

## Over expression and purification of recombinant human myocilin

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**Abstract** Human myocilin is a 55 kDa protein that is implicated in primary open angle glaucoma (POAG). Understanding the structure and folding of the native protein and the mutants that increase aggregation could lead to possible prevention of the condition. We report here the over expression and purification of the human myocilin in *E. coli*. The initial expression of recombinant myocilin in *E. coli* was found to be low. The problem of low yield was found to be due to multiple causes and was overcome using a suitable combination of vectors, tags, host background and expression protocols. The overexpressed human myocilin was purified by affinity column chromatography to yield about 8 mg of protein from 1 l of culture. The protein purity and folding were confirmed using electrophoresis, immunoblotting and fluorescence spectroscopy. Further biophysical characterization and crystallization trials using the recombinant human myocilin will pave the way for better understanding of the structure–aggregation relationship that is involved in causing POAG.

**Keywords** Human myocilin · POAG · Glaucoma · Heterologous expression · Codon usage · Purification

### Abbreviations

POAG Primary open angle glaucoma  
IOP Intra ocular pressure  
TUB Tris urea buffer  
TNB Tris NaCl buffer

### Introduction

Human myocilin precursor is a 56.9 kDa protein (Swissprot Acc: Q99972) (Nguyen et al. 1998; Stamer et al. 1998; Karali et al. 2000; Ueda et al. 2000; Caballero et al. 2000), containing 504 amino acids (Kubota et al. 1997) with an N-terminal 32 amino acid long signal peptide for secretion. Myocilin has been implicated in primary open angle glaucoma (POAG). POAG is the most common form of glaucoma in India (Balasubramanian 2002). Myocilin is predominantly hydrophobic and expressed in large amounts in various types of muscle, ciliary body, papillary sphincter, skeletal muscle, heart and other tissues and predominantly in the retina. In normal eyes, the protein is found in the inner uveal meshwork region and the anterior portion of the meshwork. In contrast, in many glaucomatous eyes, it is found in more regions of the meshwork and appears more intensively than in normal eyes, regardless of the type or clinical severity of glaucoma. The normal function of myocilin is not known and it has no known enzymatic activity. In the diseased cases myocilin aggregates and blocks the trabecular meshwork in the eye, which increases the intra ocular pressure (IOP) and leads to open angle glaucoma.

Experimental evidence has shown that mutated myocilin is not secreted but accumulates in the cells; accumulation of mutant protein suppresses secretion of normal myocilin (Jacobson et al. 2001). Patients harboring mutations in myocilin may suffer from very high IOP (Wiggs et al.

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1995; Johnson et al. 1996; Alward et al. 1998). Individuals heterozygous or homozygous for Arg46Stop in the MYOC gene do not develop glaucoma, suggesting that reduction in the amount of trabecular meshwork inducible glucocorticoid response (TIGR) protein does not cause glaucoma (Lam et al. 2000). The fact that an individual hemizygous for MYOC does not show clinical evidence of glaucoma (Wiggs and Vollrath 2001) also substantiates the same. Only 2–4% of the patients are known to have mutations in the MYOC gene (Fingert et al. 1999). Most mutations in patients with POAG are found to be localized in the olfactomedin domain (Adam et al. 1997; Polansky and Nguyen 1998; Kanagavalli et al. 2003, 2004).

In order to explain the role of known mutants that were obtained by screening the local population we have earlier (Kanagavalli et al. 2003), used a knowledge-based consensus modeling approach to predict a model for the structure of myocilin consisting of three regions—the N-terminal myosin-like coiled coil region (111–184), a flexible linker region (185–245) and the C-terminal (246–504) olfactomedin-like domain. A possible mechanism was proposed (Kanagavalli et al. 2004) involving protein aggregation arising from destabilization of interactions involving the C-terminal domain that could be caused by conformational changes due to the molecular environment or mutations. The olfactomedin domain of myocilin has been cloned, over expressed in *Pistia pastori*, purified and characterized (Nagy et al. 2003). There has been no report so far of glucosylation or the lack of it affecting the aggregation characteristics of myocilin (Park et al. 2006). Mutants which are linked to the disease do not also have any correlation with glucosylation potential. Therefore, here we report the over expression and purification of human myocilin from *E. coli* in order to biophysically and structurally characterize the normal and mutant full-length myocilin towards understanding the aggregation–structure relationship.

## Materials and methods

### Bacterial strains and culture conditions

The *E. coli* strain used in this study is Rosetta(DE3)pLysS. The recombinant human Myocilin over expression construct, pC6H-Myoc in Rosetta(DE3)pLysS (pC6H-Myoc/Rosetta(DE3)pLysS) was grown in LB containing 1.2% glucose supplemented by chloramphenicol and ampicillin.

The mRNA folding of the translation initiation region of the pRSET-Myoc, codon usage for MYOC against the low and high expression proteins of *E. coli*, occurrence of rare codons in myocilin were analyzed using mfold, correspondence and codonfrequency in GCG package (Accelrys software, USA), respectively.

The region corresponding to the signal peptide in the cDNA sequence of myocilin (GenBank Acc: HSU85257) was removed in the construct pRSET-Myoc (a kind gift from Dr M. Fautsch of Mayo Clinic, Rochester, USA). From this the myocilin insert was released with *Bam*HI and *Hind*III and recloned between the same restriction sites in pET20b<sup>+</sup>. The resulting construct pET-Myoc, containing myocilin under a pelB leader sequence, had the region corresponding the six His-tag after the stop codon of myocilin gene. An inverse PCR strategy using specific primers (Forward primer: aagcttcaccaccactgagatccg; Reverse primer: catcttgagagcttgatgtcataagtac) was used to remove the stop codon of myocilin while retaining the stop codon after the six His-tag. This resulted in the final construct, pC6H-Myoc, which was confirmed by sequencing.

The pET-Myoc construct was transformed into different strains BL21(DE3), BL21(DE3)RIL+ and GJ1158 (for NaCl induction, a kind gift from Dr Gowrishanker, CCMB, India) (Bhandari and Gowrishankar 1997) and checked for over expression (Cells grown to log phase, induced with different IPTG/NaCl concentration for different time intervals (2–10 h) at 37°C). Rosetta(DE3)pLysS host strain is a BL21 derivative designed to enhance the expression of eukaryotic proteins that contain rare codons in *E. coli* (Novagen). pC6H-Myoc was transformed into the strain Rosetta(DE3)pLysS and grown under repression with 1.2% glucose in Luria Broth (LB) medium followed by induction with IPTG in LB medium at 27°C and 37°C for 4 h.

Induced pC6H-Myoc/Rosetta(DE3)pLysS culture pellet was subjected to osmotic shock (Neu and Heppel 1965) by suspending it thoroughly in Tris–EDTA–Sucrose (TES) Buffer (20 mM Tris, 2.5 mM EDTA, 20% Sucrose) and incubating it for 30 min at 0°C followed by addition of four times Tris–EDTA (TE) Buffer (20 mM Tris, 2.5 mM EDTA), followed by mixing gently and incubating for 1 h at 0°C. This was then centrifuged at 10,000 rpm for 15 min. The pellet was treated with Tris–Urea Buffer (TUB) (20 mM Tris, 8 M urea) overnight at 4°C. Subsequently this was spun at 12,000 rpm for 15 min. The solubilized crude supernatant was loaded onto a Ni-NTA column. The matrix was then washed with five times the bed volume of TUB. This was followed by elution with imidazole (10, 50, 100, 200, 300 mM imidazole in TUB). The fractions were checked using SDS-PAGE. The trials were also made using Tris–NaCl–Buffer (TNB) (20 mM Tris, 500 mM NaCl). Samples were analyzed by SDS-PAGE. The protein bands on SDS-PAGE were checked by Colloidal Coomassie staining (Kang et al. 2002). Protein estimation was done by Bradford's method using GENEI Protein Estimation Kit (Bangalore Genei, India).

The protein was confirmed by western blot. The purified protein (5 µg) was mixed with 5× SDS sample buffer and incubated in boiling water bath for 5 min. The sample was

loaded onto 12% polyacrylamide gel and electrophoresis was performed at 100 V for 2 h. The resolved protein was transferred to a nitrocellulose membrane using semi-dry transfer system (Bio-Rad) after equilibration with TOW-BIN buffer. The membrane was blocked with 5% skim milk in 1× PBS for overnight. The membrane was washed with washing buffer containing Tween-20 in 1× PBS and incubated for 2 h with rabbit anti-myocilin antibody (1:1,000). This was followed by incubating with biotinylated goat anti-rabbit IgG (1:1,000) for 1 h. The membrane was probed with peroxidase conjugated streptavidin diluted 1:1,000 in 1× PBS and developed with the substrate 4-Chloro-1-naphtho.

Relative unfolding of the protein was checked by fluorescent emission spectroscopy using F-2500 FL spectrophotometer (Hitachi). The protein was diluted to 10 µg/ml concentration. The protein was unfolded in Tris buffer containing 2, 4, 6, and 8 M urea. Emission spectra from 250 to 800 nm were scanned after an excitation of 280 nm. Cuvette with 1 cm path length was used with the parameters; Scan speed 1500 nm min with Response of 0.08 s. The ratio between the peak obtained for protein in buffer without (340 nm for folded conformation) and with (346 nm for unfolded conformation) urea was calculated which gives us the ratio of unfolding (F340/F346). The relative unfolding ratio was calculated using the ratio of unfolding of protein in buffer without urea as a reference.

## Results and discussion

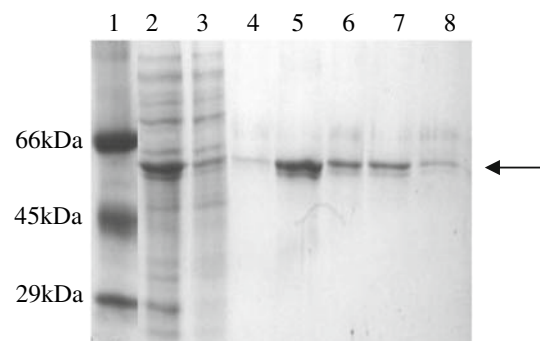
In pRSET-Myoc, myocilin coding region is cloned into pRSETb under a T7 promoter. The construct also provides an N-terminal His-tag. The protein expression was low and the protein was detected in the western blot after affinity purification (Fautsch et al. 2000). The expression of the human myocilin gene in a prokaryotic system can be affected due to codon usage bias or rare codon occurrence and translational control due to mRNA folding.

The analysis of the mRNA folding of the translation initiation region of the pRSET-Myoc suggested that the start and ribosome binding site were likely to be sequestered in the mRNA secondary structures in pRSET-Myoc. Codon usage of myocilin is closer to the codon usage of low expression proteins of *E. coli* (correspondence distance of 1.57) as against codon usage of proteins that are highly expressed (correspondence distance of 2.08). Therefore, the enhancement of translational machinery was found to be necessary. The occurrence of the rare codons of the gene which are codons found in the gene but not commonly preferred in the expression system were examined. The aminoacids for which rare codons occur and their extent of occurrence in the myocilin gene is as follows: glycine 7;

arginine 30; isoleucine 2; leucine 3; proline 7. Totally 49 rare codons are present in the myocilin gene.

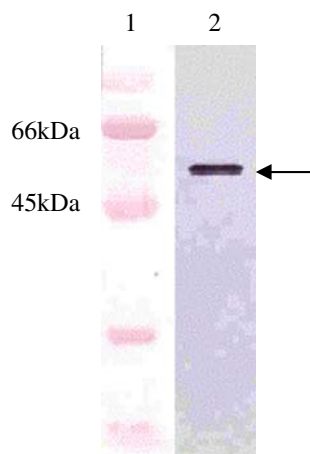
Initially over expression of myocilin using pET-Myoc construct was tried in BL21(DE3) strain. However, the myocilin expression was very low and it was detected only in dot blot. The use of pET-Myoc in the strain BL21(DE3)RIL+ which helps to overcome the rare codon problem did not help probably since myocilin has seven prolines but the strain does not have the gene-encoding tRNA for rare codon of proline. The change of induction system to NaCl in GJ1158 with both pRSET-Myoc and pET-Myoc did not help in over expressing myocilin. Both pET-Myoc and pC6H-Myoc showed over expression when repressed with 1.2% glucose in Luria Broth (LB) medium followed by induction with 0.5 mM IPTG in LB medium at 27°C for 4 h. If the protein is toxic to the cell due to basal expression of the protein, over expression is likely to be attained when it is repressed using glucose before induction. Over expression could not be attained without repression and at 37°C. Further work has been carried out using pC6H-Myoc as it has a His-tag, which makes the purification easier. The protein obtained from the pC6H-Myoc construct is called C6H-Myoc. No over expression was seen when the same conditions were tried with pRSET-Myoc suggesting that it is a combination of translational control due to mRNA folding, codon bias, occurrence of rare codons and protein toxicity in *E. coli* that inhibits the over expression.

The induced pC6H-Myoc/Rosetta(DE3)pLysS culture pellet was subjected to osmotic shock, to check if the protein is in soluble fraction. As the C6H-Myoc protein was seen found in the insoluble fraction, the insoluble fraction was treated with Urea in Tris buffer (TUB). C6H-Myoc was solubilized with TUB and purified using Ni-affinity column chromatography (Fig. 1). The denaturant

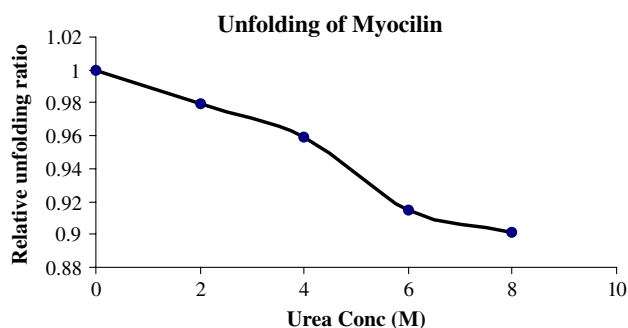


**Fig. 1** C6H-Myoc purification using Tris Urea Buffer. Lane 1. Marker, 2. Soluble fraction, 3. Flowthrough, 4. 10 mM imidazole in TUB, 5. 50 mM imidazole in TUB, 6. 100 mM imidazole in TUB, 7. 200 mM imidazole in TUB, 8. 300 mM imidazole in TUB. Colloidal Coomassie stained 12% SDS-PAGE. The arrow denotes purified recombinant human myocilin

urea was removed during elution and the protein was found to be stable in TNB. The protein was eluted using 100 and 200 mM imidazole in TNB. The purified C6H-Myoc was found to be stable as detected by SDS-PAGE. The purity and the refolding of the protein were confirmed by western blot using antibody to myocilin (Fig. 2). The purified protein is in a folded conformation as seen also by fluorescence emission spectra having a maximum at 340 nm for protein in buffer without urea and a 6 nm red shift for protein in buffer with urea. The relative unfolding ratio shows the gradual unfolding of the protein with increased urea concentration (Fig. 3). The final yield of the protein was found to be around 8 mg from 1 l of culture. Large scale production of the protein will enable screening of crystallization conditions for structure determination. Meanwhile, the availability of an over expression construct and a 3D-model (Kanagavalli et al. 2004) paves the way for site directed mutagenesis work to understand the structure–aggregation relationship of myocilin.



**Fig. 2** C6H-Myoc confirmation by Western blot. Lane 1. Marker, 2. Purified C6H-Myoc. 12.5% SDS-PAGE. The arrow denotes purified recombinant human myocilin detected by antibody to myocilin



**Fig. 3** Unfolding of C6H-Myoc in Tris Urea Buffer. Fluorescence spectroscopic analysis showing relative unfolding of C6H-Myoc using 2, 4, 6, and 8 M urea

## Conclusion

Recombinant Myocilin with C-terminal his-tag has been over expressed and purified using affinity column chromatography. As it is a human protein over expression in *E. coli* was tried in different strains and different vectors. Over expression was possible with pET20b+ vector and Rosetta(DE3)pLysS strain which overcomes the rare codon problem. However, this could be done only after basal expression was repressed using glucose before induction. Moreover, the protein appeared after induction as an inclusion body. These suggest that possibly the protein is toxic to the cell in the soluble state. The over expressed protein was solubilized in urea and purified using metal affinity chromatography. The purity and refolding of the protein was confirmed by western blot analysis and fluorescence emission analysis. Further biophysical and structural characterization is in progress.

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