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High-level expression and rapid purification of rare-codon genes from hyperthermophilic archaea by the GST gene fusion system

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Abstract

In this study, we compared two gene fusion expression strategies using two rare codon genes (Ssh10b and MtGrxM) from archaea as a model system. Both genes can be highly expressed as N- or C-terminal fusion partners to GST or the intein/chitin-binding tag. However, the fusion protein with intein tag could not be cleaved, even under stringent conditions, possibly due to steric hindrance, thus preventing further purification. In contrast, the GST fusion system could increase protein expression level and the corresponding fusion protein could be easily cleaved by thrombin. After binding to glutathione sepharose, the fusion protein was cleaved on column, and a roughly purified protein fraction was eluted. This fraction was purified by heating at 80 °C for 10 min, followed by centrifugation. The correct total mass and N-terminal primary structure were confirmed by mass spectrometry and Edman degradation. Both constructs were used for in vitro expression, and similar results were obtained, indicating higher expression levels of the GST tag vs. intein/chitin tag. Taken together, our results suggest that the GST fusion system can be used as a considerable alternative to synthetic genes for the expression of rare codon genes. The affinity chromatography purification followed by a heating step is an efficient and convenient method for thermostable protein purification.

Keywords: Hyperthermophilic archaea; Heterologous expression; Protein purification; Rare codon gene

1. Introduction

Hyperthermophiles are found in archaea and bacteria and live at temperatures near or above 100 °C, often at extreme pH conditions [1]. Enzymes purified from hyperthermophiles include proteases, amylolytic-type of enzymes, hydrogenases, and DNA polymerases, some of which are active up to 140 °C [2]. Understanding the mechanisms by which proteins and various biological cofactors and organic intermediates are stabilized at extreme temperatures is of fundamental importance [3]. Therefore, analysis of thermostable proteins not only will further the knowledge of protein stability, folding and thermodynamics in general, but also may enable researchers to adapt proteins to the often extreme conditions of industrial processes. Some examples where the biotechnological potential of thermostable enzymes has already been exploited are found in molecular biology methods (Taq-polymerase), the sugar industry (α-amylases, pullulanases, glucose-isomerases), organic synthesis (lipases, proteases, oxido-reduc-

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tases), detergents (proteases and lipases) and waste treatment [4].

Most of the hyperthermophile species known to date strictly depend on the reduction of elemental sulfur to H_2S for significant growth, a property that severely limits their large-scale culture in conventional fermentation systems [2]. At this end, protein production in hyperthermophilic hosts offers limited possibilities of obtaining sufficient amount of cells for large scale protein production. Thus heterologous overexpression of genes is still the method of choice to provide sufficient amount of recombinant protein for biophysical and functional studies.

However, this approach has its own cons. Even though many genes can be successfully expressed in *Escherichia coli* at a high level, several proteins are poorly, or not at all, expressed, partially due to the usage of rare codons. One explanation is that rare codon genes have been shown to greatly diminish expression levels of recombinant protein in *E. coli* because of translational stalling [5,6].

The two genes (Ssh10b and MtGrxM) used in our study were isolated from *Sulfolobus* and *Methanobacterium* species, and both genes contain a high proportion of rare codons and display frequencies of 33% and 29% of rare codons, respectively. Rare codon is defined as codons that tend to be in genes expressed at a low level. Here, a cut-off $<1\%$ of codon usage was used to arbitrarily define rare codons [6]. In this study, we compared two gene fusion expression strategies as an alternative to the established synthetic gene approach (i.e., the rare codons in a gene are substituted by ''normal'' host codons), using two rare codon genes (Ssh10b and MtGrxM).

Ssh10b is a small (10 kDa), basic DNA binding protein from *Sulfolobus shibatae* [7], and is possibly involved in DNA compaction and protection. The vicinity of the Ssh10b gene to *topR*, the reverse gyrase gene [7], and the ability to constrain negative supercoils using purified Ssh10b in vitro at physiological growth temperatures [8], forebode a promising role for this protein in DNA topology and adaptation to growth conditions at high temperature. MtGrxM is a Glutaredoxin (Grx)-like protein from *Methanobacterium thermoautotrophicum* (Marburg strain). MtGrxM contains the active site sequence (Cys-Pro-Tyr-Cys) shared by all Grxs and has 21% sequence identity with other Grxs [9,10]. MtGrxM was found to be inactive in the HED assay [9], however it retains the other Grx activity, by supplying reducing equivalents to ribonucleotide reductase. Here we report that both wild-type genes could be highly expressed as N- or C-terminal fusion partners to GST or the intein/chitin-binding tag, and subsequent protein purification was achieved by a combination of affinity chromatography with thermal denaturation of contaminating components.

2. Experimental

2.1. Cloning of Ssh10b and MtGrxM proteins

The Ssh10b gene was subcloned from a Ssh10bpET15b construct (derived from genomic DNA), using PCR in a total volume of 50 μl reaction buffer that contains 50 ng template DNA, dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden) at final concentrations of 20 μ*M* each, 20 pmol of 3′- and 5′-terminal primer and 1 U thermostable DNA polymerase (Taq+ long; Stratagene, Cedar Creek, TX, USA). Primers and PCR conditions are shown in Table 1. The PCR products were purified by GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech) and digested with restriction enzymes (Table 1), and then ligated into pGEX-4T-1 vector (Amersham Pharmacia Biotech). After being transformed into *E. coli* TG-1 cells (Amersham Pharmacia Biotech), the constructs were verified by DNA sequence analysis (Big Dye cycle sequencing, ABI, Foster City, CA, USA). MtGrxMpTYB1 was cloned from genomic DNA of *Methanobacterium thermoautotrophicum*, strain Marburg (kind gift of Professor Thauer, MPI, Marburg). MtGrxMpTYB11 and MtGrxMpGEX-4T-1, MtGrxpET15b were subcloned from MtGrxMpTYB1 (Table 1).

2.2. Heterologous expression Ssh10b and MtGrxM without Tag

Recombinant protein expression was carried out in *E. coli* strain BL21(DE3)LysS (Ssh10b) and BL21(DE3) (MtGrxM) (Invitrogen, Carlsbad, CA, USA). In brief, cells were grown overnight in LB medium (at 50 μg/ml ampicillin, Sigma, Steinheim,

Table 1
Cloning of Ssh10b and MtGrxM gene

Construct	5′Forward primer	5′Reverse primer	PCR	Restriction enzyme
Ssh10bpGEX-4T-1	5′-GTT CCG CGT GGA TCC ATG AGC AGC GGA ACC CCA ACT CCA-3′	5′-ATG CGG CCG CTC GAG TTA CTT TTT CCT TAT AGC AAT TTC-3′	94 °C 45 s, 58 °C 60 s, 72 °C 60 s	*Bam*HI, *Xho*I
Ssh10bpTYB1	5′-GGT GGT CAT ATG AGC AGC GGA ACC CCA ACT-3′	5′-GGT GGT TGC TCT TCC GCA CTT TTT CCT TAT AGC AAT TTC TAT TGT TGA AAC-3′	94 °C 45 s, 57 °C 60 s, 72 °C 60 s	*Nde*I, *Sap*I
MtGrxM pTYB1	5′-GTG GTG GTG CAT ATG GTT GTT AAR ATA GAG GTT TTC ACA-3′	5′-GTG GTG GTG TGC TCT TCC GCA TTC DAT CTC ATC RCT TAT GGC TTC-3′	95 °C 45 s, 52 °C 45 s, 72 °C 45 s	*Nde*I, *Sap*I
MtGrxMpTYB11	5′-GGT GGT GGT TGC TCT TCC AAC ATG GTT GTT AAG ATA GAG GAG GTT TTC ACA-3′	5′ -GGT GGT GGT CTG CAG TCA TTA TAT CTC ATC ACT TAT GGC-3′	95 °C 60 s, 58 °C 60 s, 72 °C 60 s	*Sap*I, *Pst*I
MtGrxMpGEX-4T-1	5′-GTT CCG CGT GGA TCC ATG GTT GTT AAG ATA GAG GTT TTC-3′	5′-ATG CGG CCG CTC GAG TTA TAT CTC ATC ACT TAT GGC TTC AAA-3′	95 °C 60 s, 58 °C 60 s 72 °C 60 s	*Xho*I, *Bam*HI
MtGrxMpET15b	5′-A AGG AGA TAT ACC ATG GTT GTT AAG ATA GAG GTT TTC ACA-3′	5′-TTT GTT AGC AGC C GGA TCC TTA TAT CTC ATC ACT TAT GGC TTC-3′	95 °C 60 s, 58 °C 60 s, 72 °C 60 s	*Nco*I, *Bam*HI

PCR was carried out using conditions as given under PCR, for a total of 30 cycles each. Restriction enzymes used for subsequent subcloning are given.

Germany), inoculated 1:1000 in 200 ml fresh medium, and grown at 37 °C. At an O.D. of 0.7, cells were induced by adding IPTG (Ambion, Austin, TX, USA) to a final concentration of 1 m*M* and grown at 25 °C, overnight. Cultures were collected by centrifugation.

2.3. Heterologous expression as fusion with GST and purification of fusion proteins

Recombinant protein expression was carried out in *E. coli* strain BL21 (Amersham Pharmacia Biotech). In brief, cells were grown overnight in 2×YTA medium (at 100 μg/ml ampicillin (Sigma), inoculated 1:500 in 400 ml fresh medium, and grown at 37 °C. At an O.D. of 0.6, cells were induced by adding IPTG to a final concentration of 1 m*M* and grown at 25 °C. Cultures were collected 16 h postinduction by centrifugation, and cell pellets were stored at −20 °C pending purification. Cells were resuspended in lysis buffer (20 m*M* phosphate buffer, pH 7.3, 1.0 *M* NaCl) and nuclease was added (5U Benzonase/mg cells; E. Merck, Darmstadt, Germany). After that, the sample was incubated for 30 min at room temperature, and then lysed by sonication. The supernatant was collected by centrifugation at 12 000×*g* for 10 min and mixed with Glutathione sepharose 4B (Amersham Pharmacia Biotech) for 30 min at room temperature. The slurry was transferred to a disposable column and washed with 10 volumes of lysis buffer. The GST/Ssh10b fusion protein was cleaved on-column with thrombin (1 U/mg fusion protein, Amersham Pharmacia Biotech) at room temperature overnight, and eluted in 1×PBS. The solution was heated at 80 °C for 10 min and centrifuged for 10 min at 20 000×*g*.

2.4. Heterologous expression as fusion with chitin binding domain and intein cleavage

Recombinant protein expression was carried out in *E. coli* strain ER2566 (New England Biolabs, Beverly, MA, USA). In brief, cells were grown overnight in LB medium (at 100 μg/ml ampicillin), inoculated 1:1000 in 400 ml fresh medium, and grown at 37 °C. At an O.D. of 0.7, cells were induced by adding IPTG to a final concentration of 1 m*M* and grown at 25 °C. Cultures were collected 16 h postinduction by centrifugation, and cell pellets were stored at −20 °C pending purification. The cell pellet from 1 l culture was resuspended in 50 ml ice-cold Cell Lysis Buffer (20 m*M* Tris–HCl, 500 m*M* NaCl, 1 m*M* EDTA, 0.1% Triton X-100 and Protease Inhibitor Cocktail Tablet (Complete™, Roche Diagnostics Scandinavia AB, Bromma, Sweden) and lysed by sonication. The clarified supernatant was obtained by centrifugation at 12 000×*g* for 30 min. The supernatant was collected and loaded to the equilibrated chitin beads (New England Biolabs). The column was washed with 10 bed volumes of buffer (20 m*M* Tris–HCl, pH 9.0 for pTYB1 and pH 8.0 for pTYB11). The column was flushed quickly with about three column volumes of DTT (Sigma, Steinheim, Germany) or β-mercaptoethanol (Sigma) solution (freshly diluted in cleavage buffer, 20 m*M* Tris–HCl, 1 *M* NaCl, 50 m*M* EDTA, pH 9.0). Cleavage conditions were varied, and aliquots were incubated at 4, 25, 40 and 60 °C separately for 1 h or overnight. The target protein was eluted by using additional cleavage buffer without DTT and fractions were collected.

2.5. In vitro translation

Ssh10bpGEX-4T-1 and Ssh10bpTYB1 constructs were expressed by using *E. coli* T7 S30 Extract System for circular DNA (Promega, Madison, WI, USA). Plasmid DNA (2 μg), 2.5 μl amino acid mixture minus methionine, 10 μl S30 Premix without amino acids, 0.5 μl ^{35}S methionine (1200 Ci/mmol at 15 mCi/ml, Amersham Pharmacia Biotech), 7.5 μl T7 S30 extract and nuclease free water to a final volume of 25 μl—all were mixed and incubated at 37 °C for 2 h. The resulting products were analyzed by SDS–PAGE. Following gel staining with Coomassie blue, the gel was dried by a vacuum gel drier. The fixed gel was exposed to a phosphor-imaging screen (Molecular Dynamics, Sunnyvale, CA, USA), analyzed and quantitated using the Image QuaNT software (Molecular Dynamics).

2.6. Protein analysis

Purity of protein preparations was analyzed by SDS–PAGE using 18% or 8–16% Tris–Glycine gels (Novex), stained with colloidal Coomassie dye

(NOVEX, San Diego, USA). Total mass was analyzed by MALDI-TOF mass spectrometry (Voyager DE PRO MALDI-TOF, PerSeptive Biosystems, Framingham, MA, USA). Product amount was quantitated by hydrolysis of pure protein samples and analyzed on a Pharmacia Alpha plus 4151 ninhydrin-based analyzer (Pharmacia-LKB, Uppsala, Sweden). N-terminal sequences were confirmed by Edman degradation using a 494 Procise Ht protein sequencer (Applied Biosytems, Foster City, CA, USA).

3. Results

3.1. Expression of archaeal rare codon genes in E. coli in vivo

Ssh10b and MtGrxM fusion constructs using the GST-tag or Intein/CBD were made, resulting in N-terminal (GST, Intein/CBD) or C-terminal (Intein/CBD) tags to the target genes (Table 2). Expression was achieved using *E. coli* strains BL21, or ER2566. Several culture and growth conditions were analyzed, by varying the concentration of IPTG, cultivation length and temperature (data not shown). From these initial experiments the optimal conditions for growth were selected to be 1 m*M* IPTG, 25 °C and 16 h, which resulted in the highest expression levels with these systems.

Wild type Ssh10b expression in pET15b without tag was not detected in BL21(DE3)pLysS in total lysates (Fig. 1A), which is consistent with low levels obtained after heat treatment of lysate and further purification (data not shown). GST and CBD fusion constructs were successfully expressed (Fig. 1B), resulting in clearly identifiable amounts as determined by SDS–PAGE. Similarly, wild type MtGrxM expression in BL21(DE3) without any tag was not detected either (Fig. 1C), whereas GST or CBD-fusions again increased the amounts of recombinant protein obtained in BL21 or ER 2566 (Fig. 1D). In these cases, similar levels of recombinant fusion protein could be obtained, as judged by SDS/PAGE (Fig. 1).

3.2. In vitro expression of archaeal rare codon genes in E. coli S30 extracts

The two different Ssh10b constructs (Ssh10bpGEX-4T-1 and Ssh10bpTYB1) used in this study were translated in vitro using *E. coli* S30 extract system for circular DNA, which utilizes a coupled transcription/translation protocol. The results demonstrate the suitability of rare codon gene protein production in vitro in *E. coli* (Fig. 2). Somewhat slightly higher levels using the GST vector in combination with T7 polymerase (transcription from tac promoter) were obtained over the T7 promoter based transcription of the pTYB/CBD construct.

3.3. Purification of recombinant Ssh10b and MtGrxM proteins

Recombinant Ssh10b and MtGrxM, expressed in vivo as GST or Intein/CBD-binding domain fusion proteins were subjected to purification attempts. The Intein/CBD-fused proteins were purified on a chitin resin and experiments were carried out to perform on-column intein cleavage under reducing conditions. Different temperature (4, 25, 37 and 60 °C), cleavage time (1 h or overnight) and reducing agent parameter (5% β-mercaptoethanol, 50 m*M*, 100 m*M*

Table 2
Ssh10b and MtGrxM expression with GST and Intein fusion tag in vivo and in vitro expression system

	In vivo expression			In vitro expression	
	GST tag (N-terminal fusion) PGEX-4T-1	Intein tag (C-terminal fusion) PTYB1	Intein tag (N-terminal fusion pTYB11	GST tag (N-terminal fusion) PGEX-4T-1	Intein tag (C-terminal fusion) PTYB1
Ssh10b	2.5 mg/l[a]	++	NP	++	+
MtGrxM	1.0 mg/l[a]	++	++	NP	NP

NP, not performed. +, indicate relative expression levels.
[a] The protein amount per 1 l LB medium culture.

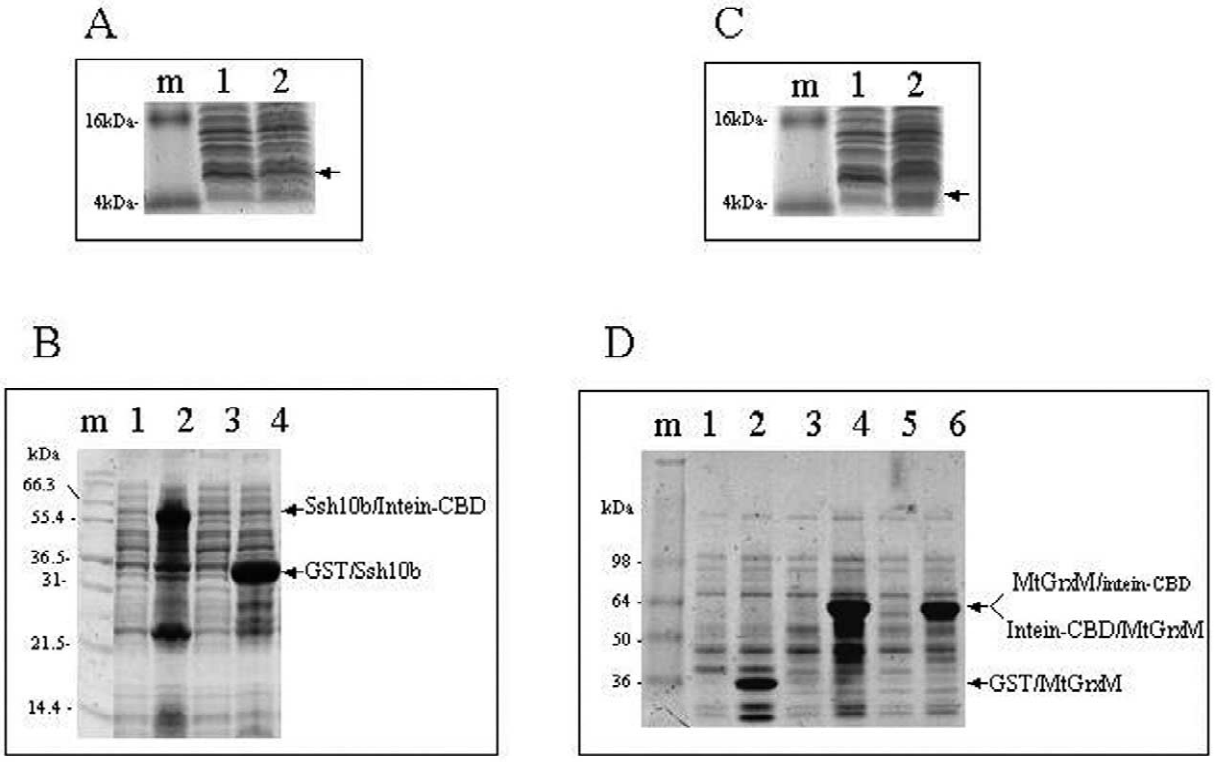

Fig. 1. In vivo expression of Ssh10b and MtGrxM constructs in *E. coli*. SDS–PAGE (18%, stained with Coomassie dye) were as follows: Panel A: Ssh10b expression without tag. The section of relevant mass region was shown, and the arrow indicates expected migration point. Lane m: SeaBluePlus2 (Pre-Stained Protein Standard, Invitrogen); 1: Ssh10bpET15b before IPTG induction; 2: Ssh10bpET15b after IPTG induction. Panel B: Ssh10b expression with tag. Lane m: Marker 12 (wide-range protein standard, Novex); 1: Ssh10/Intein-CBD before IPTG induction; 2: Ssh10/Intein-CBD after induction; 3: GST/Ssh10 before IPTG induction; 4: GST/Ssh10b after induction. Panel C: MtGrxM expression without tag. The section of relevant mass region was shown, and the arrow indicates expected migration point. Lane m: SeaBluePlus2; 1: MtGrxMpET15b before IPTG induction; 2: MtGrxMpET15b after IPTG induction. Panel D: MtGrxM expression with tag (8–16% SDS–PAGE). Lane m: SeaBluePlus2; 1: GST/MtGrxM before IPTG induction; 2: GST/MtGrxM after induction; 3: MtGrxM/Intein-CBD before IPTG induction; 4: MtGrxM/Intein-CBD after induction; 5: Intein-CBD/MtGrxM before IPTG induction; 6: Intein-CBD/MtGrxM after induction; Arrows point to translated protein products.

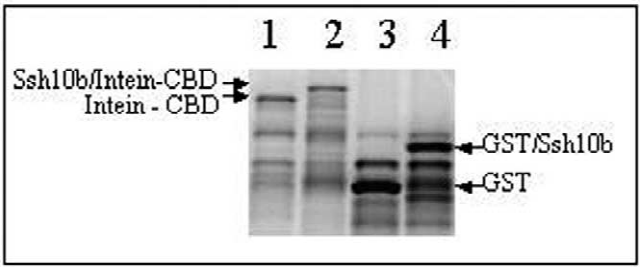

Fig. 2. In vitro expression of Ssh10b fusion constructs using *E. coli* T7 S30 System. Coupled in vitro transcription/translation reactions were carried out using the indicated constructs. pTYB and pGEX were transcribed using T7 polymerase, translation was carried out in the presence of ^{35}S-methionine. Lane 1: pTYB1 vector control; 2: Ssh10bpTYB1; 3: pGEX-4T-1 vector control; 4: Ssh10bpGEX-4T-1. Arrows point to translated fusion protein products.

DTT) were tested, however, under no condition significant cleavage (>10%) of fusion protein was obtained (data not shown). In contrast, rapid purification of GST-tagged Ssh10b or MtGrxM was achieved using a two-step protocol, which consists of affinity chromatography using glutathione sepharose resin, in combination with on-column proteolytic cleavage by thrombin, followed by a heat-denaturation step. Heating of the sample was carried out at 80 °C for 10 min (1 ml fractions) and denatured, aggregated contaminating components were removed by a 10 min centrifugation at 20 000×*g* (Fig. 3A and B).

The amounts obtained under these conditions were about 2.5 mg and 1.0 mg per l culture purified Ssh10 and MtGrxM, respectively (Table 2). In addition to SDS–PAGE analysis, purity of the samples was assessed by compositional amino acid analysis after hydrolysis of proteins, as well as by N-terminal protein sequence analysis and RP-HPLC. Judging from these experiments, the samples were homogenous and pure >95%. Furthermore, MALDI-TOF analysis revealed coincidence of determined and predicted total masses, indicating correct translation of the rare codon genes (data not shown). Circular dichroism spectroscopy reveals that both purified proteins are folded (data not shown).

4. Discussion

Recent advancements in functional and structural genomics have made high-level protein expression mandatory for successful characterization of gene products. The application areas cover among others production for pharmaceutical purposes (e.g. as drug itself or for high-throughput screens or other assays) and functional analysis to structure determinations [11–14]. A wide variety of expression vectors, including bacterial, yeast, fungal, viral, invertebrate and mammalian culture systems are available. Each of these systems has been used with a varying degree of success to produce proteins for purposes mentioned above. At this end, *E. coli* is among the best studied microorganisms, and has been established as a robust and cost-effective host for heterologous protein expression of many foreign genes [15]. However, several problems plague high-level expression, among many others, stability of mRNA and gene product, post-translational modification, correct

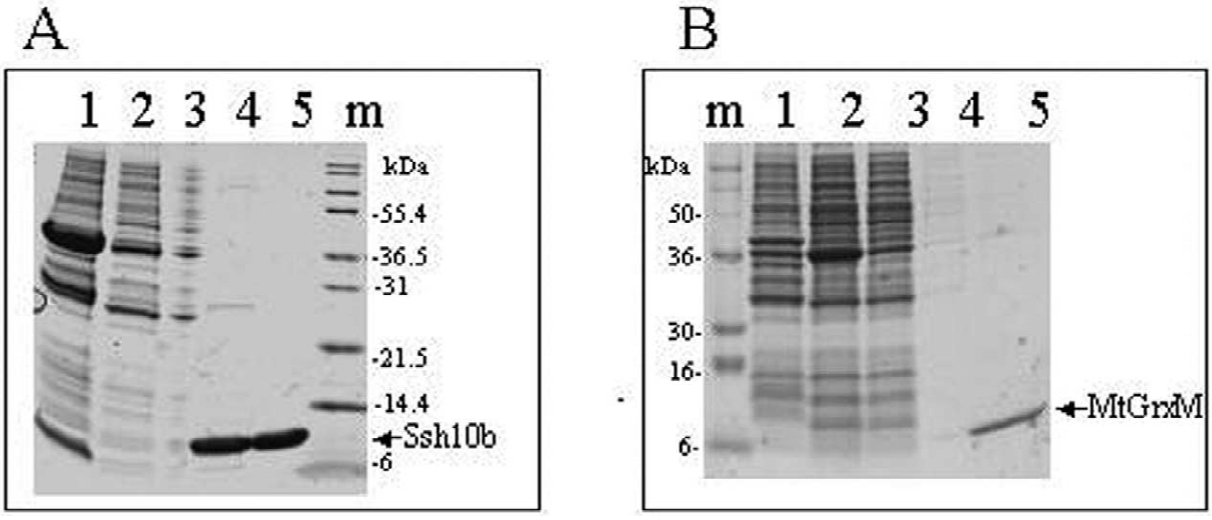

Fig. 3. Purification of Ssh10bpGEX-4T-1 and MtGrxMMpGeX-4T-1 fusion proteins by thrombin cleavage. SDS–PAGE (18%) were loaded as follows: Panel A: GST/Ssh10b purification. Lane 1: cell extract after centrifugation; 2: column flow-through; 3: column wash; 4: eluate, following on-column thrombin cleavage; 5: eluate after heat treatment; Lane m: Marker 12 (NOVEX). Panel B: GST/MtGrxM expression and purification. Lane 1: cell extract; unsoluble fraction (pellet) after centrifugation; 2: cell extract; soluble fraction (supernatant) after centrifugation; 3: column flow-through; 4: column wash; 5: purified MtGrxM protein after column elution with heat treatment.

folding or codon usage [5,6,15,16]. *E. coli* and other species show a marked bias in their utilization of codons between genes expressed at high or low levels [5,6], which explains low levels of rare-codon gene expression. Consequently, replacement of rare codons (synthetic gene approach) often is the method of choice to improve expression levels of heterologous protein. This approach has several drawbacks though, like being costly and time-consuming, as compared with routine cloning efforts. Another frequently used strategy to circumvent codon bias in *E. coli* is co-transformation or stable expression of tRNA genes for rare codons [17,18]. But it seems not always working (personal communication). In addition, not only the amount of rare codons, but also the context and distribution within a given gene appear to influence expression levels [5,6]. This prompted us to test a fusion gene approach to enhance protein expression [19], furthermore allowing affinity chromatography-based purification. In fact, our data indicate that the complex impact of rare codons on protein expression levels is probably also sequence position-dependent [5,6], since addition of fusion tags to the model rare codon genes significantly increased the low expression levels observed with the wild-type proteins. Additionally, the presence of a soluble tag might provide further aid in circumventing folding problems, degradation or aggregation.

The thermostability properties of the gene products tested, should allow to improve purification by heat denaturation and subsequent removal of contaminating compounds by centrifugation. In principle, these properties should also be ideal for rapid and efficient cleavage of intein sites within fusion proteins, as in the case of commercially available intein/CBD vectors. Interestingly, we found no differences in expression levels, regarding orientation of the fusion tag, i.e., N-terminal or C-terminal. However, we failed to observe sufficient intein cleavage of the model proteins, even though the adjacent amino acids were suitable for facilitating the release of target protein according to the reported protocols. Under the cleavage conditions employed, the target genes are presumably folded, and this compactness might prevent efficient cleavage. Steric hindrance in the chemical cleavage thus appears to be an important parameter, which might be improved through employment of suitable linker regions. The intein approach still remains a promising option, though it needs more refinement to make it a versatile approach.

Using commercially available vectors coding for GST fusion tags with integrated protease sites allowed convenient expression of target model proteins, and subsequent purification by glutathione resins. The levels obtained in our experiments are in the same order of magnitude as reported for other proteins using the pGEX system [20], and expression and purification to correct end product can be easily achieved within 2 days. Notably, in vitro expression has increasingly become a significant alternative to whole cell-based expression strategies, circumventing some of the problems encountered in *E. coli*. Here we show that by using a commercial in vitro translation system, expression can also be achieved by intein/CBD and GST-based systems.

To sum up, we propose a fusion gene approach for rare codon genes as a considerable alternative to the synthetic gene approach. This approach is quick and cost-effective, and especially suitable for high-throughput projects, as required in structural genomics efforts. The affinity chromatography purification followed by a heating step is an efficient and convenient method for thermostable protein purification.

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