

Recombinant Production of Crab Antimicrobial Protein Scygonadin Expressed as Thioredoxin and SUMO Fusions in *Escherichia coli*

Yifeng Li

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Abstract Scygonadin is an antimicrobial protein isolated from the mud crab, *Scylla serrate*. The mature protein comprises 102 amino acids and has a theoretical molecular weight of 11,272 Da. The protein's specific expression pattern strongly suggests that it plays a role in reproductive immunity. In this study, I developed a protocol for producing recombinant scygonadin in *Escherichia coli*. The target protein was expressed as both thioredoxin and SUMO fusions, and released by TEV and SUMO protease-mediated cleavages, respectively. In either case, the liberated scygonadin was separated from its carrier using a HisTrap HP column. From thioredoxin and SUMO fusion constructs, 32.7 and 29.2 mg target protein per liter of culture was obtained, respectively. The described protocol provides an effective means for producing scygonadin in relatively large quantities, which facilitates its further characterization.

Keywords Antimicrobial protein · Fusion expression · Maltose-binding protein · Scygonadin · SUMO · SUMO protease · TEV protease · Thioredoxin

Introduction

Scygonadin is a small protein originally isolated from the seminal plasma of *Scylla serrate*, which demonstrates antibacterial activity against both Gram-positive and Gram-negative

Y. Li (✉)
Protein Production Core Facility, Department of Biochemistry, University of Texas Health Science
Center at San Antonio, San Antonio, TX 78229, USA
e-mail: yifgli@gmail.com

Y. Li
e-mail: liy10@uthscsa.edu

bacteria [1, 2]. Although an earlier study showed that this protein is male-specific and is predominantly produced in the reproductive tract [2], recent data suggested that scygonadin is present in female crab as well and has a wider expression pattern [3, 4]. A similar-sized antimicrobial protein, which shares 92 % sequence similarity with scygonadin, was also identified in several tissues of both male and female crabs [5]. It has been proposed that scygonadin likely plays a role in crab fertilization and reproduction [3].

To further characterize the biological functions of scygonadin, large amount of highly purified protein is required. A system for producing recombinant scygonadin in *Escherichia coli* was previously described [6]. The target protein was expressed as a CKS fusion, which released scygonadin upon 3C protease cleavage, and as a His-tagged non-fusion. In either case, the final product contains six histidines at its C-terminus. Scygonadin obtained from CKS fusion also contains four extra residues at its N-terminus. Since extra non-native residues may have an effect on activity, it is always preferable to use protein with native sequence for functional characterization.

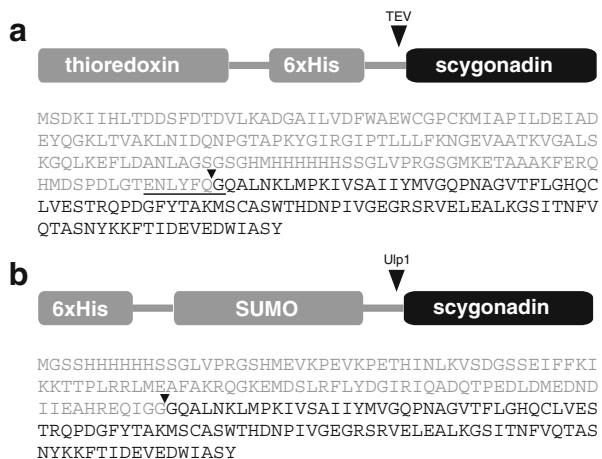
In this work, scygonadin was expressed as both thioredoxin and small ubiquitin-related modifier (SUMO) fusions (Fig. 1). The target protein was released from thioredoxin and SUMO fusions by tobacco etch virus (TEV) and SUMO protease-mediated cleavages, respectively. In both cases, scygonadin with its native sequence was generated. The liberated protein was separated from the corresponding carrier using the HisTrap HP column. The described protocol allows highly purified recombinant scygonadin to be obtained in relatively large quantities.

Materials and Methods

Materials

One Shot BL21(DE3) competent cell and dNTP mix were obtained from Invitrogen. JM109 competent cells were purchased from Promega. *Staphylococcus aureus* and *E. coli* were obtained from American Type Culture Collection (ATCC). Plasmids pET-32a and pET-28a were purchased from Novagen. The plasmids pJOE4905.1 (which encodes MBP and SUMO

Fig. 1 Schematic representation and sequence of the two constructs in which scygonadin is expressed as a fusion protein with a thioredoxin or b SUMO



dual-tagged GFP) and pJOE4847.2 (which encodes MBP tagged SUMO protease Ulp1) were kind gifts of Dr. Altenbuchner (Universität Stuttgart, Germany). TEV protease expression vector pRK793 was obtained from Addgene. Gene encoding scygonadin was synthesized by GenScript. Oligonucleotide primers were synthesized at the Nucleic Acids Core Facility at the University of Texas Health Science Center at San Antonio (UTHSCSA). Vent DNA polymerase, restriction enzymes, calf intestinal alkaline phosphatase (CIP), quick ligation kit and DNA marker were obtained from New England Biolabs. QIAquick PCR purification kit, QIAquick gel extraction kit, QIAprep spin miniprep kit, and Ni-NTA agarose were purchased from Qiagen. Difco LB broth was purchased from BD Biosciences. Ampicillin sodium salt and kanamycin sulfate were purchased from Affymetrix. Tris, glycine, SDS, 30 % acrylamide/bis, and protein standards were purchased from Bio-Rad. Isopropyl β -D-1-thiogalactopyranoside (IPTG) and L-rhamnose monohydrate were purchased from Gold Biotechnology and Sigma-Aldrich, respectively. HisTrap HP column (5 ml) was purchased from GE Healthcare.

Construction of Fusion Expression Plasmids

The gene encoding scygonadin was amplified by PCR with primers 1 and 2 (Table 1) using a synthetic gene as template. The forward and reverse primers contain *Kpn*I and *Eco*RI sites, respectively. The forward primer also contains the sequence encoding a TEV protease cleavage site. The PCR-amplified scygonadin coding sequence was digested with *Kpn*I and *Eco*RI, and ligated into pET-32a doubly digested with the same enzymes. The resultant recombinant plasmid, which allows the target protein to be expressed as a thioredoxin fusion (Fig. 1a), was named scygonadin/pET-32a.

For SUMO fusion expression (Fig. 1b), the commercial vector pET-28a was first modified by inserting a sequence encoding Smt3, the yeast SUMO protein (Fig. 2). This was achieved by amplifying the Smt3 coding sequence with primers 3 and 4 (Table 1) using plasmid pJOE4905.1 as the template and inserting the *Nde*I and *Bam*HI doubly digested PCR product into similarly digested pET-28a. A *Bsa*I restriction site was created at the end of the coding sequence for SUMO (Fig. 2b). Next, the scygonadin coding sequence was PCR

Table 1 Primers used for scygonadin coding sequence amplification and vector modification

Primers	Sequences (5'–3')
1	CGGGGTACCGAAAACCTGTATTTCCAGGGCCAGGCACTCAACAACTTATG <i>Kpn</i> I TEV
2	CCGGAATTCTTAGTAAGAAGCAATCCAGTCCTCGAC <i>Eco</i> RI
3	GGAATTCATATGGAGGTCAAGCCAGAAGTCAAGCCT <i>Nde</i> I
4	CGCGGATCCCCACTAGTGGTCTCAGCCACCAATCTGTTACGATGGGCCTCAAT <i>Bam</i> HI <i>Bsa</i> I
5	GGTCTCCTGGCGCCAGGCACTCAACAACTTATG <i>Bsa</i> I
6	CGCGGATCCTTAGTAAGAAGCAATCCAGTCCTCGAC <i>Bam</i> HI

Note. The restriction sites used for cloning are shown in italics, with the name of the enzyme under the sequence. The coding sequence for TEV protease cleavage site in primer 1 and the created *Bsa*I site in primer 4 are underlined.

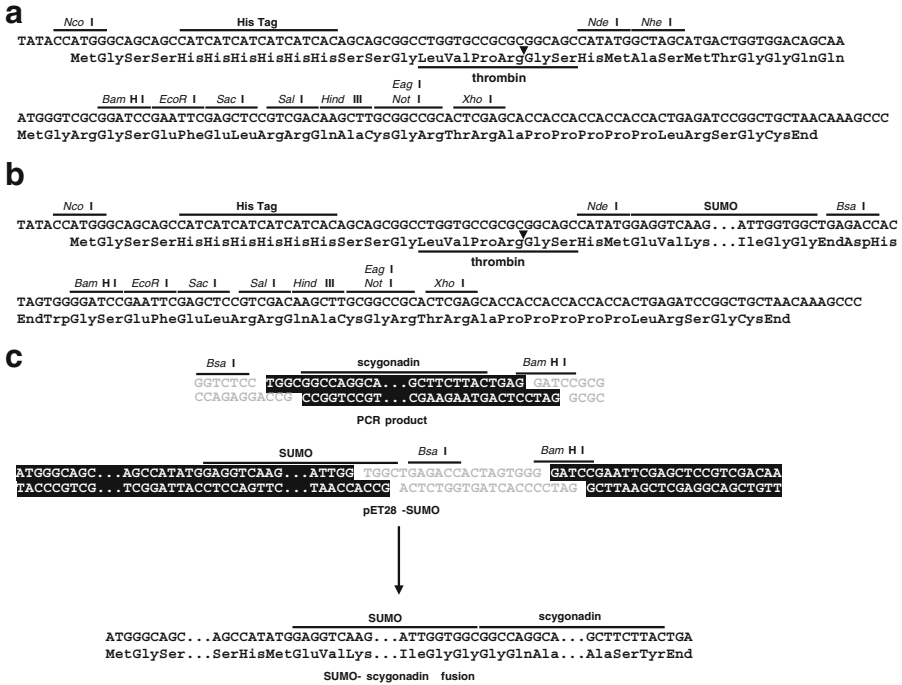


Fig. 2 Expression/cloning region of **a** original pET-28a and **b** its modified version, in which a sequence encoding yeast SUMO protein Smt3 was inserted between the *NdeI* and the *BamHI* sites. This modification allows the vector to be used for SUMO fusion expression. **c** Illustration of SUMO-scygonadin/pET-28a vector construction. PCR amplified scygonadin coding sequence was digested with *BsaI* and *BamHI* and ligated into the modified pET-28a plasmid doubly digested with the same enzymes

amplified with primers 5 and 6, and the PCR product was digested with *BsaI* and *BamHI* and ligated into the modified pET-28a plasmid doubly digested with the same enzymes (Fig. 2c). The resultant construct for SUMO fusion expression was named SUMO-scygonadin/pET-28a.

The presence and identity of the insert in both constructs was verified by diagnostic restriction digestion and DNA sequencing. The sequences for both fusion proteins are shown in Fig. 1.

Fusion Proteins Expression and Purification

After transformation of *E. coli* strain BL21(DE3) with recombinant vector scygonadin/pET-32a, the bacteria were grown overnight in 100 ml LB broth (with 100 µg/ml ampicillin) at 37 °C. This overnight culture was used to inoculate 1 L of fresh LB medium and cells were grown at 37 °C with shaking at 250 rpm. When the culture OD₆₀₀ reached 0.6, protein expression was induced by adding IPTG to a final concentration of 1 mM. After additional 5-h cultivation, cells were harvested by centrifugation at 5000 rpm for 10 min. The bacterial pellet (~5.1 g) was resuspended in 30 ml of cell lysis buffer (25 mM Tris, 200 mM NaCl, pH8.0) and cells were lysed by sonication. After centrifugation at 20,000 rpm for 40 min, the supernatant was combined with 5 ml Ni-NTA resin suspension and shaken at 4 °C for 3 h. The resin was pelleted by spinning at 1000 rpm for

5 min and the supernatant, which contains unbound proteins, was discarded. The resin was washed twice by shaking with 35 ml of lysis buffer containing 20 mM imidazole at 4 °C for 30 min. Protein bound to the resin was eluted with 20 ml of lysis buffer containing 300 mM imidazole. The fusion protein was further purified by running through a HiLoad 16/60 Superdex 75 column equilibrated with cell lysis buffer. SUMO-scygonadin fusion was produced following the same procedure, except that the bacteria were grown in medium containing 50 µg/ml kanamycin.

Production of TEV and SUMO Proteases

TEV and SUMO proteases were expressed and purified following the protocols developed by Tropea et al. [7] and Motejaded et al. [8], respectively. The procedure for making recombinant TEV protease has been briefly described in one of my recent publications [9], and therefore will not be repeated here. For the production of SUMO protease, *E. coli* strain JM109 harboring recombinant vector pJOE4847.2 was grown at 37 °C until OD₆₀₀ reached 0.4, at which point 0.2 % (w/v) L-rhamnose was added and the culture temperature was reduced to 30 °C. After 18 h, bacteria were harvested and resuspended in cell lysis buffer. Cells were lysed by sonication and the cell lysate supernatant was incubated with Ni-NTA resin for 3 h with shaking at 4 °C. After washing twice with 25 mM imidazole, the protein bound to the resin was eluted with cell lysis buffer containing 300 mM imidazole.

Scygonadin Release and Isolation

Thioredoxin-scygonadin and SUMO-scygonadin fusion proteins were cleaved with TEV and SUMO proteases, respectively, at a mass ratio of 50:1 (i.e., for 1 mg of purified fusion, 20 µg of protease was added) to release the target protein. The cleavage reaction was allowed to proceed at room temperature for 16 h without stirring. The cleavage mixture was subjected to a HisTrap HP column (5 ml). The unbound portion which contains the recombinant scygonadin was collected and concentrated for further use.

Dynamic Light Scattering Analysis

The oligomeric state of the recombinant protein was evaluated by dynamic light scattering (DLS) using a DynaPro instrument (Wyatt Technology Corporation, Santa Barbara, CA). Protein sample (~1 mg/ml) was centrifuged at 100,000 rpm for 10 min before measurements. Scattering data were analyzed using the Dynamics software.

Antibacterial Activity Assay

Antimicrobial activity of recombinant scygonadin (obtained from both constructs) was evaluated with Gram-positive *S. aureus* (ATCC 25923) and Gram-negative *E. coli* (ATCC 10798) as previously described [10]. In brief, bacteria in log phase was diluted with LB media and partitioned into a 96-well plate (90 µl per well). Dilution and partition was made in such a way that each well contains approximately 10⁵ cells (this was calculated based on the assumption that OD₆₀₀ of 1.0 equals ~10⁹ cells per ml). Scygonadin at different concentrations was prepared by making serial dilutions of concentrated sample with phosphate-buffered saline solution. To each well of bacterial

culture, 10 μ l of the protein dilution was added. At each concentration, the assay was performed in triplicate. The plate was read at 620 nm after incubation at 37 °C overnight.

Results

Purification of Scygonadin Expressed as a Thioredoxin Fusion

Thioredoxin-scygonadin fusion protein was well expressed and mainly found in the soluble portion (Fig. 3a, lanes 1–3). The fusion can be purified to a high degree using affinity and size-exclusion chromatography (Fig. 3a, lane 4). From 1 L of culture, 93.3 mg purified fusion was obtained.

TEV and SUMO proteases were both expressed as maltose-binding protein (MBP) fusions. The former underwent *in vivo* self-cleavage and was purified as a non-fusion [7], whereas the latter was purified as an intact fusion protein, which proved to be enzymatically active [8]. Both proteases can be obtained in relatively pure form after a single step affinity purification using Ni-NTA resin (Fig. 3b).

Upon TEV protease treatment, thioredoxin-scygonadin fusion released the target protein. The cleavage reaction was complete in 16 h at room temperature when 20 μ g TEV protease per mg fusion protein was used (Fig. 3a, lane 5). The released scygonadin can be readily separated from the carrier using a HisTrap HP column (5 ml). The target protein did not bind to the column and came out shortly after injection, whereas the carrier portion, which contains a His-tag, bound to the column and was eluted with increasing concentrations of imidazole (Fig. 3c). Following this approach, 32.7 mg/L scygonadin was obtained in high purity (Fig. 3a, lane 6) and its identity was confirmed by mass spectrometry (Fig. 3d). Yields of fusion protein and final product were summarized in Table 2.

Purification of Scygonadin Expressed as a SUMO Fusion

SUMO-scygonadin was also well expressed and mainly found in the soluble portion (data not shown). It was purified following the same procedure using affinity and size-exclusion chromatography. The fusion protein was efficiently cleaved upon treatment with MBP-Ulp1 (Fig. 4a), and the released target protein was separated from its carrier using the HisTrap HP column (Fig. 4b). The identity of the purified protein was confirmed by mass spectrometry (Fig. 4c). DLS studies suggested that at pH8 scygonadin forms monodisperse monomer (Fig. 5). Yields of SUMO fusion and final product were summarized in Table 2.

Fig. 3 **a** Thioredoxin-scygonadin fusion expression, purification, cleavage, and target protein isolation as followed by SDS-PAGE (13.5 %). *Lane 1*, whole cell lysate; *lane 2*, cell lysate supernatant; *lane 3*, cell lysate pellet (resuspended in 8 M urea); *lane 4*, affinity and size-exclusion chromatography purified fusion protein; *lane 5*, reaction mixture after TEV protease cleavage; *lane 6*, purified scygonadin; *lane 7*, protein standards. **b** Home-made recombinant TEV protease and MBP-Ulp1 quality checked by SDS-PAGE (12 %). *Lanes 1* and *2*, Ni-NTA resin purified TEV protease and MBP-Ulp1, respectively; *lane 3*, protein standards. **c** Imidazole gradient (indicated by a *dashed line*) elution of TEV protease cleaved thioredoxin-scygonadin fusion from a HisTrap HP column. *Inset* shows SDS-PAGE analysis of the fractions covering the two peaks. **d** Mass spectrum of recombinant scygonadin released from thioredoxin fusion. The determined molecular weight (11,271.86) is consistent with the theoretical value (11,271.76)

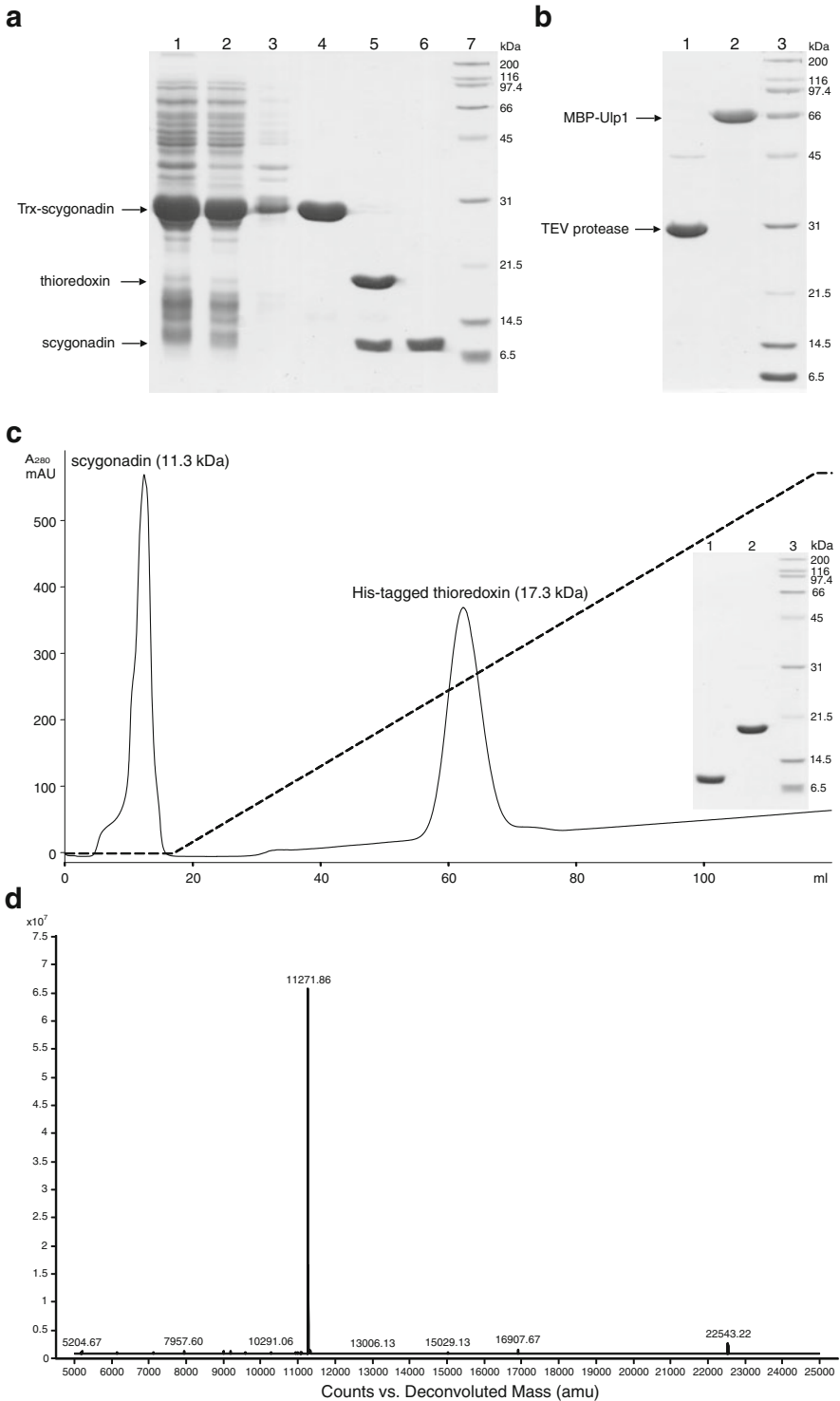


Table 2 Yields of fusion proteins and scygonadin from the two different constructs

	Thioredoxin-scygonadin	SUMO-scygonadin
Fusion protein (mg/L)	93.3	78.5
Scygonadin (mg/L)	32.7	29.2

Note. In both cases, roughly 5.1 g wet cells were obtained from 1 L of culture.

Antibacterial Activity Assay

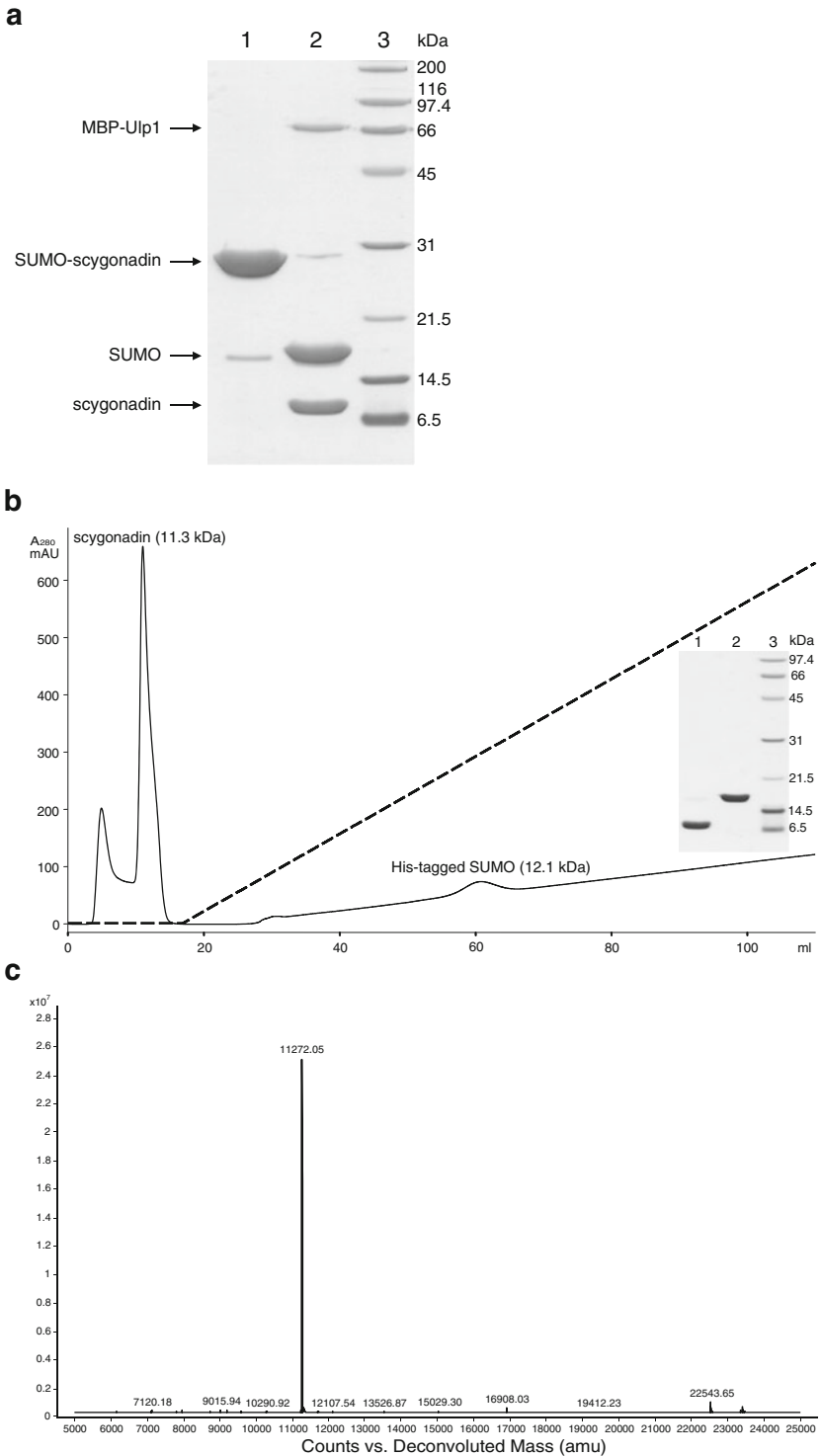
At 80 μM (the highest concentration tested), the produced recombinant scygonadin (from both constructs) did not inhibit the growth of *S. aureus* and *E. coli K-12*.

Discussion

In this work, antimicrobial protein scygonadin was expressed as both thioredoxin and SUMO fusions, and was intended to be released by TEV and SUMO protease-mediated cleavages, respectively (Fig. 1). Both carriers have a strong capacity for promoting soluble expression of the target protein, and the fusion proteins were mainly found in the soluble portion when expression was induced at 37 °C. TEV cleavage usually leaves an extra glycine at the N-terminus of the target protein. In this case, scygonadin's first residue happens to be glycine (Fig. 1a). The SUMO fusion was also carefully designed to allow scygonadin with native N-terminus to be generated upon SUMO protease cleavage (Fig. 1b). Another advantage of using TEV and SUMO proteases is that both enzymes can be produced in large quantities with relative ease [7, 8]. Both fusion proteins were efficiently cleaved upon treatment of the corresponding protease (Figs. 3a, 4a). 32.7 and 29.2 mg scygonadin per liter of culture were obtained from thioredoxin and SUMO fusion constructs, respectively.

Wang's group, who first isolated scygonadin, previously reported that the minimum inhibitory concentration (MIC) values of the recombinant protein against *S. aureus* and *E. coli* are 7.5–30 and >60 μM , respectively [5]. Surprisingly, at 80 μM the recombinant scygonadin produced in this study did not inhibit the growth of *S. aureus* and *E. coli*. Relevant information from other sources is currently not available. I notice that in the previous study the authors added 50 μl of peptide (in a 20 mM Tris–Cl buffer) to 50 μl of bacterial culture when doing the assay. The relatively large extent to which the culture media is diluted will have a negative effect on the bacterial growth by itself and this could be the reason why lower MIC values were obtained. The current data suggest that higher concentration is required for scygonadin to efficiently kill bacteria. In the case of human antimicrobial peptide LL-37, it is known that the peptide's ability to form large aggregate is critical for its antimicrobial activity [10]. DLS studies suggested that at pH8 scygonadin forms

Fig. 4 **a** Purification and cleavage of SUMO-scygonadin fusion protein as followed by SDS-PAGE (13.5 %). **Lane 1**, affinity and size-exclusion chromatography purified fusion protein; **lane 2**, reaction mixture after SUMO protease cleavage; **lane 3**, protein standards. **b** Imidazole gradient (indicated by a dashed line) elution of cleaved SUMO-scygonadin fusion from a HisTrap HP column. **Inset** shows SDS-PAGE analysis of the fractions covering the two peaks. Please note that the His-tagged SUMO has a relatively low absorbance at 280 nm and its apparent molecular weight on the SDS-gel is significantly larger than the actual value. **c** Mass spectrum of recombinant scygonadin released from SUMO fusion. The determined molecular weight (11,272.05) matches the theoretical value (11,271.76)



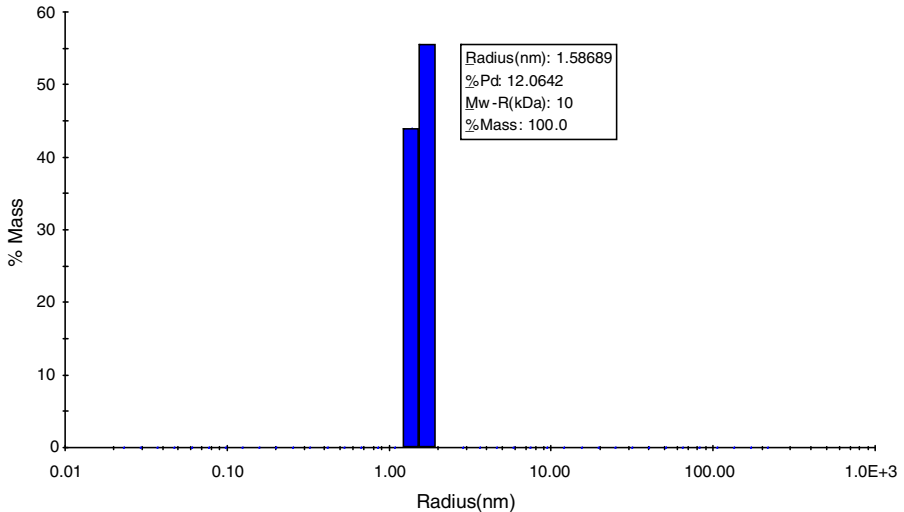


Fig. 5 DLS analysis of the produced recombinant scygonadin. At pH8 the protein forms a monodisperse monomer

monodisperse monomer, which may explain the less antimicrobial potency observed for this peptide in this study. Further studies are required to better characterize the antimicrobial potential and other biological activities of scygonadin.

In conclusion, I developed a protocol for producing recombinant scygonadin, a newly discovered antimicrobial protein that has not been fully characterized yet. High-quality protein was obtained in relatively large amounts. Scygonadin produced by this approach, which does not contain any artificial residues at either end, should be better than the previously produced version in serving the purpose of its own functional and structural characterization.

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