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PURIFICATION AND ACTIVITY DETERMINATION OF RECOMBINANT HUMAN SUMO PROTEASE'S ACTIVE REGION FUSED WITH THIOREDOXIN

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ABSTRACT

SUMO (Small Ubiquitin-related Modifier) protease is widely used for recombinant protein expression, but obtaining the protein from derivative sources is challenging due to the degradation during extraction and high costs. In a previous report, we expressed the human SUMO protease's active region (SENP2) fused with thioredoxin (Trx-SENP2) in *E. coli* BL21 (DE3). The recombinant fusion protein was soluble. In this study, we present the results of purification and activity evaluation of soluble Trx-SENP2. The enzyme was effectively purified through a single step Ni^2 affinity chromatography. Trx-SENP2 was bound to the column in a binding buffer containing 50 mM PBS, 500 mM NaCl, 50 mM imidazol, pH 7.4 and eluted in the basic buffer containing 300 mM imidazol. The purity of the enzyme obtained after purification reached over 98% and yield of Trx-SENP2 was 361 mg/liter of bacterial culture. Importantly, the Trx-SENP2 was biologically active and capable of degrading recombinant SUMO-fused proteins. The protease's bioactivity, as measured by its ability to cleave SUMO-IL11 into SUMO and IL11 fragments, was 3.33×10^5 U/mg comparable to commercial enzymes. Moreover, the Trx-SENP2 was found to be effective activity under a variety of conditions, including different buffers with high salt and imidazole concentrations and a wide range of pH values. Thus, the properties are useful and easy for the application of the enzyme to cleave recombinant SUMO-fused protein in the process of purification to obtain target proteins. The present study introduces a highly active and easily applicable enzyme for research in recombinant DNA technology.

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

INTRODUCTION

SUMO-specific protease (SENP) is used to remove SUMO from target proteins. Among the six SENP families identified in humans, SENP2 effectively cleaves SUMO3, releasing the target protein from the chimeric SUMO3-protein produced in *E.*

coli using the pE-SUMO3 vector system. Additionally, SENP2 can exhibit high activity under a wide range of cleavage conditions. Notably, SENP2 recognizes the tertiary structure of the SUMO protein thereby generating the target protein with the desired N-terminus without the addition of extra amino acid residues (Malakhov *et al.*, 2004). This specificity is a result of hydrophilic interactions and weak quantum chemical bonds generated in the salt bridge reaction with the SUMO substrate. The C-terminal diglycine of SUMO is held tightly by two Trp residues at the base of the active site. Catalytic residues including His-Asp-Cys in the active center, can be oriented in specific spatial positions for precise substrate cleavage. SENP2 cleaves at the gly-gly site on SUMO-3 (Hickey *et al.*, 2012). The active site structure of SENPs in animal cells is similar. In addition, SENP2 also directly cleaves substrate in a cis configuration by forming a knot at the C-terminus of SUMO. Because the cis configuration in the peptide molecule is a common configuration, SENP2 is determined to have the ability to cleave SUMO-3 from the fusion protein containing SUMO-3 partner (Huang *et al.*, 2006). The advantage of SUMO proteases is their ability to accurately recognize the spatial structure of SUMO in the fusion protein and cleave at specific positions to release the targeted protein. This minimizes non-specific activity on the target protein. Unlike many proteases that recognize amino acid sequences, SENP2 is active against most N-terminal amino acid residues of the fusion target protein, except the Pro residue (Butt *et al.*, 2005).

Escherichia coli is the most common expression system for recombinant protein production due to its high growth rate, easy genetic manipulation, and simple large-scale production (Maldonado *et al.*, 2007; Zhang *et al.*, 2010). Most proteins and enzymes can be produced in *E. coli*. Additionally, the purification of recombinant proteins from *E. coli* is typically straightforward because of high level production and the incorporation of a His-tag for one step purification using affinity chromatography. Obtaining SENP2 from its natural source is challenging due to degradation during extraction and high cost. Therefore, producing and purifying SENP2 through recombinant DNA technology is a suitable choice. The SUMO proteases originated from yeast and humans have been expressed in *E. coli* (Wang *et al.*, 2016; Babbal *et al.*, 2019, 2022; Fu *et al.*, 2022) but some research showed that the recombinant proteases were produced as inclusion bodies (Linova *et al.*, 2020; Patakottu *et al.*, 2023). In our previous study, SENP2 fused with thioredoxin (Trx-SENP2) was overexpressed in soluble form and exhibited high activity. In the present study, we purified Trx-SENP2 and determined the protease's activity on a model recombinant chimeric protein of SUMO fused with human interleukin 11 (SUMO-IL11).

MATERIALS AND METHODS

Materials were obtained as follows: APS, TEMED, chloroform, glycerol, glycine, ethanol, methanol, peptone, yeast extract, SDS, tris, acrylamide, bis acrylamide, agar, coomassie (Merck, Germany), ampiciline (Sigma, USA), recombinant expression vectors including pET32a-SENP2 (in previous study), *E. coli* BL21 (DE3) (Invitrogen, USA), SUMO-IL11 (Nguyen *et al.*, 2018), SUMO protease 2 (LifeSensor).

Expression of Trx-SENP2 in E. coli BL21 (DE3)

E. coli strains BL21 (DE3) harboring expression vector pET32a-SENP2 were inoculated in LBA and shaken at 200 rpm at 37°C overnight. Then, overnight cultures were transferred into fresh LBA medium to a starting OD_{600} of 0.1 and continually incubated at 37 °C, 200 rpm to the OD_{600} of the culture reached about 0.3 – 0.5. The cells were induced with 0.3 mM isopropyl β- D-thiogalactopyranoside (IPTG) (Studier *et al.*, 1990) and incubated continually at 25°C, 200 rpm for 5 hours. After fermentation, the cells were harvested by centrifugation at 5000 rpm for 5 min and suspended to OD_{600} = 10 in Tris-HCl 20 mM, pH 8.0 buffer.

For analysis of the expressed proteins, the cells at an OD_{600} of 10 (1 ml) were disrupted by sonication at 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 (insoluble fraction) was resuspended in an equivalent volume of Tris-HCl 20 mM, pH 8.0 Proteins from soluble and insoluble fractions were analyzed by SDS-PAGE using a 12.6% gel, followed by Coomassie blue staining (Laemmli, 1970).

Purification of Trx-SENP2

The recombinant cells producing Trx-SENP2 were suspended in binding buffer (50 mM PBS, 500 mM NaCl, 50 mM imidazol, pH 7.4) to an OD_{600} of 10. The cell suspension (20 ml) was sonicated at 85% amplitude for 10 minutes. Total soluble proteins containing Trx-SENP2 were separated from the pellet by centrifugation at 13000 rpm at 4 °C for 10 minutes, then passed through Ni affinity chromatography using a XK26 column (Amersharm) pre-equilibrated with the binding buffer. After washing the column with 5 column volumes (CV) of the wash buffer containing 100 mM imidazole, the bound protein Trx-SENP2 was eluted with elution buffer containing 400 mM imidazole.

Determination of SUMO protease activity of Trx-SENP2

SUMO-inteleukin11 in PBS 50 mM containing NaCl 500 mM, imidazol 150 mM was used as a substrate for determining Trx-SENP2 activity (Nguyen *et al.*, 2018). The reaction (10 µl) was carried out in PBS (10 mM) containing 1 mM DTT. To choose optimal reaction conditions, we investigated various parameters such as enzyme concentration, substrate concentration, reaction temperature, pH and incubation duration. The reaction was stopped with protein treatment buffer for checking protein by SDS-PAGE. An enzyme unit of Trx-SENP2 is defined as an amount of the particular enzyme required to digest more than 85% of 2 µg of the substrate for 1 hour (SUMO-protease, Invitrogen). Protein concentration was measured by Bradford reagent (Roth).

RESULTS AND DISCUSSION

Purification of SUMO protease Trx-SENP2

Since the Trx-SENP2 protein contains a hexahistidine tag at the C-terminal, it can be purified using immobilized metal affinity chromatography (IMAC). Various conditions for protein purification were investigated. Initially, the sample was loaded onto the column in binding buffer containing 10 mM imidazol, followed by washing with 20 CV column volume (CV) in binding

buffer. Subsequently, it was washed with 5 CV of a washing buffer containing 100 mM imidazole, and finally eluted with a buffer containing 400 mM imidazole. The results obtained from SDS-PAGE analysis showed that Trx-SENP2 was eluted in small amounts and with relatively low purity. However, by increasing the imidazole concentration in the binding buffer to 50 mM, the recovered Trx-SENP2 showed significant improvement. However, Trx-SENP2 still contaminated some proteins from the host cells. For the acquisition of higher Trx-SENP2 purity, washing buffer containing 150 mM imidazole and elution buffer containing 300 mM imidazol were investigated. As a result, Trx-SENP2 was efficiently eluted with the buffer containing 300 mM imidazole with much improved purity (Figure 1). Protein analysis on an SDS-gel followed by Quantity One software (Biorad) indicated that the purified Trx-SENP2 achieved more than 98% purity and a yield of 361 mg/l of culture. The purified enzyme solution was desalted by a dialysis membrane of 10 kDa against PBS buffer pH7.4 containing 10% glycerol and 2 mM DTT overnight at 4°C. Compared to other studies on *E. coli* recombinant protein expression and purification at a flask fermentation, the achievement of pure Trx-SENP2 is high. The purification of target protein SUMO proteases from yeast rtUlp1 has been conducted by Ni–NTA affinity chromatography and the final yield of rtUlp1 is 45 mg/l at a flask scale with a purity of up to 95% (Wang *et al.*, 2016). The ScUlp1 protease expressed in TB medium in a shake flask was 195 mg/l and purified using the Ni-NTA affinity purification with a recovery yield of 80% and the purity of 95% (Babbal *et al.*, 2019). Another study showed that approximately 285 mg of crude insoluble recombinant Ulp was recovered and about 80 mg of pure, active Ulp catalytic domain was harvested after purification and refolding from a litter of high cell density *E. coli* culture (Linova *et al.*, 2020).

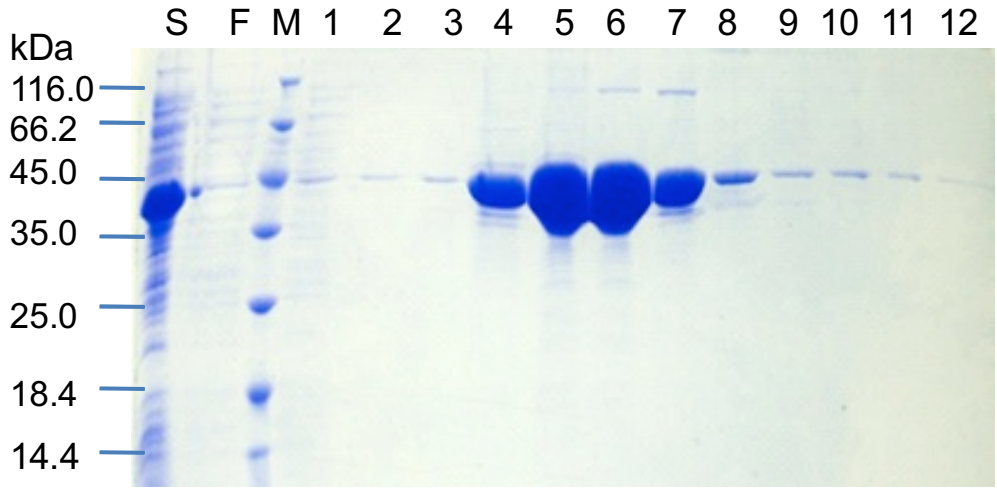

Figure 1. Analysis of protein fractions during TRX-SEP2 purification process on 12.6% SDS-PAGE gel. S: The lysate soluble fraction was loaded onto Ni affinity chromatography using an XK26 column; F: flow-through fraction; M: protein marker (Fermentas #SM0431); 1-2: washing fractions with the buffer containing150 mM imidazol; 3-7: elution fractions with the buffer containing 300 mM imidazol; 8-12: final column washing fractions.

Evaluation of biological activity of SUMO protease Trx-SENP2

Normally, a high concentration of NaCl and imidazole in the purified enzyme's will strongly affect the enzyme activity. To check the impact of the purification buffer on the enzyme activity, recombinant purified SUMO-IL11 (Nguyen *et al.*, 2018) was used as a substrate for the desalted and no-desalted Trx-SENP2 activity. The total reaction (10 µl) consisted of 2.5 µg of substrate and 0.75 or 1.5 µg of enzymes. The result (Figure 2) showed that the degradation of SUMO-IL11 into SUMO and IL11 was equal in both enzymes, whether desalted or non-desalting. The digested level reached the maximum amount when using either 0.75 or 1.5 µg of enzymes or 2 U of SUMO protease 2 (LifeSensor). SUMO-IL11 (34 kDa) was almost completely cleaved into IL11 (19 kDa) and SUMO (17 kDa). However, the SUMO-IL11 was not completely digested in all experiments, as evidenced by the presence of bands in the polyacrylamide gel. Therefore, the enzyme's activity unit is usually defined based on the calculation of 85% of the substrate being degraded. The enzyme activity was not affected by the high salt concentration (50 mM NaCl) as well as the high imidazole concentration (300 mM). Thus, the enzyme was able to be used directly after purification to cleave the substrate (Figure 2). These results are in agreement with other studies investigating the effect of various chemicals on the enzymatic activity of ULP and have stated that SUMO protease can act on high concentrations of salt and imidazole (Malakhov *et al.*, 2004).

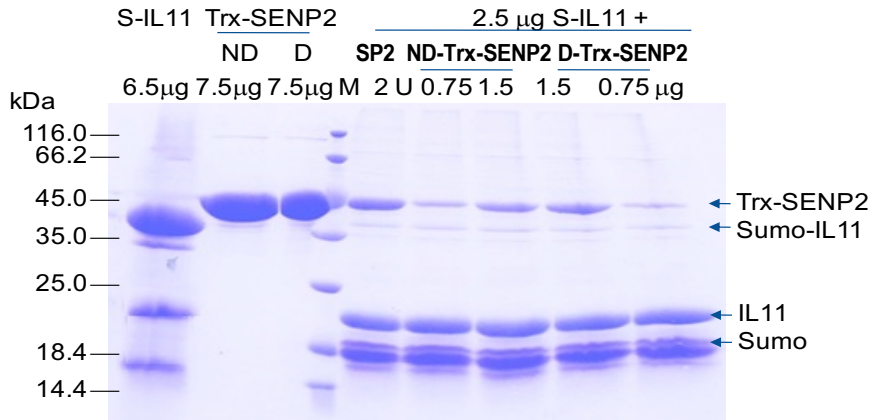

Figure 2. Analysis of SUMO-IL11 digested with different amounts of desalted and non-desalted Trx-SENP2. ND: non-desalted, D: desalted; S-IL11: SUMO-IL11; SP2: SUMO protease 2 (LifeSensor); M. protein marker (Fermentas #SM0431).

To evaluate Trx-SENP2 activity, different amounts (75, 37.5, 7.5, 3.75, and 0.75 ng) of the enzyme were used to digest 2.5 µg of SUMO-IL11 substrate at 30 °C for 3 hours. The result showed that increasing amounts of the enzyme gradually from 0.75 ng to 7.5 ng resulted in a corresponding increase in the digested SUMO-IL11 until the reaction reached equilibrium when using the 7.5 ng or higher amounts of enzyme. Thus, the amount of enzyme needed to cleave 2.5 µg of substrate was determined to be 7.5 ng (Figure 3A). After fixing the ratio of enzymeand substrate, we changed the incubation times for the enzyme and substrate reactions and the obtained result

(Figure 3B) showed that even after a short duration of 0.5 hours, 2.5 µg of SUMO-IL11 was completely cleaved into IL11 and SUMO. In the same enzyme/ substrate ratio, Trx-SENP2 had strong activity at all investigated pHs from 4.5 to 10.5 (Figure 3C). The results, shown in Figure 3C, clearly demonstrated that Trx-SENP2 cleaves effectively in buffers ranging from pH 4.5 to 10.5. Optimal cleavage was observed between pH 7.5 and pH 10.5. These findings are in accordance with another study investigating conditions for ULP activity cleavage, which found that ULP effectively cleaves substrates over a broad pH range from 5.5 to 10.5 (Malakhov *et al.*, 2004). Thus, Trx-SENP2 possesses the ability to cleave substrates over a wide pH range, from acidic to alkaline pH, making it suitable for the application of cleaving SUMO-fused recombinant proteins.

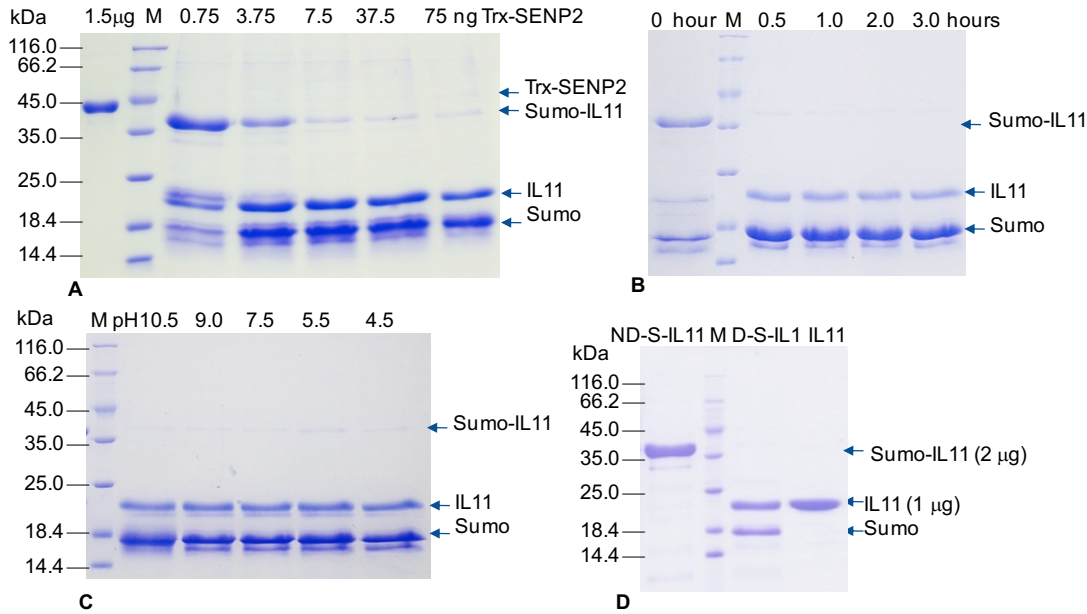

Figure 3. Investigation of some parameters including enzyme concentration (A), reaction duration times (B), pH (C) and substrate concentration (D) impact on Trx-SENP2 activity. The reactions were conducted in total volume of 10 µl, while Trx-SENP2 was fixed 7.5 ng/reaction in experiments B, C and 6 ng in experiment D; Sumo-IL11 was fixed at 2.5 µg/reaction in experiments A, B, C and 2 µg in experiment D; M: Protein marker (Fermentas #SM0431). ND-S-IL11: non-digested Sumo-IL11; D-S-IL11: Sumo-IL11 was digested with Trx-SENP2.

To calculate enzyme units, the Trx-SENP2 was incubated for different durations ranging from 0.5 hours to 3 hours. The result showed that with an incubation of 0.5 hours, 0.0075 µg of the enzyme digested more than 85% of 2.5 µg of SUMO-IL-11 to generate SUMO and IL-11. Thus, if one unit of the enzyme is defined as the amount enzyme of that cleaves 85% of 2 µg of the substrate in one hour, then the Trx-SENP2 activity was calculated as 3.33×10^5 U/mg (Table 1). The enzyme activity was confirmed by the last experiment, which used 6 ng of Trx-SENP2 to digest 2 µg of Sumo-IL11 at 30 °C for 3 hours. The product was electrophoresed by SDS-PAGE alongside 1 µg of IL-11. The result (Figure 3D) showed that the SUMO-IL11 was completely digested, and the

released IL-11 band was the same size and amount as the standard IL-11, which was purified and confirmed by N-terminal amino acid sequencing (Nguyen *et al.*, 2018). Expression of codon-optimized recombinant truncated Ulp1 (Leu403-Lys621) (rtUlp1) from *Saccharomyces cerevisiae* in *E. coli* yielded 45 mg/l of rtUlp1 in flask fermentation with a purity of up to 95% and the a specific activity of up to 2.8 × 10^4 U/mg (Wang *et al.*, 2016). At flask scale and under optimal conditions in TB medium, the *S. cerevisiae* Ulp1 protease catalytic domain (402–621 aa) (ScUlp1) was expressed in *E. coli* to a yield of 195 mg/l with a purity of 95% and a recovery yield of 80%. The specific activity of the purified ScUlp1 was 3.986 × 10^5 U/mg (Babbal *et al.*, 2019). Extracellular production of Ulp1403-621 using a leaky *E. coli* BL21(DE3) strain obtained a specific activity of extracellular Ulp1403-621 reaching 2.0 × 10^6 U/l (Fu *et al.*, 2022).

Table 1. Summary of purification and bioactivity of recombinant Trx-SENP2 from 1 liter of cell culture *Escherichia coli* BL21 (DE3) overexpressing Trx-SENP2.

Sample	Purified Trx-SENP2 (mg)	Total activity (U)	Specific activity (U/mg)	Purity of Trx-SENP2 (%)
Purified Trx-SENP2	361	1.2 x 10^8	3.33 x 10^5	98%

CONCLUSION

The recombinant SUMO protease Trx-SENP2 was purified with a purity of 98% and yielded 361 mg from a liter of culture. The enzyme demonstrated activity under high salt and imidazole concentrations and over a wide pH range from 4.5 to 10.5. The specific activity of the Trx-SENP2 enzyme was measured at 3.33 × 10^5 U/mg.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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