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Production of recombinant thermostable proteins expressed in *Escherichia coli*: completion of protein synthesis is the bottleneck

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Abstract

Heterologous expression and high yield purification of proteins are frequently required for structural and functional investigations. Purification of recombinant thermostable proteins is essentially trivial since unwanted mesophilic host protein can efficiently be removed by heat denaturation. However, heterologous expression in *E. coli* often results in truncated protein forms. In many cases, this is a consequence of abundant codons in heterologous genes, which are decoded by rare tRNAs in *E. coli*—a combination that can be responsible for translational stalling and termination during protein biosynthesis. Other complications may originate from potential initiation codons and ribosomal binding sites present inside the open reading frame of the target gene or from other less well defined phenomena such as mRNA instability. Separation of full-length protein from truncated forms is a serious chromatographic problem that can be solved in the expression step. We have investigated the heterologous expression and purification of two translation initiation factors from the hyperthermophilic sulphate-reducing archaeon, *Archaeoglobus fulgidus*. Expression in *E. coli* was optimised to avoid truncated forms completely by complementation with the plasmids pSJS1244, pRIG, pCODON+ and pLysSR.A.R.E harbouring and expressing genes encoding rare tRNAs corresponding to the codons AGA, AGG, AUA, CUA, GGA, AAG and CCC. Two expression strains, C41(DE3) and C43(DE3) were found highly advantageous when combined with rare tRNA encoding plasmids as compared to BL21(DE3). We have also investigated the effects of site directed mutagenesis on rare lysine encoding AAG doublets as well as two methionine residues preceded by potential ribosomal binding sites. The expression approach presented here has enabled us to purify gram quantities of full-length protein by one step of ion-exchange chromatography and is generally applicable to many other heterologously expressed thermostable proteins.

Keywords: *Escherichia coli*; Recombinant thermostable proteins

1. Introduction

Purification of recombinant proteins from hyperthermophiles is a simple process when pure heterologous expression without generation of truncated products can be obtained. Heat denaturation and precipitation of mesophilic host proteins are fast, cheap and convenient steps which often result in an almost pure sample prior to chromatograpy—the thermostable fraction. *Escherichia coli* is a highly advantageous host for the expression of recombinant proteins from a variety of heterologous sources. Variables such as vectors, promoters and signals for transcriptional termination and translation initiation can be successfully regulated. However, expression of heterologous genes is not always a routine matter, since factors such as mRNA stability and secondary

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structure, target protein toxicity, proteolytic degradation and codon usage are more difficult to control [1,2]. These processes present a central chromatographic problem since the separation of truncated forms from full-length protein is difficult and require an elaborate purification procedure.

In *E. coli*, the levels of charged tRNAs are in high correspondence to codon usage. Rare *E. coli* codons are often found to be preferred in organisms from which the recombinant protein of interest originates [3]. Limited availability of cognate charged tRNAs can give rise to translational stalling and an increased probability for a shift in reading frame, thus resulting in translational termination prior to complete protein synthesis. The outcome of this is a poor expression of full-length heterologous target protein as well as a number of truncated expression products [4]. Absence of tRNA has influence on protein quality caused by amino acid misincorporation, as has been demonstrated by the insertion of lysine for arginine at AGA codons [5]. Two alternative strategies have been described for overcoming this codon bias and thus increasing expression levels and avoiding truncated protein products. One approach is to use site-directed mutagenesis in order to generate codons in the target sequence, which reflect the tRNA pool in the host system. A less time-consuming method is the co-transformation of the host with a plasmid harbouring a gene encoding the tRNA cognate to the problematic codons [6]. Another factor yielding truncated expression products is the presence of secondary sites for translation initiation, which can be present when the translated mRNA contains a sequence resembling the ribosomal binding site with appropriate spacing to a downstream AUG codon [7].

New tools and optimisation procedures for expression of heterologous genes are essential, since the generation and purification of recombinant protein products is a highly important discipline within the study of protein structure and function. *Archaeoglobus fulgidus* (*Afu*) (DSM 4304) is a sulphate-reducing hyperthermophilic archaeon with an optimum growth temperature of 83 °C [8]. The complete genome sequence from this organism is known and include genes encoding the two universally distributed translation initiation factors IF1/eIF1A and IF2/eIF5B [9,10]. Here we demonstrate that the recombinant expression of *Afu*IF1A and *Afu*IF5B can be drastically improved using site directed mutagenesis, various plasmids encoding rare tRNAs and special strains of *E. coli*.

2. Experimental

2.1. Strains and plasmids

Plasmids containing rare tRNAs were pSJS1244 (spectinomycin resistance, 50 μg/ml) a kind gift from Professor Sung-Hou Kim [11], pRIG (chloramphenicol resistance, 34 μg/ml) a kind gift from Professor W.G.J. Hol [12], pCODON+ (chloramphenicol resistance, 34 μg/ml) from Stratagene and pLysSR.A.R.E (chloramphenicol resistance, 34 μg/ml) from Novagen. The expression vector pET-15b (ampicillin resistance, 100 μg/ml) and the plasmid pLysS (chloramphenicol resistance, 34 μg/ml) were from Novagen. Host strains BL21(DE3) and Rosetta(DE3) were from Novagen. The mutant strains C41(DE3) and C43(DE3) have been published previously by Miroux and Walker [13]. Strains and plasmids are summarized in Table 1.

2.2. Cloning and site directed mutagenesis

Genomic DNA was obtained from *A. fulgidus* cells as described [14]. Fragments of 701 and 1866 bp containing the *Afu*IF1A and *Afu*IF5B encoding genes were PCR amplified using recombinant *Pfu* DNA polymerase and *A. fulgidus* genomic DNA as template. The *Afu*IF1A encoding gene was amplified using 5′ atgacgtattatcatgaggttaccggacaggaaga 3′ (−13 forward) and 5′ atctcccccagcgggatccccaacaatg 3′ (688 reverse) oligonucleotides, introducing *Rca*I and *Bam*HI sites (underlined), in a 30-cycle PCR reaction (1 min at 94 °C, 1 min at 60 °C, and 1.30 min at 72 °C). The *Rca*I site was used in order to keep the second amino acid in the sequence (arginine) unchanged. Oligonucleotides 5′-tgacgggatccggatatagaaggaaccatgggcaaaaaaaaagaagaagc-3′ (−27 forward) and 5′-caaaaggggatccagtaggggcagtatttgctg-3′ (1839 reverse) were used as forward and reverse primers (recognition sites *Nco*I and *Bam*HI underlined), in a 30-cycle PCR (1 min at 94 °C, 1 min at 63 °C, and 2.30 min at 72 °C) to

Table 1
Bacterial strains and plasmids

	Genotype and relevant characteristics	Reference or source
Strain:		
BL21(DE3)	F$^-$ *ompThsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
Rosetta(DE3)	F$^-$ *ompThsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1* (DE3)	Novagen
C41(DE3)/C43(DE3)	Selected from BL21(DE3)	[13]
Plasmid:		
pLysS	pACYC184 containing T7 lysozyme gene, Cam^R	Novagen
pET-15b	T7 RNA polymerase promoter, Amp^R	Novagen
pSJS1244	*argU* (AGA/AGG), *ileX* (AUA), *lys* (AAG), Spec^R	[11]
pRIG	*argU* (AGA/AGG), *ileX* (AUA), *glyT* (GGA), Cam^R	[12]
pCODON+	*argU* (AGA/AGG), *ileX* (AUA), *leuW* (CUA), Cam^R	Stratagene
pLysSR.A.R.E	*argU* (AGA/AGG), *ileX* (AUA), *leuW* (CUA), *glyT* (GGA), *proL* (CCC), LysS, Cam^R	Novagen

amplify the *Afu*IF5B encoding gene. Both fragments were restriction endonuclease digested and ligated between the *Nco*I and *Bam*HI site of pET-15b to obtain the constructs pET-15b-*Afu*IF1A and pET-15b-*Afu*IF5B. Substitution of Met152 with Ile and Met157 with Lys were made by PCR using oligonucleotides 5′-gcccttcattaagagctacgcgaaacaggaggact-3′ (447 forward) and 5′-agtcctcctgtttcgcgtagctcttaatgaagggc-3′ (481 reverse)—the construct was called pET-15b-*Afu*IF5Bmet (mutated positions underlined). PCR mutations in two areas of *Afu*IF5B rich in lysines encoded by rare codons were introduced using the oligonucleotides 5′-cccattaaaaaagcagaagtgggggatgttgataaacgcgacgtt-3′ (1146 forward), 5-aacgtcgcgtttatcaacatcccccacttctgcttttttaatggg-3′ (1191 reverse), 5′-gtcaaacttcttcccggcgttgaggaggaggcgaaaaaaatacgg-3′ (1245 forward) and 5′-ccgtatttttttcgcctcctcctcaacgccgggaagaagtttgac-3′ (1289 reverse) giving rise to the construct pET-15b-*Afu*IF5Blys (mutated positions underlined). All constructs were verified by sequencing.

2.3. Small scale overexpression

Newly transformed cells were grown at 37 °C in 2×TY medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.5) containing appropriate antibiotics. Cultures were induced by adding isopropyl-2-D-thio-galactopyranoside (IPTG) to a final concentration of 0.5 m*M* when the optical density reached 0.6–0.8 at 550 nm. The cells were grown for a further period of 5 h and the optical density was measured every hour. A volume corresponding to $120/OD_{550}$ was centrifuged (2000 *g*, 25 min), cells were resuspended in 1.5 ml of 50 m*M* Hepes, 10 m*M* $MgCl_2$, pH 7.6, and disrupted by sonication on ice. The supernatant (obtained by centrifuging at 15 000 *g* for 25 min) was heated at 75 °C for 15 min followed by cooling on ice for 15 min. Heat-denatured *E. coli* proteins were removed by centrifugation at 15 000 *g* for 25 min and the thermostable fraction was analyzed in Coomassie brilliant blue R250 stained 15% SDS–polyacrylamide gels.

2.4. Large scale overexpression and protein purification

*Afu*IF1A was overexpressed in 2×TY medium at 37 °C using the Rosetta(DE3) strain containing the plasmid pLysSR.A.R.E and the expression construct pET-15b-*Afu*IF1A. *Afu*IF5B was overexpressed under the same conditions, but in the C41(DE3) strain containing the plasmids pSJS1244 and pET-15b-*Afu*IF5B. Cultures of 1600 ml containing ampicillin and chloramphenicol or spectinomycin were grown to an optical density of 0.6–0.8 and induced by adding IPTG to a final concentration of 0.5 m*M*. Cultures were grown for 4 h and harvested by centrifugation (2000 *g*, 25 min). Cells were resuspended in 50 m*M* Hepes pH 7.6, 10 m*M* $MgCl_2$, 15 m*M* NaN_3, 0.1 m*M* phenylmethylsulfonylfluoride

(PMSF), 1 m*M* 1,4-dithioreitol (DTT) and disrupted by passing the suspension through a French pressure cell at 1500 p.s.i (10 350 kPa), 4 °C. The lysed cells were centrifuged at 15 000 *g* for 30 min and the supernatant transferred to COREX glasstubes and heated at 75 °C for 60 min followed by 15 min cooling on ice. Fresh PMSF (0.1 m*M*) was added immediately after the heating step. Thermostable proteins was separated from the denatured *E. coli* proteins by centrifugation at 10 000 *g* for 30 min. The thermostable fraction was filtered through a 0.45-μm filter. *Afu*IF1A and *Afu*IF5B were purified using the ÄKTAexplorer 100 system (Amersham Biosciences). Both proteins could be bound to a SP Sepharose high-performance ion-exchange column (Amersham Biosciences) and eluted using an increasing gradient of NaCl (*Afu*IF1A elutes at 850 m*M* and *Afu*IF5B at 350 m*M* NaCl). Proteins were more than 95% pure after this step and were effectively desalted using a Sephadex G-25 gelfiltration column. Yields of 30–40 and 40–50 mg from 1 l of culture could be obtained for *Afu*IF1A and *Afu*IF5B, respectively.

3. Results and discussion

The T7 RNA polymerase driven pET expression vectors have proven highly advantageous and the pET vector system are now one of the most widely used for recombinant protein expression [15]. Among several other tools developed in order to facilitate expression in this system are the expression strain BL21(DE3) and the plasmid pLysS. BL21(DE3) is the standard expression host, deficient in the *lon* protease and lacking the *ompT* outer membrane protease [16]. Plasmid pLysS provides low level expression of bacteriophage T7 lysozyme, a natural inhibitor of T7 RNA polymerase, which reduce basal activity from an inducible T7 RNA polymerase thus avoiding possible toxic effects arising from the expression of heterologous proteins [17]. We have used the pET system in combination with a variety of non-standard supplementary tools to investigate the heterologous expression and purification of *Afu*IF1A and *Afu*IF5B, a small and a large protein from the sulphate reducing hyperthermophilic archaeon *A. fulgidus*.

3.1. Expression and purification of AfuIF1A

*Afu*IF1A is a 10.4-kDa translation initiation factor structurally composed of a single β barrel oligomer binding (OB) domain. The protein is involved in translation initiation where it occupies the A-site of the small ribosomal subunit [18]. Expression from the construct pET-15b-*Afu*IF1A could be obtained in the standard host strain BL21(DE3) and inhibition of background T7 RNA polymerase expression by inclusion of pLysS results in increased cell growth (Fig. 1A, lanes 5, 6 and Fig. 1C, curves 5, 6). Inspection of the sequence demonstrates a high occurrence of rare tRNA codons (11.24% AGG/AGA, 1.12% CCC, 1.12% AUA, 3.37% GGA and 7.87% AAG) (Fig. 2A). Therefore we have tried to increase expression levels by the inclusion of plasmids harbouring genes for these rare tRNAs. Plasmid pSJS1244 provides resistance to spectinomycin and carries the tRNA genes *argU*, *ileX* and a gene encoding $tRNA^{Lys}UUU$ with the U wobble base substituted to C [11]. In addition, three other plasmids, pRIG (*glyT*, *argU*, *ileX*), pCODON+ (*leuW*, *argU*, *ileX*) and pLysSR.A.R.E (*proL*, *argU*, *ileX*, *leuW*, *glyT*), all providing chloramphenicol resistance, were tested (Table 1). Expression levels from pET-15b-*Afu*IF1A could not be improved by inclusion of any of the four tRNA plasmids (Fig. 1A, lanes 7–10). However, amino acid misincorporation at rare codons can be avoided when rare tRNAs are expressed and more homogeneous protein obtained. Therefore, we used cells transformed with pLysSR.A.R.E and pET-15b-*Afu*IF1A for large-scale protein production. Purification of the protein was uncomplicated since *Afu*IF1A is a thermostable protein which denatures at 96 °C. At 75 °C most *E. coli* proteins were effectively denatured and a very pure thermostable fraction could be prepared. This approach allowed us to produce highly pure and homogeneous protein in one single step on ion-exchange chromatography (Fig. 3). The purified protein could be crystallized under several conditions in a standard crystal screen. This procedure illus-

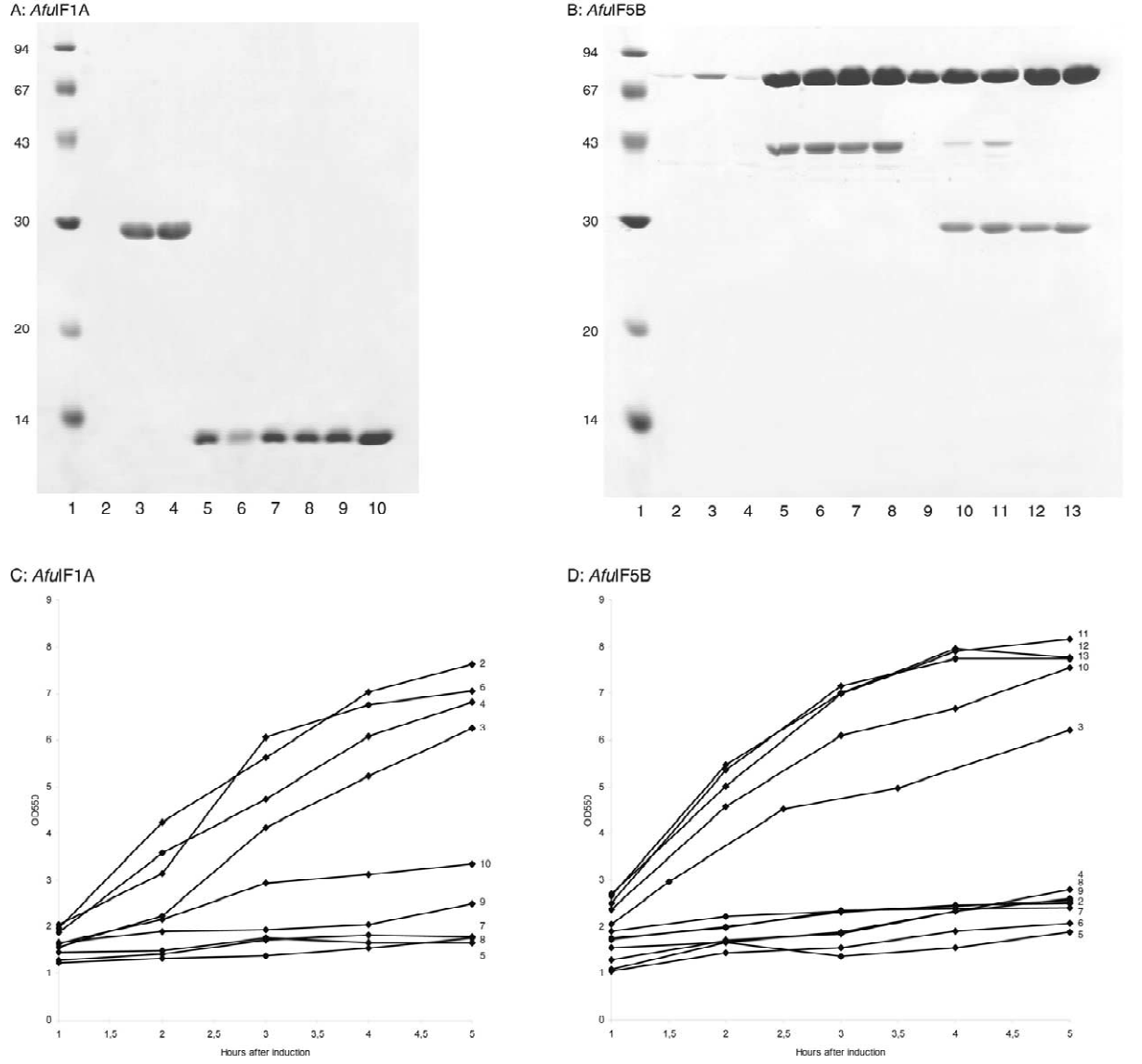

Fig. 1. Expression of *Afu*IF1A and *Afu*IF5B. (A) 15% SDS–PAGE of thermostable fractions. All samples have been treated as described in Section 2.3. Lanes: (1) low-molecular mass marker (LMW, Amersham Biosciences); (2) BL21(DE3); (3) C41(DE3); (4) C43(DE3); (5) BL21(DE3)-[pET-15b-*Afu*IF1A]; (6) BL21(DE3)-[pET-15b-*Afu*IF1A]-[pLysS]; (7) BL21(DE3)-[pET-15b-*Afu*IF1A]-[pSJS1244]; (8) BL21(DE3)-[pET-15b-*Afu*IF1A]-[pCODON+]; (9) BL21(DE3)-[pET-15b-*Afu*IF1A]-[pRIG]; (10) Rosetta (DE3)-[pET-15b-*Afu*IF1A]-[pLysSR.A.R.E]. (B) 15% SDS–PAGE of thermostable fractions. Lanes: (1) LMW; (2) BL21(DE3)-[pET-15b-*Afu*IF5B]; (3) BL21(DE3)-[pET-15b-*Afu*IF5B]-[pLysS]; (4) BL21(DE3)-[pET-15b-*Afu*IF5B]-[pSJS1244]; (5) BL21(DE3)-[pET-15b-*Afu*IF5B]-[pCODON+]; (6) BL21(DE3)-[pET-15b-*Afu*IF5B]-[pRIG]; (7) Rosetta(DE3)-[pET-15b-*Afu*IF5B]-[pLysSR.A.R.E]; (8) Rosetta (DE3)-[pET-15b-*Afu*IF-5Bmet]-[pLysSR.A.R.E]; (9) Rosetta (DE3)-[pET-15b-*Afu*IF5Blys]-[pLysSR.A.R.E]; (10) C41(DE3)-[pET-15b-*Afu*IF5B]; (11) C43(DE3)-[pET-15b-*Afu*IF5B]; (12) C41(DE3)-[pET-15b-*Afu*IF5B]-[pSJS1244]; (13) C43(DE3)-[pET-15b-*Afu*IF5B]-[pSJS1244]. (C, D) Growth curves showing absorbance at 550 nm. Curve numbers correspond to the lane numbering in (A) and (B).

A: *Afu*IF1A

MRLPDRKKGELFGVVTSMLGAGHIKVRCEDGVERLARIPGKMRKKIWIREGDVVIVVPWSFQKDRADIVWRTNPQVEWLERK
GYLKF

B: *Afu*IF5B

MSKKKEEAKALRTPIVAVLGHVDHGKTTLLDRIRKSKVVAKEAGGITQHIGATEVPLDVIKQICKDIWKVEVKIPGLLFIDT
PGHKAFTNLRRRGGALADLAILIVDINEGFKPQTEEALSILRTFKTPFVVAANKIDRIPGWQSHEDTPFMKSYAMQEDFAKQ
NLENRLYNLIAELYQRGFSAERFDRISDFTRTVAVVPISALKGEGIPELLLLILVGLAQRYLEKNLRLHIEGKGRGTVLEVKE
ERGLGVTCDAILYDGTLKVGDRIAIAGKDEVIVTNVKAILKPPPVREMRVESKFQSVKSVTAAAGIKIVAPNLENVLAGSEF
EVVESEEDIKKFEERVRKEYEEIAIRTDEEGVVLKTDTLGSLEALINELRQEGIPIKKAEVGDVDKRDVVDASANKDELNKV
VLAFNVKLLPGVEEEAKKYGVRIFSHEIIYTLIESFVKWREEERLARERQKVEALIKPGKIKLLKEFIFRRSKPAIVGVRVL
AGELRRGVDLIKPDGTKVGAVRTMQKEGKNVAIASAGDELAIAIEDVTIGRQLEGDEELYVDVPERHAKVIERDLLDSLDEE
TKRAFKEFLEIKRKDNPFWGK

C: *Afu*IF5B - Methionine mutant

```
455                                   474
agccacgaagacacgcccttcatgaagagctacgcgatgcag  Wild type
S  H  E  D  T  P  F  M  K  S  Y  A  M  Q

455                                   474
agccacgaagacacgcccttcattaagagctacgcgaaacag  Methionine mutant
S  H  E  D  T  P  F  I  K  S  Y  A  K  Q
```

D: *Afu*IF5B - Lysine mutant

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1146                                  1191        1245                                  1289
cccataaagaaggcagaagtgggggatgttgataagagggacgtt.......gtcaagcttcttcccggagttgaggaggaggcgaagaagtacgg  Wild type
cccattaagaaagcagaagtgggggatgttgataaacggacgtt.......gtcaaacttcttcccgggttgaggaggaggcgaaaaagtacgg  Lysine mutant
P  T  K  K  A  E  V  G  D  V  D  K  R  D  V         V  K  L  L  P  G  V  E  E  E  A  K  K  Y  G
```

Fig. 2. (A,B) Primary sequences of *Afu*IF1A and *Afu*IF5B. Amino acids encoded by rare codons are marked in bold. (C) Sequence of pET-15b-*Afu*IF5B and pET-15b-*Afu*IF5Bmet nucleotides and encoded residues in the mutated area. (D) Sequence of pET-15b-*Afu*IF5B and pET-15b-*Afu*IF5Blys nucleotides and encoded residues in the mutated area. Mutant positions are underlined.

trates the desired standard situation for a small protein.

3.2. Expression of AfuIF5B

A 66.7-kDa homolog of bacterial translation initiation factor IF2 has been identified in *A. fulgidus* where it is designated *Afu*IF5B. This factor is a functional homolog involved in the adjustment of initiator tRNA to the ribosomal P-site, hydrolysis of GTP and joining of ribosomal subunits [9,19,20]. Initial attempts to express *Afu*IF5B from pET-15b-*Afu*IF5B in BL21(DE3) to a satisfactory level with or without pLysS failed (Fig. 1B, lanes 2, 3). Primary sequence analysis identifies several rare codons (6.2% AGA/AGG, 1.2% AUA, 8.5% AAG, 0.7% CUA, 2% CCC and 1.7% GGA). Especially the tRNAs encoded by *ArgU* and the lysine codon AAG are abundant and therefore the plasmid pSJS1244 was tested. Surprisingly, altered expression levels could not be observed (Fig. 1B, lane 4). Subsequently the plasmids pRIG, pCODON+ and pLysSR.A.R.E were used and it appears that they are able to induce high level expression of *Afu*IF5B (Fig. 1B, lanes 5–7). However, truncated expression of the protein occurs with several bands at about 40–50 kDa. Expression of protein without generation of truncated forms was necessary, since chromatographic methods failed to separate them from full-length protein. Several attempts using size-exclusion chromatography revealed that full-length protein make complexes with the truncated forms (data not shown). Results presented below demonstrate that proteolytic degradation is not responsible for the observed truncations. Looking at the primary sequence, there are two obvious reasons for the truncated expression pattern. One is a secondary translation initiation site at two inframe methionine codons present at positions 152 and 157 which would result in fragments of 40–50 kDa. A double mutant containing substitutions M152I and M157K was created (Fig. 2C). Expression was tested in the presence of pLysSR.A.R.E, but no change in the truncation pattern could be observed (Fig. 1B, lane

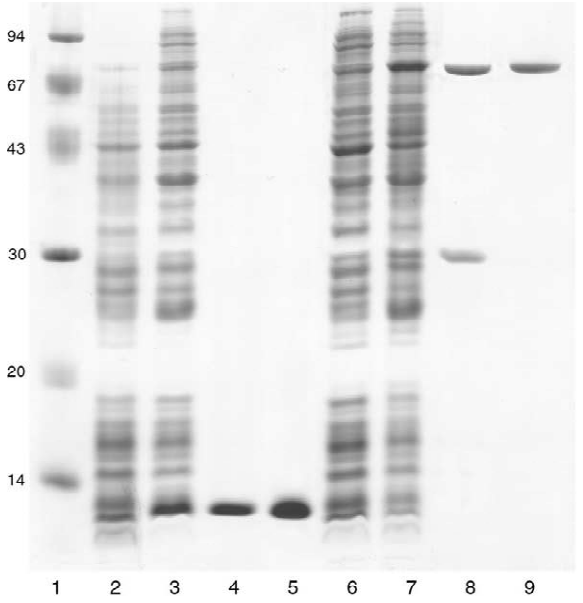

Fig. 3. *Afu*IF1A and *Afu*IF5B purification stages shown on a 15% SDS–PAGE gel. Lanes: (1) LMW; (2–5) *Afu*IF1A expressed from Rosetta (DE3)-[pET-15b-*Afu*IF1A]-[pLysSR.A.R.E]; (6–9) *Afu*IF5B expressed from C41(DE3)-[pET-15b-*Afu*IF5B]-[pSJS1244]; (2,6) pre-induction; (3,7) sample after 4 h of induction; (4,8) thermostable fraction; (5,9) Desalted protein purified on SP-Sepharose High-Performance.

8). Another possibility is the presence of consecutive rare codons in the sequence. Three lysine doublets can be found in the area corresponding to a fragment size of 40–50 kDa. In *E. coli* AAG is decoded by $tRNA^{Lys}UUU$, which is enabled to wobble to G by the $xm^5s^2U_{34}$ modification [21]. Since UUU reads AAG less efficient there is a problem when a target sequence contain consecutive AAG codons. This should be compensated for by the inclusion of pSJS1244, but as mentioned earlier, there was no change in the expression pattern when pSJS1244 was used. A mutant exhibiting the AAG→AAA substitutions shown in Fig. 2D were generated and tested for overexpression (Fig. 1B, lane 9). In combination with pLysSR.A.R.E this mutant resulted in completely non-truncated expression in Rosetta(DE3). Therefore it may be concluded that the presence of consecutive lysine AAG codons is responsible for the truncated polypeptides seen when the wild-type protein is expressed.

In 1996, Miroux and Walker reported the selection of two *E. coli* strains, C41(DE3) and C43(DE3), which are highly advantageous for the expression of toxic recombinant proteins [13]. Expression of *Afu*IF5B in these two strains were tested and as demonstrated in Fig. 1B, lanes 10, 11 and Fig. 1D, curves 10, 11, yielded less truncated product as well as highly increased growth rates. Subsequently, pSJS1244 and pET-15b-*Afu*IF5B were cotransformed into C41(DE3) and C43(DE3) resulting in completely non-truncated expression of the *Afu*IF5B product and high growth rates (Fig. 1B, lanes 12, 13). The sudden effect of pSJS1244 is a result of properties possessed by C41(DE3) and C43(DE3). Gram quantities of *Afu*IF5B could be purified from the thermostable fraction of C41(DE3) harbouring pSJS1244 and pET-15b-*Afu*IF5B on SP-Sepharose High Performance in one single step (Fig. 3). Consequently, a fast and inexpensive method for the production of crystallization grade thermostable protein has been developed. Here we used temperature, IPTG concentration and media compositions which are usually considered as standard conditions. Our results demonstrate that optimal improvement of expression can be obtained without changing these standard conditions. Inclusion of the appropriate plasmid and strain supplements is therefore of outstanding importance for the expression of difficult recombinant proteins.

3.3. Properties of C41(DE3) and C43(DE3)

Our study is among one of many demonstrating the value of the strains C41(DE3) and C43(DE3) in heterologous protein expression. The strains have been suggested to function by limiting the accumulation of transcripts from an expressed target gene and thereby decrease mRNA toxicity and allow higher expression levels. Expression difficult proteins such as membrane-bound cytochrome b_5, membrane-bound cytochrome P450 2B4 and acyl–acyl carrier protein synthase has been successfully expressed in these strains [22–24]. Furthermore, it has been shown that the strain C43(DE3), originally selected from C41(DE3), is forming intracellular membrane structures when recombinant b subunit of F_1F_0 ATP synthase is highly expressed [25]. However, more detailed knowledge about the properties of C41(DE3) and C43(DE3) has not as yet been

presented. A protein of approximately 30 kDa is found in the thermostable fraction of these two strains but not in BL21(DE3) from which they were selected (Fig. 1A, lanes 2–4). We have identified this protein as the periplasmic ribose binding protein (RBP). Further investigations of this finding will be presented elsewhere.

4. Conclusion

A serious chromatographic problem, namely the isolation of full-length protein from truncated forms can be solved by engineering and optimisation of the preceding expression steps. Elaborate chromatographic procedures can be replaced by simple single-step purifications if truncated products are completely avoided during recombinant expression. Expression of *Afu*IF1A and *Afu*IF5B demonstrate the advantages of several available tools and the utilisation of protein thermostability in heterologous protein expression. Two proteins of considerable different molecular mass were both expressed in gram quantities under optimised conditions. We conclude that the methods and tools presented here enable the expression and purification of a wide range of thermostable proteins under standard conditions.

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