Expression, purification and characterization of Human γD crystallin
Protein expression and purification studies were carried out essentially according to already published work with slight modification (Flaugh et al., 2005 and reference there in).

**Expression of Human γD crystallin**

Studies of protein expression have routinely been carried out with *E. coli* BL21(λ.D3)pLys strains as this strain is known to prevent the leaky expression of the protein from the T7 promoter and facilitates the lysis of the cells to release the expressed protein (Kruse et al., 1996). But the strain puts an extra pressure on cell growth due to the presence of ampicillin and chloramphenicol as double selection markers. Human γD crystallin was expressed in both *E. coli* BL21(λ.D3) and its pLys strain as hosts so as to distinguish between the expression profile of the two strains. Both the hosts were found to express the protein equally well with an added advantage of fast and larger biomass in case of BL21(λ.D3) strain, further expressions were proceeded with *E. coli* BL21(λ.D3) strains (figure 3.1). Analysis of the protein revealed a prominent band at ~20 kDa (lane 2 and 4 in figure 3.1) in the induced bacterial culture in comparison to the uninduced cell lysates (lane 1 and 3 in figure 3.1).

![Image](image-url)

**Figure 3.1:** Expression of Human γD Crystallin in *E. coli*: lane 1 and 2- BL21(λ.D3) uninduced and induced, lane 3 and 4 BL21(λ.D3) pLys uninduced and induced, M- molecular weight marker, lane 5 and 6- expression in LB medium (uninduced and induced), lane 7 and 8-expression in TB medium (uninduced and induced).
Media composition is crucial for recombinant protein production as it affects the expression of recombinant protein in several ways. An ideal medium should be able to support cellular growth to maintain high specific growth rates and finally give high recombinant protein yields. As specific growth rate is a function of availability of substrates, hence the availability of nutrients especially during the induction phase of the cells is critical for recombinant yield (Covalt et al., 2005). The composition of media can affect the specific growth rate of cells as well as the biomass yield. Thus it becomes an important factor for recombinant protein production. Various strategies are reported where media optimization improved production of recombinant protein (Niccolai et al, 2003, Tong et al, 2001, Li et al., 1994). We therefore studied the expression of γD crystallin in different complex and highly enriched TB medium which usually promised higher biomass in culture.

The culture was grown in TB medium until it reaches an OD$_{600}$ of 4 and thereafter was induced by adding 1 mM IPTG. The cells were harvested 4hrs post induction and samples were analyzed on SDS PAGE for the expression of γD crystallin. The expression levels were also compared with those observed in LB medium. The results showed that there was no significant change in the expression levels of the protein in both the media (lane 6 and 8 in figure 3.1). Although the specific yield of the cells did not change with media but the volumetric product yield obtained in TB medium higher than that in LB medium. This increase in volumetric product yield was because of the higher biomass achieved in TB medium. We therefore decided to use TB medium for the production of recombinant Human γD crystallin for the purpose of conducting aggregation, folding and stability studies.

**Purification of Human γD crystallin**

Expressed cells were centrifuged and resuspended in lysis buffer containing protease inhibitor cocktails, DNase I and lysozyme. The mixture was then subjected to sonication/French press to lyse the cells leading to release of expressed protein in soluble form. Thus obtained protein mixture was then centrifuged again to get rid of cell debris and other high molecular weight impurities. The 90% of the protein was recovered in the supernatant.
Gel filtration chromatography

Supernatant was collected and subjected to gel filtration chromatography on a superdex 200HR FPLC column. Eluted protein fractions were analysed on the SDS-PAGE for the presence of Human γD Crystallin. Intense bands of crystallin could be observed along with few faint bands of high molecular weight contaminating proteins indicating partial purity of the protein. Fractions enriched with relatively pure protein were collected and used for further purification steps.

Cation exchange chromatography

Human γD crystallin has a pI of ~5.7 and has a network of charged residues on its surface and therefore at acidic pH, it is sufficiently charged. Charged status of the protein at acidic pH (pH 4.8) was therefore exploited further to purify and concentrate the protein by cation exchange chromatography on SP sephadex C-50. A linear NaCl gradient was applied on the column to purify the protein against remaining impurities (figure 3.3 A).
Figure 3.3: Cation exchange chromatography of partially purified protein on SP-Sephadex C-50 column obtained after gel filtration chromatography. A. Elution profile and B. SDS-PAGE analysis of Human γD crystallin protein fractions after a gradient of increasing NaCl concentration was applied. Lane 1. molecular weight markers. Lanes 2-14 are the samples containing crystallin at ~0.35M NaCl.
Purified fractions of the protein (lane 12 in figure 3.3 B) were then dialysed against 20 mM sodium phosphate buffer, pH 7, concentrated and quantified spectrophotometrically. The protein stock was stored at 4°C and was ready for further characterization.

**Spectroscopic characterization of the purified Human γD crystallin**

![Figure 3.4: Characterization of Human γD crystallin: A. Far UV-CD spectrum B. fluorescence spectrum of Native protein in 10mM phosphate buffer and denatured fluorescence spectra in 6 M GdmCl in phosphate buffer, pH 7, 25 °C. equilibrium denaturation-renaturation curves in presence of GdmCl, pH 7, 25°C.](image-url)
Properties of purified protein were characterized and confirmed by CD and fluorescence spectroscopy. Predominantly \( \beta \) sheet characteristics of the protein were confirmed in far UV CD spectrum of the protein where a clear minimum at 218nm could be observed (figure 3.4 A) (Pande et al., 2000). Since the protein did not have any molecular signature for fluorescence, therefore a combination of fluorescence spectral characteristics for both native and chemically denatured state was carried out to verify the tertiary structural content of the protein. Nativity of the tertiary content was confirmed by the emission maximum at 325nm in the fluorescence spectrum which corresponded to quenched fluorescence characteristics of buried tryptophans (Kosinski-Collins et al., 2004, Chen et al., 2006) which when completely exposed in chemically denatured protein show its fluorescence spectrum red shifted with emission maximum at 355nm (figure 3.4 B). The reliability and the nativity of the protein was further supported by equilibrium denaturation/renaturation profile (figure 3.4C), unfolding kinetics in 5.5 M GdmCl (figure 3.5 A) and refolding kinetics of chemically denatured protein in 1.5 M GdmCl (figure 3.5 B). All the spectroscopic characteristics were found similar to already
published literature on Human γD crystallin thereby confirming the purity of recombinant Human γD crystallin (Kosinski-Collins and King, 2003).