Chapter 2

SUMO Fusion Technology for Enhanced Protein Expression and Purification in Prokaryotes and Eukaryotes

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Abstract

The preparation of sufficient amounts of high-quality protein samples is the major bottleneck for structural proteomics. The use of recombinant proteins has increased significantly during the past decades. The most commonly used host, *Escherichia coli*, presents many challenges including protein misfolding, protein degradation, and low solubility. A novel SUMO fusion technology appears to enhance protein expression and solubility (www.lifesensors.com). Efficient removal of the SUMO tag by SUMO protease in vitro facilitates the generation of target protein with a native N-terminus. In addition to its physiological relevance in eukaryotes, SUMO can be used as a powerful biotechnology tool for enhanced functional protein expression in prokaryotes and eukaryotes.

Key words: SUMO, Smt3, SUMO protease1, protein expression, protein solubility, protein purification SUMOstar, SUMOstar protease.

1. Introduction

SUMO proteins are covalently attached to and removed from specific protein substrates in eukaryotic cells. SUMOylation as a reversible post-translational modification process has been shown to be involved in many cellular processes, such as nuclear-cytosolic transport (1), apoptosis (2), protein activation (3) and stability (4), response to stress (5), and progression through the cell cycle (6). A SUMO fusion system using yeast SUMO (*Saccharomyces cerevisiae* Smt3) as the N-terminal tag appears to enhance the expression and solubility of partner proteins and decrease proteolytic degradation (*see* **Fig. 2.1**) (7–13). After expression in *E. coli*, an N-terminal His 6 SUMO tag facilitates purification of the fusion protein. This tag can be efficiently removed by SUMO

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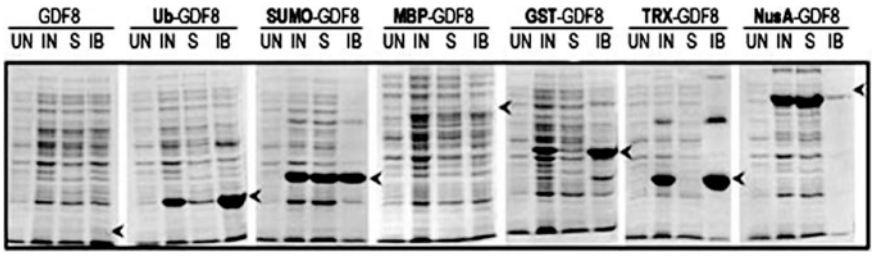

Fig. 2.1. Comparison of protein expression and solubility properties among GDF8 derivatives containing various N-terminal fusions (SUMO, GST, MBP, TRX, NusA, and Ub). All genes were expressed in a pET24 background. Equal amounts of protein from uninduced culture (UN), induced (IN), soluble fraction (S), and inclusion bodies (IB) were analyzed by 10% SDS-PAGE. Gels were stained with Coomassie blue. For details, please see the text. GDF8 fused with SUMO or NusA consistently showed higher amount of expression and solubility, whereas the GDF8 derivatives tagged with GST, MBP, or TRX were least effective in expression and solubility properties. GDF8 is expressed relatively poorly in *E. coli* as an unfused protein.

protease 1 (*S. cerevisiae* Ulp1), which recognizes the 3D structure of SUMO as well as the C-terminal sequence. Cleavage after the conserved C-terminal Gly–Gly motif of SUMO generates a partner protein with a native N-terminus and the capability of being re-purified and used for many biomedical or biopharmaceutical purposes.

SUMO fusion technology is an excellent tool for prokaryotic expression systems; however, the SUMO tag will be cleaved by SUMO proteases in a eukaryotic organism. LifeSensors, Inc. recently engineered a novel mutant SUMO tag, called SUMOstar, which is resistant to cleavage by SUMO protease in eukaryotic expression systems. This SUMOstar tag also maintains enhanced protein expression and solubility as shown in yeast (*S. cerevisiae* and *Pichia pastoris*, unpublished data), insect, and mammalian cells (14, 15). In addition, a novel SUMOstar-specific protease has been developed by LifeSensors, Inc. which is able to cleave the SUMOstar tag in vitro from the fusion protein. This novel SUMOstar fusion technology can be utilized for a variety of prokaryotic and eukaryotic expression systems.

Here, we will provide detailed protocols on how to construct a SUMO tag with a gene of interest, express this gene fusion in *E. coli* and purify the gene product from either the soluble fraction or inclusion bodies. We will also demonstrate how to cleave the SUMO tag in vitro using a SUMO-specific protease and purify the target protein with a native N-terminus.

2. Materials

2.1. Cloning into the pSUMO Vector

1. pSUMO vector (www.lifesensors.com) (*see* **Fig. 2.2**)
2. DNA containing the gene of interest

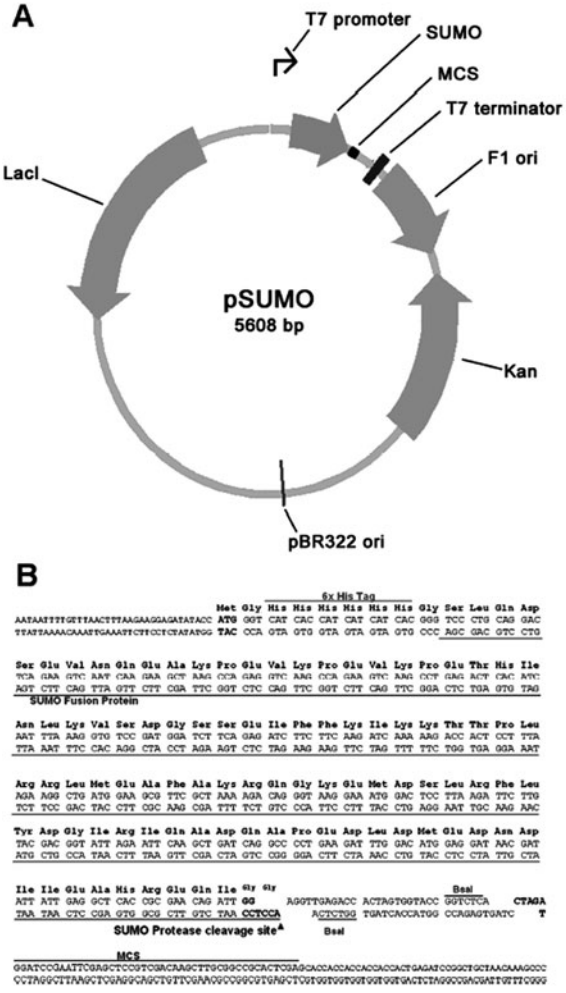

Fig. 2.2. The cloning vector pSUMO. (**a**) A feature map of the vector. Expression of a SUMO fusion protein is driven by an inducible T7 promoter. The pSUMO vector is a low copy plasmid with kanamycin selection. (**b**) The DNA sequence encoding the SUMO tag. The restriction digestion site of enzyme *Bsa*I is illustrated. Digestion with *Bsa*I results in two unique overhangs for directional cloning of the gene of interest.

3. PCR kit (High Fidelity DNA polymerase, 10x reaction buffer, dNTP nucleotide mix)
4. Appropriate DNA restriction enzymes and reaction buffers
5. PCR purification kit
6. 50°C water bath
7. DNA gel extraction kit
8. TAE buffer: 40 mM Tris, 1 mM EDTA, 20 mM acetic acid, pH 8.5

9. Agarose
10. T4 DNA ligase and reaction buffer
11. Competent *Escherichia coli* TOP10 cells
12. LB medium: 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0
13. LB agar plates with appropriate antibiotic
14. Plasmid DNA miniprep kit

2.2. E. coli Transformation and Induction

1. SOC medium: 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 20 mM glucose, pH 7.0
2. LB medium: 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0
3. Appropriate antibiotics
4. Propane torch and flint striker
5. Bucket of ice
6. Shaking 37°C incubator
7. Sterile 2.5 l flasks
8. Water bath heated to 42°C
9. Sterile spreader
10. Automatic pipettor
11. Centrifuge with a rotor capable of holding 250 or 500 ml bottles

2.3. Preparation of Soluble and Inclusion Body (IB) Fractions

1. Sterile 35 ml centrifuge tubes
2. PBS: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 8.0
3. Lysis buffer: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 287 mM NaCl, 2.7 mM KCl, 10 mM imidazole, 1% (v/v) Triton X-100, pH 8.0
4. IB wash buffer: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 287 mM NaCl, 2.7 mM KCl, 10 mM imidazole, 0.5% (v/v) Triton X-100, 1 mM EDTA, 1 M urea, pH 8.0
5. IB solubilization buffer: 50 mM CAPS, 0.3 M NaCl, 0.3% (w/v) *N*-laurylsarcosine, 1 mM DTT, pH 11
6. DNase: 50 mg/ml stock of deoxyribonuclease I from bovine pancreas (store at –20°C)
7. RNase: 50 mg/ml stock of ribonuclease A from bovine pancreas
8. PMSF: 1 M phenylmethylsulfonyl fluoride
9. IPTG: 1 M isopropyl β-D-thiogalactopyranoside

10. Dialysis buffer: 20 mM Tris, 150 mM NaCl, 10% (v/v) glycerol, pH 8.0

2.4. Affinity Purification

1. Ni-NTA resin
2. Lysis buffer: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 287 mM NaCl, 2.7 mM KCl, 10 mM imidazole, 1% (v/v) Triton X-100, pH 8.0
3. Wash buffer 1: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 287 mM NaCl, 2.7 mM KCl, 5 mM imidazole, and 1% (v/v) Triton X-100, pH 8.0
4. Wash buffer 2: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 287 mM NaCl, 2.7 mM KCl, 15 mM imidazole, pH 8.0
5. Elution buffer: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 287 mM NaCl, 2.7 mM KCl, 300 mM imidazole, pH 8.0
6. Strip buffer: 20 mM Tris, 100 mM EDTA, and 0.5 M NaCl, pH 7.9
7. IB solubilization buffer: 50 mM CAPS/KOH, 300 mM NaCl, and 0.3 % (w/v) *N*-laurylsarcosine, pH 11
8. Charge buffer: 50 mM $NiCl_2$
9. 20% (v/v) ethanol
10. Dialysis buffer: 20 mM Tris, 150 mM NaCl, 10% (v/v) glycerol, pH 8.0

2.5. SUMO Tag Cleavage

1. SUMO protease 1: 10 unit/μl (LifeSensors, Inc.)
2. PBS: 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 8.0
3. Refolding buffer: 20 mM Tris, 150 mM NaCl, 1% (w/v) CHAPS, 10% (v/v) glycerol, pH 8.0
4. 1 M DTT
5. 3.5 kDa MWCO (*m*olecular *w*eight *c*ut-*o*ff) dialysis tubing

3. Methods

3.1. Directional Cloning into pSUMO Vector Using BsaI

Here we describe the strategy of generating a SUMO tag fused gene of interest using the pSUMO vector and a single restriction enzyme for in-frame cloning (*see* **Notes 1** and **2**):

1. Design PCR primers to amplify the gene of interest. For the forward and reverse primers, use the sequences 5′-NNN **GGT CTC** NAG GTX XXX XXX XXX XXX XX–3′ and 5′-NNN **GGT CTC** ***TCT AGA*** TCA YYY YYY YYY YYY YYY-3′ as templates, respectively. Within these PCR

templates, "X" corresponds to nucleotides at the 5′-end of the gene of interest and "Y" to the reverse complement of nucleotides at its 3′-end. "N" is any nucleotide, the *Bsa*I site is in bold, and the *Xba*I site is in bold and italics (*see* **Note 3**).

2. Amplify the gene of interest with the designed primers in a PCR reaction using a thermostable high-fidelity DNA polymerase.
3. Clean up the PCR reaction using a PCR purification kit.
4. Digest the pSUMO vector and the generated PCR product separately in reaction tubes with *Bsa*I restriction endonuclease (10 U) for 1 h at 50°C (*see* **Notes 4** and **5**) and separate the reaction samples on a 1% (w/v) TAE agarose gel by running for 30 min at 10 V/cm.
5. Isolate (excise) both restricted DNA molecules, the pSUMO vector and the PCR fragment, from the agarose gel using a DNA gel extraction kit.
6. Mix the vector and the PCR fragment in the ratio 1:3 (mol:mol) and set up a 20 μl ligation reaction.
7. Incubate at room temperature for 2 h.
8. Use 5 μl of the ligation mixture to transform 50 μl of competent *E. coli* TOP10, DH5α, or other strains suitable for cloning according to the transformation protocol in **Section 3.2**.
9. Inoculate 3 ml culture with the positive colonies on LB-based selection plates and grow with shaking at 37°C overnight.
10. Spin down bacteria at 4000×*g* for 5 min and discard the supernatant.
11. Isolate the plasmid using a plasmid DNA miniprep kit. After sequence confirmation proceed to the transformation of the *E. coli* expression strain.

3.2. Transformation and Protein Expression

In this section, we describe how to express the SUMO fusion construct in *E. coli* BL21(DE3) cells. This strain is commonly used for inducible, T7 RNA polymerase-driven, high-level gene expression. Genes of two major proteases, OmpT and Lon, are deleted from the genome of this strain in order to decrease cellular degradation.

1. Gently thaw chemically competent cells on ice and keep the cells as cold as possible at all times. Always work aseptically when transforming and culturing *E. coli* cells.

2. Add 1 μl of DNA (approximately 0.1 μg) to 50 μl of chemically competent cells while on ice in a sterile 1.5 ml microfuge tube.
3. Mix gently with a pipette tip and let incubate on ice for 15–30 min.
4. Following incubation on ice, heat shock the cells by removing tube from the ice and immediately immersing in a 42°C water bath for 40 s. Place them back on ice after this heat shock period.
5. After 2 min on ice, add 200 μl of SOC pre-warmed to 37°C.
6. Leave the cells for recovery in the 37°C shaker for 1 h.
7. Aseptically transfer 0.1 ml of transformation culture to an LB plate containing 30 μg/ml kanamycin.
8. Spread transformation culture evenly with sterile spreader.
9. Place the plate into a 37°C incubator and incubate overnight.
10. Pipette 5 ml LB medium containing 30 μg/ml kanamycin into sterile, 15 ml snap cap tubes.
11. Inoculate the 5 ml LB with a single *E. coli* colony using an inoculation loop.
12. Incubate with shaking (250 rpm) at 37°C overnight.
13. Transfer 1 l of LB containing 30 μg/ml kanamycin to sterile 2.5 l flask.
14. Inoculate the 2.5 l flask with the 5 ml starter culture
15. Incubate with shaking (rpm = 250) at 37°C for approximately 3 h until the cell density reaches an $OD_{600\,nm}$ of 0.6–0.8.
16. When the $OD_{600\,nm}$ of 0.6–0.8 is reached, remove 1 ml of culture to serve as a negative induction control. Store this culture at –80°C.
17. Add IPTG to a final concentration of 1 mM. Incubate either at 37°C with shaking for 3 h or at 20°C for 16 h. Harvest cells by centrifugation at 4000×*g* for 15 min at 4°C. Store the pellet at –80°C. The typical wet weight of cultured cells is approximately 10–12 g^{-1}/l culture.

3.3. Cell Lysis and Protein Preparation

Overexpressed proteins in *E. coli* usually accumulate in the soluble fraction or form insoluble inclusion bodies. The SUMO fusion system significantly increases protein solubility and expression; therefore, SUMO-tagged proteins are usually present in the soluble fraction. However, in some cases, even SUMO-tagged proteins form inclusion bodies. Preparation of a soluble fraction and

insoluble inclusion bodies from bacterial cells will be described here.

3.3.1. Preparation of Soluble Protein Fraction from E. coli Cells

1. Resuspend the cell pellet in lysis buffer (approximately 3 ml lysis buffer per gram cell paste).
2. Lyse cells by sonication (75% output for 10 × 15 s, with 30 s intervals between the pulse cycles) on wet ice (*see* **Note 8**).
3. Add DNase and RNase (20 μg/ml) to the lysates and incubate for 20 min on wet ice (*see* **Note 9**).
4. Add Triton X-100 to the sample to a final concentration of 1% (v/v) and incubate at 4°C for 1 h.
5. Centrifuge the sample (20,000×*g*, 30 min at 4°C) and pool the supernatant as the soluble protein fraction and keep the pellet for preparation of insoluble proteins (*see* **Section 3.3.2**).

3.3.2. Preparation of Insoluble Protein Fraction from Inclusion Bodies

1. Wash the pellet prepared above from the 1 l cultured cells with 30 ml IB wash buffer by resuspension and centrifugation at 10,000×*g* for 10 min at 4°C.
2. Discard the wash supernatant and repeat wash steps twice as described above.
3. Add 30 ml IB solubilization buffer to the pellet and incubate with shaking for 1 h at room temperature to extract insoluble proteins.
4. Centrifuge the sample at 15,000×*g* for 30 min at 4°C and collect the supernatant as the insoluble protein fraction.
5. Analyze the soluble and insoluble fractions prepared above using an SDS-PAGE according to the molecular weight of the gene product.
6. Use the protein samples immediately for purification or store them at 4°C for a short period of time (less than 10 days). For long-term storage keep the protein samples at –80°C and avoid repeated freeze–thaw cycles.

3.4. Affinity Purification Using Ni-IMAC Resin

The N-terminal His 6 tag of SUMO allows the affinity purification of SUMO fusion proteins using a Ni-IMAC resin (*see* **Fig. 2.3**). This method is an efficient and inexpensive way of purifying proteins from bacterial lysates. In this section, we will demonstrate the procedures of purifying SUMO fusion proteins from the soluble fraction and insoluble inclusion bodies.

3.4.1. Purification from a Soluble Extract

1. Pipette 25 ml Ni-IMAC resin into a column and allow to drain by gravity (here and in all subsequent purification steps allow the column to drain by gravity).
2. Wash the column with 5 column volumes (CV) of water.

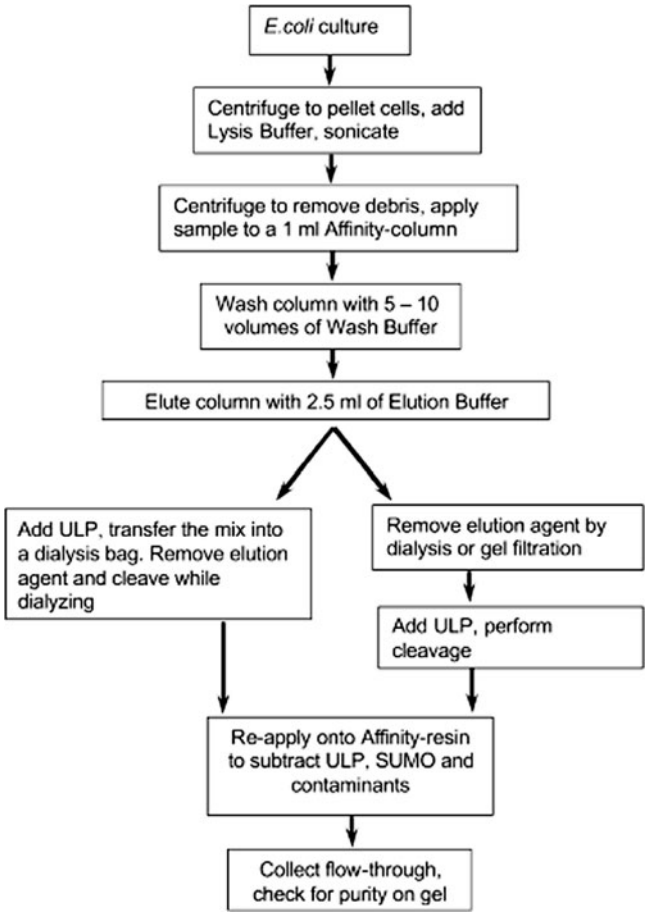

Fig. 2.3. Flow chart for purification and cleavage of His 6-SUMO-tagged proteins.

3. Charge the column with 5 CV charge buffer. If using a new Ni resin, the resin is pre-charged and this step may be omitted.
4. Equilibrate column with 10 CV of wash buffer 1.
5. Load sample onto Ni-IMAC resin. Make sure that the protein extract contains 5 mM imidazole. If not, add 1.7% (v/v) elution buffer to the protein sample and mix gently (*see* **Note 10**).
6. Wash column with 10 CV of wash buffer 1.
7. Wash with 10 CV of wash buffer 2.
8. Elute the bound SUMO fusion protein with 4 CV of elution buffer.
9. Strip column by adding 5 CV of strip buffer.
10. Wash column with 5 CV water.

11. Apply 5 CV 20% (v/v) ethanol to the column, allow $^1/_2$ the volume to drain by gravity, and store the column at 4°C.

3.4.2. Purification from an Insoluble Extract

1. Equilibrate the pre-charged column with 10 CV of IB solubilization buffer containing 5 mM imidazole.
2. Load sample onto Ni-IMAC resin. Make sure the protein extract contains 5 mM imidazole as mentioned in **Section 3.4.1**.
3. Wash column with 10 CV IB solubilization buffer containing 5 mM imidazole.
4. Wash with 10 CV IB solubilization buffer containing 15 mM imidazole.
5. Elute with 4 CV IB solubilization buffer containing 300 mM imidazole.
6. Strip column by adding 5 CV strip buffer.
7. Wash column with 5 CV water.
8. Wash the column with 5 CV storage buffer as described in **Section 3.4.1**.

3.5. Removal of SUMO Tag

Unlike other proteases, SUMO protease 1 not only recognizes its specific amino acid sequence "x-Gly-Gly|x," but also the tertiary structure of the SUMO tag. SUMO proteases have been found to completely cleave a wide range (6–110 kDa) of proteins fused to SUMO and approximately 100 SUMO fusions have been cleaved without erroneous digestion (7, 9, 11). Variable conditions have also been tested on SUMO protease activity (7) (*see* **Fig. 2.4**). SUMO protease is capable of cleaving the SUMO-GFP fusion under a wide range of conditions such as in the presence of up to 2 M urea, 0.1 M guanidine-HCl, 300 mM imidazole, 1% Triton,

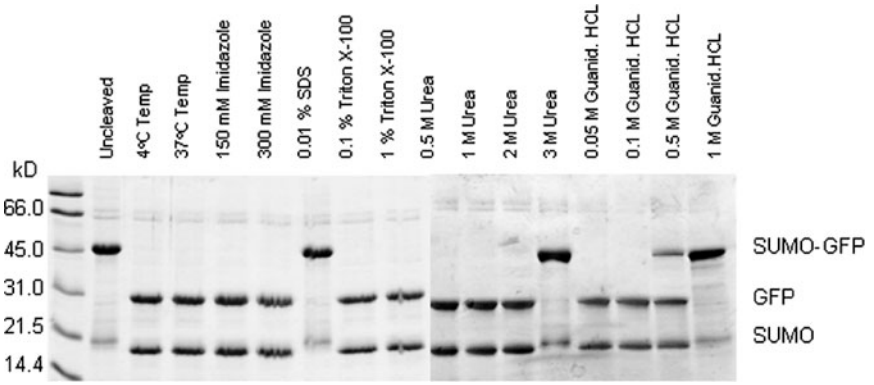

Fig. 2.4. Effect of temperature and various chemicals on SUMO protease 1 activity. Purified SUMO-GFP fusion (15 μg) and one unit of SUMO protease were combined in PBS buffer with indicated additives and the reactions were stopped after 20 min. Reaction products were resolved by SDS-PAGE and stained with Coomassie blue.

0.5 M NaCl, temperature from 4 to 37°C, and pH from 6 to 9 (7). Another important advantage of using SUMO as a fusion tag is the capability of generating the native N-terminus of a target protein which is particularly critical for producing proteins whose activity relies on their specific N-terminus (e.g. chemokines). The recombinant form of SUMO protease includes an N-terminal His 6 tag and so it can be easily removed using Ni-IMAC chromatography.

1. Dialyze a purified SUMO-fused protein using 3.5 kDa MWCO dialysis tubing against either 500 ml PBS for soluble fusions or 500 ml refolding buffer for insoluble SUMO-fused proteins for 24 h at 4°C with at least four fresh buffer exchanges.
2. Add SUMO protease 1 to the SUMO fusion protein sample in appropriate buffer (conditions are listed in **Table 2.1**) at a ratio of 1 unit enzyme per 100 μg substrate and incubate at 30°C for 1 h (*see* **Note 11**).

Table 2.1
Influence of various chemicals on the activity of SUMO protease 1

Chemical	Concentration	Percent of cleavage
Phosphate-buffered saline (PBS)	*See* **Section 2.3**	100
DTT or β-mercaptoethanol	20 mM	100
NaCl	150 mM	100
	500 mM	60
	1 M	30
Urea	1 M	100
	2 M	95
	3 M	5
Guanidine hydrochloride	500 mM	60
	1 M	0
Triton X-100	1%	100
Imidazole	300 mM	100
GSH (reduced glutathione)	20 mM	100
Maltose	20 mM	100
Glycerol	20% (v/v)	100
Ethylene glycol	20% (v/v)	100
Sucrose	20% (w/v)	100
Ethanol	10% (v/v)	100

3. Add DTT to the enzyme–substrate mixture to a final concentration of 2.0 mM. Do not exceed 2 mM if nickel affinity resin (Ni-IMAC) will be used for subtracting SUMO in the downstream purification, because high concentrations of DTT can disassociate the metal from the resin.
4. Incubate the mixture at 30°C for 1 h with slight shaking. Typically, >95% of SUMO fusions can be cleaved under these conditions. (To maximize cleavage, continue to incubate the mixture at 4°C overnight.)
5. Check the cleavage using SDS-PAGE. If the SUMO fusion is not approx. 95% cleaved, add more SUMO protease 1 and incubate for a longer time.
6. Dialyze the cleaved SUMO fusion with proper buffer for the next purification step and subtraction of SUMO and SUMO protease 1 for pure target proteins (*see* **Note 12**).
7. For the subtraction step, reapply the digestion mixture to the 5 ml Ni-IMAC column. Add two column volumes of PBS to the column. While the gene product of interest, which does not harbor a His 6-tag, flows through the column, both

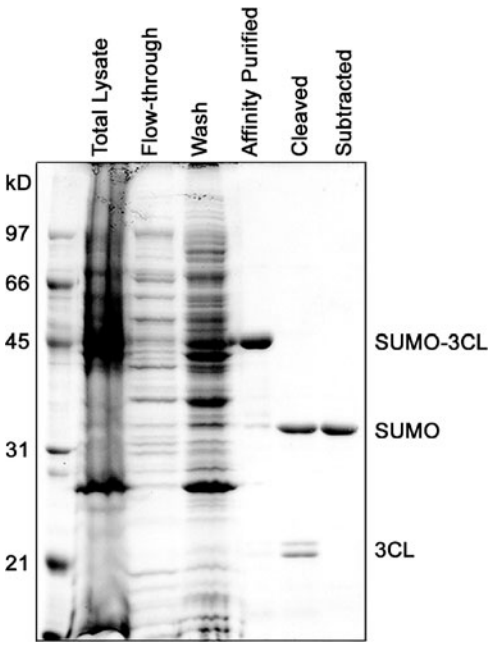

Fig. 2.5. Expression and purification of His 6-SUMO-3CL and protein. *Escherichia coli* grown in LB medium was induced at 20°C for 6 h. Proteins were purified on Ni-IMAC resin and eluted (lanes as described in **Section 3.4**). Cleavage and dialysis of 2 mg of each fusion were performed overnight at 4°C with 10 units of SUMO protease 1. Following dialysis, His 6-SUMO and SUMO protease 1 were subtracted by passing the proteins through a miniature Ni-IMAC column. Protein fractions were resolved by SDS-PAGE and stained with Coomassie blue.

the SUMO fusion and SUMO protease contain a His 6-tag and thus bind to the Ni-IMAC resin and become subtracted from the mixture.

8. Analyze the protein samples by SDS-PAGE (see a sample purification and cutting analysis in **Fig. 2.5**).

4. Notes

1. To take full advantage of SUMO fusion technology including the removal of the SUMO tag by SUMO protease, it is critical to make a fusion protein without any additional sequence between SUMO and the desired gene. SUMO protease recognizes the structure of the SUMO moiety and cleaves at the C-terminal end of a conserved – Gly–Gly sequence (16). Therefore, SUMO protease never cleaves inside the partner protein.
2. SUMO protease can cleave the SUMO moiety in any amino acid context except that in which a proline residue follows immediately after the last two glycines of SUMO.
3. In designing DNA primers for the amplification of the open reading frame, make sure to add at least two extra nucleotides at the 5′-end of the primer. Otherwise the restriction enzymes will not cleave PCR-amplified DNA efficiently.
4. If the gene of interest contains an inherent *Bsa*I site, one can use an alternate type IIS restriction endonuclease. Below are recommended ways to incorporate these enzymes into the DNA primers.

*Aar*I: 5′ – **CACCTGC**NNNNAGGTXXXXXXXXXXXXXXXX – 3′

*Bbs*I: 5′ – **GAAGAC**NNAGGTXXXXXXXXXXXXXXX – 3′

*Bbv*I: 5′ – **GCAGC**NNNNNNNNAGGTXXXXXXXXXXXXXXX – 3′

*Bfu*AI: 5′ – **ACCTGC**NNNNAGGTXXXXXXXXXXXXXXX – 3′

*Bsa*I: 5′ – **GGTCTC**NAGGTXXXXXXXXXXXXXXXX – 3′

*Bsm*AI: 5′ – **GTCTC**NAGGTXXXXXXXXXXXXXXXX – 3′

*Bsm*BI: 5′ – **CGTCTC**NAGGTXXXXXXXXXXXXXXX – 3′

*Bsm*FI: 5′ – **GGGAC**NNNNNNNNNNAGGTXXXXXXXXXXXXXXX – 3′

*Btg*ZI: 5′ – **GCGATG**NNNNNNNNNNAGGTXXXXX XXXXXXXXXX – 3′

*Fok*I: 5′ – **GGATG**NNNNNNNNNAGGTXXXXXXX XXXXXXXX – 3′

*Sfa*NI: 5′ – **GCATC**NNNNNAGGTXXXXXXXXXXXX XXX – 3′

where N represents any nucleotide and X represents the sequence of the gene of interest. The enzyme recognition sequence is underlined and in bold.

5. When performing the restriction digest, make sure not to use too much enzyme for an extended period of time. Over-digestion of the vector could result in inefficient cloning.
6. For the protein expression use *E. coli* expression strain freshly transformed with expression plasmid (<2 weeks old). Using older plates entails the risk of significant reduction in protein expression as compared with expression using freshly transformed plates.
7. When inducing the culture for protein expression it is important to know that even with the SUMO fusion tag some proteins are insoluble when expressed at 37°C. Lowering the temperature during the induction ensures a higher yield of soluble protein. Induction can be performed at 20°C overnight, in which case the preinduction cell density must be 0.8 OD instead of 0.5.
8. When sonicating, it is critical to not overheat the lysate. If a large bacterial pellet is being sonicated, it is wise to use a metal container for the best heat transfer from the lysate to the iced water.
9. The DNase I solution must be freshly prepared because freeze/thaw cycles significantly decrease DNase I enzymatic activity and the cell lysate might be too viscous after centrifugation.
10. During column purification it is critical to have imidazole in the lysis and wash buffers to ensure a clean protein preparation. However, if the protein starts to elute during the second wash, replace the wash buffer II with wash buffer I.
11. SUMO protease is a very robust enzyme and it can cleave in a variety of buffers and additives. **Figure 2.4** and **Table 2.1** summarize some tested conditions for the cleavage efficiency.
12. It is noteworthy that with SUMO the fusion partners are more soluble; however, after cleavage with SUMO protease the cleaved off protein might fall out of solution.

13. Due to the lack of endogenous SUMO protease in *E. coli* cells, SUMO fusion expression system is well established in prokaryotic cells (LifeSensors, Inc.). However, SUMO tag will be cleaved by endogenous SUMO protease after translation in eukaryotic cells (9). We recently engineered a novel SUMO tag, called SUMOstar, which is not removed from the fusion partner in eukaryotic cells. We have shown that the SUMOstar system enhances expression of proteins in *yeast, P. pastoris*, insect cells, and mammalian cells. In addition to enhancing intracellular expression, novel SUMOstar tags have been developed that enhance secretion of proteins in insect and mammalian cells. Thus, the SUMOstar fusion could be utilized for enhanced expression of functional proteins not only in prokaryotes but also in eukaryotic systems. After affinity purification of fusion proteins, SUMOstar tag can be cleaved in vitro by a specific SUMOstar protease (LifeSensors, Inc.).

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