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Effect of osmotic stress and heat shock in recombinant protein overexpression and crystallization

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Abstract

Overexpressed recombinant proteins in bacteria often tend to misfold and accumulate as soluble aggregates and/or inclusion bodies. A strategy for improving the level of expression of recombinant proteins in a soluble native form is to increase the cellular concentration of osmolytes or of chaperones. This can be accomplished by growing the bacterial cells in the presence of high salt, sorbitol, and betaine as well as exposing the cells to a heat shock step. Our results suggest that by growing the cells under varied conditions one may be able to express targets as soluble proteins (from previously insoluble targets) and to improve the chances of their crystallization.

Keywords: Recombinant protein; Solubility; Crystallization; Osmotic stress

The growing demands from proteomics, drug development and biotechnology programs for recombinant proteins are often met by expression and purification of proteins in *Escherichia coli* [1] because of the simplicity of the system. However, many proteins, particularly mammalian proteins, do not express well in a useful form in *E. coli* due to the absence of a proper folding environment or post translational modification. For the latter a new strategy, confirmed by successful production of glycosylated protein, has been proposed to obtain certain posttranslational protein modification in *E. coli* [2–4].

As for the proper folding environment, it is well known that bacteria have defense mechanisms to protect native proteins from misfolding and aggregation caused by osmotic and/or heat stress. These mechanisms include intracellular accumulation by synthesis or uptake of osmolytes and synthesis of heat shock proteins [5,6]. Heat shock proteins are molecular chaperones, some of which actively drive folding processes, whereas others prevent protein aggregation [7]. Native bacterial chaperones co-expressed or in fusion with target proteins help prevent aggregation and correctly arrange disulfide bonds of recombinant proteins in several cases [8–11]. It has been shown that the solubility of some recombinant proteins can be enhanced by applying heat-shock prior to induction, possibly due to chaperone induction [12]. Adding ethanol to the growth media mimics heat-shock response in *E. coli* and enhances solubility of some recombinant proteins [13–15].

Osmolytes accumulated in cells have also been found to stabilize the native state of proteins [16–18]. Although the stabilization mechanism is not completely understood, there is more evidence favoring an osmophobic theory: in the presence of an osmolyte, the backbone of a protein in the denatured state is highly exposed to the osmolyte; causing more destabilization of the denatured state due to unfavorable interaction between the osmolyte and the protein backbone than the destabilization of the native state. This results in enhancement of protein stability [19,20]. It has been shown with several proteins that some osmolytes, when added to the growth media, assist protein folding resulting in increased protein solubility [21–23]. Preadapta-

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tion of *E. coli* cells to increasingly high concentrations of salt in the media leads to crosstalk between osmolytes and heat-shock proteins and decreases the tendency of soluble proteins to form insoluble aggregates during heat-shock [24].

In this work, we investigated the effect of heat-shock, osmotic stress, and osmolyte supplementation on the production of several highly insoluble proteins. We show that a combination of osmolytes and high salt concentration in the growth media enhances protein solubility. In addition, expressing recombinant proteins under these conditions can improve their crystallization properties.

Materials and methods

Cloning

The MPN330 (1260B) [MP506/MPN330, BSGCAIR30529 gi number 1674200, annotation MG237 homolog, from *Mycoplasma genitalium*] gene was amplified by PCR using *Mycoplasma pneumoniae* genomic DNA template and primers designed for ligation-independent cloning (LIC)[1] [25]. The amplified PCR product was prepared for vector insertion by purification, quantitation and treatment with T4 DNA polymerase (New England Biolabs, Beverley, MA) in the presence of 1 mM dTTP. The prepared insert was annealed into the LIC expression vector pB2, a derivative of pET21a (Novagen, Madison, WI) that expresses the cloned gene fused to a non-cleavable N-terminal His_6-sequence and transformed into chemical competent DH5α cells to obtain fusion clones. The frwD (1016B) gene [BSGCAIR30326, gi number 1790390, annotation PTS system fructose-like IIB component 2] was amplified by PCR using *E. coli* K12, genomic DNA template and the M5005_Spy1602 (1326B) gene [BSGCAIR30595, gi number 15675702, annotation conserved hypothetical protein] was amplified using *Streptococcus pyogenes* genomic DNA template. These two inserts were annealed into the LIC expression vector pB3, a derivative of pET21a (Novagen, Madison, WI) that expresses the cloned gene with an N-terminal His_6 tag-TEV (tobacco etch virus) cleavage sequence. Proteins were expressed in *E. coli* strain BL21(DE3) Star/pSJS1240 [26].

Growth of cultures

Escherichia coli BL21(DE3)Star/pSJS1240 transformed with pB3.frwD, pB3.M5005_Spy1602, or pB2.MPN330 were grown on Luria Bertani (LB) agar plates containing 100 μg/ml ampicillin and 100 μg/ml spectinomycin. Cells were adapted to the high salt concentration by overnight growth in Luria Broth (LB) in the presence of 0.5 M NaCl. Overnight cultures were diluted 1:100 into fresh LB media containing 0.2% glucose, 0.5 M NaCl, 1 mM betaine (LBNB) and incubated at 37 °C with shaking until the OD 600 was 0.6–0.8. Cells were then cooled to 20 °C and induced at a final concentration of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures were incubated with shaking overnight.

For the heat-shock experiment, the overnight culture was diluted 1:100 into LBNB and allowed to grow to an OD 600 of 0.9, then the culture was transferred to a 47 °C water bath until the same temperature was achieved in the media. The flask was then transferred to an Infors shaker (Infors AG, Switzerland) previously set at 47 °C and induced with 0.5 mM IPTG. Cells were kept at this temperature for 20 min and shaken at 210 rpm. The flask was then transferred to another shaker set at 20 °C for overnight growth. Similar growth conditions were carried out for cells grown in the presence of 0.5 M sorbitol (S) and 1 mM betaine (B) as described above.

Auto-inducing media (ZYP and PASM) was prepared as described by Studier [27]. Seleno-methionyl (Se-met) protein was prepared according to the method of Doublie [28] using the methionine auxotroph B834 (DE3)/pSJS1240 background strain.

Cell lysis and purification

Cell paste (20 g) was resuspended in 100 ml of 50 mM Hepes, pH 8, 100 mM NaCl, 1 mM PMSF, 10 μg/ml DNase I, and Roche protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and disrupted by microfluidization (Microfluidics, Newton, MA). Cell debris was pelleted by centrifugation at 15,000 rpm for 20 min in a Sorvall centrifuge. The supernatant was then spun in a Beckman ultracentrifuge Ti45 rotor at 35,000 rpm for 30 min at 4 °C and affinity purified using a HisTrap Chelating column on an AKTA Explorer (GE Healthcare, Piscataway, NJ). The target was eluted with a gradient from 20 mM to 1 M imidazole. Fractions were pooled and in the case of pB3.frwD, the protein was dialyzed overnight at room temperature against 50 mM Hepes, pH 8, 100 mM NaCl, in the presence of TEV protease. After centrifugation, the supernatant was applied again onto the HisTrap Chelating column. The target was found in the flow through. With pB2.MPN330 and pB3.M5005_Spy1602, the His-tags were not cleaved off. pB2.MPN330 was further purified by ion-exchange chromatography using a 5 ml Hi Trap Q-Sepharose (GE Healthcare, Piscataway, NJ) column in buffer containing 50 mM Hepes, pH 8, 50 mM NaCl using a linear salt gradient from 50 to 500 mM NaCl. The purified samples were screened using dynamic light scattering (DynaPro 99, Wyatt Technology Corp., Santa Barbara, CA).

Crystallization

Screening for crystallization conditions was performed using the sparse matrix method [29] with several screens

[1] *Abbreviations used:* LIC, ligation-independent cloning; LB, Luria Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; S, sorbitol; B, betaine; Se-met; selenomethionyl; DLS, dynamic light scattering; OSS, optimum solubility screen.

from Hampton Research (Hampton Research, Aliso Viejo, CA) and the Wizard Screen (Emerald Biosystems, Oak Lawn, IL). The crystallization robot Hydra Plus-One (Matrix Technologies, Hudson, NH) was used to set the screens using the sitting drop vapor diffusion method at room temperature.

Single crystals of MPN330 were obtained in 1.5 M Li_2S0_4 and 0.1 M Tris–HCl, pH 8.5. The structure was determined at 2.5 Å by single-wavelength anomalous diffraction [30]. Crystals of frwD were obtained in 0.1 M Tris–HCl, pH 8.5, 20% w/v polyethylene glycol monomethyl ether 2000, and 0.01 M nickel (II) chloride hexahydrate. Crystals were soaked in K_2PtCl_4. Two data sets at 2.8 Å using synchrotron radiation tuned for the Pt of K_2PtCl_4 and sulfur of cysteine and methionine residues were collected. Crystals of M5005-Spy1602 were obtained in 0.1 M Tris–HCl, pH 8.5, 10 mM nickel chloride, 1 M lithium sulfate.

Results

We have screened different parameters to improve the solubility of our targets. Optimal conditions were then chosen for expression of the selected targets. The experiments were first done in small scale (2–5 ml) and then at a 2 L scale once the optimum conditions were obtained. We present three cases in which the solubility of the targets had been a problem but when growth conditions were altered, the proteins became more soluble and we were able to crystallize them for structural studies (Table 1).

MPN330

Expression of pB2.MPN330 in *E. coli* grown in LB and induced with 0.5 mM IPTG yielded 20 mg/L soluble and 40 mg/L insoluble target protein (Table 2). In the auto-inducing ZYP media, the amount of soluble protein expressed was 84 and 300 mg/L insoluble target. Both of these soluble samples were purified as described in Materials and methods using a HisTrap metal chelating column and the protein was concentrated to 33 mg/ml. Dynamic light scattering (DLS) suggested high polydispersity (3.37 nm radius/42% polydispersity). Crystallization trials were set up but yielded no crystals.

After multiple attempts, conditions were modified by growing the cells in LB and 0.5 M NaCl thus increasing the extracellular osmotic pressure as well as inducing a heat shock response by exposing the cells to 47 °C for 30 min. These growth conditions yielded expression of 50 mg/L soluble and 20 mg/L insoluble target. The soluble MPN330 was purified as above. The DLS measurements showed some improvement in the monodispersity of the sample (2.4 nm radius/32% polydispersity). This sample crystallized and diffracted to 2.5 Å.

Table 1
Three proteins were expressed under different growth conditions

Gene	Molecular weight (Da)	Gene source	Number of cysteines
MPN330	34,135	*M. pneumoniae*	1
frwD	12,637	*E. coli*	3
M5005_Spy1602	59,140	*S. pyogenes*	1

Table 2
Effect of growth conditions on crystallization of MPN330 protein

Media	Soluble/ insoluble (mg/L)	His-tag	Crystals
LB[a]	20/40	Uncleaved	No
ZYP[b]	84/300	Uncleaved	No
LBN + heat shock[c]	50/20	Uncleaved	Yes, 2.5 Å *
SMG.NB[d] + heat shock	12/0	Uncleaved	Yes, structure solved

Cells were grown in 1 L of media. Amount of soluble and insoluble protein target was estimated based on SDS/PAGE.
[a] LB, Luria Broth.
[b] ZYP, auto-inducing media.
[c] LBN + heat shock: LB media containing 0.5 M NaCl; heat shock was applied for 20 min (see Materials and methods) prior to induction.
[d] SMG.NB, seleno-methionyl media containing 0.25 M NaCl; heat shock step was applied for 30 min prior to induction.
* Crystal diffraction.

The same approach was used to prepare Se-Met labeled MPN330 protein by using the methionine auxotroph B834(DE3) as the background strain and growing them according to the method of Doublie [27]. The level of soluble SeMet labeled protein was lower than in LBN + heat shock (12 mg/L soluble expression) but there was no insoluble protein detected (Table 2). The SeMet sample was purified as described in Materials and methods. Crystallization attempts were successful and the three-dimensional structure was determined at 2.5 Å by single-wavelength anomalous diffraction [30].

frwD

The pB3.frwD target was expressed under many different conditions in order to produce a sample that would crystal-

Table 3
Effect of growth conditions and purification on solubility and crystallization of frwD protein

Media	Soluble/insoluble (mg/L)	His-tag	Crystals
LB[a]	0/90	N/A[e]	N/A
ZYP[b]	5/120	N/A	N/A
LBSB[c] + heat shock	32/40	Cleaved	Yes
PASMNB[d]	32/27	Cleaved	Yes, 2.5 Å*

Cells were grown in 1 L of media. Amount of soluble and insoluble protein target was estimated based on SDS/PAGE.
[a] LB, Luria Broth.
[b] ZYP, auto-inducing media.
[c] LBSB + heat shock: LB media containing 0.5 M sorbitol, 1 mM betaine, heat shock was applied for 30 min (see Materials and methods).
[d] PASMNB, PASM media (auto-inducing) containing 0.5 M NaCl, 1 mM betaine.
[e] N/A, not applicable.
* Crystal diffraction.

lize. Using the normal conditions of growth (LB + IPTG or ZYP media), the protein was found mainly in inclusion bodies (Table 3). The same growth conditions (LBNB + heat shock) used on pB2.MPN330 was attempted on pB3.frwD. Although the level of soluble protein increased, and the purified protein could be concentrated to 26 mg/ml, no crystals could be obtained. A different condition was then tried by using LB with sorbitol and betaine as described in Materials and methods. The protein was purified using a metal chelating resin, cleaved with TEV protease and purified a second time through the metal chelating column, yielding a prep that could be concentrated to 10 mg/ml. Crystals were obtained that diffracted to 2.5 Å (Fig. 1).

Table 4
Effect of growth conditions on crystallization of M5005_Spy1602 protein

Media	Soluble/insoluble (mg/L)	His-tag	Crystals
LB[a]	10/80	Uncleaved	No
ZYP[b]	10/80	Uncleaved	No
LBNB[c] + heat shock	50/10	Uncleaved	Yes

Cells were grown in 1 L of media. Amount of soluble and insoluble protein target was estimated based on SDS/PAGE.

[a] LB, Luria Broth.

[b] ZYP, auto-inducing media.

[c] LBNB + heat shock: LB media containing 0.5 M NaCl, 1 mM betaine, heat shock was applied for 30 min (see Materials and methods) prior to induction.

Fig. 1. Crystals of pB3.frwD. Initial screening for crystallization with a sparse matrix sampling method [29] was performed at room temperature by using a hanging drop vapor-diffusion method, by mixing 1 μl of the protein with an equal volume of reservoir solution. Crystals (30 × 40 × 20 microns) were obtained in 0.1 M Tris–HCl, pH 8.5, 20% w/v polyethylene glycol monomethyl ether 2000, and 0.01 M nickel (II) chloride hexahydrate, 0.1 M cesium chloride.

M5005_Spy1602

The pB3.M5005_Spy1602 target was grown in the presence of LBNB plus heat shock. Protein expressed under these conditions yielded 50 mg/L soluble target whereas when grown in LB and ZYP the protein was mainly insoluble (Table 4). Cells did not grow well in LBN even when pre-adapted to the media overnight and when grown in LB plus 1 mM betaine, the target was expressed mainly as insoluble protein (unpublished data). The soluble portion was purified, yielding a sample that could be concentrated to 10 mg/ml and the DLS was 21 nm radius/35% polydispersity. In order to improve monodispersity, the optimum solubility screen (OSS) was performed [28], the buffer was exchanged to 100 mM *N*-(carbamoylmethyl)iminodiacetic acid; *N*-(2-acetamido)iminodiacetic acid, pH 6.5, and the DLS improved to 1.9 nm/0% polydispersity. Crystallization trials were performed and crystals were obtained.

Nine targets grown in LB or LBNB

On the basis of the results presented in Tables 1–3, nine target proteins were grown in LB containing 0.5 M NaCl and 1 mM betaine (LBNB) in order to obtain better protein solubility. Out of the nine targets, three showed equal levels of soluble expression whether grown in LB or LBNB while the other six targets showed much more soluble target expression when grown in LBNB (Fig. 2).

Discussion

The overproduction of recombinant proteins in *E. coli* often yields improper folding of the expressed proteins. This often leads to insoluble aggregates. Although there is no way of predicting from the amino acid sequence

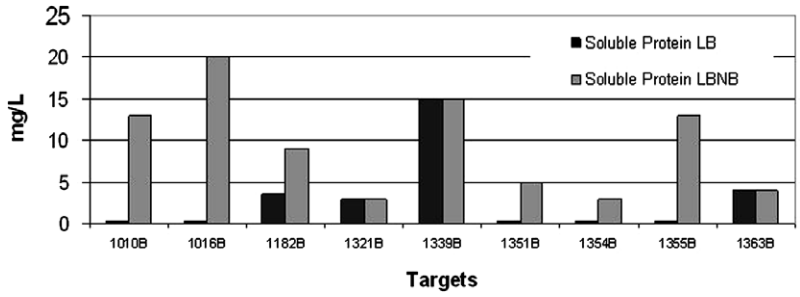

Fig. 2. Effect of combination of high salt and betaine on the soluble expression of nine recombinant proteins. Cells were grown in 2 ml of culture using LB and LB media containing 0.5 M NaCl and 1 mM betaine (LBNB). Amount of expressed protein was calculated based on SDS–PAGE.

whether the protein will aggregate *in vivo*, it is known that small changes in primary structure can sometimes affect solubility. It is also known that altering the conditions of growth can affect the level of soluble protein expressed.

Growth and induction of cells under osmotic stress in the presence of salt, sucrose, sorbitol, ethanol, and glycyl betaine have been shown to increase the level of soluble protein [13,21,31]. Bacteria adapts to high external osmotic pressure by accumulating small organic compounds known as osmolytes. These osmolytes can act as "chemical chaperones" by increasing the stability of native proteins and possibly assisting the refolding of unfolded polypeptides. Exposure of the cells to heat stress also triggers the expression of heat-shock proteins, many of which act as molecular chaperones. These molecular chaperones assist protein refolding, disassemble aggregated proteins, or prevent protein aggregation.

As part of the Berkeley Structural Genomics Center (BSGC) sponsored by the Protein Structure Initiative of the National Institutes of Health, we were challenged by many targets that either expressed as insoluble aggregates or those that although soluble, did not crystallize. Experiments were therefore performed to evaluate the effect of heat shock and increased osmolarity on the production of soluble proteins or better behaving proteins.

Two targets (frwD and M5005_Spy1602) were basically insoluble when grown in LB or ZYP media, but when subjected to the heat and/or osmotic shock the level of soluble expression was greatly improved. A third target had expressed some soluble protein (MPN330) but upon exposure to high salt and a heat shock step, soluble expression was greatly improved. In all three cases crystals were obtained, whereas when grown in LB and ZYP crystallization was not possible even after multiple attempts.

Out of nine targets tested, six of them showed improved soluble expression when grown in the presence of salt and betaine; three did not show any improvement (Fig. 2). This type of screening is easy to do and can be helpful in finding the best conditions for expressing soluble target proteins.

For these BSGC targets we did not have any functional assays to test since these were hypothetical proteins of unknown functions, but we were able to test the monodispersity of the samples as well as their ability to crystallize.

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