Protein Expression and Purification 62 (2008) 21–28

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Enhanced protein expression in the baculovirus/insect cell system using engineered SUMO fusions

Li Liu [1], Joshua Spurrier [1], Tauseef R. Butt [1], James E. Strickler [1,*]

Lifesensors, Inc., Research and Development, 271 Great Valley Parkway, Malvern, PA 19355, USA[2]

ARTICLE INFO

Article history:
Received 22 April 2008
and in revised form 25 June 2008
Available online 5 August 2008

Keywords:
SUMOstar tag
SUMOstar protease
Protein expression
Insect cells
Desumoylase

ABSTRACT

Recombinant protein expression in insect cells varies greatly from protein to protein. A fusion tag that is not only a tool for detection and purification, but also enhances expression and/or solubility would greatly facilitate both structure/function studies and therapeutic protein production. We have shown that fusion of SUMO (small ubiquitin-related modifier) to several test proteins leads to enhanced expression levels in *Escherichia coli*. In eukaryotic expression systems, however, the SUMO tag could be cleaved by endogenous desumoylase. In order to adapt SUMO-fusion technology to these systems, we have developed an alternative SUMO-derived tag, designated SUMOstar, which is not processed by native SUMO proteases. In the present study, we tested the SUMOstar tag in a baculovirus/insect cell system with several proteins, i.e. mouse UBP43, human tryptase beta II, USP4, USP15, and GFP. Our results demonstrate that fusion to SUMOstar enhanced protein expression levels at least 4-fold compared to either the native or His_6-tagged proteins. We isolated active SUMOstar tagged UBP43, USP4, USP15, and GFP. Tryptase was active following cleavage with a SUMOstar specific protease. The SUMOstar system will make significant impact in difficult-to-express proteins and especially to those proteins that require the native N-terminal residue for function.

The conjugation and de-conjugation of SUMO[3] (small ubiquitin-like modifier) to target proteins is similar to the pathways described for ubiquitin [1–3]. Essentially, three ligating enzymes, E1, E2, and E3 work sequentially, in an ATP-dependent cascade to couple SUMO through an isopeptide bond to the ε-NH_2-group of lysine residues of the acceptor protein. Unlike ubiquitin, however, SUMOylation does not result in targeting the protein for degradation by the 26S proteosome. Rather, SUMOylation appears to be involved in regulating transport to different intracellular compartments, e.g. the nucleus. In addition, some plasma proteins have been found to be SUMOylated [4]. As an integral part of regulating intracellular trafficking, SUMO specific proteases (isopeptidases) are involved in maturation of, removal of and recycling of SUMO. In yeast, the SUMO (Smt3) specific protease Ulp1, removes the C-terminal tripeptide AlaThrTyr of preSUMO to generate mature SUMO with GlyGly at the C-terminus. The carboxylate of the terminal glycine residues is attached to lysyl-ε-NH2-groups in the target protein. Ulp1 and Ulp2 release SUMO from the target protein [5–7].

Recombinant fusion of yeast SUMO (Smt3) to the N-terminus of heterologous proteins for expression in bacteria has been shown to enhance both protein expression and solubility [8,9]. This SUMO "tag" can be removed by digestion with Ulp1 to generate the native N-terminus of the fusion partner [10]. Because Ulp1 recognizes exosites in the tertiary structure of SUMO as well as the Gly-Gly dipeptide sequence, it does not cleave other peptide bonds within the fusion partner. While this system works well in prokaryotes, it is likely that endogenous desumoylases would limit its utility in eukaryotes. We have created an R64T, R71E double mutant of yeast SUMO (smt3), termed SUMOstar, which is not recognized by native desumoylases as well as a mutant Ulp1, SUMOstar protease, that is capable of processing SUMOstar (Panavas et al., manuscript in preparation). We have previously used this mutant and analogous mutants of human SUMOs 1 and 3 to obtain enhanced expression of sPLA2 in transiently transfected HEK293T and CHO-K1 cells [11]. This study was designed to test the utility of SUMOstar as an expression tag in a baculovirus/insect cell system and determine if it would yield the same enhancement in expression levels and solubility that we have shown with SUMO in bacteria and mammalian cells.

To this end, several proteins were chosen as test objects, green fluorescent protein (GFP), UBP43, USP4, USP15, and tryptase β.

* Corresponding author. Fax: +1 610 644 8616.
E-mail address: strickler@lifesensors.com (J.E. Strickler).

[1] The authors are employees of LifeSensors, Inc. which produces and markets SUMO-based expression systems and reagents.

[2] www.lifesensors.com

[3] *Abbreviations used:* SUMO, small ubiquitin-like modifier; GFP, green fluorescent protein; NBD C_6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; USP, ubiquitin specific protease; UBP, ubiquitin protease.

1046-5928/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2008.07.010

UBP43, USP4, USP15, and tryptase were selected because they are the subject of active drug discovery programs. UBP43 is the specific de-conjugating enzyme for ISG15, another small ubiquitin-like modifier. Both ISG15 and UBP43 are induced by viral or bacterial infection and by interferon [12–15]. UBP43 has not been expressed in *E. coli* in an active form [16]. USP4 (UNP/Unph) is a deubiquitinase that shows both nuclear and cytoplasmic localization. It has been shown to be constitutively associated with the retinoblastoma protein (pRb), the autoantigen Ro52 (an E3 ubiquitin ligase), and the A2a adenosine receptor. Deubiquitination of each of these proteins prolongs its intracellular lifetime. USP4 is also considered an oncogenic protein although the mechanism of oncogenesis is not understood. Elevated USP4 levels have been found in adenocarcinomas of the lung and injection of cells overexpressing USP4 leads to tumors in nude mice [17–20]. USP15 is a Zinc-finger containing deubiquitinase associated with the ubiquitin proteasome regulatory COP9 signalosome (CSN). It shows 61% identity with USP4 [21–26,20]. Tryptase is a trypsin-like protease with a unique tetrameric structure. It is stored as an active enzyme in mast cell secretory granules and has been implicated in the etiology of asthma and other allergic and inflammatory disorders. Both human tryptase α and β form a ring-like tetramer with active sites facing an oval central pore. The tetrameric structure of tryptase α is stabilized by sulfated polysaccharides, e.g. heparin. In the absence of such polymeric anions, tryptase α reversibly converts to an inactive conformation and dissociates into monomers. Tryptase β is more stable although it also requires a polymeric anion for maximal activity. Tryptase β has previously been expressed and isolated in active form from insect cells [27–33]. GFP was used as an easily detected control protein [34].

Our results show that the native SUMO was indeed cleaved from the fusion proteins *in vivo* while the SUMOstar tag was not. Both SUMO and SUMOstar lead to enhanced expression of all of the test proteins. USP4 and USP15 were all active with the SUMOstar tag intact; however, tryptase β was only active after the SUMOstar tag was removed.

Materials and methods

Gene cloning

A series of pFastBac vectors with His_6, SUMO, His_6Sumo, gp67His_6, gp67SUMO, SUMOstar, His_6SUMOstar, gp67SUMOstar, and gp67His_6SUMOstar was prepared by primer annealing, PCR and subcloning. The SUMOstar template was derived from a bacterial vector generated previously at LifeSensors (Cat. 1101). The gene for Tryptase β II was the kind gift of Dr. Norman Schechter. The UBP43 gene was obtained from OpenBiosystem. USP4 and USP15 genes were kindly provided by Dr. Rohan Baker from The Australian National University. These genes were amplified by PCR using the forward and reverse primers shown below. Following purification, the genes were subcloned into pFastBac vectors.

UBP43–BveI	5′-CGCGACCTGCATCGAGGTATG GGCAAGGGGTTTGGGCTCCTGAGG 5′-CGCGACCTGCATGTCTAGATTAGG ATCCAGTCTTCGTGTAAACCAAG
Tryptase–BpiI	5′-TTTGAAGACGAAGGTATC GTCGGGGGTCAGGAGG 5′-TTTGAAGACGAAGCTTATTAC GGCTTTTTGGGGACATAGTG
GFP–Eco31I/HindIII	5′-ATGATGGGTCTCTAGGTATGGT GAGCAAGGGCGAGGAGCT 5′-CGCAAAGCTTGAGCTCTTACTTGT ACAGCTCGTCCATGCCGA
USP4–Esp3I/Esp3I–XbaI	5′-CGATCGTCTCTAGGTATGG CGGAAGGTGGAGGCTG 5′-GCGCCGTCTCTCTAGA TTAGTTGGTGTCCATGCTGCAAGCC
USP15–Esp3I/Esp3I–XbaI	5′-CGTTCGTCTCTAGGTATGGCGG AAGGCGGAGC 5′-CGCCGTCTCTCTAGATTA GTTAGTGTGCATACAGTTTTCATTTTC

PCR was carried out with 0.5 U Platinum® *Tag* DNA Polymerase High Fidelity according to the manufacturer's instructions (Invitrogen, St. Louis, Mo). The amplified DNA was kept at 4 °C prior to final purification which was accomplished on a Epoch miniprep column.

The vectors for UBP43, USP4 and USP15 were digested with Esp3I and XbaI. The vectors for tryptase and GFP were digested with Esp3I and HindIII. The purified PCR fragments were cut with restriction enzymes and isolated by agarose electrophoresis. The vector and insert fragments were ligated directionally and the resulting plasmid was transformed into XL10Gold competent cell (Stratagene, La Jolla, CA). Multiple colonies were screened for the correct ligation. The genes were confirmed by DNA sequencing (Agencourt, Beverly, MA).

Insect cell expression

pFastBac-genes were transformed into DH10Bac cells and plated onto agar plates containing kanamycin, gentamycin, tetracycline, and IPTG according to the manufacturers' instructions (Invitrogen). White colonies were used for isolating Bacmid DNA by a modified procedure. Briefly the MX1, MX2, and MX3 buffers from GenCatch plasmid DNA miniprep kit (Epoch BioLabs) were used for extract DNA from 3 ml cultures of DH10Bac cells. Bacmid were precipitated by adding 70% isopropanol followed by a 30 min incubation at −20 °C. The Bacmid DNA was collected by centrifugation at 16.1 Krcf for 15 min at room temperature. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 40 μl of 1× TE buffer (10 mM Tris–HCl, pH 8.5, 10 mM EDTA) by gently tapping the 1.5 ml tube. The Bacmid DNA was stored at 4 °C. Two micrograms of bacmid DNA were transfected into 2×10^6 Sf9 cells in a T25 cell culture flask combined with PEI (polyethylenimine) at N/P ratio of about 40. After 5 days, 1 ml of this P1 virus was transferred to a new 4 ml sf9 cell culture in a T25 flask for P2 virus production. After 3 days, the P2 virus was harvested by centrifugation at 600 rcf for 5 min spin. An aliquot (50–100 μl) of the clarified P2 virus preparation was used for P3 virus production which was harvested after 3 days of culture. The titer of the baculovirus was obtained by plague assay. Multiplicity of infection (MOI) studies were performed and cells and conditions medium were harvested 3–6 days post-infection in Sf9 cells as described in the figure legends. Sf9 cells were maintained in Sf900II medium (Invitrogen) with antibiotics and anti-mycotics (Invitrogen) at 0.75 to 10×10^6 cells per ml in 125 ml shake flask in 40 ml cultures.

Protein isolation

Sf9 cells (1×10^8) were infected with baculovirus harboring the gp67His_6SUMOstar-tryptase gene at an MOI of 4×10^{-4} and cultured for 90 h. One hundred ml of conditioned medium were harvested for the isolation of secreted tryptase. Briefly, the cells