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Enhancing recombinant protein solubility with ubiquitin-like small archeal modifying protein fusion partners

Sándor Varga [a], Ganesh Ramnath Pathare [b,1], Erzsébet Baka [a], Marius Boicu [b], Balázs Kriszt [c], András Székács [a], Luca Zinzula [b], József Kukolya [a], István Nagy [b,*]

[a] Agro-Environmental Research Institute, National Agricultural Research and Innovation Centre, Department of Applied and Environmental Microbiology, H-1022 Budapest, Hungary
[b] Max Planck Institute of Biochemistry, Department of Molecular Structural Biology, D-82152 Martinsried (Planegg), Germany
[c] Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Aquaculture and Environmental Safety, Department of Environmental Safety and Ecotoxicology, H-2100 Gödöllő, Hungary

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ABSTRACT

A variety of protein expression tags with different biochemical properties has been used to enhance the yield and solubility of recombinant proteins. Ubiquitin, SUMO (small ubiquitin-like modifier) and prokaryotic ubiquitin like MoaD (molybdopterin synthase, small subunit) fusion tags are getting more popular because of their small size. In this paper we report on the use of ubiquitin-like small archaeal modifier proteins (SAMPs) as fusion tags since they proved to increase expression yield, stability and solubility in our experiments. Equally important, they did not co-purify with proteins of the expression host and there was information that their specific JAB1/MPN/Mov34 metalloenzyme (JAMM) protease can recognize the C-terminal VSGG sequence when SAMPs fused, either branched or linearly to target proteins, and cleave it specifically. SAMPs and JAMM proteases from *Haloferax volcanii*, *Thermoplasma acidophilum*, *Methanococcoides burtonii* and *Nitrosopumilus maritimus* were selected, cloned, expressed heterologously in *Escherichia coli* and tested as fusion tags and cleaving proteases, respectively. Investigated SAMPs enhanced protein expression and solubility on a wide scale. *T. acidophilum* SAMPs Ta0895 and Ta01019 were the best performing tags and their effect was comparable to the widely used maltose binding protein (MBP) and N utilization substance protein A (NusA) tags. Moreover, *H. volcanii* SAMP Hvo_2619 contribution was mediocre, whereas *M. burtonii* Mbur_1415 could not be expressed. Out of four investigated JAMM proteases, only Hvo_2505 could cleave fusion tags. Interestingly, it was found active not only on its own partner substrate Hvo_2619, but it also cleaved off Ta0895.

1. Introduction

With the advantages of recombinant gene technology a plethora of heterologous gene expression systems has been developed, aiming to obtain high amounts of pure and properly folded proteins suitable for X-ray crystallography, enzymatic assays and many other applications (Bell et al., 2013). However, the success rate of expressing soluble proteins at high yield is low, whereas the likelihood of obtaining aggregated, precipitated or misfolded proteins is much higher, if the proteins are expressed at all (Unzueta et al., 2015). Therefore, when starting a protein expression project many factors have to be considered, including expression host and vector, affinity purification tag, codon usage/sequence optimization, inducer concentration, expression temperature, solubilization fusion tag, protease cleavage site for affinity tag and fusion partner removal as well as optimization of purification buffer composition (Chen, 2012). In most of the cases *E. coli* is mostly preferred over other prokaryotic or eukaryotic expression systems, due to short generation time, well developed and reliable genetic tools and inexpensive maintenance (Bell et al., 2013). In order to enhance the expression level and to prevent precipitation one solution is to fuse solubility protein tag(s) to the target protein either N- or C-terminally (Chen, 2012; Rosano and Ceccarelli, 2014). In fact, solubility tags can stabilize the protein structure at various chemical conditions and can enhance the expression level as well (Sørensen and Mortensen, 2005). However, given that such fusion partners may have an impact on the folding of the recombinant protein and since how the tertiary structure will be distorted cannot be reliably predicted, their effects should be investigated in every case (Arnau et al., 2006; Costa et al., 2013; Yuan et al., 2014). Thus far, there is no single affinity and solubility tag combination that can be used for every protein in order to eliminate solubility issues. Therefore, existing fusion tags suitable to solve problems should be tested, whereas novel solubility protein tag candidates can be searched,

Abbreviations: SAMP, small archaeal modifier protein; AEBSF-HCl, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; SEC, size exclusion chromatography; LC-MS, liquid chromatography–mass spectrometry; NiNTA, nickel-nitriloacetic acid.

* Corresponding author.
E-mail address: nagy@biochem.mpg.de (I. Nagy).
[1] Present address: Friedrich Miescher Institute for Biomedical Research, CH-4058 Basel, Switzerland.

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thus further increasing the chance to find the optimal purification conditions for any given target protein.

When expressing the Rpn11 subunit of the 19S regulatory particle of the 26S proteasome from *Saccharomyces cerevisiae* (ScRpn11) for X-ray crystallography studies we came across a severe inclusion body formation (Pathare et al., 2014). Despite of all expression optimization trials and implementation of glutathion S-transferase (GST) and SUMO solubility tags, inclusion bodies prevented successful purification. To overcome this hurdle we wanted to identify a small and stable protein fusion partner that could be expressed at high level in soluble form without interacting with proteins of the host organism, also having a specific cleavage site recognized by a cleavage partner for subsequent tag removal. Recently, a group of ubiquitin-like (Ubl) proteins was discovered, namely ubiquitin-like small archaeal modifier proteins (SAMPs), which are common to all taxa within Archaea and, besides being involved in sulfur metabolism, they also take part in protein degradation (Humbard et al., 2010; Maupin-Furlow, 2013). It has also been demonstrated that the *H. volcanii* (Hvo) JAMM protease homolog Hvo_2505 could specifically cleave the linearly fused SAMP Hvo_2619 from the target protein MoaE (molybdopterin synthase, large subunit). Based on this report we focused our study on homologs of this newly discovered group found in *T. acidophilum* (Ta) – namely Ta0895, Ta1019 and Ta1442 – and the cold tolerant *M. burtonii* (Mbur) Mbur_1415. In addition, the Hvo_2619 was used as a control. SAMP solubilizing effect was compared to well known fusion partners such as GST, MBP, NusA, SUMO and thioredoxin (Trx), which were also fused to ScRpn11.

Although best performing SAMPs had multiple beneficial impact on target proteins, it is known that occasionally tag proteins interfere with the folding and/or activity of target protein, thus their removal is required (Arnau et al., 2006). Overall, endopeptidases such as enteropeptidase, thrombin, tobacco etch virus (TEV), rhinovirus C3 protease and exopeptidases (e.g. carboxypeptidases and aminopeptidases) are used to remove N- or C-terminal tags (Waugh, 2011). To supersede added protease cleavage sites required for these proteases we aimed to find an enzyme that was capable to specifically cleave off SAMPs similarly to Hvo JAMM protease, but at lower temperatures (4–10 °C). Hvo JAMM1 protease has maximum activity at 40–50 °C and cleaves the linearly fused Hvo SAMP from MoaE at the VSGG sequence (Hepowit et al., 2012). Therefore, JAMM1 homologs of the Hvo JAB domain metalloprotease family were selected from *T. acidophilum*, *N. maritimus* and *M. burtonii* and, as control, the Hvo JAMM1 protease was used to investigate their potential to cleave off linearly fused SAMPs from target proteins.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1.

E. coli strains used for cloning purposes were routinely grown in Luria-Bertani (LB) medium (tryptone 10 g; yeast extract 5 g; NaCl 10 g in 1.000 ml dH_2O). Chloramphenicol, kanamycin, ampicillin and spectinomycin selection agents were used at 34, 50 and 100 mg/ml concentrations, respectively. *E. coli* and *Rhodococcus* strains used for protein expression were cultured in terrific broth (TB) medium (tryptone 12 g; yeast extract 24 g; KH_2PO_4 2.3 g; K_2HPO_4 12.5 g; glycerol 8.0 g in 1.000 ml dH_2O) supplemented with appropriate antibiotics. Typically small scale protein expression experiments were carried out by inoculating 2 ml TB medium with expression strains and after over night (ON) incubation at 37 °C with 180 rpm shaking the pre-culture was added to 10 ml TB medium in a 50 ml flask. For large scale expression 2 ml starter culture was grown over 12 h at 37 °C then transferred to 20 ml medium for ON incubation at same conditions. After incubation the culture was added to the final 200 ml medium and shaken at 200–220 rpm for 2 h. In case of auto-induction in the first step 2 ml of LB medium was inoculated and after ON incubation at 37 °C it was transferred into 10 ml auto-induction medium (Na_2HPO_4 25 mM; KH_2PO_4 25 mM; NH_4Cl 50 mM; Na_2SO_4 5 mM; $MgSO_4$ 2 mM; glucose 0.05%; lactose 2.0%; glycerol 0.5%; tryptone 2.0%; yeast extract 3.0%; trace metal solution 1×) for expression (Burroughs et al., 2007; Li et al., 2011).

2.2. Protein expression conditions

Culture conditions used to express proteins of interest are described in Table S1. Recombinant protein production in *E. coli* cells was induced with 1 mM final concentration of IPTG, and 50 μg/L thiostrepton in case of *Rhodococcus* cells. Five conditions (A, B, C, D and E) were applied regarding cell culture volume, induction temperature and time interval, as follows: 220 ml cell cultures were used in methods A, B, C, E and 50 ml in method D. Cultures were incubated at (A) 24 °C for 7 h, (B) 37 °C for 4 h, (C/1) 16 °C for 12 h, (C/2) 10 °C for 24 h,(D) 24 °C for 5 h and (E) 30 °C for 24 h. Recombinant protein expression was monitored by Western analysis using antibodies against His-, or Strep-tag, and by LC-MS.

2.3. Transformation methods

2.3.1. E coli transformation

50 μl of *E. coli* DH5 alpha and *E. coli* BL21 (DE3) chemically competent cells (New England Biolabs) thawn on ice was mixed with 5 μl (30–35 ng/μl) plasmid (dissolved in Quiagen EB buffer) and incubated on ice for 30 min. After incubation the cells were heated to 42 °C in an Eppendorf Thermomixer Comfort for 30 s. After heat shock cells were chilled on ice for 2 min then mixed with 450 μl „super optimal broth with catabolite repression" (SOC) medium (Invitrogen) and regenerated for 45 min at 37 °C, 750 rpm. Following the regeneration step 200 μl of cell suspension was spread on LB agar plate containing the appropriate antibiotic.

2.3.2. Rhodococcus transformation

400 μl of competent *R. erythropolis* L88 cells was mixed with 3 μl (30–35 ng/μl) plasmid in a 0.2 mm cuvette and electroporated by using GenePulser XCell (BioRad), operated at the following conditions: output: 2500 V, capacitance: 25 μF, resistance: 400 Ω. Following the electroshock 1 ml SOC medium was added and the cell suspension was transferred to a culture tube containing 2 ml SOC medium. Cells were regenerated at 30 °C for 4 h with shaking and 200 μl of the mixture was spread on an LB agar plate containing 34 μg/ml chloramphenicol.

2.4. PCR amplification methods

Genes of interest were amplified in PCR reactions (Table S2) using primers with *Nde*I-*Xho*I or *Ase*I-*Sal*I protruding ends (Table S3) and *Pfu* DNA polymerase according to the supplier recommendations (Thermo Scientific). In the reactions genomic DNA served as template. To prepare samples for downstream applications PCR products were cleaned up with Qiagen Gel extraction kit (Qiagen) according to manufacturer recommendations and digested with the appropriate FastDigest restriction endonucleases (Thermo Scientific) for 2 h. The reaction mixtures were loaded on a 1% agarose gel in 1× TAE buffer (Applichem) with SYBR Safe DNA dye (Invitrogen). Visualized bands were cut after electrophoresis in TAE buffer (100 V for 50 min) and cleaned up with Qiagen Gel extraction kit (Qiagen).

2.5. General cloning methods

For cloning purposes both vector and insert were digested with restriction endonuclease pairs regularly FastDigest *Nco*I/*Xho*I or *Nde*I/*Xho*I (Thermo Scientific) for correct gene orientation. DNA bands of digested plasmids and PCR products (Table S2 and Table S3) were