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ORIGINAL ARTICLE

Optimization of expression and purification of HSPA6 protein from *Camelus dromedarius* in *E. coli*

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Abstract The HSPA6, one of the members of large family of HSP70, is significantly up-regulated and has been targeted as a biomarker of cellular stress in several studies. Herein, conditions were optimized to increase the yield of recombinant camel HSPA6 protein in its native state, primarily focusing on the optimization of upstream processing parameters that lead to an increase in the specific as well as volumetric yield of the protein. The results showed that the production of cHSPA6 was increased proportionally with increased incubation temperature up to 37 °C. Induction with 10 μM IPTG was sufficient to induce the expression of cHSPA6 which was 100 times less than normally used IPTG concentration. Furthermore, the results indicate that induction during early to late exponential phase produced relatively high levels of cHSPA6 in soluble form. In

Abbreviations: amp, ampicillin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani; 2× LB, double strength Luria–Bertani; NB, nutrient broth; Ni–NTA, nickel–nitrilotriacetic acid; OD_{600}, optical density at 600 nm; PMSF, phenylmethylsulfonyl fluoride; rpm, rotations per minute; TB, terrific broth

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addition, 5 h of post-induction incubation was found to be optimal to produce folded cHSPA6 with higher specific and volumetric yield. Subsequently, highly pure and homogenous cHSPA6 preparation was obtained using metal affinity and size exclusion chromatography. Taken together, the results showed successful production of electrophoretically pure recombinant HSPA6 protein from *Camelus dromedarius* in *Escherichia coli* in milligram quantities from shake flask liquid culture.

1. Introduction

Heat shock proteins (molecular chaperones) belong to a very essential group of proteins involved in the stability and control of structure–function of proteins under unfavorable conditions. Heat shock activity was first discovered in Drosophila in response to high temperature (Ritossa, 1962). Under thermal stress, the protein activity dramatically increased at transcription level (Wu, 1995). It has previously been observed that the open reading frames and regulatory sequences of heat shock genes are highly conserved (Lindquist, 1986). Research on different heat shock protein classes in various organisms indicated that heat shock genes are induced by many environmental factors such as high temperature, osmotic stress, oxidative stress, heavy metals, alcohol, damaged/aggregated proteins, exposure to toxins, different deficiencies and diseases (Jakob et al., 1999; Spiess et al., 1999; Santoro, 2000; Foit et al., 2009; Quan et al., 2011; Quan and Bardwell, 2012). Arabian camel (*Camelus dromedarius*) successfully survives under extremely stressful environment of heat and osmotic stresses. The cellular factor that helps in attaining a three-dimensional structure of the proteins and protects them from inactivation and misfolding during extremely hot and dry summer days in camel is largely unknown.

HSPA6 also known as HSP70B' (70 kDa), has been involved in maintaining cellular proteostasis (Heldens et al., 2010). The mRNA of HSPA6 was found to be significantly increased at transcription level under different stress conditions and could be used as a useful biomarker (Leung et al., 1990; Xu et al., 2000; Wada et al., 2005; Wang et al., 2005; Noonan et al., 2007). Both, HSPA6 and Human HSP72 are evolutionarily related but unlike HSP72, HSPA6 is stringently inducible, resulting in no significantly detectable level in most cells (Leung et al., 1990; Parsian et al., 2000).

The camel's HSPA6 nucleotide and amino acid sequences have shown a high degree of similarity with other mammalian species. Moreover, the predicted three dimensional structure of camel HSPA6 showed 87% and 82% similarity with human and mouse HSPs (Elrobh et al., 2011). However, detailed biophysical, structural and functional studies using pure camel heat shock proteins are lacking. To perform such studies, highly pure homogenous preparations of a protein in milligram quantity are needed. Keeping this in mind, this study was aimed at expressing, optimizing and producing a large quantity of pure recombinant cHSPA6 in *Escherichia coli*. We have selected *E. coli* to express the recombinant cHSPA6 because it has been proven to be a very good host for the heterologous expression of recombinant proteins, and for many purposes *E. coli* is the best host (Baneyx, 1999; Pines and Inouye, 1999; Lebendiker and Danieli, 2014; Rosano and Ceccarelli, 2014). The recent advances in physiology and genetics of the *E. coli* at molecular level offer a great opportunity for the rapid and economical production of recombinant proteins (Malik et al., 2006). It has previously been shown that genetically fused purification tag enhances the level of purity and protein recovery (Malik et al., 2006, 2007). Here in this study, we have optimized various growth parameters (cultivation temperature, inducer concentration, culture media, pre- and post-induction incubation), we were successful in obtaining highly pure cHSPA6 in milligram quantities from shake flask experiments.

2. Materials and methods

2.1. Chemicals and instruments

The ORF of cHSPA6 cloned on pET15 vector and expression host *E. coli* BL21 (DE3) pLysS were kindly provided by Elrobh et al. (2011). IPTG and ampicillin were obtained from Biobasic. Benzonase was purchased from Sigma, Chicken egg lysozyme from USB Corporation. Superdex 75, Ni–NTA resin, low molecular weight markers and prepacked columns were from Amersham Biosciences. All other chemicals used in this study were of reagent grade. Ultrospec 2100 pro Spectrophotometer, AKTA purification system, SDS–PAGE assembly were from Amersham Biosciences. Thermomixer, electroporator and benchtop cooling centrifuge were from Eppendorf. Lamp sterilizer from Cole-Parmer, shaking incubator from Jeio Tech, South Korea, gel scanner from Epson and pH meter was from Sentron.

2.2. Expression of cHSPA6 in E. coli

In this study, *E. coli* BL21 (DE3) pLysS was used for expression of cHSPA6. 1 µl of pET15-cHSPA6 plasmid was transformed into high efficiency chemical competent cell of *E. coli* BL21 (DE3) pLysS (Swords, 2003; Sambrook and Russell, 2006). We have used 200 µg/ml ampicillin in the solid and liquid medium to maintain plasmid throughout this study. Glycerol stocks of the transformed cells were made by mixing 1:1 ratio of the freshly grown overnight culture with 30% (w/v) sterilized glycerol solutions and stored at −80 °C. Single colony of *E. coli* BL21 (DE3) pLysS strain harboring pET15-cHSPA6 plasmid was inoculated into 20 ml LB_{amp} grown overnight in shaking incubator at 37 °C. Pre-inoculum (0.2 ml) was inoculated into 20 ml LB_{amp} and culture was grown at 37 °C until OD_{600} was reached 0.65. The expression of cHSPA6 was induced with isopropyl β-D-thiogalactopyranoside (IPTG) as described (Studier et al., 1990). In the preliminary expression experiment, culture at the mid exponential phase was induced with 1 mM IPTG. The culture was grown for 3 h post induction at 37 °C, 200 rpm. Before harvesting culture, final growth of the culture was calculated by measuring OD at 600 nm. To harvest, 1 ml

induced culture was centrifuged at 13,000 rpm for 5 min. Supernatant was discarded and pellet was resuspended in appropriate volume of 1× loading dye to achieve OD1/ml. The sample was boiled for 3 min and loaded on 12% SDS–PAGE. In the parallel experiment, uninduced culture was grown, harvested and sample analyzed on SDS–PAGE. In the control experiment, untransformed *E. coli* BL21 (DE3) pLysS strain was cultivated in the identical conditions. Equal amounts (equal number of cells) of transformed and untransformed culture with and without IPTG induction were loaded on 12% SDS–PAGE (Laemmli, 1970).

2.3. Optimization of cHSPA6 expression in E. coli

To increase the specific as well as volumetric yield of recombinant cHSPA6, a variety of independent cultivation parameters such as post induction incubation temperature, types of culture media, inducer concentration, pre-induction growth and post-induction incubation time were optimized.

2.3.1. Temperature optimization

To optimize cultivation temperature, fresh culture of *E. coli* BL21 (DE3) pLysS cells containing pET15-cHSPA6 plasmid was streaked on $LB_{amp\ plate}$. Single isolated colony was inoculated in 20 ml LB_{amp} and grown overnight at 37 °C, 150 rpm. Fresh inoculation (1%) was made in 20 ml LB_{amp} and incubated at 37 °C until the mid-exponential growth phase was achieved (OD_{600} = 0.9). After inducing with 100 μM IPTG at mid exponential phase, cultures were further incubated at three different temperatures (24, 30 and 37 °C) for 3 h at 150 rpm. Final growth of the cultures was measured in the induced and uninduced cultures. Two ml of cultures was harvested for soluble protein extraction. An equal amount of soluble crude extract was analyzed on SDS–PAGE (Laemmli, 1970).

2.3.2. Culture media optimization

To optimize culture media, overnight cultures of *E. coli* BL21 (DE3) pLysS harboring pET15-cHSPA6 were made in 20 ml LB_{amp} at 37 °C. From pre-inoculum culture, 1% was transferred into four different media (NB_{amp}, LB_{amp}, 2× LB_{amp} and TB_{amp}) in duplicate. When OD_{600} was reached in the mid exponential phase, one set of culture was induced with 100 μM IPTG and incubated at 37 °C at 150 rpm. After 3 h of incubation, final OD_{600} were measured. Two ml from each culture was harvested and pellet was resuspended in appropriate volume of lysis buffer to make OD2/ml. An equal amount of extracted soluble proteins was analyzed on 12% SDS–PAGE.

2.3.3. Inducer concentration optimization

Freshly prepared pre-inoculum culture was transferred into 8 flasks containing 20 ml LB_{amp} and grown at 37 °C until mid exponential phase. All cultures were induced with varying concentrations of IPTG (0, 10, 25, 50, 100, 250, 500 and 1000 μM) and further expressions were made for 3 h at 37 °C. After measuring final growth of all the cultures, 2 ml from each was harvested. Pellet was resuspended in appropriate volume of lysis buffer to make OD3/ml. An equal amount of soluble protein extract was analyzed on 12% SDS–PAGE.

2.3.4. Pre-induction growth optimization

E. coli cells harboring pET15-cHSPA6 plasmid were grown overnight in LB_{amp} at 37 °C. Fresh culture (0.2 ml) was inoculated in 4 flasks containing 20 ml LB_{amp} and incubated for different time at 37 °C. When the OD_{600} of the cultures reached 0.3, 0.6, 1.2 and 1.8, induction was made with 25 μM IPTG. After induction, each culture was incubated for 3 h at 37 °C, 150 rpm. Two ml culture was harvested in duplicate. Pellet was resuspended in appropriate volume of lysis buffer to make OD2/ml. An equal amount of soluble extract was analyzed by SDS–PAGE.

2.3.5. Post-induction incubation optimization

To evaluate maximum yield of cHSPA6, incubation time after induction was studied. Again, freshly grown 1% overnight culture was inoculated in 200 ml LB_{amp} and incubated at 37 °C to reach mid exponential phase. When OD_{600} reached 0.43, 25 μM IPTG was added to induce expression. Five ml culture was withdrawn post-induction at different time (0, 1, 2, 3, 4, 6 and 24 h) intervals. Two ml culture was centrifuged at 13,000 rpm and pellets were resuspended in lysis buffer to make OD2/ml. Equal volume from each samples (20 μl) was analyzed on 12% SDS–PAGE.

2.4. Biomass preparation and extraction of soluble cHSPA6

To produce biomass of *E. coli* expressing cHSPA6 in gram quantity, liter scale shake flask experiment was set up. Freshly grown pre-inoculum was transferred (1%) into 1 liter LB_{amp} in six liter conical flasks. Culture was induced with 25 μM IPTG when growth reached the mid exponential phase (OD_{600} = 0.6). After 5 h of post-induction incubation, cells were harvested at 5000 rpm for 30 min. Wet biomass was stored at −80 °C. About 2.5 g biomass was resuspended in 15 ml of lysis buffer (50 mM Tris, 200 μM PMSF, 3 mM DTT, 2 mM Magnesium chloride and 300 μg/ml lysozyme, pH 7.5) (De Mey et al., 2008). The biomass was homogenized in a mechanical homogenizer to uniform slurry and then 1 μl benzonase (250 U/μl) was added to the slurry and incubated at room temperature for 1 h. The slurry was then subjected to mild sonication (Soniprep 150, MSE (UK) Ltd,) twice for 10 s at 5 μm amplitude at 4 °C. The slurry was then centrifuged for 30 min at 13,000 rpm at 4 °C. Supernatant was collected and was filtered through 0.45 μm filter. To this sodium chloride was added to a final concentration of 500 mM and Imidazole to a final concentration of 10 mM.

2.5. Purification of cHSPA6

Homogenous preparation of cHSPA6 in two chromatographic steps.

2.5.1. Ni–NTA chromatography

HisTrap column (1 ml) was equilibrated with 20 ml equilibration buffer (50 mM Tris, 10 mM imidazole and 500 mM sodium chloride, pH 7.5) at 1 ml/min. The filtered supernatant was then loaded onto the column at 1 ml/min, connected with AKTA FLPC. Flow-through was collected. The column was washed with equilibration buffer at

1 ml/min till the absorbance at 280 nm reached basal level and the wash was collected. To elute bound protein, gradient was set 0 to 50%B (50 mM Tris, 500 mM imidazole and 500 mM sodium chloride, pH 7.5) at 0.5 ml/min and the protein was fractionated. Presence of cHSPA6 in crude extract, flow through, wash and different fractions were analyzed on 12% SDS–PAGE.

2.5.2. Size exclusion chromatography

The fractions containing the protein of interest were pooled and loaded onto superdex 75 column 26/60, connected with AKTA FPLC. The column was pre-equilibrated with (25 mM Tris, 250 mM sodium chloride, and pH 7.5). Flow rate was 1.5 ml/min. Highly enriched cHSPA6 was loaded using superloop. The eluted protein fractions were analyzed for protein content on 12% SDS–PAGE.

2.6. Protein quantification

Fractions containing pure cHSPA6 were pooled. Total protein was quantified by Bradford method (Bradford, 1976).

2.7. Silver staining

To analyze the purity of pooled protein fractions eluted from gel exclusion chromatography, 25 ng protein was run on SDS–PAGE. The gel was stained with silver staining by following the procedure of Tunon and Johansson, 1984. This protocol allows very sensitive detection (1–10 ng of protein per band) with negligible background staining.

3. Results and discussion

3.1. Expression of recombinant cHSPA6

The schematic diagram of cHSPA6 is shown in Fig. 1. Briefly, a highly specific TEV protease cut site was placed between N-terminal His-tag and cHSPA6 to remove tag from fusion protein after purification. In the preliminary expression experiment, *E. coli* BL21 (DE3) pLysS cells containing expression plasmid pET15-cHSPA6 were induced with 1 mM IPTG when growth was reached in the mid-log phase ($OD_{600} \sim 0.75$) in LB_{amp} medium at 37 °C. Post-induction culture was incubated for 3 h at 150 rpm. Negative control experiments (uninduced cells with plasmid and induced cells without plasmid) were performed in parallel. The growth of induced culture expressing cHSPA6 was significantly reduced (Table 1). SDS–PAGE analysis of the total cell lysate revealed that culture induced with 1 mM IPTG showed over expression of cHSPA6 fusion protein band at the expected size (72.7 kDa) while in the negative control experiments no overexpression band was observed (Fig. 2).

Table 1 Typical growth of cultures under standard expression condition.

Culture	Cell density at the time of induction (OD_{600})	Cell density after 3 h of post-induction (OD_{600})
Induced cells without plasmid	0.8	3.5
Uninduced cells with plasmid	0.75	3.1
Induced cells with plasmid	0.77	1.9

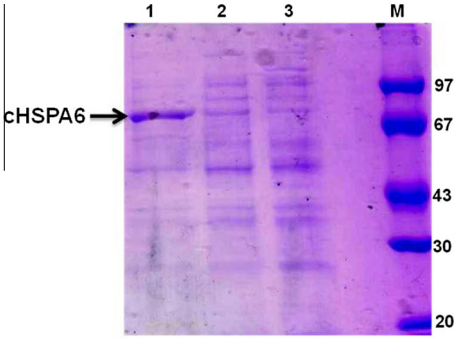

Figure 2 Expression of cHSPA6 fusion protein. cHSPA6 was expressed using standard expression parameters such as 1 mM IPTG, 37 °C pre- and post-induction temperatures and 3 h post-induction incubations. Overexpressed cHSPA6 band was seen at expected size in the lane 1 while in the negative controls (lane 2 and 3; uninduced culture and induced cells without plasmid, respectively) corresponding band was not present.

3.2. Optimization of cHSPA6 overexpression in E. coli

The aim of optimization parameters for folded cHSPA6 expression was to achieve the highest amount of specific yield (units of proteins per unit cell) in shortest possible time (per unit time) in the shake flask experiment. To achieve this goal, important growth and protein folding parameters were optimized.

3.2.1. Effect of temperature on the overexpression of cHSPA6

To evaluate the effect of growth temperature on the expression of cHSPA6 in soluble state, cultures were incubated at three different temperatures (24, 30 and 37 °C) for 3 h after induction with IPTG. Final growth at different temperatures is listed in Table 2. The effect of temperature on the level of

Figure 1 Schematic diagram of the hexa-histidine tagged cHSPA6 fusion protein. cHSPA6 fusion protein was cloned under strong T7 promoter. Amino acid numbering starts with the N-terminus of His-tag (1–10), followed by highly specific TEV protease site (11–18) labeled as X-site and cHSPA6 (19–661).

soluble cHSPA6 is clearly seen (Fig. 3). The expression level of cHSPA6 increases with an increase in temperatures. Total cell lysate was fractionated and nearly all the cHSPA6 was found in the soluble fraction.

Camel HSPA6 gene was cloned on pET15 vector, which had pUC origin of replication. pUC origin of replication is similar to pBR322 origin of replication except for a single point mutation in the replication primer (RNA II) which made pUC origin of replication temperature sensitive, leading to dramatically several fold increase in copy number of plasmids as a function of temperature (Lin-Chao et al., 1992). Another important feature was cloning of HSPA6 under T7 promoter, one of the strongest known promoters (Makrides, 1996; Bandwar et al., 2002; Sorensen and Mortensen, 2005). The *E. coli* cells grow over wide range of temperatures (~15–42 °C), but the growth rate of the *E. coli* cells increases proportionally in response to an increase in growth temperature between 20 °C and 37 °C (Farewell and Neidhardt, 1998). It has been observed that in the temperature range of 23–42 °C, the number of ribosomes per cell and level of rRNA remain constant (Herendeen et al., 1979; Ryals et al., 1982). In fact, at normal temperature range, peptide chain elongation rate increases with respect to an increase in temperatures (Farewell and Neidhardt, 1998) which results in higher synthesis of proteins. Therefore, at higher temperatures, the rate of cHSPA6 synthesis was faster due to an increase in plasmid copy number, transcription rate (mRNA synthesis) and peptide chain elongation rate (Fig. 3).

Every protein folds differently depending upon its characteristics such as size, hydrophobicity, post-translational modifications, isoelectric points, etc. (Doig and Williams, 1991; Lilie et al., 1998; Baneyx and Mujacic, 2004; Kong and Guo, 2014; Lebendiker and Danieli, 2014). If the rate of protein synthesis supersedes protein folding machinery, the recombinant protein will then aggregate and result in inclusion body formation (Rudolph and Lilie, 1996; Fahnert et al., 2004; Gopal and Kumar, 2013; Klint et al., 2013). In this experiment, the recombinant protein expressions at different temperatures were evaluated to determine the optimum temperature where faster growth rate (higher biomass production) did not compromise folding of cHSPA6 protein. It was observed that cHSPA6 was folded well and remained in the soluble fractions at all the tested temperature. It was also found that the specific as well as volumetric yield of cHSPA6 increased at higher temperature. Therefore, 37 °C was found to be the optimum temperature and further optimization was performed at this temperature.

Table 2 Effect of temperature on the growth of induced and uninduced culture.

Growth temperature (°C)	IPTG (μM)	Cell density at the time of induction (OD_{600})	Cell density after 3 h of post-induction (OD_{600})
24	0	0.65	1.67
24	100	0.66	1.47
30	0	0.7	1.28
30	100	0.72	1.19
37	0	0.69	3.04
37	100	0.68	2.17

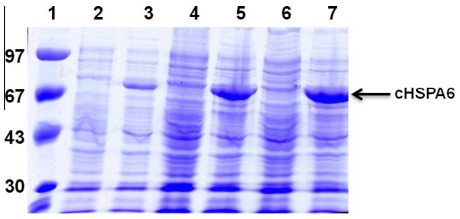

Figure 3 Effect of temperature on the overexpression of cHSPA6. Lane 1, low molecular weight marker; 2, uninduced culture at 24 °C; 3, induced culture at 24 °C; 4, uninduced culture at 30 °C; 5, induced culture at 30 °C; 6, uninduced culture at 37 °C; 7, induced culture at 37 °C.

3.2.2. Culture media optimization

To achieve the optimum expression and production of folded soluble cHSPA6 in *E. coli*, we evaluated four different media formulations. To examine the effect of different media compositions, overnight culture was made in LB_{amp} at 37 °C. Overnight cultures (1%) were transferred into four different media (NB_{amp}, LB_{amp}, 2× LB_{amp} and TB_{amp}) in duplicate. When OD_{600} was reached in the exponential growth phase, one set of culture was induced with 100 μM IPTG. After the induction, the cultures were grown at 37 °C for 3 h at 150 rpm to get the recombinant protein expression. Table 3 lists the typical final growth. The final growth varied due to different growth rates under each media. TB medium showed the highest growth rate under uninduced conditions while lowest under induced culture condition. Therefore, induced culture in the TB medium expressing cHSPA6 showed the lowest volumetric yield. On the other hand, the LB media showed relatively higher growth rate under induced culture conditions resulting in higher volumetric yield. Relative level of recombinant cHSPA6 protein accumulation in the soluble fractions of the cell lysate under induced and uninduced cultural conditions was observed by SDS–PAGE (Fig. 4). Overall, the four different uninduced cultures showed no significant leakiness whereas all induced cultures showed similar cHSPA6 expression levels. Because the strength (richness) of

Table 3 Effect of media on the cell density of induced and uninduced culture.

Growth medium	IPTG (μM)	Cell density at the time of induction (OD_{600})	Cell density after 3 h of post-induction (OD_{600})
Nutrient broth	0	0.56	1.39
Nutrient broth	100	0.55	1.14
LB broth	0	0.6	2.27
LB broth	100	0.59	1.52
2× LB broth	0	0.63	1.92
2× LB broth	100	0.64	1.53
Terrific broth	0	0.68	3.13
Terrific broth	100	0.67	0.92

medium had no apparent effect on the yield of cHSPA6 in liquid cultures, further experiments were carried out in LB medium at 37 °C.

The aim of optimizing media was to produce the highest amount of folded soluble recombinant cHSPA6 protein per unit volume and time. The production of secondary metabolites in the bacterial cells are largely known to depend upon the constituents of the medium in which, they are grown (Sheldon et al., 2001; Sahdev et al., 2008); however, little is known about the effect of media formulations on protein folding and accumulation of soluble fractions (Lee, 1996; Rispoli and Shah, 2007).

3.2.3. Inducer concentration optimization

The evaluation of the different concentrations of inducer for the expression of recombinant cHSPA6 protein was done in liquid cultures. Upon achieving the exponential growth phase (OD_{600} ~0.6), cultures were induced with 0, 10, 25, 50,100, 250, 500 and 1000 μM IPTG and were allowed to express for 3 h at 37 °C. Results presented in Fig. 5a indicate the cell growth rates at different IPTG concentrations. The results further showed that the growth of induced cultures was reduced proportionally at lower (up to 400 μM) IPTG concentrations. The effect of IPTG concentrations on the soluble fractions of cHSPA6 was compared on 12%SDS–PAGE gels by loading an equal amount of soluble crude extract (Fig. 5b). Results showed that all IPTG concentrations produced the soluble cHSPA6 crude extracts. It was also observed that the above 250 μM IPTG concentration, the band corresponding to cHSPA6 was found in the pellet fraction when analyzed on SDS–PAGE, indicating inclusion body formation at higher inducer concentration (data not shown), however at lower IPTG concentrations, the presence of cHSPA6 in the pellet fraction was insignificant. Interestingly it was found that even a low 10 μM IPTG concentration was enough to induce the cHSPA6 protein expression. The level of recombinant cHSPA6 expression in soluble fraction of *E. coli* was very similar at IPTG concentrations between 10 and 1000 μM (Fig. 5b, lane 3–9) (Neubauer et al., 1992; Donovan et al., 1996, 2000; Candan et al., 1998). This result indicates that a higher IPTG concentration has no significant effect on the yield of cHSPA6 protein. Therefore, for further optimization experiments a 40-fold lower IPTG concentration than the normal was used.

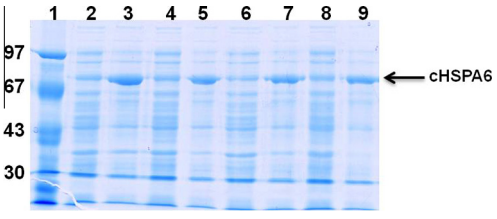

Figure 4 Effect of media on the overexpression of cHSPA6. Four different rich mediums (nutrient broth, NB; Luria–Bertani, LB; double strength Luria–Bertani, 2× LB; terrific broth, TB) were tested for optimum expression of cHSPA6. Lane 1, low molecular weight marker; 2, uninduced in NB; 3, induced in NB; 4, uninduced in LB; 5, induced in LB; 6, uninduced in 2× LB; 7, induced in 2× LB; 8, uninduced in TB; 9, induced in TB.

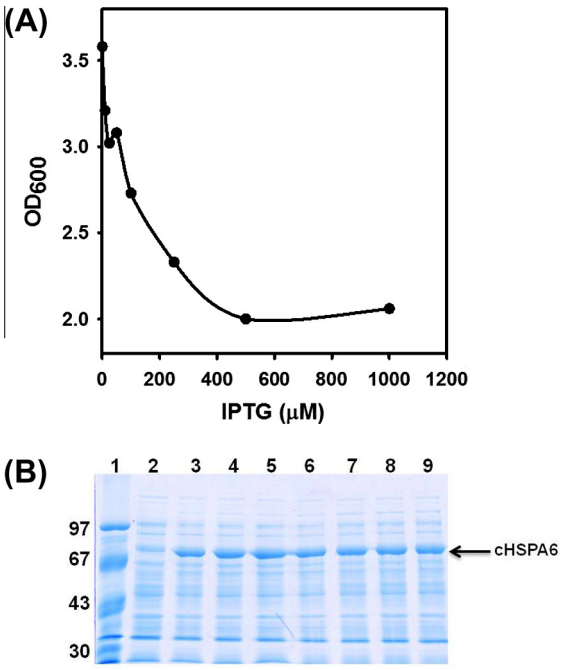

Figure 5 Growth vs inducer concentration. (a) Effect of IPTG concentration on the final growth of *E. coli* cells expressing cHSPA6. As the concentration of IPTG increases up to 400 μM, the final growth decreases proportionally. (b) SDS–PAGE analysis of effect of inducer concentration on the overexpression of cHSPA6. Here, effect of 0–1000 μM IPTG was examined. Lane 1, LMW marker; lane 2, 0 μM; lane 3, 10 μM; lane 4, 25 μM; lane 5, 50 μM; lane 6, 100 μM; lane 7, 250 μM; lane 8, 500 μM and lane 9, 1000 μM IPTG were added in the cultures.

In this study, *E. coli* Bl21 (DE3) pLysS strain was used which is lacY1$^+$ strain and does not allow titration of IPTG into the cells. *E. coli* Tuner (DE3) strain (Novagen, Madison, USA) which is a *lacZY* deletion strain, allows a concentration-dependent induction with IPTG in the *E. coli* cells and will be helpful in tuning accurately the level of inducer for optimal production of recombinant proteins in *E. coli*. The lac permease (lacY) mutation permits steady transport of IPTG uniformly into all cells, which allows uniform induction with IPTG in a true concentration-dependent manner. Therefore, by tuning the IPTG concentration, production of recombinant proteins can be regulated from very low to fully induced expression levels. Hence, inducing at low expression rate may boost the production of difficult proteins in the native folded state (Candan et al., 1998; Turner et al., 2005; Hartinger et al., 2010).

3.2.4. Pre-induction growth optimization

After optimization of IPTG concentration, relationship between cellular growth at the time of induction and cHSPA6 production was studied. In order to determine the optimum growth at the time of induction, four liquid culture experiments were performed in parallel. Each culture was induced at different growth phases. Cells were initially allowed to reach different growth stages. Cultures were then induced with 25 μM IPTG when OD_{600} of the cultures reached 0.3, 0.6, 1.2 and 1.8, representing early exponential, exponential,

late exponential and stationary growth phases, respectively. After induction, protein production was further allowed to express for 3 h. The results showed that the yield of cHSPA6 remained same when induced at early exponential to late exponential stage (Fig. 6, lanes 2–4) but induction level was reduced when cells reached in the stationary growth phases (Fig. 6, lane 4). The final growth of the cultures is shown in Table 4. These results showed that the optimal induction at the mid exponential phase produced high levels of soluble proteins with high cell density.

This result is in agreement with earlier studies, where it has been reported that the induction level of recombinant protein increases up to the late exponential phase but expression level reduces in the stationary growth phase (Candan et al., 1998; Maldonado et al., 2007). This result also indicates that, cHSPA6 production could be induced at any stage between early exponential and late exponential growth stage.

3.2.5. Post-induction incubation optimization

Effect of post-induction incubation period affects the overall folding, accumulation and productivity of recombinant proteins in *E. coli* (Shin et al., 1997; Wong et al., 1998). The duration of post-induction incubation period in the shake flask culture of *E. coli* is affected by several factors such as strength of promoter, inducer concentration, post-induction incubation time, solubility of recombinant protein and intrinsic properties of recombinant protein (Babaeipour et al., 2013). In order to determine the optimum time of post-induction incubation for higher yield of cHSPA6, shake flask culture was induced with 25 μM IPTG at the exponential growth phase. After the addition of IPTG, culture was incubated at 37 °C and samples were removed at different time intervals. The level of soluble cHSPA6 was determined by SDS–PAGE in the crude extract. As shown in Fig. 7a, the level of cHSPA6 expression reached at its maximal level within 1 h of induction and incubation at 37 °C. The level of cHSPA6 remained unchanged up to 24 h of post-induction incubation at 37 °C, indicating that the camel HSPA6 is well folded, soluble and resistant to *E. coli* cytosolic proteases. The growth of cells at different intervals of post-induction incubation is shown in Fig. 7b. (Palomares et al., 2004; Jana and Deb, 2005; Lee et al., 2006).

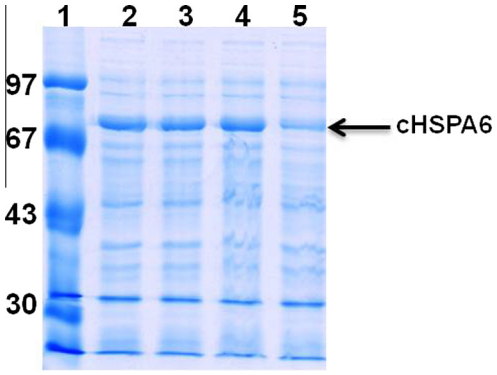

Figure 6 Effect of pre-induced growth on the expression of cHSPA6. Culture was induced at different growth phases. Crude soluble extract was loaded on SDS–PAGE. Lane 1, low molecular weight marker; lane 2, induction at OD 0.3; lane 3, induction at OD 0.6; lane 4, induction at OD 1.2 and lane 5, induction at OD 1.8.

Table 4 Effect of pre-induction growth on the final cell density.

IPTG (μM)	Cell density at the time of induction (OD_{600})	Cell density after 3 h of post-induction (OD_{600})
25	0.3	2.14
25	0.6	2.34
25	1.2	2.6
25	1.8	2.5

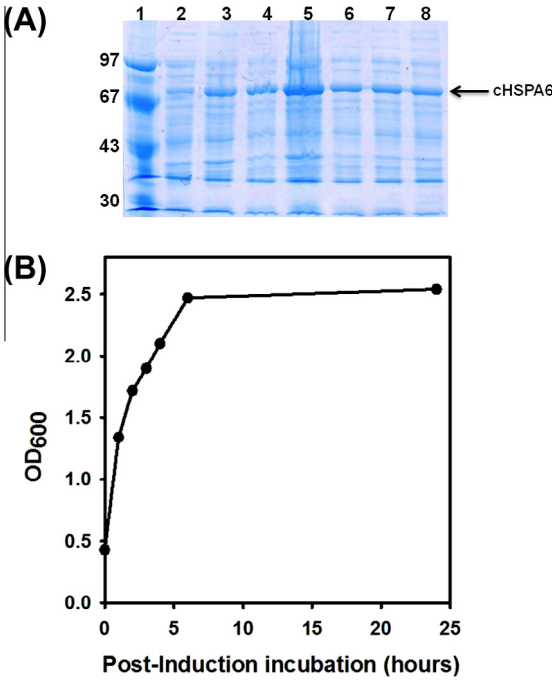

Figure 7 (a) Effect of post-induced incubation on the expression of cHSPA6. After induction, *E. coli* culture expressing cHSPA6 was incubated at 37 °C. Aliquot of culture was removed at different time intervals. Soluble protein was extracted and analyzed on SDS–PAGE. The yield of soluble camel protein remained same up to 24 h of incubation. (b) Post-induction incubation vs growth in the shake flask culture. The growth of induced culture increases with time but the growth was ceased after 5 h of incubation, resulting in no further increase in biomass after 5 h of post-induction incubation.

3.3. Biomass preparation

Under optimized condition for expression of folded cHSPA6, *E. coli* biomass was produced. As shown in Fig. 7b, the growth of induced culture was ceased approx. at OD_{600} = 2.5. Therefore, ~3 g wet biomass per liter culture was obtained.

3.4. Extraction and purification of cHSPA6

Crude extract was isolated by treating biomass with sixfold excess of lysis buffer followed by gentle sonication. Viscosity of the solution was removed by treatment with a strong nuclease (Benzonase) and slurry was cleared by high-speed centrifugation and filtration through 0.45 μm syringe filter. cHSPA6

fusion protein contains N-terminal $(His)_6$-tag. To suppress non-specific binding, 10 mM Imidazole and 500 mM NaCl were added in the crude extract which was then passed though equilibrated HisTrap FF Ni–NTA column (Fig. 8a). The bind, wash and elute fractions were analyzed by SDS–PAGE, indicating that cHSPA6 efficiently bound on column and was eluted by imidazole. As shown in Fig. 8b, lanes 8–10, cHSPA6 was highly enriched. Therefore, eluted fractions (19–26) containing relatively pure camel were pooled. After Ni–NTA chromatography, ~17 mg highly enriched cHSPA6 was obtained which corresponds to 6.4 mg per gram wet biomass.

Ni–NTA chromatography system is a rapid and convenient purification technique. Proteins fused with His-tag at either ends (N- or C-terminus) in native or denatured state bind tightly with high affinity on immobilized nickel ions. The strong binding between tagged protein and matrix allows easy washing of contaminants but gentle and efficient elution of bound His-tagged protein by competition with Imidazole or by slightly reducing pH in the acidic range (Crowe et al., 1994).

The HSP70 family of proteins has a propensity to dimerize and oligomerize (Schonfeld and Behlke, 1998; Borges and Ramos, 2006). Therefore, to obtain homogenous preparation

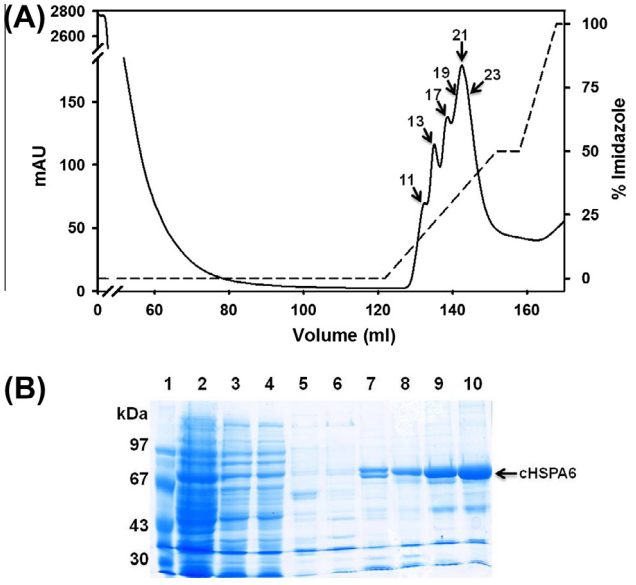

Figure 8 (a) Ni–NTA purification of (His)6-tagged cHSPA6 fusion protein. Crude extract was subjected to Ni–NTA chromatography. The crude extract was passed through the column, equilibrated with 50 mM Tris, 10 mM imidazole and 500 mM sodium chloride pH 7.5. The column was washed with 50 mM Tris, 10 mM imidazole and 500 mM sodium chloride pH 7.5. His-tagged cHSPA6 was eluted by a linear 0–50% gradient of 50 mM Tris, 500 mM imidazole and 500 mM sodium chloride, pH 7.5 (dashed line, ---). The protein chromatogram is shown with the solid line. Fractions indicated by arrows were analyzed on SDS–PAGE. (b) The protein separation was done on 12% SDS–PAGE. Lane 1, low molecular weight marker; lane 2, crude extract; lane 3, flow through; lane 4, wash; lane 5, fraction 11; lane 6, fraction 13; lane 7, fraction 17; lane 8, fraction 19; lane 9, fraction 21; lane 10, fraction 23.

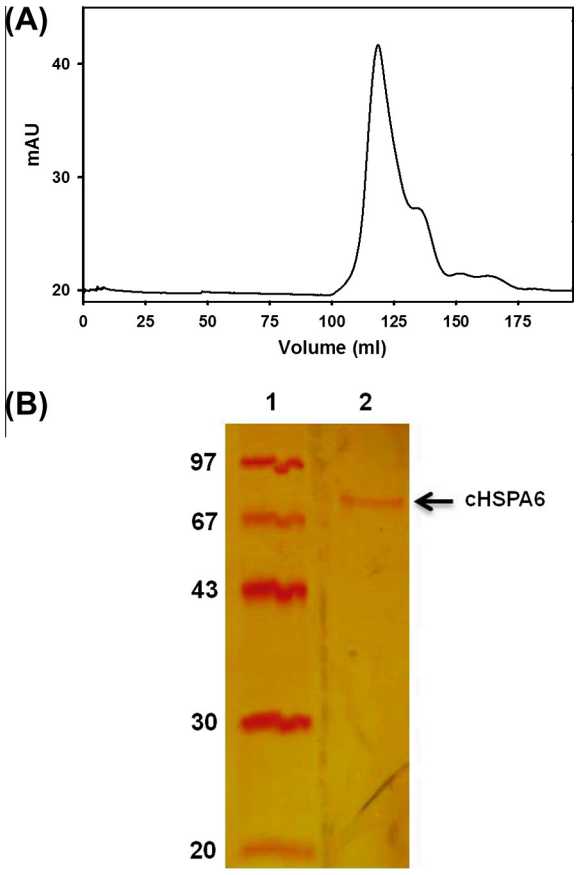

Figure 9 (a) Final purification of cHSPA6 via size exclusion chromatography. Gel filtration column (Superdex 75) was pre-equilibrated with 25 mM Tris, 250 mM sodium chloride, pH 7.5. The purity of the fractions indicated with arrow was analyzed on SDS–PAGE. (b) Analysis of purity of eluted protein from Superdex 75 column by silver staining. Lane 1, low molecular weight marker; lane 2, Pool of fractions obtained from size exclusion chromatography.

of cHSPA6 in monomeric state, the Ni–NTA elute was subjected to size-exclusion chromatography. In the gel filtration chromatogram (Fig. 9a), shoulder peak and small peaks were present. The fractions were analyzed for the purity of cHSPA6 and highly pure fractions were pooled. The silver staining of the pooled cHSPA6 showed a single band (Fig. 9b). After polishing step, 9 mg highly pure cHSPA6 was obtained, corresponding to 3.6 mg per gram wet biomass.

4. Conclusion

Following the thorough utilization of optimization parameters, our results show that a 100-fold less than the usual inducer concentration (10 μM) which is routinely used in expression experiments was sufficient to express and produce the optimum amount of cHSPA6 in *E. coli*. In addition, the lower concentration of IPTG had no adverse effect on the growth rate and showed higher biomass. Induction between early and late exponential growth phase results in similar yield of recombinant proteins. Moreover, five-hour post-induction incubation at 37 °C was sufficient to produce higher level of cHSPA6 in native state.

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