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EXPRESSION OF SOLUBLE RECOMBINANT HUMAN SUMO PROTEASE'S ACTIVE REGION FUSED WITH THIOREDOXIN IN *ESCHERICHIA COLI*

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ABSTRACT

The human's SUMO protease is commonly used in gene expression studies. However, it is challenging to extract this enzyme from its natural host due to degradation during extraction and high cost. Recombinant expression using *Escherichia coli* is considered for its high production from the fast growth rate and feasible manipulation. In this study, we aimed to express the human SUMO protease's active region from the catalytic domain ranging from 364 to 589 amino acids (SENP2) in *E. coli*. The *SENP2* gene sequence was optimized to improve the codon adaptation index from 0.63 to 0.88. It was artificially synthesized and cloned into both pET22b (+) and pET32a (+) expression vectors for the production of unfused SENP2 protein and thioredoxin fused SENP2 protein (Trx-SENP2) in *E. coli* BL21 (DE3). In the pET22b(+) vector, SENP2 was highly expressed at temperatures ranging from 20 to 37°C but accumulated insoluble fractions and did not exhibit activity. The fusion protein Trx-SENP2 was soluble in pET32a(+) and was overexpressed up to 75% of total recombinant proteins at 25°C. For soluble Trx-SENP2 production 0.3 mM of IPTG was used. Upon applying the soluble protein fusion Trx-SENP2 to the SUMO-IL11 complex, two fragments corresponding to the size of SUMO and IL-11 were obtained. To the best of our knowledge, this is the first report showing that the catalytic domain of humnan's SUMO protease was overexpressed in the fusion form with Trx in *E. coli* and exhibited high activity. The recombinant Trx-SENP2 will be purified and characterized for application of SUMO-fused protein research.

Keywords: thioredoxin, SUMO protease, recombinant expression, *E. coli*.

INTRODUCTION

The small ubiquitin-like modifier (SUMO) is a 100-residue protein thatmodulates protein structure and function by covalently modifying target proteins (Tatham *et al.*, 2001). SUMO is widely used as a fusion protein in recombinant protein expression

with the aim of enhancing target protein expression and stability. It acts as a chaperone, preventing the aggregation of intermediate proteins, and keeping them long enough to form proper configurations, thereby improving the solubility of proteins (Bis *et al.*, 2014). In *E. coli*, the SUMO fusion system helps the recombinant proteins express efficiently as well as improve solubility (Malakhov *et al.*, 2004). This system has been shown to enhance the expression and solubility of difficult-to-express proteins such as matrix metalloproteinase 13 (Malakhov *et al.*, 2004); the capsid protein VP1 of enterovirus EV71 and the capsid protein VP3 of foot-and-mouth disease virus, the yeast RecA and Rad51 (Lee *et al.*, 2009); SARS-CoV 3CL protease (Butt *et al.*, 2005); the green fluorescent protein (Marblestone, 2006); the human fibroblast growth factor (FGF21) (Wang *et al.*, 2010), interferon-consensus (Peciak *et al.*, 2014) and interferol α-2a (Bis *et al.*, 2014). SUMO can act as an efficient agent for the solubilization of recombinant proteins (Bird, 2011; Kim *et al.*, 2019). Moreover, the expression level of SUMO fused proteins in soluble form is very high, and can attain up to more than 30% of total soluble protein in *E. coli* (Huang *et al.*, 2009; Wang *et al.*, 2010).

The SUMO-specific protease (SENP) is used to remove SUMO from the target protein. Among six SENP families identified in humans, SENP1 and SENP2 are present in both extra nuclear compartments and the cytoplasm (Mukhopadhyay and Dasso, 2007; Hickey, Wilson and Hochstrasser, 2012). SENP2 effectively cleaves the target protein fused with the SUMO3 partner that is expressed in *E. coli* by the pE-SUMO3 vector system. Notably, SENP2 recognizes the tertiary structure of the SUMO protein, thereby releasing the target protein with the desired N-terminus without the addition of amino acid residues (Malakhov *et al.*, 2004). The commercial SENP2 from EMD Milipore and LifeSensors was expressed in *E. coli* in the fusion form with an atomic mass of 27.7 kDa. Bioinformatics tools developed by Oklahoma University (https://biotech.ou.edu/) and SPpred software predicted SENP2 to be insoluble. Even though, the SUMO proteases originated from yeast and human have been produced in *E. coli* (Wang *et al.*, 2016; Babbal *et al.*, 2019, 2022; Fu *et al.*, 2022), some findings suggest that recombinant expression of SUMO protease is toxic to the *E. coli* host and form as inclusion bodies (Linova *et al.*, 2020; Reddy Patakottu, Vedire and Reddy, 2023). We optimized the codons of the catalytic domain of human SENP2 to be compatible with codon usage in *E. coli*. The optimized gene was inserted into pET22b (+) and pET32a (+) expression vectors to produce SENP2 and Trx-SENP2 (a fusion form with thioredoxin).

MATERIAL AND METHODS

Amonium persulfate (APS), TEMED, chloroform, ethidium bromide, glucose, glycerol, glycine, isoamyl-alcohol, ethanol, methanol, peptone, yeast extract, sodium dodecyl sulphate (SDS), tris, acrylamide, bis acrylamide, agar, agarose, coomassie were provided by Merck (Germany), dNTP, Taq DNA polymerase, dnase I, T4 DNA – ligase, restriction enzymes from Fermentas (USA), ampiciline from Sigma (USA), QIAquick PCR purification kit (Qiagen, USA), pUC57 containing the optimized codon sequence of the SENP2 gene was synthesized by Genscript (USA), expression vectors including pET22b(+) and pET32a(+) (Novagen, USA), strain *E. coli* DH10b from

Invitrogen (USA), *E. coli* BL21 (DE3) from Invitrogen (USA).

Selection of SENP2 gene region for recombinant expression and optimization of codons

The human *SENP2* gene of 1770 bp in length encodes the SENP2 enzyme contains 589 amino acids. Based on data from UniProtKB and Nextprot, the enzyme's active region (catalytic domain) spans between residues 396 to 560. The region spanning amino acid residues 363 to 589 was selected for recombinant expression to retain SENP2's enzyme activity. The recombinant SENP2 was calculated to weight 26 kDa and have a pI of 8.9. The *SENP2* gene was codon optimized, synthesized then cloned into pUC57 by Genscript (USA).

Construction of expression vector pET22b-SENP2 and pET32a-SENP2

Vectors pET22b(+), pET32a(+) and pUC57-SENP2 were digested with *Nco*I and *Xho*I restriction enzymes. The *SENP2* fragment and the cleaved vectors were purified using the QIAquick PCR purification kit and ligated together using T_4 DNA ligase to generate pET22b-SENP2 and pET32a-SENP2 expression vectors. The ligated products were transformed into *E. coli* DH10b by the heat shock method. The potential colonies were selected on LB plates supplemented with 100 µg/ml ampicillin (LBA) (Sambrook, Fritsch and Maniatis, 1989). Plasmids isolated from selected transformants were checked for harboring the insert of SENP2 gene using restriction enzymes *Nco*I + *Xho*I, *Pst*I and *Eco*RV. Finally, the constructed expression vectors pET22b-SENP2 and pET32a-SENP2 were separately introduced into the expression strain *E. coli* BL21(DE3) for overexpression of recombinant proteins.

Expression of SENP2 and Trx-SENP2

E. coli strains BL21 (DE3) harboring expression vectors pET22b-SENP2 or pET32a-SENP2 were inoculated into LBA (Luria–Bertani supplemented with ampicillin100 µg/ml), shaking at 200 rpm at 37°C overnight. After that, the overnight cultures were inoculated into fresh medium LBA at OD_{600} about 0.1 and continually carried out at 37°C, shaking at 200 rpm up to OD_{600} about 0.3 – 0.5, then induced with isopropyl β- D- thiogalactopyranoside (IPTG) (William Studier *et al.*, 1990) and continually shaken at 200 rpm for 5 hours. Some conditions, including IPTG concentration, temperatures for producing SENP2 (by pET22b-SENP2 vector) and Trx-SENP2 (by pET32a-SENP2 vector), from the recombinant *E. coli* cells were investigated. After fermentation, the cells were harvested by centrifugation at 5000 rpm for 5 min and suspended in buffer Tris-HCl 20 mM, pH 8 to OD_{600} = 10 and subsequently disrupted by sonication with 85% amplitude for 10 minutes. Total soluble proteins were separated from the pellet by centrifugation at 13000 rpm at 4°C for 10 minutes. The pellet was resuspended in an equivalent volume in buffer of Tris HCl 20 mM, pH = 8.0. Proteins from soluble and insoluble fractions were estimated by SDS-PAGE (12.6% gel) followed by Coomassie blue staining (Laemmli, 1970).

Assessment of Trx-SENP2 activity on SUMOIL-11 substrate

The cleavage activity of Trx-SENP2 in the total soluble fraction was preliminary evaluated using a SUMO-tagged fusion

protein, SUMO-IL11. The reaction mixture (10 µl) contained 2 µg of SUMO-IL11 with different amounts of the Trx-SENP2 sample in PBS supplemented with 1 mM DTT. The mixture was incubated at different temperatures for an hour. stopped by adding treatment buffer and then analyzed by SDS-PAGE (Laemmli, 1970). One enzyme unit of Trx-SENP2 is defined as the amount of enzyme to digest more than 85% of 2 µg of the substrate for 1 hour (SUMO-protease, Invitrogen).

RESULTS AND DISCUSSION

Codon optimization of target gene

We chose the region encoding the catalytic domain of SENP2 (amino acid residues 364-589) for recombinant expression (Figure 1).

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        10         20         30         40         50         60         70         80         90
MYRWLVRILG TIFRFCDRSV PPARALLKRR RSDSTLFSTV DTDEIPAKRP RLDCFIHQVK NSLYNAASLF GFPFQLTTKP MVTSACNGTR
       100        110        120        130        140        150        160        170        180
NVAPSGEVFS NSSSCELTGS GSWNNMLKLG NKSPNGISDY PKIRVTVTRD QPRRVLPSFG FTLNSEGCNR RPGGRRHSKG NPESSLMWKP
       190        200        210        220        230        240        250        260        270
QEQAVTEMIS EESGKGLRRP HCTVEEGVQK EEREKYRKLL ERLKESGHGN SVCPVTSNYH SSQRSQMDTL KTKGWGEEQN HGVKTTQFVP
       280        290        300        310        320        330        340        350        360
KQYRLVETRG PLCSLRSEKR CSKGKITDTE TMVGIRFENE SRRGYQLEPD LSEEVSARLR LGSGSNGLLR RKVSIIETKE KNCSGKERDR
       370        380        390        400        410        420        430        440        450
RTDDLLELTE DMEKEISNAL GHGPQDEILS SAFKLRITRG DIQTLKNYHW LNDEVINFYM NLLVERNKKQ GYPALHVFST FFYPKLKSGG
       460        470        480        490        500        510        520        530        540
YQAVKRWTKG VNLFEQEIIL VPIHRKVHWS LVVIDLRKKC LKYLDSMGQK GHRICEILLQ YLQDESKTKR NSDLNLLEWT HHSMKPHEIP
       550        560        570        580
QQLNGSDCGM FTCKYADYIS RDKPITFTQH QMPLFRKKMV WEILHQQLL
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Figure 1. Amino acid sequence of SENP2. The highlighted region is active zone of SUMO protease SENP2. It was constructed to express in *E. coli* in this study.

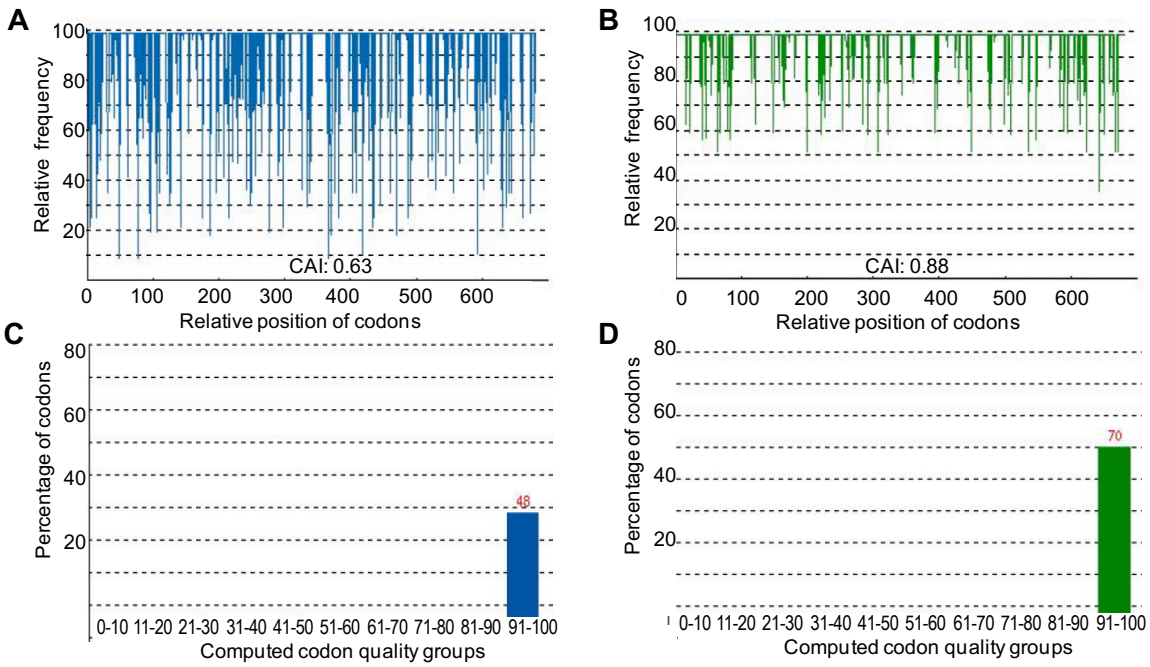

Figure 2. Analysis of condon usage frequency along *SENP2* gene sequences (A, B) and optimal codon frequency (C, D) of the original (A, C) and optimized *SENP2* (B, D) genes. Codon adaptation index (CAI) of 1.0 is considered to be the most suitable and codon quality of 100 is the highest usage frequency for a given amino acid in *E. coli*.

The prediction of codons usage in *E. coli* using Genscript's software showed that ratio of rare codons in SENP2 gene was accounted 13%. Codon adaptation index (CAI) of the origin gene was 0.63. Thus, the suitability of the gene with the host strain was low due to the high percentage of rare codons. Meanwhile, the overall gene distribution of GC is in the range of 30-50% which is relatively consistent with *E. coli*. The nucleotide sequences of *SENP2* gene were modified to increase the suitability of the gene to match with the host strain. After coding optimization, the CAI was increased to 0.88 and percentage distribution of codons in GC content and unfavorable peaks have also been optimized (Figure 2).

Construction of recombinant expression vectors

Trx has been demonstrated to stabilize and improve the solubility of recombinant protein. To improve the solubility of the fusion proteins, we constructed unfused SENP2 and Trx-SENP2 fusion proteins (Figure 3A). The structures predicted with Alphafold2 showed that the protein structures contain alpha helixes and beta sheets (Figure 3B).

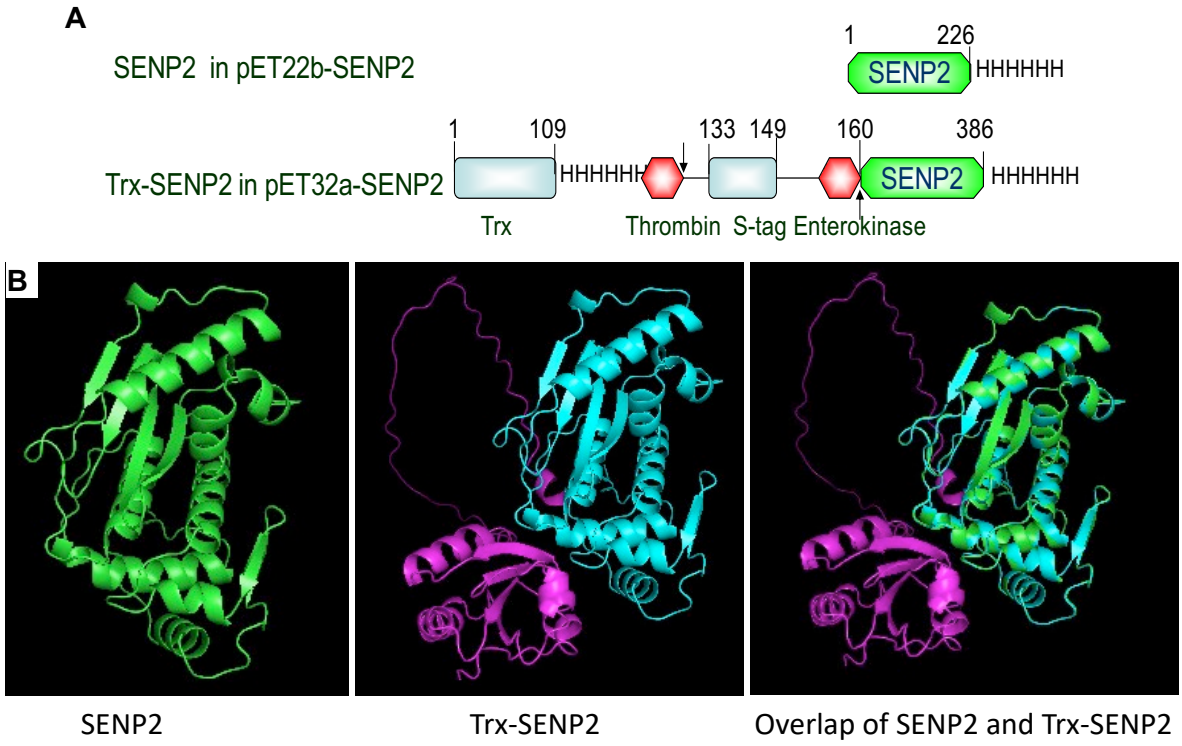

Figure 3. Construction of recombinant SENP2, Trx-SENP2 expressed in *E. coli* using pET22b(+) and pET32a(+) expression vector systems (A); The structure of proteins was predicted with Alphafold2 (B).

The optimized *SENP2* gene was synthesized and cloned into pUC57 (pUC57-SENP2). The *SENP2* gene fragment was constructed with pET22b(+) or pET32a to generate recombinant vectors pET22-SENP2 and pET32-SENP2.

Expression of SENP2 with/without Thioredoxin (Trx)

Recombinant expression vectors pET22b-SENP2 and pET32a-SENP2 were introduced into *E. coli* BL21 (DE3). For each expression construct, two clones were assessed for the expression of the targeted protein. As expected, in both constructs, recombinant SENP2 and Trx-SENP2 with molecular weights of 27 kDa and 43 kDa, respectively, were produced (Figure 4A). At 20°C and 37°C, the SENP2 was expressed mostly in the inclusion body (Figure 4B), while Trx-SENP2 was partly soluble depending on temprature. Increasing in the temperature leads to a decrease in Trx-SENP2 production and significantly reduces the solubility of the fusion protein. A small amount of soluble Trx-SENP2 was obtained when produced at 37°C. The accumulation of soluble Trx-SENP2 accounted for 50% of total Trx-SENP2 produced at 30°C and about 75% of total recombinant protein at 25°C (Figure 4C). Thus, a temperature of 25°C was used to obtain the high soluble recombinant protein production. This result was in agreement with other findings that lowering temperature can improve the solubility of recombinant proteins (Dragosits *et al.*, 2011; Seyfi *et al.*, 2019; Nguyen Thi Binh *et al.*, 2022).

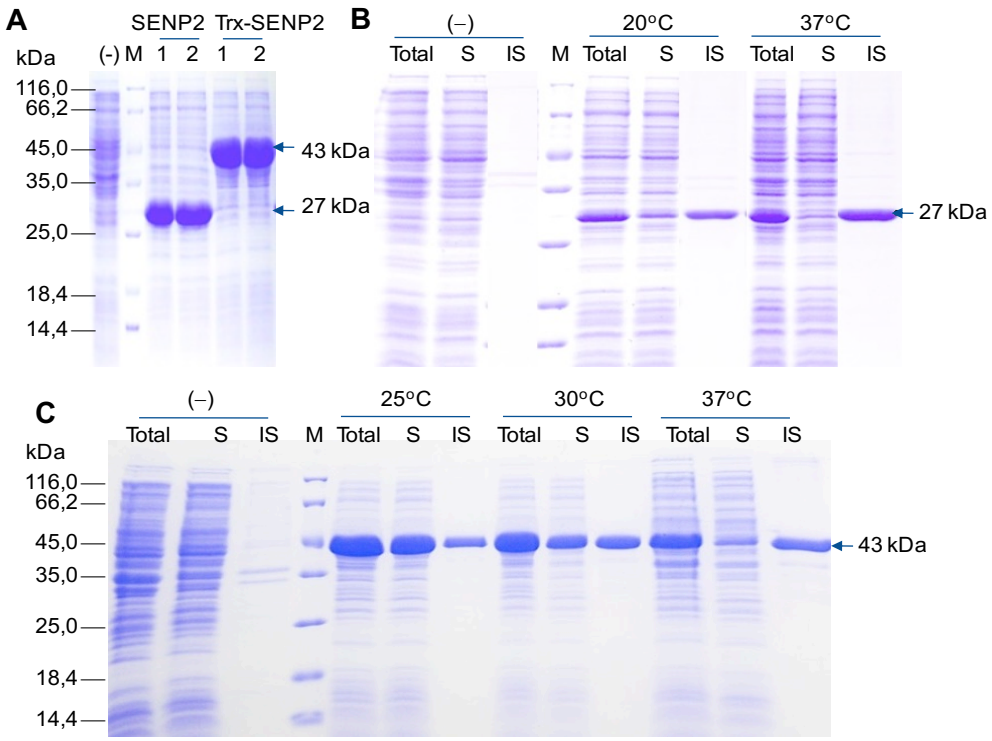

Figure 4. Analysis of expressed SENP2 and Trx-SEP2 fusion proteins in *E. coli* BL21 (DE3) (A) and total, soluble (S), and insoluble (IS) protein factions of the cells harboring *SENP2* (B) and *trx-SENP2* (C) induced and cultured at different temperatures by SDS-PAGE. The recombinant strain was fermented in LBA medium with 0.5 mM IPTG. The cells harvested after 6h of induction with IPTG were disrupted by sonication. Soluble and insoluble protein fractions were separated by centrifugation. Protein samples were separated on SDS-PAGE and detected with Coomasie blue. M: protein marker (Fermentas #SM0431), (-): The cells were not induced with IPTG.

The gene encoding SENP2 was cloned in the pET32a(+) vector under the control of the T7 promotor system. IPTG was used to induce the production of the recombinant protein. A high level of IPTG can affect the yield of recombinant proteins as it is a toxic chemical and is harmful to host cells, leading to a lower yield of recombinant proteins (Villaverde and Carrió, 2003; Gutiérrez-González *et al.*, 2019). The optimal IPTG concentration was investigated for heterologous proteins. As a result, IPTG strongly affected the growth of *E. coli* harboring pET32-SENP2. The increase in IPTG concentrations from 0 to 0.1 mM reduced harvested cell masses after 6 hours of cultivation at 25°C from an OD_{600} of 7.8 to 3.35. In contrary, Trx-SENP2 was initially synthesized in a medium containing very low IPTG concentration (0.02 mM) and steadily increased from 0.02 to 0.3 mM. At this point, IPTG then reached the stationary phase. Higher IPTG concentrations, from 0.5 mM to 1 mM, yield a small amount of soluble Trx-SENP2 protein (Figure 5). Thus, the induction conditions of 0.3 mM IPTG and 25ºC were suitable to express soluble Trx-SENP2.

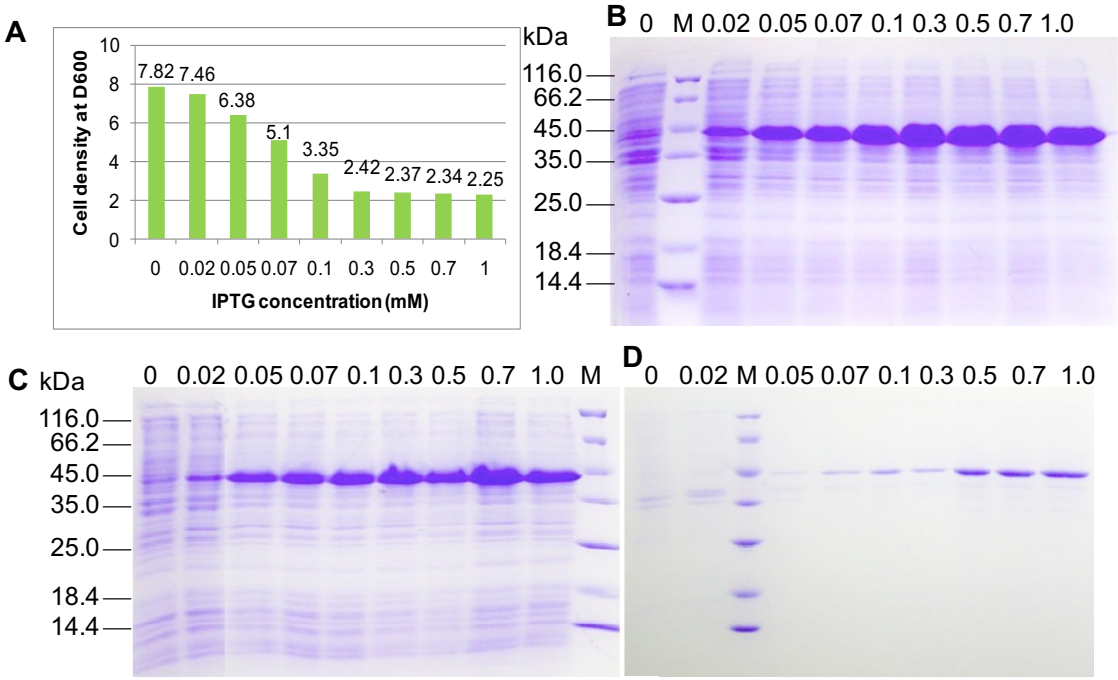

Figure 5. Effect of IPTG concentration on the growth of *E. coli* harboring pET32-SENP2 (A) and on the expression of Trx-SENP2 in the cells. B-D: Analysis of Trx-SENP2 in the total, soluble, and insoluble protein fractions, respectively, by SDS-PAGE. M: Marker protein (Fermentas); (0, 0.02, 0.05, 0.07, 0.1, 0.3, 0.5, 0.7, 1.0) indicating expression of proteins at IPTG concentrations of 0, 0.02, 0.05, 0.07, 0.1, 0.3, 0.5, 0.7, 1.0 mM, respectively.

Initial assessment of the SENP2 protease on the substrate of the fusion protein SUMO-IL11 showed that the insoluble SENP2 had no biological activity. The soluble fusion Trx-SENP2 was capable of degrading SUMO-IL11 into two peptide fragments, IL-11 (19 kDa) and SUMO (17 kDa) (Figure 6). At a lower amount of 1 µl, Trx-SENP2 was

not able to completely cleave the substrate. A larger amount of enzymes (3 or 5 µl), is required for full cleavage. This shows the recombinant enzyme Trx-SENP2 exhibits biological activity on the SUMO fused protein substrate.

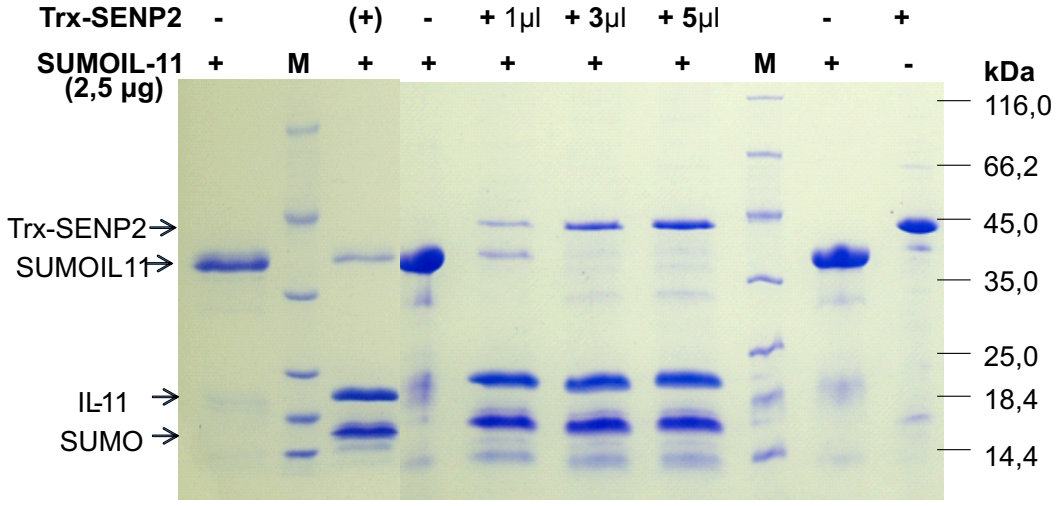

Figure 6. Analysis of the cleavage product of 2.5 µg SUMO-IL11 with 1U LifeSensors SUMO protease and Trx-SENP2 at 30°C. M: Marker protein (Fermentas); (+): Positive control (1U SUMO protease of Lifesicence).

CONCLUSION

In conclusion, we have constructed and successfully expressed the recombinant enzyme SUMO protease SENP2 fused to thioredoxin. This was expressed at high levels and in soluble form. The induction conditions of 0.3 mM IPTG and 25°C were selected for optimal soluble Trx-SENP2 expression in *E. coli* BL21. The recombinant protein obtained had biological activity and was able to cleave the substrate SUMO IL-11.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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