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

Li Liu¹, Joshua Spurrier¹, Tauseef R. Butt¹, and James E. Strickler^{1,2}

Lifesensors, Inc., 271 Great Valley Parkway, Malvern, PA 19355, www.lifesensors.com

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 *E. coli*. In eukaryotic expression systems, however, the SUMO tag could be cleaved by endogenous de-SUMOylases. 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 His₆ 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.

Keywords

SUMOstar tag; SUMOstar protease; protein expression; insect cells; desumoylase

The conjugation and de-conjugation of SUMO (small ubiquitin-related modifier) to target proteins is similar to the pathways described for ubiquitin [1,2,3]. 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 -NH₂-group of lysine residues of the acceptor protein. Unlike ubiquitin, however, SUMOylation does not result in targeting the protein for degradation by the 26S proteasome. 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

²to whom correspondence should be sent, Tel: 610-644-8845 ext 307, Fax: 610-644-8616, strickler@lifesensors.com.

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

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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[5,6,7].

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 . 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 [12,13,14,15]. 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, itself an E3 ubiquitin ligase, and the A2a adenosine receptor. Deubiquitination of each of these proteins prolongs their 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. [17,18, 19,20]. USP15 is a Zincfinger containing deubiquitinase associated with the ubiquitin proteasome regulatory COP9 signalosome (CSN). It shows 61% identity with USP4 [21,22, 23,24,25,26,20]. Tryptase is a tetrameric 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 [27,28,29,30,31,32,33]. 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 His₆, SUMO, His₆Sumo, gp67His₆, gp67SUMO, SUMOstar, His₆SUMOstar, gp67SUMOstar, and gp67His₆SUMOstar 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 - Bvel	5'-CGCGACCTGCATCGAGGTATGGGCAAGGGGTTGGGCTCCTGAGG
	5'-CGCGACCTGCATGTCTAGATTAGGATCCAGTCTTCGTGTAACCAAG
Tryptase - BpiI	5'-TTTGAAGACGAAGGTATCGTCGGGGGTCAGGAGG
	5'-TTTGAAGACGAAGCTTATTACGGCTTTTGGGGACATAGTG
GFP - Eco31I/HindIII	5'-ATGATGGGTCTCTAGGTATGGTGAGCAAGGGCGAGGAGCT
	5'-CGCAAAGCTTGAGCTCTTACTTGTACAGCTCGTCCATGCCGA
USP4 - Esp3I/Esp3I-XbaI	5'-CGATCGTCTCTAGGTATGGCGGAAGGTGGAGGCTG
	5'-GCGCCGTCTCTCTAGATTAGTTGGTGTCCATGCTGCAAGCC
USP15 - Esp3I/Esp3I-XbaI	5'-CGTTCGTCTCTAGGTATGGCGGAAGGCGGAGC
	CGCCGTCTCTCTAGATTAGTTAGTG TGCATACAGTTTTCATTTC

PCR was carried out with 0.5 units 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 plate 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 μ l of 1 $\times$ 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 μ l) 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 plaque assay. The multiplicity of infection (MOI) studies were performed and cells and condition media were harvested 3 to 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-mytotics (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 gp67His₆SUMOstar-tryptase gene at an MOI of 4×10^{-4} and cultured for 90 hours. One hundred ml of conditioned medium were harvested for the isolation of secreted tryptase. Briefly, the cells were removed by centrifugation at $3700 \times g_{av}$ for 10 min.

The supernatant was isolated, made 1 mM in PMSF, and 1/10 volume of 10X PBS was added. Four mL of Ni-NTA resin was added and the resulting slurry was mixed at room temperature for 90 min. The beads were precipitated by centrifugation at $600 \times g$ for five min, then washed by suspension and centrifugation as above with 50 mL PBS containing 25 mM imidazole and 0.35 M NaCl (WB). The resin was then suspended in WB and poured into a column. The column was washed and packed by passing an additional 20 mL of WB through. Tryptase was eluted with 5 mL of NaKPO₄ buffer, pH 6 containing 0.5 M imidazole. The eluted tryptase was pooled and concentrated in an Amicon Ultra centrifugal filter device using a YM-30 membrane. The buffer was exchanged to NaKPO₄ buffer pH 6.0 containing 1 M NaCl by dilution and reconcentration. USPs 4 and 15 were isolated from Sf9 cell lysates by Ni-NTA as described above. The lysate was prepared by incubating the cells with WB (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% NP-40 and 1 mM PMSF for 30 min on ice followed by sonication. The lysate was clarified by centrifugation at $16,000 \times g_{av}$ for 30 min. The supernatant was isolated and brought to 40 mL with WB before proceeding with the batch binding protocol described above. The bound protein was washed with WB containing 0.5M NaCl, 25 mM imidazole and eluted with WB containing 0.5M NaCl, 0.5 M imidazole. The pooled, concentrated enzymes were stored at -80°C in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20% glycerol and 0.5 mM DTT.

Protein analyses

SDS-polyacrylamide electrophoresis was carried out on homogeneous 12% or 15% polyacrylamide gels. The separated proteins were stained with Coomassie brilliant blue or transferred to nitrocellulose membranes in a BioRad semi-dry transfer apparatus for immunoblot analysis. Blotted proteins were localized with an monoclonal anti-His₆ antibody (Amersham Biosciences), followed by anti-mouse conjugated to HRP (Rockland). Chemiluminescence was detected using a Fujifilm LAS3000 (Edison, NJ). Coomassie gels were photographed using the Fujifilm LAS3000. Densitometric analysis of both Western blots and Coomassie blue stained gels was carried out using the Multigauge software supplied with the LAS3000. Protein concentrations were estimated using the Coomassie blue dye binding assay (BioRad, Hercules, Ca.)

Deubiquitinase Assays

As described in the text, the assay is based on the release of inhibition caused by fusion of a ubiquitin-like (UBL)-tag to PLA2 [35]. For UBP43, the UBL is ISG15, whereas, for USPs 4 and 15, the UBL is ubiquitin (Ub) itself. ISG15-PLA2 and Ub-PLA2 were kindly provided by Dr. Ben Nicholson of Progenra. Assays were carried out at room temperature in Greiner black, medium binding 96 well assay plates (E and K Scientific, Santa Clara, CA). Briefly, the deubiquitinases were diluted into freshly prepared assay buffer (50 mM Tris-HCl, pH 8.0, 2 mM CaCl₂, 2 mM β -mercaptoethanol) containing either 20 nM ISG15-PLA2 or 20 nM Ub-PLA2 and 20 μM NBD C₆-HPC (Invitrogen N3786). Negative controls contained either Sf9 cell crude extract (UBP43) or Ni-elution buffer (USPs 4 and 15). The release of active PLA2 was measured by the increase in fluorescence intensity of free NBD (exc. 460 nm; emm. 538 nm) using a Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA) at either 20 or 60 s intervals over 1 hour. Data for each enzyme were fit to the integrated rate equation 1 using non-linear least squares.

$$A_{405} = V_s(t) + (V_0 - V_s)(1 - e^{(-k_{obs}(t)})}/k_{obs} + c \quad [1]$$

In this equation, V_0 is the initial velocity of substrate turn over by PLA2 prior to removal of the SUMOstar tag ($\cong 0$), V_s is the steady state velocity following removal, c is the initial Abs at 405nm, and k_{obs} is the pseudo-first order rate constant of activation. Under appropriate conditions, V_s is dependent on the amount of active PLA2 generated, while k_{obs} is dependent on the amount of deubiquitinase used.

Tryptase Assay

Purified tryptase was diluted into assay buffer (NaKPO₄ buffer, pH 6.0; 1M NaCl; 2 mg/ml heparin). Tryptase substrate Chromozym TH (Tosyl-Gly-Pro-Arg-*p*-nitroaniline, Cat. No. 10206849001, Roche Applied Science) dissolved in 5% DMSO was added to give a final concentration of 1 mM. SUMOstar specific protease, 0.5 μ g with or without DTT, was added to remove the SUMOstar tag and initiate the assay. The absorbance at 405 nm was measured every 60s at room temperature in a Multiskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA). The data were fit to equation 1 as described above.

Results

Enhanced Intracellular Protein Expression Level by Attachment to the SUMOstar Tag

We have previously shown that the use of the Small Ubiquitin-like MOdifier, SUMO, as a fusion partner for recombinant expression of heterologous proteins in *E. coli*, resulted in increased expression levels and solubility. Because we anticipated that this benefit would be, at least partially, lost due to endogenous deSUMOylase activity in eukaryotic cells, we developed the R64T,R71E double mutant SUMO-tag that is not recognized by eukaryotic enzymes. We have termed this modified SUMO, SUMOstar (Panavas, et al., manuscript in preparation). In the present study, we examined whether fusion of heterologous proteins to SUMOstar would lead to enhancement of expression levels in a baculovirus/insect cell expression system. Conditions were optimized by varying the multiplicity of infection (MOI) and the time post infection for harvest. Protein expression levels were determined by SDS-PAGE. In the first set of experiments, GFP was expressed as a fusion with a panel of tags – SUMOstar (S*), His₆-SUMOstar (HS*), the gp67 signal sequence (gp67)-His₆-SUMOstar (GHS*), wild type SUMO (S), gp67 (G), gp67- His₆ (GH), and gp67-SUMO (GS). Expression levels were compared to results obtained with untagged GFP. Surprisingly, despite the presence of the gp67 leader sequence, most of the GFP expressed was retained in the cell (see discussion). Based on densitometry of Coomassie-stained SDS-gel, constructs containing SUMOstar exhibited over 3-fold higher levels of expression when compared to the non-SUMOstar constructs (Fig. 1A); however, this analysis is complicated by the presence of an Sf9 protein which co-migrated with GFP. By Western blot analysis using an anti-His₆ antibody, which allows better quantitative comparison than Coomassie staining, GHS*-GFP levels were found to be > 6 fold higher than the level of GH-GFP (Fig. 1B.) Similar results were obtained for HS*-UBP43; a 3-fold enhancement (corrected for the increased molecular weight of the fusion protein) in expression compared to H-UBP43 (Fig. 2.)

We next wanted to determine whether fusion to SUMO or SUMOstar would lead to enhanced expression of secreted proteins. For this purpose, we examined conditioned culture medium from Sf9 cells infected with baculoviruses containing either GFP or tryptase fused to the gp67 signal sequence with either no tag or the native SUMO or SUMOstar tags by SDS-PAGE (Fig. 3A.). The results of this analysis are shown in Figure 3. Densitometric analysis of the Coomassie Blue staining intensity of the GFP or tryptase bands, indicated that at an MOI of 1, GS*-GFP was expressed at a higher level than either S-GFP or G-GFP. At MOIs of 0.1, 1 and 5, GS*-tryptase was expressed at a level 3-fold higher than that of G-tryptase. From these

data, it is clear that fusion to SUMOstar led to increased levels of these recombinant proteins accumulating in the growth medium. On the other hand, comparison of the cell lysate results (Fig. 1B) for GHS*-GFP to the medium results (Fig. 3A, GS*-GFP) suggested that some of the expressed protein was being retained intracellularly despite the presence of the gp67 leader. To determine if trypsin was also being retained intracellularly, we analyzed cell lysates from cells expressing G-trypsin and GS*-trypsin (Fig. 3B). Based on this analysis, it is clear that a portion of the expressed trypsin was not secreted from the cells.

Isolation of active enzymes from insect cells

In order to determine if the SUMO-modified baculovirus/insect cell expression system would be useful for the isolation of purified proteins for biological or structural studies, we isolated GHS*-trypsin from conditioned medium and USPs 4 and 15 from cell lysates and tested their activities (Figs. 4 and 5). In order to increase the accumulation of secreted trypsin in the medium, we infected at an extremely low MOI (4×10^{-4}) which, in some instances, has been shown to lead to increased expression and secretion of proteins [36,37,38,39]. The secreted trypsin was readily isolated from conditioned medium by chromatography on a Ni-NTA column as described in the Materials and Methods section (Fig. 4A). Although we did not test it, we assume that the gp67 leader sequence was correctly processed during secretion. The resulting secreted HS*-tagged protein was not active as isolated but required removal of the HS*-tag. Hence, we incubated the purified trypsin in a phosphate buffer containing 1M NaCl, 10% glycerol, 2 mg/mL heparin, and 25 μ g/mL SUMOstar protease at pH 6. Activation of trypsin was measured simultaneously by measuring the release of *p*-nitroaniline from Tosyl-Gly-Pro-Arg-*p*NA (Fig. 4B). The profile was consistent with a slow activation phase followed by increased turnover of the trypsin substrate as active enzyme was released from inhibition by SUMO.

Recombinant USP4 and USP15 were also readily isolated from insect cells as His₆-SUMOstar fusions (Figs. 5A and 5C). Progress curves demonstrating the isopeptidase activity of both enzymes are shown in Figure 5B and 5D. This assay takes advantage of the requirement of PLA2 to have a free NH₂-terminus for activity [35]. For USP4 and USP15, a ubiquitin-PLA2 fusion protein was used as the substrate and DUB activity was determined by measuring the activation of PLA2 following removal of the ubiquitin-tag (Fig. 5B and 5D). As isolated, both enzymes were active, without the need of removing the SUMOstar fusion partner.

Discussion

Expression of recombinantly derived, eukaryotic proteins in the baculovirus/insect cell system has seen increased usage in recent years in an attempt to circumvent problems associated with the incorrect folding, lack of disulfide bond formation, and lack of post-translational modifications found in prokaryotic systems. Based on our observation that fusion of the ubiquitin-like molecules in the SUMO family to a variety of proteins led to increased expression and solubility of many of these proteins when expressed in *E. coli*, we wanted to determine if fusion to SUMO would yield similar results in insect cells [22,40,41,18,42,43, 44,45,46]. However, we anticipated that fusions to native SUMO would be subject to intracellular processing by endogenous deSUMOylases; hence, in addition to native SUMO, we tested a new SUMO mutant, termed SUMOstar, that is resistant to cleavage by yeast and human deSUMOylases. In addition to enhancing expression levels of recombinant proteins, use of the SUMO-fusion technology has the advantage that removal of SUMO by deSUMOylases results regenerates the native N-terminus of the target protein. The SUMO proteases recognize not only the Gly-Gly structure found at the C-terminus of SUMO, but also tertiary structure elements only present on correctly folded SUMO. As a result, digestion of SUMO-fusion proteins is limited to the junction between SUMO and the target protein. Since

the SUMOstar tag is no longer recognized by native deSUMOylases, we were able to develop a mutated Ulp1 protease which binds to and processes SUMOstar as well as native SUMO although not as efficiently (Panavas et al. manuscript in preparation). As expected, our first series of experiments confirmed that fusion proteins containing native SUMO are processed by endogenous isopeptidases in insect cells (*cf.* lanes 1,2,4 of Fig. 1 with lanes 3 and 5). In addition, while fusion to SUMOstar did indeed lead to enhanced intracellular expression of soluble GFP and UBP43, fusion to native SUMO only gave expression levels equivalent to either untagged or His₆-tagged proteins. The enhancement in expression seen with SUMOstar is equivalent to that previously seen with native SUMO in *E. coli* [9].

Fusion to SUMOstar also led to increased levels of soluble recombinant proteins in the growth medium of the cells. Furthermore, significant amounts of SUMOstar-tryptase and SUMOstar-GFP were found to be retained in the cells despite the presence of the gp67 leader sequence. Defects in the secretion of overexpressed recombinant proteins in baculovirus infected cells have been described in the literature [47,48] and it has been proposed that reduced secretion may be a direct result of the overexpression and retention in the endoplasmic reticulum of the viral chitinase gene [49]. Tryptase was also partly retained within the cell, was soluble, and we were able to isolate and activate it by incubation with SUMOstar protease (data not shown). Purification of the secreted His₆-SUMOstar-tryptase from insect cell conditioned medium was facile and the enzyme could be reactivated by incubation with heparin and SUMOstar protease (Fig. 4 ●). Removal of SUMOstar from the enzyme was absolutely required for activity recovery since incubation of the His₆SUMOstar-tryptase with heparin alone did not generate active enzyme (Fig. 4, ◆). Human mast cell tryptase has been expressed in *Pichia pastoris* and in the baculovirus/insect cell system by others [50,51,52,31,33]. The enzyme was expressed as fusion with either the natural propeptide [51], a KEX2 propeptide [52], a truncated form of the propeptide [50] or as an N-terminal ubiquitin fusion [31,33]. In each case, an enterokinase (EK) site was engineered between the C-terminus of the fusion partner and the N-terminus of tryptase. Activation of the enzyme required removal of the fusion partner by digestion with EK; however, at least in the case of the ubiquitin fusion protein, an additional EK cleavage was found within tryptase itself. For efficient recovery of active tryptase, Selwood and colleagues were required to introduce a mutation in tryptase in order to eliminate this site. This concern is obviated by use of the SUMOstar/SUMOstar protease system.

Finally, we expressed members of the deubiquitinase family in active form in insect cells, USP4 and USP15. These enzymes were easily isolated in active form from crude lysates and did not require removal of SUMOstar to be active. Each of these enzymes has proven to be difficult to express as soluble active proteins in prokaryotes either due to their size or complex structures. As can be seen from Fig. 5, both USP4 and USP15 can be obtained in good yields from this system and, with minor optimization, would be suitable for biochemical and structural characterization.

In conclusion, we demonstrate that the SUMOstar-system enhances the expression and solubility of difficult-to-expressed recombinant proteins in insect cells. This tag was specially designed for eukaryotic expression systems in order to avoid premature processing of SUMO-fusion proteins by endogenous SUMO hydrolases. Since processing by SUMOstar protease is highly specific for the C-terminus of SUMO and is largely insensitive to the nature of the amino acid residue in the P1' position, this system has the potential to allow the facile production of proteins which require well defined and specific amino acids at the NH₂-terminus to achieve proper activity, *e.g.* cytokines [53,54] or chemokines [55,56]. Also, precise processing by SUMOstar protease would lead to a more homogeneous product. Thus the SUMOstar system in insect cells will be a powerful tool for structural and functional studies of variety of proteins.

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References

1. Hochstrasser M. SP-RING for SUMO: New Functions Bloom for a Ubiquitin-like Protein. *Cell* 2001;107:5–8. [PubMed: 11595179]
2. Johnson ES. Protein modification by SUMO. *Ann Rev Biochem* 2004;73:355–82. [PubMed: 15189146]
3. Kim KI, Baek SH, Chung CH. Versatile protein tag, SUMO: its enzymology and biological function. *J Cell Physiol* 2002;191:257–68. [PubMed: 12012321]
4. Lalioti VS, Vargarajauregui S, Pulido D, Sandoval IV. The Insulin-sensitive Glucose Transporter, GLUT4, Interacts Physically with Daxx. TWO PROTEINS WITH CAPACITY TO BIND Ubc9 AND CONJUGATED TO SUMO1. *J Biol Chem* 2002;277:19783–19791. [PubMed: 11842083]
5. Li SJ, Hochstrasser M. A new protease required for cell-cycle progression in yeast. *Nature* 1999;398:246–51. [PubMed: 10094048]
6. Li SJ, Hochstrasser M. The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. *Mol Cell Biol* 2000;20:2367–77. [PubMed: 10713161]
7. Schwienhorst I, Johnson ES, Dohmen RJ. SUMO conjugation and deconjugation. *Mol Gen Genet* 2000;263:771–86. [PubMed: 10905345]
8. Butt TR, Edavettal SC, Hall JP, Mattern MR. SUMO fusion technology for difficult-to-express proteins. *Prot Expr Purif* 2005;43:1–9.
9. Marblestone JG, Edavettal SC, Lim Y, Lim P, Zuo X, Butt TR. Comparison of SUMO fusion technology with traditional gene fusion systems: enhanced expression and solubility with SUMO. *Prot Sci* 2006;15:182–9.
10. Malakhov MP, Mattern MR, Malakhova OA, Drinker M, Weeks SD, Butt TR. SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. *J Struct Funct Gen* 2004;5:75–86.
11. Peroutka RJ, Elshourbagy N, Piech TL, Butt TR. Enhanced protein expression in mammalian cells using engineered SUMO fusions: Secreted phospholipase A₂. *Prot Sci* 2008;10810.1110/ps.035576
12. Farrell PJ, Broeze RJ, Lengyel P. Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. *Nature* 1979;279:523–525. [PubMed: 571963]
13. Loeb KR, Haas AL. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. *J Biol Chem* 1992;267:7806–7813. [PubMed: 1373138]
14. Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J Biol Chem* 2002;277:9976–81. [PubMed: 11788588]
15. Ritchie KJ, Zhang DE. ISG15: the immunological kin of ubiquitin. *Sem Cell Dev Biol* 2004;15:237–246.
16. Kim KI, Zhang DE. UBP43, an ISG15-specific deconjugating enzyme: expression, purification, and enzymatic assays. *Meth Enzymol* 2005;398:491–9. [PubMed: 16275353]
17. Blanchette P, Gilchrist CA, Baker RT, Gray DA. Association of UNP, a ubiquitin-specific protease, with the pocket proteins pRb, p107 and p130. *Oncogene* 2001;20:5533–7. [PubMed: 11571651]
18. Gilchrist CA, Gray DA, Baker RT. A ubiquitin-specific protease that efficiently cleaves the ubiquitin-proline bond. *J Biol Chem* 1997;272:32280–5. [PubMed: 9405433]
19. Milojevic T, Reiterer V, Stefan E, Korkhov VM, Dorostkar MM, Ducza E, Ogris E, Boehm S, Freissmuth M, Nanoff C. The ubiquitin-specific protease Usp4 regulates the cell surface level of the A2A receptor. *Mol Pharmacol* 2006;69:1083–94. [PubMed: 16339847]
20. Soboleva TA, Jans DA, Johnson-Saliba M, Baker RT. Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme. *J Biol Chem* 2005;280:745–52. [PubMed: 15494318]
21. Angelats C, Wang XW, Jermini LS, Copeland NG, Jenkins NA, Baker RT. Isolation and characterization of the mouse ubiquitin-specific protease Usp15. *Mamm Genome* 2003;14:31–46.

22. Baker RT, Catanzariti AM, Karunasekara Y, Soboleva TA, Sharwood R, Whitney S, Board PG. Using deubiquitylating enzymes as research tools. *Meth Enzymol* 2005;398:540–54. [PubMed: 16275357]
23. de Jong RN, Ab E, Diercks T, Truffault V, Daniels M, Kaptein R, Folkers GE. Solution structure of the human ubiquitin-specific protease 15 DSUP domain. *J Biol Chem* 2006;281:5026–5031. [PubMed: 16298993]
24. Hetfeld BK, Helfrich A, Kapelari B, Scheel H, Hofmann K, Guterman A, Glickman M, Schade R, Kloetzel PM, Dubiel W. The zinc finger of the CSN-associated deubiquitinating enzyme, USP15, is essential to rescue the E3 ligase Rbx1. *Curr Biol* 2005;15:1217–1221. [PubMed: 16005295]
25. Peth A, Boettcher JP, Dubiel W. Ubiquitin-dependent proteolysis of the microtubule end-binding protein 1, EB1, is controlled by the COP9 signalosome: possible consequences for microtubule filament stability. *J Mol Biol* 2007;368:550–563. [PubMed: 17350042]
26. Schweitzer K, Bozko PM, Dubiel W, Naumann M. CSN controls NF- κ B by deubiquitinylation of I κ B α . *EMBO J* 2007;26:1532–1541. [PubMed: 17318178]
27. Addington AK, Johnson DA. Inactivation of human lung tryptase: evidence for a re-activatable tetrameric intermediate and active monomers. *Biochem* 1996;35:13511–8. [PubMed: 8885830]
28. Fukuoka Y, Schwartz LB. Human beta-tryptase: detection and characterization of the active monomer and prevention of tetramer reconstitution by protease inhibitors. *Biochem* 2004;43:10757–64. [PubMed: 15311937]
29. Hallgren J, Karlson U, Poorafshar M, Hellman L, Pejler G. Mechanism for activation of mouse mast cell tryptase: dependence on heparin and acidic pH for formation of active tetramers of mouse mast cell protease 6. *Biochem* 2000;39:13068–77. [PubMed: 11041873]
30. Pereira PJ, Bergner A, Macedo-Ribeiro S, Huber R, Matschiner G, Fritz H, Sommerhoff CP, Bode W. Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. *Nature* 1998;392:306–11. [PubMed: 9521329]
31. Selwood T, Wang Z-M, McCaslin DR, Schechter NM. Diverse Stability and Catalytic Properties of Human Tryptase alpha and beta Isoforms are Mediated by Residue Differences at the S1 Pocket. *Biochem* 2002;41:3329–3340. [PubMed: 11876641]
32. Sommerhoff CP, Schaschke N. Mast cell tryptase beta as a target in allergic inflammation: an evolving story. *Curr Pharm Des* 2007;13:313–32. [PubMed: 17313363]
33. Wang Z, Walter M, Selwood T, Rubin H, Schechter NM. Recombinant expression of human mast cell proteases chymase and tryptase. *Biol Chem* 1998;379:167–74. [PubMed: 9524068]
34. Heim R, Cubitt AB, Tsien RY. Improved green fluorescence. *Nature* 1995;373:663–4. [PubMed: 7854443]
35. Nicholson B, Leach CA, Goldenberg SJ, Francis DM, Kodrasov MP, Tian X, Shanks J, Sterner DE, Bernal A, Mattern MR, Wilkinson KD, Butt TR. Characterization of ubiquitin and ubiquitin-like protein isopeptidase activities. *Prot Sci* 2008;17:1035–1043.
36. Chen L, Butters JTM, Hardwick JP, Tamura S, Penman BW, Gonzalez FJ, Crespi CL. Coexpression of cytochrome P450A6 and human NADPH-P450 oxidoreductase in the baculovirus system. *Drug Metabol Disposit* 1997;25:399–405.
37. Maranga L, Brazão TF, Carrondo MJT. Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. *Biotechnol Prog* 2003;84:245–253.
38. Wong KTK, Peter CH, Greenfield PF, Reid S, Nielsen LK. Low multiplicity infection of insect cells with a recombinant baculovirus: The cell yield concept. *Biotechnol Bioeng* 1996;49:659–666. [PubMed: 18626861]
39. Zhang YH, Enden G, Merchuk JC. Insect cells-Baculovirus system: Factors affecting growth and low MOI infection. *Biochem Eng J* 2005;27:8–16.
40. Butt TR, Jonnalagadda S, Monia BP, Sternberg EJ, Marsh JA, Stadel JM, Ecker DJ, Crooke ST. Ubiquitin fusion augments the yield of cloned gene products in *Escherichia coli*. *Proc Natl Acad Sci USA* 1989;86:2540–4. [PubMed: 2539593]
41. Catanzariti AM, Soboleva TA, Jans DA, Board PG, Baker RT. An efficient system for high-level expression and easy purification of authentic recombinant proteins. *Prot Sci* 2004;13:1331–9.
42. Hunter AM, Kottachchi D, Lewis J, Duckett CS, Korneluk RG, Liston P. A novel ubiquitin fusion system bypasses the mitochondria and generates biologically active Smac/DIABLO. *J Biol Chem* 2003;278:7494–9. [PubMed: 12511567]

43. Pilon A, Yost P, Chase TE, Lohnas G, Burkett T, Roberts S, Bentley WE. Ubiquitin fusion technology: bioprocessing of peptides. *Biotechnol Prog* 1997;13:374–9. [PubMed: 9265776]
44. Pilon AL, Yost P, Chase TE, Lohnas GL, Bentley WE. High-level expression and efficient recovery of ubiquitin fusion proteins from *Escherichia coli*. *Biotechnol Prog* 1996;12:331–7. [PubMed: 8652120]
45. Tcherniuk SO, Chroboczek J, Balakirev MY. Construction of tumor-specific toxins using ubiquitin fusion technique. *Mol Ther* 2005;11:196–204. [PubMed: 15668131]
46. Wang T, Evdokimov E, Yiadom K, Yan Z, Chock PB, Yang DC. Biotin-ubiquitin tagging of mammalian proteins in *Escherichia coli*. *Prot Expr Purif* 2003;30:140–9.
47. Kaba SA, Salcedo AM, Wafula PO, Vlask JM, van Oers MM. Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. *J Virol Meth* 2004;122:113–118.
48. van Oers MM, Thomas AA, Moormann RJ, Vlask JM. Secretory pathway limits the enhanced expression of classical swine fever virus E2 glycoprotein in insect cells. *J Biotechnol* 2001;86:31–38. [PubMed: 11223142]
49. Possee RD, Thomas CJ, King LA. The use of baculovirus vectors for the production of membrane proteins in insect cells. *Biochem Soc Trans* 1999;27:928–932. [PubMed: 10830130]
50. Chan H, Elrod KC, Numerof RP, Sideris S, Clark JM. Expression and characterization of recombinant mast cell tryptase. *Prot Expr Purif* 1999;15:251–257.
51. Huang C, Li L, Krilis SA, Chanasyk K, Tang Y, Li Z, Hunt JE, Stevens RL. Human tryptases α and β are functionally distinct due, in part, to a single amino acid difference in one of the surface loops that forms the substrate-binding cleft. *J Biol Chem* 1999;274:19670–19676. [PubMed: 10391906]
52. Niles AL, Maffitt M, Haak-Frendscho M, Wheelless CJ, Johnson DA. Recombinant human mast cell tryptase β : stable expression of *Pichia pastoris* and purification of fully active enzyme. *Biotechnol Appl Biochem* 1998;28:125–131. [PubMed: 9756742]
53. Kirkpatrick RB, Grooms M, Wang F, Fenderson H, Feild J, Pratta MA, Volker C, Scott G, Johanson K. Bacterial production of biologically active canine interleukin-1 β by seamless SUMO tagging and removal. *Prot Expr Purif* 2006;50:102–10.
54. Liu B, Novick D, Kim SH, Rubinstein M. Production of a biologically active human interleukin 18 requires its prior synthesis as PRO-IL-18. *Cytokine* 2000;12:1519–25. [PubMed: 11023667]
55. Proudfoot AEI, Power CA, Hoogewerf AJ, Montjovent M-O, Borlat F, Offord RE, Wells TNC. Extension of Recombinant Human RANTES by the Retention of the Initiating Methionine Produces a Potent Antagonist. *J Biol Chem* 1996;271:2599–2603. [PubMed: 8576227]
56. Proudfoot AEI, Buser R, Borlat F, Alouani S, Soler D, Offord RE, Schroder J-M, Power CA, Wells TNC. Amino-terminally Modified RANTES Analogues Demonstrate Differential Effects on RANTES Receptors. *J Biol Chem* 1999;274:32478–32485. [PubMed: 10542293]

Abbreviations

SUMO	small ubiquitin-like modifier
GFP	green fluorescent protein
NBD C₆-HPC	2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl- <i>sn</i> -glycero-3-phosphocholine
USP	ubiquitin specific protease
UBP	ubiquitin protease

Figure 1.

Analysis of the expression of GFP in Sf9 cells. Sf 9 cells (2×10^6) were infected at an MOI of 0.05 with baculovirus harboring various GFP constructs. The cells were harvested at 120 hours post infection and lysed. (A) Equal amounts total, soluble protein were analyzed by SDS-PAGE and the separated proteins stained with Coomassie Blue. (B) Cell lysates expressing gp67His₆SUMOstar- and gp67His₆-GFP constructs were also analyzed by western blot using an anti-His₆ antibody as the primary antibody. Images of the stained gels or western blots were made using a Fuji imager. Fuji image analysis software was used to estimate the relative amounts of each protein. Molecular weights, in kDa, of the marker proteins are shown next to each image. *N.b.* although the calculated MW of SUMO is 11.5 kDa, it migrates with an apparent MW of ~20 kDa on SDS-PAGE. The positions of native GFP and His₆SUMOstar-GFP are indicated. Gp67 = the gp67 signal sequence, His₆ = the hexahis tag, SUMO = the SUMO fusion partner; and SUMO* = the SUMOstar fusion partner.

Figure 2.

Analysis of the expression of UBP43 in Sf9 cells. Cells (10^4) in 24-well plates were infected with baculovirus harboring either His₆-UBP43 (H), SUMOstar-UBP43 (S*), or His₆SUMOstar-UBP43 (HS*) at different MOI as indicated. The cells were harvested at 140 hours post infection and SDS-PAGE analysis as described in the Materials and Methods. Equivalent amounts of cells were loaded in each lane. Following electrophoresis, the separated proteins were stained with Coomassie Blue. Image collection and analysis was carried out as described in the legend to Fig. 1.

Figure 3.

Analysis of the secretion of Tryptase β II and GFP into the medium by Sf9 cells. (A) Cells (10^4) were infected with baculovirus harboring either gp67-Tryptase (G), gp67SUMO-Tryptase (GS) or gp67SUMOstar-Tryptase (GS*) at MOIs of 0.1, 1 or 5 in 6 well plates. The corresponding fusions to GFP were only analysed at an MOI of 1. Conditioned medium from each well was collected at 72 hr post infection and clarified by centrifugation at $300 \times g$ for 10 min. The clarified samples were concentrated using a 0.5 ml Microcon concentrator (MWCO 10 kDa, Millipore, Billerica, MA) at $14,000 \times g$ for 40 min. Equal amounts of the concentrated media were loaded in each lane. (B) Aliquots of the cells expressing either G-Tryptase or GS*-tryptase were lysed and analyzed by SDS-PAGE in order to determine if the fusion proteins were being retained within the cells. Following electrophoresis, the separated proteins were stained with Coomassie Blue. Image collection and analysis was carried out as described in the legend to Fig. 1.

Figure 4.

Isolation secreted SUMOstar-Tryptase from Sf9 conditioned medium. Secreted gp67His₆SUMOstar-Tryptase from 100 mL of conditioned medium was captured in batch mode by mixing with Ni-NTA as described in the Materials and Methods section. (A) SDS-PAGE analysis of aliquots of the starting condition medium (L), the unbound material (FT), the Wash (W) and pooled and concentrated eluate fractions (E). (B) The pooled and concentrated eluate fraction was assayed for tryptase activity as with and without removal of the SUMOstar fusion partner by incubation with SUMOstar protease using Chromozym TH as a substrate for tryptase as described in the Materials and Methods section. (●) Complete reaction mixture with SUMOstar protease, heparin, and SUMOstar-tryptase. (◆) Reaction mixture with heparin and SUMOstar-tryptase but without SUMOstar protease. (▲) Reaction mixture minus SUMOstar-tryptase and SUMOstar protease. In a separate experiment SUMOstar protease was found to have no activity against the tryptase substrate (data not shown.)

Figure 5.

Isolation of intracellular USP4 and USP15 and activity determination. Isolation of His₆SUMOstar-enzymes from Sf9 cell lysates was carried out as described in the Materials and Methods. (A) SDS-PAGE analysis of aliquots of samples from the isolation of USP4, L = the clarified cell lysate, FT = the unbound fraction from the Ni-NTA column, W = the low imidazole wash from the column and E = the pooled and concentrated elution fractions. The expected position of His₆SUMOstar-USP4 is indicated. (B) SDS-PAGE analysis of aliquots of samples from the isolation of USP15. Samples labeled as per (A). The expected position of His₆SUMOstar-USP15 is indicated. The ability of each purified enzyme to process Ub-PLA2 to release active PLA2 was carried out as described in the Materials and Methods. (C) Ub-PLA2 processing activity of either 5 (▲) or 10 (●) μL of the concentrated pool of USP4. (D) Ub-PLA2 processing activity of either 5 (▲) or 10 (●) μL of the concentrated pool of USP15. For both, the line represents the fit of the data to equation 1. Boxed points were excluded from the calculation due to depletion of the PLA2 substrate.