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Study of native SMAC protein production in the pUbiq expression system: Molecular cloning, biosynthesis and molecular modelling

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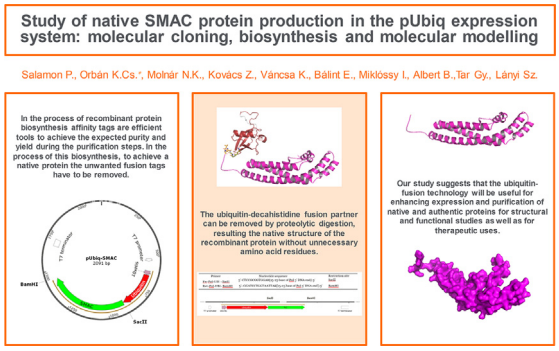
GRAPHICAL ABSTRACT



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ABSTRACT

Background: In the process of recombinant protein biosynthesis affinity tags are efficient tools to achieve the expected purity and yield during the purification steps. Nonetheless these tags might alter enzyme specificity and activity, therefore in functional assays it is recommended to use authentic or native proteins. Several ubiquitin fusion systems have been developed for *E. coli*-based recombinant protein expression that provide high levels of expression, with simple purification, and allow the production of various proteins with authentic N-terminus for subsequent applications.
Results: In the present research, we describe an ubiquitin fused bacterial biosynthetic system (pUbiq) for the production of the native Second mitochondria-derived activator of caspases (SMAC) recombinant protein. Using this system, the recombinant protein is expressed with an ubiquitin-decahistidine fusion partner, then purified from the cell-forming proteins by affinity chromatography. The fusion partner is then removed by proteolytic digestion, resulting the native structure of the recombinant protein without unnecessary amino acid residues. Following proteolysis, another affinity chromatography method is used to separate the native protein from the fusion partner and the proteolytic enzyme. The folding of the protein of interest was verified by a pull-down assay.
Conclusions: Based on our results, the presented pUbiq system was successfully applied in the production of native SMAC recombinant protein, where the affinity tag required for purification was completely

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Ubiquitin

removed. Our study suggests that the ubiquitin-fusion technology will be useful for enhancing expression and purification of native and authentic proteins for structural and functional studies as well as for therapeutic uses.

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1. Introduction

The second mitochondria-derived activator of caspases (SMAC) protein is known for its regulation of apoptosis induction by derepressing the IAP-mediated caspase inhibition through physical interaction with IAPs [1]. In apoptosis regulation studies and enzymatic assays it is relevant to use authentic or native proteins. The SMAC protein has to be produced without any unwanted fusion tags, mainly because its N-terminal end starts with the AVPI amino acids, which mediate the interaction with IAPs [2,3]. For the biosynthesis of such proteins it is critical to select a corresponding expression system optimized for eukaryotic protein expression.

Heterologous expression in standard expression host cells, such as *Escherichia coli*, often results in low yields or insoluble recombinant proteins [4,5]. There are many opportunities to optimize these *E. coli* expression systems to enhance the quality and quantity of the target product: selection of host strain, improving mRNA stability, bypassing codon bias, use of fusion protein technology and site-specific proteolysis, or compartment directed secretion and co-overexpression technology [4,6,7,8,9,10].

The ubiquitin fusion system has been applied to the production of several recombinant proteins in *E. coli* systems [4,11,12,13], like cecropinA(1–8)–magainin 2(1–12) [14], heparinase I [15], human urodilatin [16], lacticin Q [17]. Ubiquitin is extremely resistant to proteolytic degradation and has a long half-life (180 min) in *Escherichia coli* [18]. Also it is a highly stable protein as previous studies have shown that it sometimes acts as a translational enhancer, improving protein folding, solubility, and the expression level of target protein when fused to it. Its most relevant biotechnological use can be attributed to its TLHL aa sequence, which is part of the C terminal β strand, making it a potential target for enhanced affinity chromatography purification [11,19].

The pUbiq expression vector contains the ubiquitin (GenBank accession number: X05731.1) protein coding gene and a decahistidine group which is found at the N-terminal end [20]. This simple and versatile expression system is reported to be efficient in the biosynthesis of small isotope-enriched peptides and large enhanced proteins [12,21].

Several ubiquitin fusion systems have been developed for *E. coli*-based recombinant protein expression that provide high levels of expression, include a simple purification mode, and allow the production of various proteins with authentic N-terminus for subsequent applications [13,22]. In the process of this biosynthesis, to achieve a native protein the unwanted fusion tags have to be removed. This can be achieved by a specific cleavage at the N-terminal end of the protein. In the described systems an ubiquitin fusion partner is attached after the protein or between the protein and the affinity tag. After purification, using a yeast ubiquitin hydrolase enzyme (YUH1, GenBank accession number: DAA08884.1), also called ubiquitin carboxyl-terminal hydrolase enzyme the ubiquitin fusion partner along with the affinity tag can be removed entirely. The YUH1 enzyme proved to be very active due to the fact that the N-terminal and intersecting loop show high dynamics in terms of ubiquitin recognition and proteolysis surrounding the active site. The large movement surrounding the active site contributes greatly to the efficiency of the enzymatic activity [23].

In this study we use the pUbiq system for the biosynthesis of the second mitochondrial caspases activator (SMAC) protein. The SMAC protein was identified in the early 2000s as having a role in inducing apoptosis through the activation of caspases (caspase-9) [3]. The protein binds to members of the IAP (Inhibitor of Apoptosis Protein) family of proteins that act as inhibitors of apoptosis, thereby activating the caspase-9 protein, which induces apoptosis in the cytochrome C/APAf-1/caspase-9 pathway. SMAC interacts with IAPs via its N-terminal AVPI binding motif [1]. The pUbiq expression system allows the formation of the authentic N-terminal active binding site of SMAC [20]. The success of the native SMAC protein production was verified by a GST pull-down assay where the bait was a GST-XIAP complex and the SMAC participated as the prey. In this assay the XIAP-SMAC direct interaction indicates the authenticity of the purified protein, which was previously described in literature [24,25,26].

2. Materials and methods

All chemicals, bacterial growth media used for our experiments were purchased from commercial sources (Sigma Aldrich, Merck, and Thermo Scientific). Bacterial strains were also obtained from commerce (AddGene).

2.1. Construction of SMAC expression plasmid

Designed oligonucleotides (primers) were purchased from GeneriBiotech, Debrecen. Amplification of the SMAC gene from pET20b_SMAC [27] construction was performed by PCR using a thermocycler (PRO FLEX PCRS System, Life Technologies) with corresponding primers (Table 1). Snapgene software (https://www.snapgene.com/; version 1.1.3) was used to visualize and edit the DNA sequence. The composition of the PCR reaction is shown in Table 2. The thermal cycling condition was 90 s at 95°C, followed by 35 cycles of 10 s at 95°C, 30 s at 54°C and 60 s at 72°C, and then 10 min at 72°C.

The amplified sequence was digested with SacII (Thermo Scientific) and BamHI (Thermo Scientific) restriction endonucleases. Phosphodiester binding to the sticky ends of the SMAC insert was accomplished with the sticky ends of the pUBK plasmid using T4 DNA ligase (Thermo Scientific) to form the pUbiq-SMAC vector DNA construct.

The vector construct was verified on a 1% agarose gel by separation (figure not shown) and the capillary sequencing of the vector construct was performed by GeneriBiotech, Debrecen.

2.2. Expression and purification of recombinant protein

The culture was grown in a Sartorius BIOSTAT® A plus 1L bioreactor, using the following operating parameters: cells were grown at 37°C in Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) with 50 mg/L kanamycin for the selection and maintenance of *E. coli* transformants; mixing rate of 300 rpm;

Table 1
Designed oligonucleotides (cloning of SMAC protein).

Primer	Nucleotide sequence	Mp (°C°)	Restriction site	G-C Ratio (%)
Fw-SMAC-UBI–SacII	5′CTCCGCGGTGGAGCGGTTCCTATTGCACAGAAATC	70	SacII	57
R-SMAC-UBI-BamHI	5′CCGGATCCTGGTAATTAATCCTCACGCAGGTAGGCC	67	BamHI	56

Table 2
Composition of the PCR reaction.

Component	Quantity
10x Phusion HF puffer (Thermo Scientific)	5 μL
10 mM dNTP mix(Thermo Scientific)	1.5 μL
10 μM Fw-SMAC-UBI–SacII	0.5 μL
10 μM R-SMAC-UBI-BamHI	0.5 μL
SMAC template (21.23 g/mL)	1 μL
Pfu DNA polymerase (Thermo Scientific)	1 μL
dH_2O	40.5 μL
In total	50 μL

air flow rate of 0.2 vvm. The cellular growth was monitored by optical density measurements at λ = 600 nm using a UV–Vis spectrophotometer (GeneQuant pro Spectrophotometer, GE Healthcare). Expression of 10xHis-UBI_SMAC was induced by the addition of 0.5 mM IPTG at optical density of 0.8.

The culture was harvested by centrifugation (SL-40R, Thermo Scientific) at 4000 g for 10 min. Cells were resuspended in 20 mL lysis buffer (Table 3), and then lysed by sonication, and centrifuged (Sorvall Lynx 6000, Thermo Scientific) at 60,000 g for 60 min. The supernatant was stored at 4°C during the purification process. Proteins from supernatants were separated by SDS-PAGE (12% acrylamide). Protein Marker I (unstained), peqGOLD (VWR) ladder was used to identify protein by size.

Purification of the 10xHis-UBI_SMAC recombinant protein expressed in the bioreactor was performed by FPLC (ÄKTA purifier, GE Healthcare) on a HisTrap (GE) 5 mL affinity chromatography column. The buffer system used is shown in Table 3. The absorbance of the eluted proteins was monitored by a UV detector (UPC-900, GE Healthcare) at 280 nm of wavelength.

After successful purification of 10xHis-UBI_SMAC, we digested the protein complex with YUH1. This way the ubiquitin along with the 10xHis fusion partner was removed and we obtained the native SMAC protein. The YUH1 digestion buffer contained 20 mM Tris-HCl, 1 mM DTT, and 0.5 mM EDTA. The digestion reaction was verified by SDS-PAGE and the resulted image was analyzed using GelAnalyzer 19.1 software.

2.3. Prediction of recombinant protein structure

The 3D structure of the 10xHis-UBI_SMAC protein complex was calculated with homology modelling algorithms. Phyre2 online search engine tool was used to search for homologues of the fusion recombinant protein sequence and to construct the model. PyMOL2.0 software was used to visualize the 3D structure. From the appropriate templates, based on existing known crystal structures, the Phyre2 can determine (with high accuracy) the spatial structure of the fusion protein [28,29]. For molecular modelling, the following structures were used as templates: 3L0W, 5Y3T, 1FEW, 3AI5, 1OGW, 1OQY, 6PX3, 1YX5.

Table 3
Buffers used to purify decahistidine fusion (10xHis-UBI_SMAC) recombinant protein.

Lysis buffer	Wash I. Buffer	Wash II. Buffer	Elution buffer
pH = 8	*pH = 8*	*pH = 8*	*pH = 8*
50 mM $Na_2HPO_4{}^*2H_2O$	50 mM $Na_2HPO_4{}^*2H_2O$	20 mM Tris	20 mM Tris
300 mM NaCl	300 mM NaCl	1 M NaCl	200 mM NaCl
20 mM imidazole	40 mM imidazole	20 mM imidazole	400 mM imidazole
			10% glycerin
0.2% CHAPS	2 mM β-merk.	2 mM β-merk.	2 mM β-merk.
2 mM β-merk.			0.2% BOG

2.4. In vitro interaction between GST-XIAP and SMAC

The recombinant GST-XIAP complex was synthesized in *E. coli* BL21 (DE3) Rosetta plysS expression system described elsewhere. Glutathione Sepharose 4b resin was equilibrated using reaction buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, pH 7.4). GST-pulldown experiments were performed at room temperature. GST-XIAP proteins (8 μg) and recombinant SMAC (8 μg) were incubated with glutathione beads for 1–2 h in reaction buffer, followed by three washes. The bound proteins were eluted by 10 mM free reduced glutathione in Tris buffer. GST (8 μg) is adopted as a negative control. Eluted samples were verified on an 8% SDS–PAGE.

3. Results

3.1. Construction of SMAC expression plasmid

The expression vector was constructed as shown on Fig. 1. Both the agarose gel separation and capillary sequencing results confirmed the successful construction of the vector. The SMAC protein coding gene is found between the SacII and BamHI restriction sites.

3.2. Expression of recombinant protein

Biomass growth in the bioreactor was monitored hourly by OD600 measurements. The change in the optical density (OD600) during expression is shown in Fig. 2. Recombinant protein production was induced in the 2nd hour of fermentation at OD600 = 0.8, marked with "▲" in Fig. 2.

SDS-PAGE analysis of the recombinant protein production is shown in Fig. 3. The recombinant protein is increasingly produced in the soluble fraction with the time of fermentation.

3.3. Purification of the recombinant protein

Purification of the recombinant protein was successful based on SDS-PAGE gel image on Fig. 5, lane 1. During the purification, the 10xHis-UBI_SMAC protein was separated from cellular proteins and other impurities. Fig. 4 contains the affinity chromatography results exported from UNICORN 5.0. software (AKTA Explorer, GE Healthcare).

Hereinafter we removed the 10xHis-UBI fusion partner from the protein complex by digestion with YUH1 enzyme, obtaining the authentic SMAC protein as a result. The digestion was verified by an SDS-PAGE analysis shown on Fig. 5. The reaction was performed for 3 h and as it can be observed on Fig. 5 from the second hour, the efficiency of digestion does not improve. On lanes 3 to 5, the band found at 30 kDa represents the 10xHis-UBI_SMAC complex, the band at 26 kDa, 23 kDa and 8 kDa (according to GelAnalyzer 19.1 analysis) represents the YUH1, native SMAC and ubiquitin respectively.

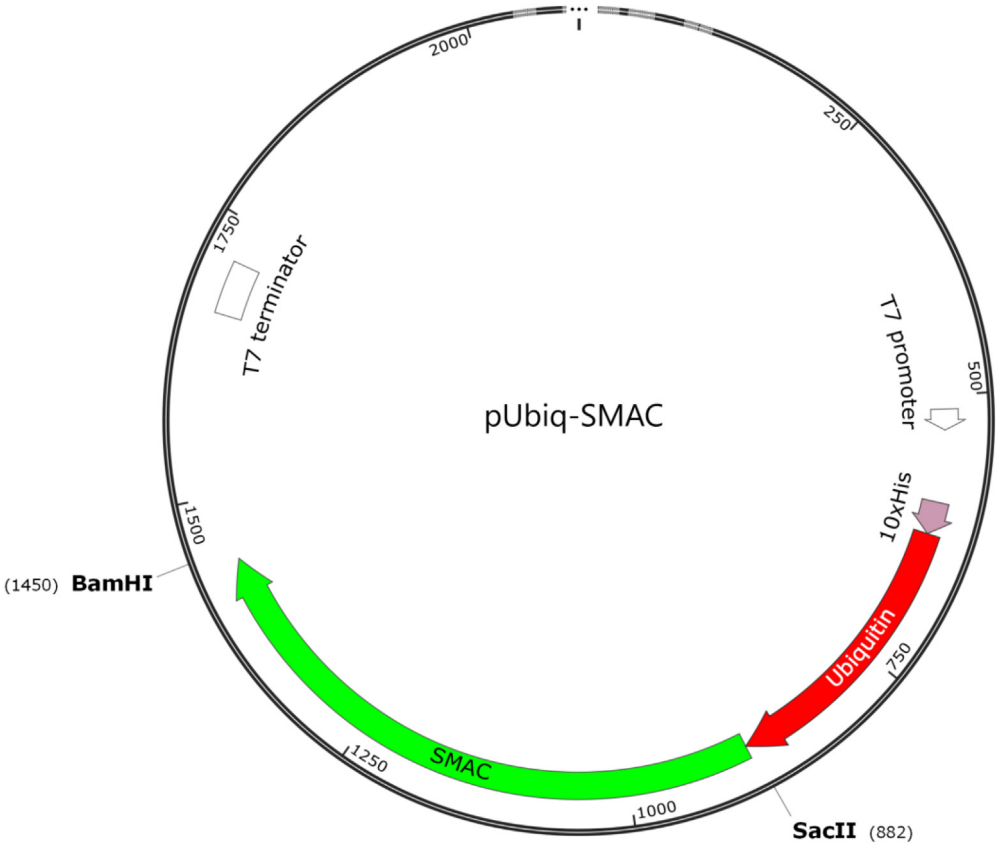

Fig. 1. pUbiq-SMAC vector construction. (The vector construct was visualized using Snapgene 1.1.3).

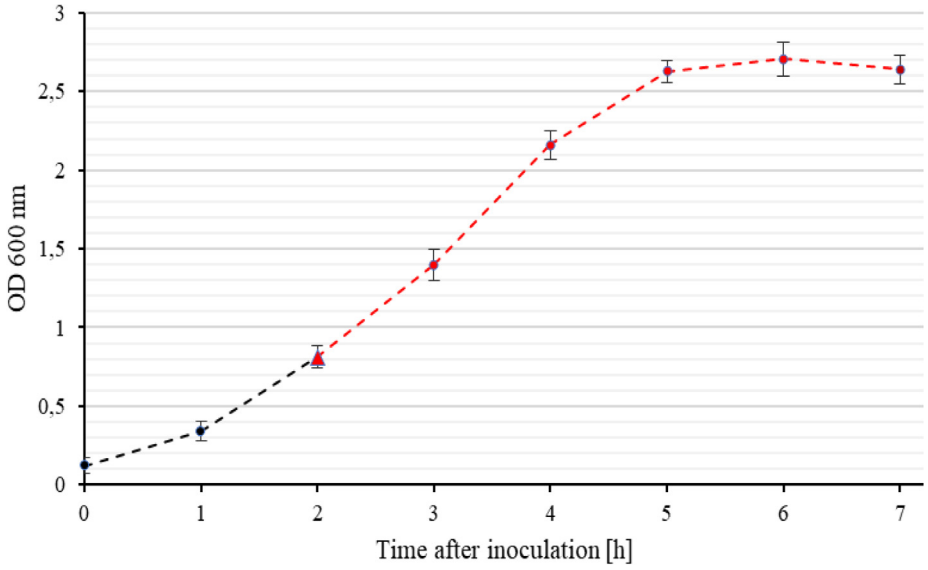

Fig. 2. Analysis of the bacterial growth (Recombinant protein production was induced in the 2nd hour of fermentation for 5 h, marked with "▲").

The separation of the native SMAC was realized with another affinity chromatography, where the 10xHis-UBI residues bonded to the column leaving the SMAC proteins in the flow-through, results not shown.

3.4. Recombinant protein structure

For further studies of the native protein biosynthesis process we built the 3D structural model of the 10xHis-UBI_SMAC complex. Confidence in the model: 277 residues (98%) modelled at >90% accuracy. With the advent of new molecular modelling platforms, the agreement between predicted values of the protein properties and experimental data in the literature is satisfactory.

Using the expression system, the native protein can be produced without the fusion segment as shown in the model of the complex in Fig. 6. Using the decahistidine fusion partner, the recombinant protein can be purified from the cell-forming proteins by affinity chromatography. As illustrated in Part C of Fig. 6, the

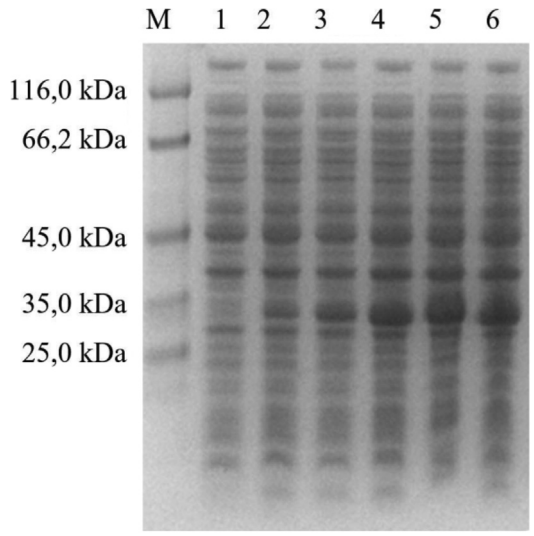

Fig. 3. Analysis of the recombinant protein production, supernatant on a 12% SDS-PAGE gel. Contains 10xHis-UBI_SMAC protein (32.1 kDa) (Lane M: standard protein marker (PeqGold Protein marker); Lane 1: sample before induction; Lane 2: 1st hour after induction; Lane 3: 2nd hour after induction; Lane 4: 3rd hour after induction; Lane 5: 4th hour after induction; Lane 6: 5th hour after induction; per hour,).

recombinant construct has a specific sequence that is recognized by the enzyme YUH1 and cleaves the purified recombinant protein. Following the proteolysis, another affinity chromatography method can be used to separate the native protein from the fusion partner and the YUH1 enzyme.

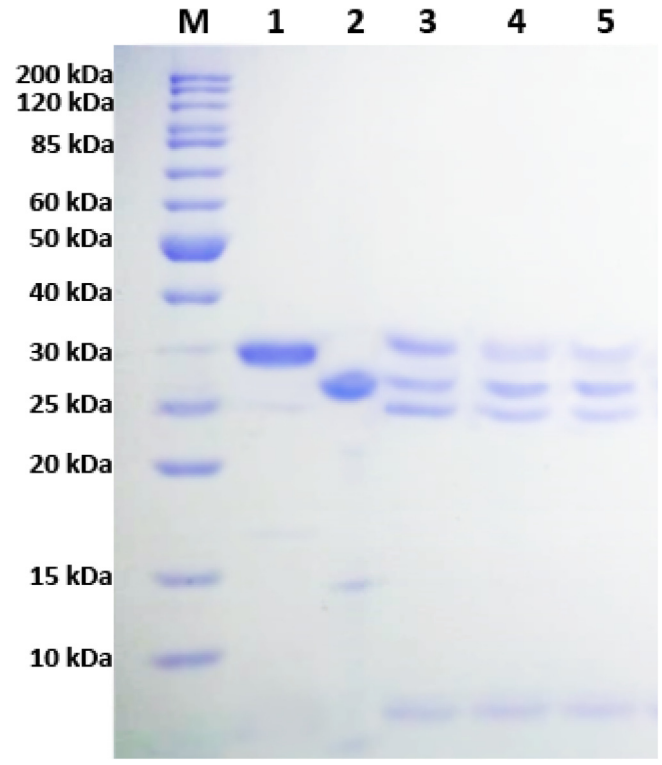

Fig. 5. Result of the YUH1 digestion of the 10xHis-UBI_SMAC complex on a 12.5% SDS-PAGE gel. Lane M, standard protein marker (PeqGold Protein II marker); Lane 1, 10xHis-UBI_SMAC; Lane 2, YUH1; Lane 3–5, digestion of 10xHis-UBI_SMAC construct with YUH1 at 1st, 2nd and 3rd hour.

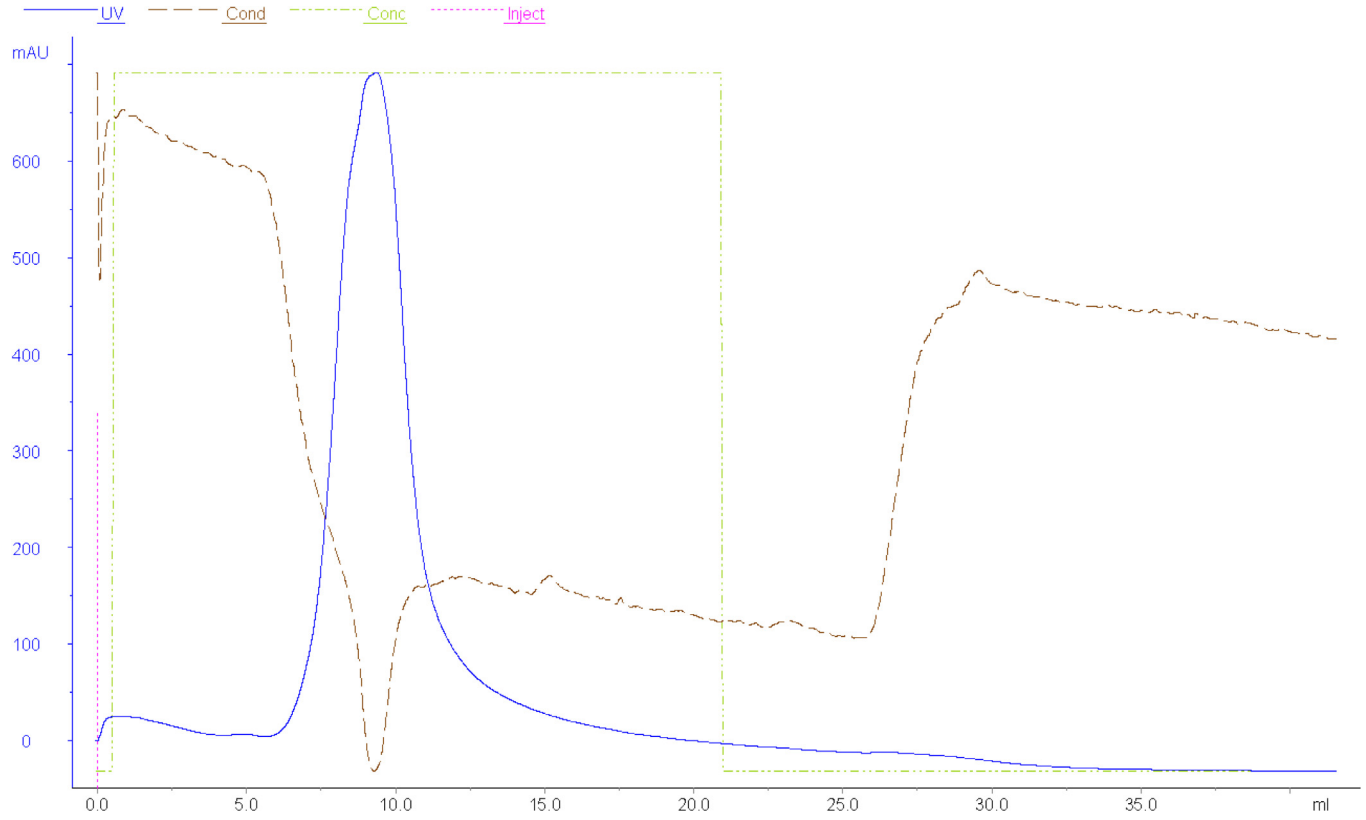

Fig. 4. Affinity chromatography results (AKTA Explorer) of 10xHis-UBI_SMAC protein purification; a single peak was obtained. The blue line represents the absorbance at 280 nm. The chromatogram also shows the conductivity and the conditions used during the elution. Elution was performed with 400 mM imidazole.

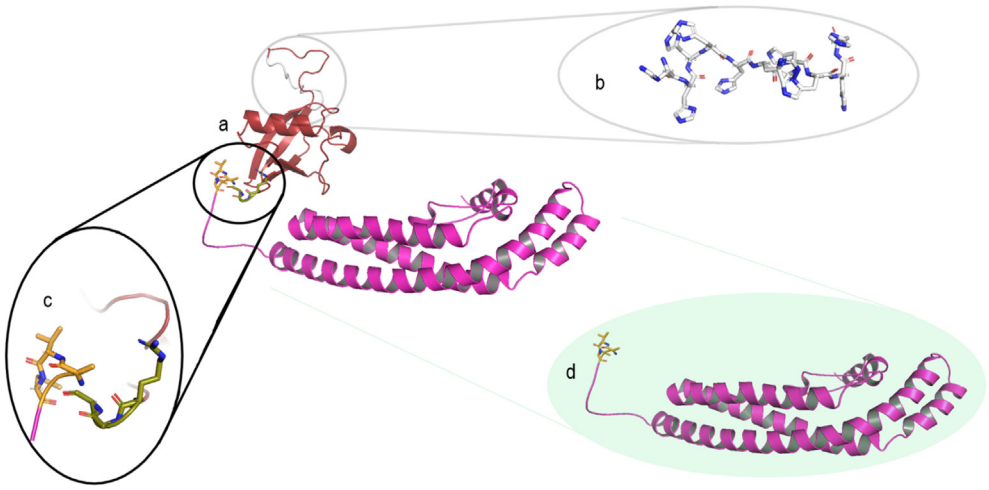

Fig. 6. 10xHis-UBI_SMAC structure ((a) recombinant SMAC protein after biosynthesis, (b) decahistidine fusion segment, (c) YUH1 enzyme cleavage site, (d) Ubiquitin removed, native SMAC protein.

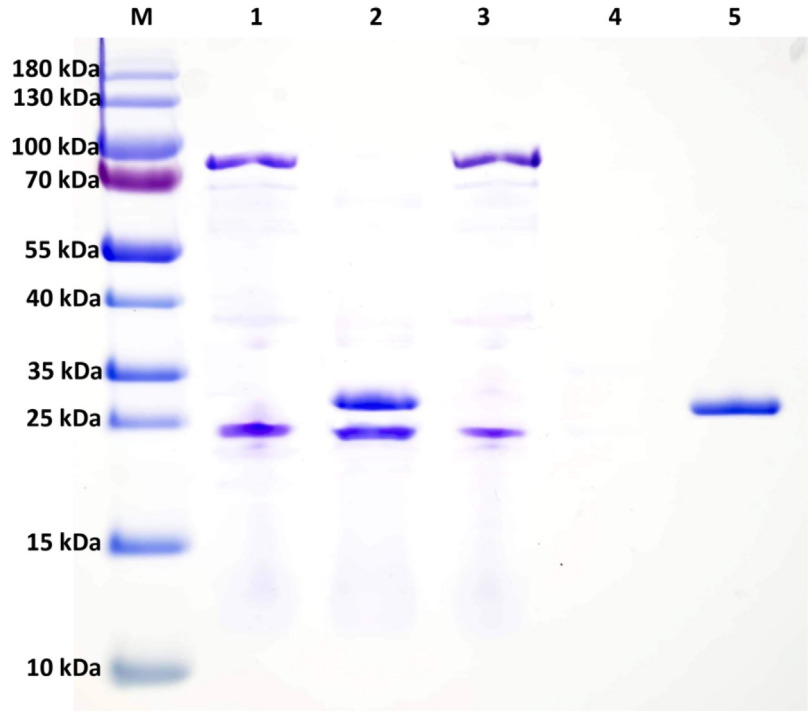

Fig. 7. GST pull-down binding assay of the recombinant native SMAC with GST-XIAP. Lanes 1 and 2 show input of protein samples in pull-down binding assays of SMAC as prey and GST-XIAP as bait. Lane 1: GST-XIAP and SMAC; Lane 2: GST and SMAC as control; Lanes 3 to 5 shows pull-down binding assays of the prey protein with the bait proteins. Lane 3: interaction complex of GST-XIAP and SMAC; lane 4: SMAC as negative control; lane 5: GST control.

3.5. In vitro interaction between GST-XIAP and SMAC

To investigate the active form and function of native SMAC, we performed a pull-down experiment with a known interaction partner, GST-XIAP. The results are shown on Fig. 7. The pull-down assay is a suitable method for examining protein–protein direct interactions between two purified proteins using affinity purification. The ability of GST-XIAP fusion protein to bind on the GST Sepharose beads was essential to perform GST pull-down experiments. This method may provide decisive data in terms of whether the study is suitable for structure determination.

During the interaction complex, the first band was identified as XIAP (78 kDa), while the second band was recognized as SMAC (approximately 23 kDa). According to the results presented above, the detection of SMAC-GST-XIAP interaction was successful which proves the authenticity of the recombinant SMAC protein. This can also be considered as a proof that native SMAC proteins interact with XIAP.

4. Discussion

Using the constructed pUbiq expression system for the biosynthesis of the native second mitochondrial caspases activator protein (SMAC) proved successful which is justified by the GST pull-down binding assay.

Table 4
Designed oligonucleotides (cloning of target protein (PoI)).

Primer	Nucleotide sequence	Restriction site
Fw-PoI-UBI-SacII	5′-CTCCGCGGTGGA&[15-25 base of PoI 5′ DNA end]-3′	SacII
Rev-PoI-UBI-BamHI	5′-GGATCCTGGTAATTA&[15-25 base of PoI 3′ DNA end]-3′	BamHI

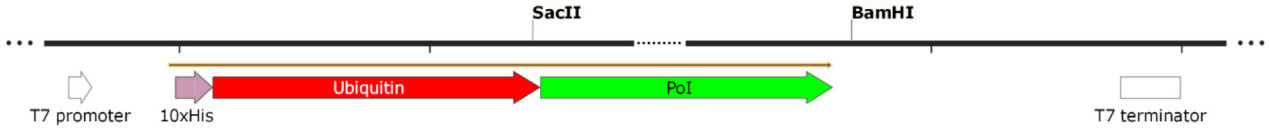

Fig. 8. Schematic structure of pUbiq system constructed in this study.

By examining the 3D structure of the desired recombinant protein complex, the efficiency of proteolysis and the removal of the fusion partner can be predicted. Molecular modeling of the 3D structure of the recombinant protein should be one of the most important steps in the design of biosynthesis processes [30].

Using multiple molecular biology tools and techniques, the development of an efficient expression system to produce industrial enzymes is a necessary process [31,32]. In this study the biosynthesis of human SMAC recombinant protein *E. coli* was realized successfully. Using *E. coli* as an expression host and the pUbiq expression construct, despite the lack of expression optimization an adequate protein production was carried out for further studies. Experimental results demonstrated that a high purity 10xHis-UBI_SMAC protein complex was achieved by affinity chromatography. Furthermore the native SMAC was successfully isolated after removing the fusion partners by YUH1 digestion. The authenticity of the target protein was proved by GST pull-down assay using GST-XIAP as bait. The successful expression and pull-down assay of the active form of SMAC protein will provide us the opportunity to study the binding dynamics of the protein *in vitro*.

In conclusion, the streamlined protein expression and purification approach we described here can be used to produce medium-sized, actively produced recombinant proteins in a time-saving and cost-effective manner. We assume that this approach is also suitable for producing enzymes for high-throughput studies [33,34], where simplicity and economy are critical enabling factors [35].

The key results of this study was synthesizing the ubiquitin-fused recombinant SMAC protein for the first time and producing authentic SMAC protein with the described method. Instead of the whole protein structure, we only cloned and expressed regions with features involved in interactions.

5. Conclusions

The pUbiq expression system can be widely used to produce foreign proteins in bacteria with ubiquitin fusion partners. The universal primers for cloning the target protein to be fused are summarized in simplified form in Table 4. In the case of forward and reverse primers, the constant parts and the variable, target protein-dependent DNA segment are also marked: the constant sequence is preceded by a "&", followed by the appropriate end sequence of the target protein. Schematic structure of pUbiq system constructed in this study are shown on Fig. 8. The T7 expression system provides controlled production of fusion recombinant protein [22,36].

Using the primers in Table 4, the genetic material of the corresponding target protein can be cloned into the product of interest (PoI) site as shown on Fig. 8.

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Conflict of interest

The authors declare that they have no conflict of interest.

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