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Large-scale production of recombinant Saw1 in *Escherichia coli*

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ABSTRACT

Saccharomyces cerevisiae Saw1 is an essential gene in single-strand annealing – the DNA repair pathway that repairs double-strand breaks when they occur between homologous repeats. Saw1 interacts with the structure-specific nuclease Rad1-Rad10 and this results in the recruitment of this nuclease to 3′ non-homologous DNA tailed recombination intermediates. Saw1 is unstable in the absence of the Rad1-Rad10 nuclease and, hence, it has been difficult to study its specific function *in vitro*. In the present work, we present the combination of dynamic light scattering and differential scanning fluorimetry techniques to optimize the stability and homogeneity of recombinant Saw1. The protein expression and purification conditions identified in this study allow for higher recovery of soluble Saw1 and enable the biochemical characterization of the protein.

1. Introduction

Cellular DNA is susceptible to endogenous and environmental damaging agents that compromise genetic integrity. Therefore, cells have developed complex DNA repair networks that prevent the accumulation of damage. One of the main challenges that repair proteins face is identifying the lesions specifically, so that each type of lesion is processed correctly. This task is especially challenging for DNA repair nucleases because they create repair intermediates that are often more toxic than the original damage. *Saccharomyces cerevisiae* Rad1-Rad10 (XPF-ERCC1 in mammals) is a structure-specific nuclease that functions in nucleotide-excision repair by incising 5′ to a distorting lesion [1,2]. Beyond this well-characterized role, Rad1-Rad10 is also involved in other DNA repair pathways including interstrand crosslink repair [3,4], telomere length control [5,6], homologous recombination repair [7,8], and gene-targeting events [9,10]. In nucleotide-excision repair, Rad14 (XPA in mammals) interacts with Rad1-Rad10 and recruits it to UV lesions [11,12]. However, the targeting mechanisms in other repair pathways are not as clear.

Single-strand annealing (SSA) repairs double-strand breaks when they occur between direct repeats [13]. During SSA, double strand breaks between repeats are resected 5′ to 3′ to expose homologous sequence. Rad52 can then anneal the homologous repeats, forming a recombination intermediate with 3′ non-homologous DNA tails that must be removed in order to complete repair. Rad1-Rad10 processes these 3′ non-homologous DNA tails, thereby preparing these intermediates for DNA synthesis and ligation [14]. Repair of a break through single-strand annealing causes the loss of the intervening sequence, as well as one of the homologous repeats. Given the high content of repetitive DNA in the human genome, and the fact that homologous repeats are prone to recombination by single-strand annealing, recombination events between homologous repeats can be a major source of genome instability [15–19]. Therefore, single-strand annealing, and specifically targeting of Rad1-Rad10 to recombination intermediates, must be tightly regulated [15].

In mammals, Rad52 interacts with ERCC1 and this interaction stimulates the processing of 3′ non-homologous DNA tails [20,21]. In *Saccharomyces cerevisiae*, Rad52 does not interact directly with Rad1-Rad10. Instead, the Saw1 (Strand-Annealing Weakened 1) protein bridges the interaction between Rad52 and Rad1-Rad10 [22,23]. Like Rad1-Rad10, Saw1 is a structure-specific DNA binding protein [22]. *In vitro*, Saw1 has high affinity for branched DNA substrates including 5'- and 3'-flap DNA, splayed and fork-like DNA structures, but binds only weakly to double-stranded or recessed-end DNA substrates. However, the relative affinities for each substrate have not been measured, due to difficulties preparing homogeneous and consistently active Saw1 preparations. DNA binding of Saw1 is required for the recruitment of Rad1-Rad10 to 3′

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non-homologous DNA tails [22]. *In vivo* Saw1 is unstable in the absence of Rad1-Rad10 [23]. Therefore, the biochemical characterization of Saw1, including its effect on the nuclease activity of Rad1-Rad10, has been limited. Using differential scanning fluorimetry, we have identified buffer conditions that stabilize Saw1 and have developed a robust strategy for producing large quantities of recombinant Saw1 in the absence of Rad1-Rad10.

2. Materials and methods

2.1. Protein expression and solubility

The expression plasmid encoding full-length *SAW1* (pAG8815) allows for expression of the Saw1 protein (residues 1–261) with an N-terminal hexa-histidine tag removable with thrombin (Li et al., 2013). We tested protein expression and solubility of Saw1 by transforming the pAG8815 plasmid in BL21-CodonPlus (DE3), as well as ArticExpress (DE3) and BL21^{STAR}(DE3), previously transformed with a plasmid encoding rare codons (pRARE). We grew each cell culture to OD_{600} of 0.5 (sample '-') and induced protein expression by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were grown at 30 and 20 °C for four and 6 h, respectively (and, in the case of Arctic pRARE cells, 12 °C for 16 h), with orbital agitation (sample '+'). To test for protein solubility, cell pellets were resuspended in lysis buffer (20 mM TRIS pH 8, 1.4 mM 2-mercaptoethanol) and lysed in two steps. First, cells were incubated in 90 mM KCl, 10 mM $MgCl_2$, 0.05% lauryldimethylamine-oxide (LDAO) supplemented with lysozyme (1 mg/mL). After a 30-minute incubation, 20 units of DNaseI were added to shear chromosomal DNA and incubation continued for another 30 min. Cell debris and insoluble proteins were removed by centrifugation and soluble proteins remained in solution (sample 's'). Samples '-', '+' and 's' were resolved in 15% SDS-polyacrylamide gels to assess the amount of soluble versus total protein expressed.

For large-scale protein expression, competent BL21^{Star} DE3 pRARE cells were transformed with the pAG8815 plasmid and transformed cells were selected with 30 μg/mL Kanamycin and 25 μg/mL Chloramphenicol in LB media. Cell cultures were grown to an OD_{600} of 0.4, induced with 0.5 mM IPTG and incubated for 4 h at 20 °C with orbital agitation. Cells were harvested by centrifugation and stored at -80 °C until ready to be purified.

2.2. Protein purification

Cell pellets (1L) were resuspended in 20 mL Ni Buffer (50 mM Tris pH 8, 500 mM NaCl, 5 mM 2-mercaptoethanol, 10 mM imidazole, 10% glycerol) supplemented with protease inhibitors (157 μg/mL benzamidine, 174 μg/mL PMSF, 0.7 μg/mL pepstatin A, and 5 μg/mL leupepteine) and 0.05% LDAO and lysed by sonication. Lysates was cleared by centrifugation at 39,000 g and the supernatant loaded onto a HiTrap Nickel-Chelating HP column (GE Healthcare) equilibrated with Ni Buffer. Saw1 was eluted with a linear imidazole gradient (53.5–300 mM). Fractions containing his-tagged Saw1 were pooled, diluted to adjust the salt concentration to 200 mM NaCl, and loaded onto a Mono S 5/50 GL exchange column (GE Healthcare) equilibrated with Mono S buffer (50 mM Tris pH 8, 200 mM NaCl 0.5 mM EDTA pH8, 5 mM 2-mercaptoethanol, 10% glycerol). His-Saw1 was eluted with a linear gradient to 500 mM NaCl.

2.3. Dynamic light scattering

Protein samples (0.3 mM) were diluted 1:15 in test buffer (50 mM Buffering agent, 50 mM Arg-Glu mix, 500 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1.4 mM 2-mercaptoethanol) and incubated for 1 h at 4 °C. Samples were then centrifuged at 1,570 × g for 10 min, and 18 μL used for the measurement. Buffers tested include: K_2HPO_4 (pH 5), K_2HPO_4 (pH 6), MES (pH 6), Tris (pH 7), HEPES (pH 7), Tris-HCl (pH 7.5), HEPES (pH 7.5), Tris-HCl (pH 8), Tris-HCl (pH 8.5), and CHES (pH 9). All measurements were recorded at 4 °C on a Zetasizer NanoS with a 633 nm laser (Malvern Instruments).

2.4. Differential scanning fluorimetry

Buffer conditions were prepared in 96-well PCR plates by adding 5 μL of a 5X stock of each component of the buffer and diluting to a final volume of 23 μL with filtered, autoclaved double deionized water. Conditions were pre-chilled before 2 μL of 50 μM 6XHis-Saw1 and 2 μL 62X SYPRO orange dye are added simultaneously at 4 °C. The plate was immediately placed in a Thermocycler with a CFX96 Real-time optical unit (BioRad). Samples were then heated from 4 to 95 °C in steps of 1 °C/min. Fluorescence intensity was recorded using a SYBR-Green filter.

2.5. Preparation of DNA substrates

Oligonucleotides were purchased Integrated DNA Technologies;

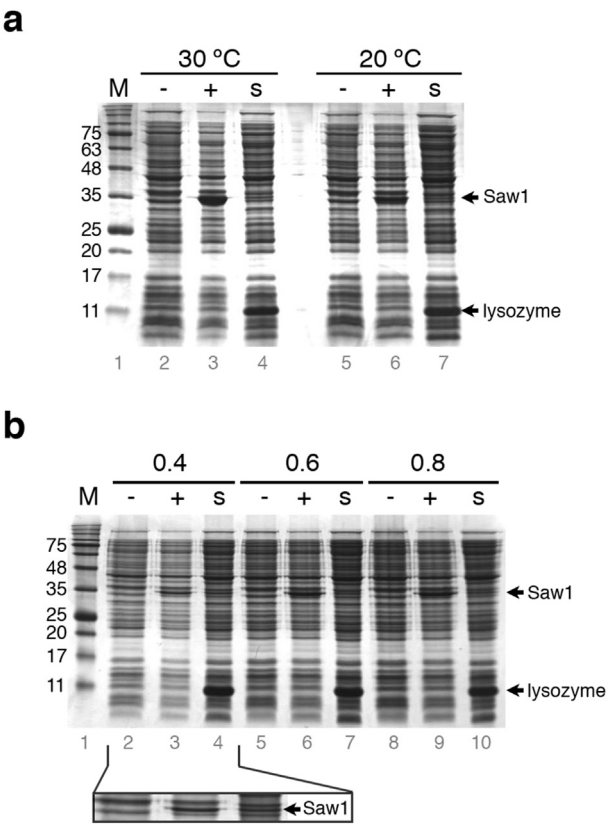

Fig. 1. Expression and Solubility of Saw1 expressed in BL21^{STAR} DE3. (a) Solubility assay for Saw1 in BL21^{STAR} DE3 at two growth temperatures. Gel is loaded: 1) molecular weight markers (M, in kDa), 2–4) protein content of the pre-induction (-), post-induction (+) and soluble (s) fractions of the cultures grown at 30 °C; 5–7) protein content of the pre-induction (-), post-induction (+) and soluble (s) fractions of the cultures grown at 20 °C. **(b)** Solubility assay for BL21^{STAR} DE3 cells induced at different OD_{600}. Gel is loaded: 1) molecular weight markers (M, in kDa), 2–4) protein content of the pre-induction (-), post-induction (+) and soluble (s) fractions of the cultures induced with IPTG at OD_{600}~0.4; 5–7) protein content of the pre-induction (-), post-induction (+) and soluble (s) fractions of the cultures induced with IPTG at OD_{600}~0.6; 8–10) protein content of the pre-induction (-), post-induction (+) and soluble (s) fractions of the cultures induced with IPTG at OD_{600}~0.8.