level on SDS-PAGE. Recombinant asparaginase was expressed as 37 kDa protein. Expression level of asparaginase was approximately 15 % of total cellular proteins. The maximum turbidities of cells (at 600 nm) at the end of 6 hours culture for un-induced and induced *E. coli* cells were 1.616 and 1.544 respectively. It was observed that expression level of asparaginase got saturated between 3 to 4 hours of post induction period (Figure 3.1b).
Figure 3.1a Growth kinetics of *E. coli* cells expressing asparaginase. Arrow indicates the induction point.

Figure 3.1b SDS-PAGE analysis of *E. coli* cells expressing asparaginase. Lane 1, un-induced cells; lane 2-5, induced cells isolated at 1, 2, 3 and 3.5 hours duration after induction with IPTG; lane M, LMW marker (97, 66, 45, 30, 20.1 and 14.4 kDa). Arrow indicates the expressed asparaginase (37 kDa).
Growth and expression profiles of *E. coli* cells expressing enolase, hGH and Cu-Zn SOD were analyzed (Figure 3.2 to 3.4). They were expressed as inclusion body aggregates. Optimal inducer (IPTG) concentration for gene expression was 1 mM. Induction of *E. coli* expressing enolase led to sharp decrease in growth rate (Figure 3.2a). The lag phase was approximately 1.5 hours and log phase of 3.5 hours. SDS-PAGE of enolase showed protein band at of 45 kDa (Figure 3.2b). Maximum expression level of enolase was 25 % of total cellular proteins and there was substantial difference between turbidity (at 600 nm) of un-induced (1.5) and induced (1.16) cell cultures. Human growth hormone was expressed as 22 kDa protein band on SDS-PAGE (Figure 3.3b). Expression level was 12-15 % of total cellular proteins. Induction of *E. coli* cells led to decrease in cell growth (Figure 3.3a). Expression of hGH saturated after 3 hours of induction. Maximum turbidities of cultures (at 600 nm) at the end of 6.75 hours growth for un-induced and induced recombinant hGH were found 1.474 and 1.375. *E. coli* cells expressing Cu-Zn superoxide dismutase growth curve showed long lag phase of 2 hour and log phase of 4 hour durations (Figure 3.4a). Induction of *E. coli* cells expressing SOD led to the decrease in cell growth. Expression of SOD saturated after 2.5 hours of post induction period. Expression level of SOD was 10 % of total cellular proteins. The turbidities (at 600 nm) of un-induced and induced cell culture of recombinant SOD were 1.376 and 1.276. SDS-PAGE of cell lysate of expressed SOD showed protein band at 22 kDa position contrary to its molecular weight (16 kDa). It could be due to incomplete denaturation, linearization or lowering in stoichiometric ratio of SDS binding to SOD polypeptide; even in presence of 10 % SDS and high concentration of β-mercaptoethanol in sample loading buffer.

Expression studies of all these proteins indicated reduction in cell growth of *E. coli* after induction, independent of whatsoever genes are expressed. However intensity of this response varied depending on the nature of recombinant proteins being expressed. In this study, *E. coli* cells expressing enolase showed maximum decrease in cell growth rate after induction in comparison to other proteins. There were differences in duration of lag and log phase for different recombinant clones. This was also related to the expression level of recombinant proteins. Enolase showed highest expression level of 25 %
of total cellular proteins. Cell growth reduction during enolase expression was also maximum.

**Figure 3.2a** Growth kinetics of *E. coli* cells expressing r-enolase. Arrow indicates the induction point.

**Figure 3.2b** SDS-PAGE analysis of *E. coli* cells expressing r-enolase. Lane 1, un-induced cell; lane 2-9, induced cells isolated at 30, 60, 90, 120, 150, 165, 180 and 210 minutes duration after induction with IPTG; lane M, LMW marker (97, 66, 45, 30, 20.1 and 14.4 kDa). Arrow indicates the expressed r-enolase (45 kDa).
Figure 3.3a Growth kinetics of *E. coli* cells expressing r-hGH.

Figure 3.3b SDS-PAGE analysis of *E. coli* cells expressing r-hGH. Lane 1, uninduced cell; lane 2-9, induced cells isolated at 15, 45, 75, 105, 135, 165, 195 and 225 minutes duration after induction with IPTG; lane M, LMW marker (97, 66, 45, 30, 20.1 and 14.4 kDa). Arrow indicates the expressed r-hGH (22 kDa).
Figure 3.4a Growth kinetics of *E. coli* cells expressing r-SOD.

Figure 3.4b SDS-PAGE analysis of *E. coli* cells expressing r-SOD. Lane 1, uninduced cell; lane 2-9, induced cells isolated at 30, 60, 90, 120, 150, 165, 180 and 210 minutes duration after induction with IPTG; lane M, LMW marker (97, 66, 45, 30, 20.1 and 14.4 kDa). Arrow indicates the expressed r-SOD (16 kDa).
3.3.2 Isolation and purification of inclusion bodies from *E. coli*

Inclusion bodies of recombinant proteins from *E. coli* were harvested by sonication and centrifugation protocols as described in methods (Flow Chart 3.1 to 3.4). Inclusion body isolation and purification protocol for all recombinant protein were optimized with respect to pH, ionic strength and detergent concentration. Inclusion bodies of asparaginase, enolase, hGH and Cu-Zn SOD were isolated to maximum purity (Figure 3.5 to 3.8). During sonication steps significant amount of proteinaceous and non-proteinaceous impurities get attached to the inclusion bodies. Removal of these impurities was necessary for obtaining pure and homogeneous inclusion bodies. This was achieved using sodium deoxycholate (DOC) detergent during sonication and washing steps. Finally the purity level of inclusion bodies was checked by SDS-PAGE analysis.

Asparaginase inclusion bodies were isolated by sonicating the cells in pH 8.5, 50 mM Tris buffer. To remove impurities, 0.1 % DOC was used during sonication and washing steps. Use of higher concentration (1-2 %) of DOC resulted in solubilization of proteins from inclusion body pellet. Purified asparaginase inclusion bodies consisted of 80 % of recombinant protein (Figure 3.5).

![Figure 3.5 SDS-PAGE analyses of samples during asparaginase IB purification.](image)

(a) lane 1, un-induced cell lysate; lane 2, induced cell lysate. (b) lane 1-5, pellets obtained after each step of IB isolation; lane 6-8, supernatants obtained at 1st and 2nd sonication steps and 1st washing step; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrows indicate asparaginase inclusion bodies (37 kDa).
Enolase was expressed as 45 kDa protein upon induction of *E. coli* cells with IPTG (Figure 3.6a). Isolation and purification of enolase inclusion bodies were carried out at 50 mM Tris-HCl buffer of pH 7.5 (Flow chart 3.2). Use of Tris-HCl buffer at pH 8.5 resulted in loss of enolase during sonication and washing steps (Figure 3.6b). Detergents were not used in isolation process as they led to solubilization of enolase from inclusion bodies (Figure 3.6c).

**Figure 3.6** SDS-PAGE analyses of samples during enolase IB purification. (a) lane 1, un-induced cell lysate; lane 2, induced cell lysate. (b) Isolation of IBs at pH 8.5. Lane 1-4, supernatants obtained during isolation steps; lane 5, purified IBs. (c) Isolation of IBs at pH 7.5. Lane 1-3, supernatants obtained during isolation steps; lane 4, purified IBs; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrows indicate the enolase inclusion bodies (45 kDa).
E. coli cells expressing hGH were induced with 1 mM IPTG and expression level was analyzed on SDS-PAGE (Figure 3.7a). Inclusion body of hGH was isolated using Tris-HCl buffer of pH 8.5 consisting of 1 mg/ml lysozyme solution as described in flow chart 3.3. Use of lysozyme helped in improving the cell lysis as well as purity of hGH inclusion bodies. Lysozyme was not used in isolation of other inclusion bodies as it was very difficult to remove it from inclusion bodies during washing steps. The possible reason could be its aggregation or association with inclusion body pellets. In case of hGH, it was removed during sonication and washing steps using 2 % deoxycholate (Figure 3.7b). Maximum purity of hGH inclusion bodies isolated using this method was around 90 % (Figure 3.7b).

![Figure 3.7 SDS-PAGE analyses of samples during hGH IBs purification. (a) lane 1, un-induced cell lysate; lane 2, induced cell lysate. (b) Isolation of IBs at pH 8.5. Lane 1 and 3, supernatants obtained during isolation steps; lane 2, 4 and 5, IB pellets obtained at sonication steps; lane 6, purified IBs; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrows indicate hGH Inclusion bodies (22 kDa).](image)

Tris-HCl buffer of pH 8.5 with 1 % DOC solubilized the SOD inclusion bodies during sonication and washing steps. Thus, Cu-Zn SOD inclusion bodies isolation was carried out at pH 7.5 Tris-HCl buffer without detergents. There was
higher amount of impurities in SOD inclusion bodies in comparison to others (Figure 3.8). The expression level and final yield of SOD was also less in comparison to other proteins. It indicated higher expression of heat shock proteins and toxic nature of SOD aggregates in bacterial cells.

![Figure 3.8 SDS-PAGE analyses of samples during SOD IB purification. Lane 1, un-induced cell lysate; lane 2, induced cell lysate; lane 3-5, supernatants obtained during isolation steps; lane 6, purified IBs; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrow indicates r-SOD inclusion bodies (16 kDa).](image)

3.3.3 Effect of induction time on cell growth and recombinant protein expression

This study was performed to find out the effect of induction at different phases of *E.coli* growth on expression of recombinant proteins. *E.coli* culture expressing asparaginase, enolase, hGH and SOD were induced at early, mid and late exponential phase of growth. The turbidity of culture was recorded at 600 nm and plotted against time to study the growth kinetics. Cells were grown for 3 hours duration after induction. Inclusion bodies were isolated from these cultures and protein concentration was estimated to determine the yield of recombinant proteins.

Induction of cells during early log phase resulted in sharp reduction in cell growth in comparison to the late log phase induction. This type of pattern was
observed for all the recombinant clones (asparaginase, enolase, hGH and SOD). Figures 3.9a to 3.12a show the growth kinetics of these cells. Biomass yield was lower in case of the cells induced during early log phase (0.2-0.3 OD 600nm). This was very prominent for *E. coli* culture expressing SOD. Comparative analyses of slopes of growth curves indicated that early induction hampers the growth of *E. coli* more drastically than late log phase induced culture. It could happen due to differences in metabolic status of cells during different phases of growth. Early log phase induction can also lead to production of heat shock proteins in large quantity, intolerable to cells. SDS-PAGE analysis of IBs isolated after induction of cells at different phase of growth is shown in figure 3.9b to 3.12b. IBs isolated from equal biomass of these induced cells were loaded on SDS-PAGE. Protein concentration was estimated by micro BCA method (Table 3.2). Results showed that yield of recombinant proteins per unit biomass was lower for early log phase induced cell culture comparison to mid or late log phase induced cell culture. Beside this, IBs from early log phase induced culture were associated with more impurities in comparison to mid or log phase induced cells.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Proteins</th>
<th>Induction points</th>
<th>IBs per unit biomass (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asparaginase</td>
<td>0.211</td>
<td>22.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.407</td>
<td>28.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.593</td>
<td>31.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.876</td>
<td>30.94</td>
</tr>
<tr>
<td>2</td>
<td>Enolase</td>
<td>0.220</td>
<td>36.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.402</td>
<td>41.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.593</td>
<td>42.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.940</td>
<td>41.76</td>
</tr>
<tr>
<td>3</td>
<td>hGH</td>
<td>0.230</td>
<td>17.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.365</td>
<td>28.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.651</td>
<td>34.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.815</td>
<td>34.39</td>
</tr>
<tr>
<td>4</td>
<td>Cu-Zn SOD</td>
<td>0.303</td>
<td>11.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.453</td>
<td>14.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.645</td>
<td>17.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.952</td>
<td>18.16</td>
</tr>
</tbody>
</table>

**Table 3.2** Inclusion body protein yields from culture induced during different growth phases.
Figure 3.9a Growth kinetics of *E. coli* cells expressing asparaginase, induced at different phases of growth. Arrows indicate the time of induction.

Figure 3.9b SDS-PAGE of asparaginase IBs isolated after 3 hours of induction at different cell OD at 600 nm. Lane 1, 0.211; lane 2, 0.407; lane 3, 0.593; lane 4, 0.876; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrow indicates r-asparaginase (37 kDa).
Figure 3.10a Growth kinetics of *E. coli* cells expressing enolase, induced at different phases of growth. Arrows indicate time of induction.

Figure 3.10b SDS-PAGE of enolase IBs isolated after 3 hours of induction at different cell OD at 600 nm. Lane 1, 0.220; lane 2, 0.402; lane 3, 0.593; lane 4, 0.940; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrow indicates the r-enolase (45 kDa).
Figure 3.11a Growth kinetics of *E. coli* cells expressing enolase, induced at different phases of growth. Arrows indicate the time of induction.

Figure 3.11b SDS-PAGE of hGH IBs isolated after 3 hours of induction at different cell OD at 600 nm. Lane 1, 0.23; lane 2, 0.365; lane 3, 0.651; lane 4, 0.815; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrow indicates the r-hGH (22 kDa).
Figure 3.12a Growth kinetics of *E. coli* cells expressing r-SOD, induced at different phases of growth. Arrows indicate time of induction.

Figure 3.12b SDS-PAGE of r-SOD IBs isolated after 3 hours duration of induction at different cell OD at 600 nm. Lane 1, 0.303; lane 2, 0.453; lane 3, 0.645; lane 4, 0.952; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa). Arrow indicates the r-SOD. Arrow indicates r-SOD (16 kDa).
3.3.4 Purification of inclusion bodies by sucrose density gradient ultracentrifugation

Though sonication and detergent based washing methods yielded enriched IBs mostly consisting of recombinant proteins, in certain cases it retains some membranous and cellular proteins released during sonication. These loosely attached impurities on IB surface can be separated using density gradient method; as they have lower density than inclusion body aggregates. All the recombinant protein IBs (asparaginase, enolase, hGH and SOD) were purified using sucrose density gradient ultracentrifugation methods (Figure 3.13). Density gradient pattern for asparaginase, enolase, hGH and SOD IBs were different. Densities of these IBs were calculated by determining the density of sucrose solutions in the regions where IBs bands were present. The asparaginase IBs showed one distinct band of density 1.315 g/ml and another dispersed band with density between 1.31 to 1.29 g/ml. The density of hGH IBs was maximum (1.347 g/ml). Enolase IBs showed dispersed band of density range 1.305 to 1.286 g/ml. While SOD IBs showed homogeneous nature with sharp distinct band in sucrose density tube and had density of 1.29 g/ml. Thus, asparaginase and enolase IBs consist of heterogeneous mixture of different sized and density aggregates while nature of hGH and SOD inclusion body aggregates were quite homogeneous. Membranous impurities were present on the top of gradient tube owing to lower density. The order of densities of different inclusion body aggregates were hGH > Asparaginase > Enolase > SOD (Figure 3.13a). SDS-PAGE of purified IBs was carried out to check the purity (Figure 3.13b).

From SDS-PAGE analysis, it was evident that sucrose density gradient ultracentrifugation helped in removing the impurities attached to IBs surfaces and other protein aggregates. Removal of impurities was better for asparaginase, enolase and SOD inclusion body samples. As the IB samples loaded on the gradient tubes were isolated by single steps sonication without detergent and washing steps, there were large amount of impurities present on them. However, if the inclusion bodies were isolated by methods described in flow charts, then sucrose density gradient method can further improve the purity of inclusion bodies.
Figure 3.13a Sucrose density gradient picture of IB proteins. Tube 1-4, asparaginase, hGH, enolase and SOD IBs respectively. The dense band in each tube corresponds to the inclusion body aggregates.

Figure 3.13b SDS-PAGE of pure inclusion bodies isolated after sucrose density gradient ultracentrifugation. (a) Lane 1, L-asparaginase II. (b) Lane 1, enolase. (c) Lane 1, hGH and (d) lane 1, SOD; lane M, LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa).
3.3.5 Electron microscopy and surface charge analysis of inclusion bodies

The size and refractive properties of pure inclusion bodies of asparaginase, enolase, hGH and SOD, purified by sucrose density gradient ultracentrifugation methods were studied by transmission electron microscopy (Figure 3.14 to 3.15). Asparaginase inclusion bodies were less refractive and of smaller size (100-200 nm). They were ovoid and circular in shapes with extended appearance (Figure 3.14a). TEM of enolase inclusion bodies showed their dense refractivity and size in range of 0.6 to 0.8 μm. (Figure 3.14b). The size of hGH inclusion bodies were approximately 0.6-0.9 μm (Figure 3.14 c and d).

Figure 3.14 Transmission electron micrograph of recombinant protein inclusion bodies. (a) Asparaginase. (b) r- Enolase. (c) and (d) hGH inclusion bodies.
The SOD inclusion bodies were strongly refractive under electron beam and had size in 0.6 to 0.9 μm range (Figure 3.15a and 3.15b). TEM of fixed *E. coli* cells producing recombinant proteins showed the presence of two IBs at the poles of bacterial cells (Figure 3.15 c and d). Generally, they showed heterogeneity in sizes and shapes, but most of them were circular and ovoid.

![Figure 3.15](image-url)

**Figure 3.15** Transmission electron micrograph of recombinant SOD IBs and *E. coli* cells expressing hGH as IBs. (a) and (b) Cu-Zn SOD inclusion bodies. (c) and (d) *E. coli* cells showing dense black regions at poles of cells. IBs can be seen at poles in dividing cells (arrow).
Surface potential of asparaginase, enolase, hGH and SOD IBs were determined by zeta nano-sizer Malvern, UK. It was observed that all IBs proteins had net negative potential. There were slight differences in their surface potential.

<table>
<thead>
<tr>
<th>Results</th>
<th>Mean (mV)</th>
<th>% PDI</th>
<th>Width (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta Potential (mV): -12.57</td>
<td>Peak 1: -12.57</td>
<td>100</td>
<td>11.12</td>
</tr>
<tr>
<td>Std. Dev (mV): 11.12</td>
<td>Peak 2: 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Conductivity (mS/cm): 24.34</td>
<td>Peak 3: 0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

![Zeta Potential Distribution](a)

![Zeta Potential Distribution](b)

**Figure 3.16** Zeta potential plots of inclusion bodies. (a) Asparaginase inclusion bodies. (b) r-enolase inclusion bodies.
Figure 3.17 Zeta potential plots of inclusion bodies. (a) hGH inclusion bodies. (b) SOD inclusion bodies.

Thus from electron microscopy and surface potential studies of IBs, it was evident that IBs are dense refractive aggregates with negative surface potential. They were mostly ovoid or circular in shapes. Generally, the IBs occupied the bacterial cytoplasm, mostly at the poles.
3.3.6 Specificity of *in vitro* aggregation of recombinant proteins

It was observed from sucrose density gradient study that density of hGH IBs was higher than asparaginase IBs (Figure 3.13a). To study whether their *in vitro* aggregates were also of similar nature, aggregates of hGH and asparaginase were made by solubilizing their IBs at high pH condition and aggregating them by rapid change of pH. Mixed aggregates of hGH and asparaginase were also made to understand the specificity of aggregation. All the *in vitro* aggregates were analyzes by density gradient ultracentrifugation. It was observed that densities of *in vitro* aggregates (Figure 3.17) were higher than their IBs aggregates (Figure 3.13a). Also, the density of mixed aggregate was in between of their individual densities. Thus, it indicated that *in vitro* aggregation is non-specific in nature. Further studies are required to validate it. However, recent study by Maji *et al.* suggests similar behavior for *in vivo* aggregation (22). Transmission electron micrograph revealed their irregular morphology and refractivity (Figure 3.18a to 3.18c). It could be due to differences in unfolded intermediates formed during *in vitro* denaturation and *in vivo* misfolding.

![Figure 3.18](image)

**Figure 3.18** Sucrose density gradient ultracentrifugation of *in vitro* aggregates. Tube 1, hGH; tube 2, hGH + asparaginase mixed aggregates; tube 3, asparaginase.
Figure 3.19 Transmission electron micrograph of *in vitro* aggregates of solubilized inclusion body proteins. (a) hGH *in vitro* aggregates (b) asparaginase *in vitro* aggregate and (c) mixed *in vitro* aggregates of hGH and asparaginase proteins.
3.4 Conclusions

From this study, the following conclusions were drawn about inclusion body proteins:

1. Expression of recombinant proteins in *E. coli* as inclusion bodies resulted in reduction in cell growth.
2. Induction of culture in early log phase brought sharp reduction in cell growth in comparison to late mid or late log phase induced culture. Specific protein yield (protein/unit cell biomass) was low when cells were induced at early log phase.
3. Induction time plays major role in regulating the expression level and yield of recombinant protein. Duration of lag phase was different for various recombinant clones.
4. Use of optimal pH buffers and detergent concentration during isolation process improved the yield and purity of IBs.
5. Buffers of pH higher than 7.5 led to the loss of recombinant asparaginase, enolase and SOD from inclusion bodies.
6. Lysozyme treatment of *E. coli* cells during sonication helped in enhancing the purity of hGH IBs.
7. Use of 1% deoxycholate (DOC) during sonication and washing steps resulted in loss of recombinant asparaginase, enolase and SOD proteins from inclusion bodies.
8. Purification of inclusion bodies by sucrose density gradient ultracentrifugation improved its purity and quality.
9. Inclusion bodies of different proteins were ovoid or circular in shape with varying sizes (0.2 to 1.0 µm) and densities (1.28 to 1.35 g/ml).
10. Surface potential analysis revealed that inclusion body aggregates are negatively charged.
11. Different proteins co-aggregated during *in vitro* aggregation. The morphologies of *in vitro* aggregates were different than those of IBs (*in vivo* aggregates).
3.5 References


storage of peptide hormones in pituitary secretory granules. Science 325:328-32