Expression and antibody preparation of Small Ubiquitin-like Modifier (SUMO) from *Aspergillus flavus*

W Z Wu¹, S Ahmad¹, S Wang¹, Y F Zhang¹, H Yang¹, S H Wang¹, ² and Y Wang¹, ²

¹Key Laboratory of Mycotoxins and Pathogenic Fungi of Fujian Province Fuzhou, Key Laboratory of Chemical Biology and Biopesticide of Education Ministry, School of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou. 35002 China
²These two authors equally contributed in this work.

E-mail: wshyyl@sina.com (S H Wang); wangyu@fafu.edu.cn (Y Wang)

Abstract. *Aspergillus flavus* is a ubiquitous filamentous fungus, which contaminates crops, also causes severe disease in animals. The well conserved representative of the ubiquitin-like family SUMO (small ubiquitin-like modifier) is associated with the superfamily of ubiquitin-like polypeptides. In this study, the recombinant protein SUMO from *A. flavus* was cloned and expressed in *E. coli* BL21 (DE3). The protein was purified by nickel column affinity chromatography method and identified by mass spectrometry with molar mass was 10296 Da. The yield of recombinant protein was > 90% pure. The SUMO protein was immunized into BALB/c mice, and its potency was measured by western blot. The predicted superposition model of SUMO protein was analyzed by ITASEER server. These results indicated that SUMO proteins and its polyclonal antibody might provide scientific basis to study the SUMOylation in *A. flavus*.

1. Introduction

*Aspergillus flavus* is a widespread pathogen which is mainly found in tropical and subtropical regions for numerous economically important crops, as well as maize, cotton peanut is sources of yield losses all over the world [1, 2]. This kind of pathogenic fungi can also produce mycotoxins-aflatoxins, which is a class of strong carcinogen [3], Aflatoxins B1 is the most toxic toxins among these metabolites [4]. Moreover, it is lethal for human and animals that could cause myocardial, kidney tissue damage and liver cancer [5, 6].

Ubiquitination of proteins for proteasomal degradation is a common mechanism to ensure degradation of key factors for cellular regulation in eukaryotic organisms, which is necessary for removing misfolded proteins [7]. SUMOylation is directed by an enzymatic cascade similar to that elaborated in ubiquitination [8]. Furthermore, SUMOylation is not employed to tag proteins for degradation comparison with ubiquitination [9]. Depending on their function, the SUMOylating of proteins has several consequences for the target substrates [10]. In higher eukaryotes, it was shown that SUMOylating played an important role in signal transduction, transcription regulation, stability of genome, repair of DNA, and progression of cell cycle [11].

SUMO (Small Ubiquitin-like Modifier) proteins are associated with the superfamily of ubiquitin-like polypeptides which covalently attached to several intracellular target proteins to controls proteins sub-cellular localization and is involved in protein-protein interactions [12]. Moreover, the SUMOylating of proteins was also shown to be elaborated in ubiquitin-dependent breakdown by the
proteasome [13]. SUMO could block the access for further putative collaboration partners, which SUMO could help as a binding interface to alleviate the relationship with further proteins or DNA and could influence the conformation of target protein’s through binding to a another non-covalent site of SUMO [14, 15]. Furthermore, numerous controlling mechanisms for SUMOylating were reported, such as phosphorylation capability act as positive as well as negative signal for sumoylation [16]. In this study, the purified SUMO protein from A. flavus (NRRL3357), which was expressed by a prokaryotic expression system, molar mass was identified by mass spectrometry and the predicted superposition model of SUMO protein was analyzed. The polyclonal antibody against SUMO was prepared for further detection of the potential SUMOylating sites.

2. Material and methods

2.1. Multiple sequence alignment
Comparison of SUMO protein with other known fungal species. Sources of SUMO (OQ_E24410.1), Penicillium steckii (XP_020056676.1), Aspergillus aculeatus, (OX_S03525.1), Aspergillus thermostatus (GA_O88816.1), Aspergillus udagawae (XP_015405215.1), and Aspergillus nomius (OX_N33420.1), were obtained from NCBI. The multiple alignments was done by DNAMAN software [17].

2.2. Molecular cloning and protein expression
The SUMO coding gene (accession NO. XM_002380391.1) found in NCBI was used for the construction of the recombinant plasmid, and the primers (upstream primer: 5'-ATGGCCGTAGCAGGACTC-3', downstream primer: 5'-CTAACCACCGATCTGCTCCT-3') were used for PCR amplification from cDNA of A. flavus. The PCR products were cloned into pET28α containing a 6×His tag monitored by a PreScission protease cleavage at the site of N-terminus of SUMO, and the recombinant plasmid was moved into E. coli BL21 (DE3). The transformed cells were cultured in 10 ml LB medium which contains 50mg/L kanamycin at 37°C, 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After that cells were incubated on ice for 10 min then for overnight at 37°C. Then the cells were stored at 4°C with centrifugation of 6000 rpm for 5 min [18].

2.3. Protein purification
The cells were resuspended and sonicated on ice sage of binding buffer A (50mM Tris-HCl, 500 mM NaCl, 20mM imidazole pH 8.0). The suspension was shatted for 3 seconds interval (total 40 min). The lysate of the cell was centrifuged 3 times at 15000 rpm/min for 20 minutes at temperature 4°C then the supernatant was loaded into the Ni-NTA column (nickel-charged resin). The protein was eluted from the column with Buffer A, B (imidazole concentration 20 mM, 50 mM, 100 mM and 300 mM), Buffer C (500 mM NaCl, 50 mM Tris-HCl, 300 mM imidazole pH 8.0), then the 6×His tag (polyhistidine-tag) was removed and incubation of fusion protein with buffer C at temperature 4°C for 16 h. After that mixed sample was dialyzed against buffer D (500 mM NaCl, 50mM Tris) for the purpose to separate the unveage SUMO (cleaved by PreScission protease) from the cleaved mixture. After that loaded into another Ni-NTA column[18, 19]. Totally fractions that contain the purified SUMO were analyzed by SDS-PAGE.

2.4. Mass spectrometry
Mass spectrometry examination of SUMO was done by Beijing Protein Innovation. The SDS gel band was erased, and digested into decolorizing reagent contains acetonitrile (50%) and 25 mM ammonium bicarbonate. For the whole absorption of colloidal elements, DTT (dithiothreitol) was used and incubated at 50°C for 1 hr. After DTT was detached, 55mM IAM (iodoacetamide) was added after that incubated at 24°C for 45 min. Then excess IAM was removed and twice wash away with 25 mM ammonium bicarbonate for 10 min. Furthermore, enzyme was diluted as 1μg/μL up to 15 folds with 25mM ammonium hydrogen carbonate then addition with the dehydrated colloidal particles.
colloidal particles were immersed with 25mM ammonium hydrogen carbonate and incubated into a water bath at 37°C overnight for digestion purpose. The reaction of digestion was completed with 0.1% concentrated FA (formic acid), in addition sample was identified through Q-TOF (Quadrupole Time-of-Flight). The identification protein was performed with the Mascot software [20].

2.5. Immunization and iELISA assay of anti-serum
In the initial immunization, old female BALB/c mice have age six months (Wushi animal laboratory Shanghai, China) was administrated primarily by purified SUMO protein (60 μg/mL) slightly modified and added with the similar volume of Freund’s complete adjuvant at interparatoneal injection of the final water-in-oil emulsion. Booster injections of purified protein (60μg/mL) additional with the same volume of Freund’s incomplete adjuvant were injected, intervals at fourteen days. After three times injection, the anti-serum was collected from the tails of the two mice and assayed by ELISA. The ELISA plate contains 96-well was coated with coating antigen (purified SUMO protein, 100 μL/well), then incubated overnight at 4°C. The plate was wash-down away by PBS (phosphate buffered saline), jammed with PBSM (skim milk powder 200 μL/well), then incubated at 37°C for 1 h. Then plate was wash away by PBS and PBST (0.5% Tween 20 in PBS) three times. The serum of the mice was included to the plate, and then incubated at 37°C for 2h. Anti-mouse of goat IgG-HRP (1:8000, 100μl/well) was added after washing then incubated 37°C for 1h. These chemicals were purchased from (Sigma-Aldrich Chemical St. Louis, USA) after washing, TMB (100 μL/well) was added then incubated 37°C for 15 mint. Then 2M H2SO4 (50 μL/well) was added, then yellow colour was formed for measuring at 450nm by microplate reader [21, 22].

2.6. Western blot
After SDS-PAGE electrophoresis, the proteins on the gel were shifted to a PVDF (Polyvinylidene difluoride) and the membrane was placed in a closed solution (5% skimmed milk powder) with shaking at room temperature for 1h. Primary antibody incubation, with 5% nonfat dry milk diluted each film is about 4ml; closed PVDF membrane after hybridization carefully placed. Then added with a good antibody solution, slow shaker at room temperature and incubated with shaking 1.5h; the membrane washed with TBST (Tris-buffered saline-Tween) three times shaking every 5min, to remove residual antibody. The second antibody incubation: with TBS diluted with the instructions by the ratio of the secondary antibody (typically 1: 4000). The PVDF film is carefully placed in hybridization box. Added 4ml of each film with a good II antibody solution, hybridization with a mouth sealed then incubated shaking at room temperature for 1.5h. The film was taken after incubation; the membrane washed with TBST shaking three times 10min. After the secondary antibody incubation membranes finished membrane washed with TBST, a luminescent solution A, B in the darkroom exposure [23].

2.7. Predicted model
To choose the final models, I-TASSER uses the program (SPICKER) which was built on the pair-wise structure resemblance. The assurance of the model was calculated by C-score which was created on the importance of threading template alignments and the merging endowments of the structure gathering simulations. After assembly of structure simulation, I-TASSER was used the program TM-align structural alignment for the purpose to match the model to the entire structures in the PDB [24]. The structural analogue identified in PDB i.d IwzOA, so we had combined the predicted model and structure analogue using 1.12.Chimera software [25].

3. Results and discussion

3.1. Molecular cloning
The cDNA coding gene for SUMO was successfully amplified by PCR as in (Figure 1A), The PCR fragment was consistent with the size of the target gene (279 bp). The PCR product was cloned into the pET28a and transformed into the E. coli BL21 (DE3). The sequencing of DNA was confirmed that
recombinant plasmid was constructed successfully. The SUMO open reading frame encodes a predicted protein of 92 amino acids, which belongs to ubiquitin superfamily. Comparisons of amino acid sequences, it revealed that SUMO showed the highest similarity to other Aspergillus members (Figure 1B).

Figure 1. Cloning and sequence alignment (A) Cloning of SUMO gene from cDNA of *A. flavus*. M: DNA marker, Lane 1: cloned PCR fragment of 279 bp. (B) Comparison of SUMO protein with other known fungal species. The identical residues are in black colour.

3.2. Protein purification
The yield of SUMO which expresses in *E. coli* expression system was very high, and the protein showed excellent solubility. The recombinant SUMO was initially purified by Ni-NTA column successfully (Figure 2A). The 6×His-tag of the recombinant protein was removed by PreScission protease at 4°C, about 90% His-tag was removed from SUMO protein (Figure 2B). The untagged SUMO protein from the proteolytic digestion was isolated with the Ni-NTA column by collecting imidazole-free, and low concentration of imidazole (10 mM imidazole) wash fractions. Our results were mostly similar as the previous study about the purity of nucleoside diphosphate kinase (NDK) proteins nearly >98% [18].
Figure 2. SUMO protein expression and purification. (A) SUMO protein purification with 6xHis tag after cell extract by column chromatography (Ni-NTA). Where M showed marker Lane 1: supernatant; Lane 2: fractions of elution by Ni-NTA Column; Lane 3: fractions of elution with 50 mM imidazole concentration and Lane 4 fractions of elution with 100 mM imidazole concentration. (B) Purification of untagged SUMO by a proteolytic reaction of preScission protease. Where M indicated marker, Lane 1 SUMO protein (control): Lane 2 fraction flow by Ni-NTA Column (imidazole 10 mM).

3.3. The verification of peptides through Mass spectrometry

The recombinant target protein was purified and analyzed by liquid chromatography-mass spectrometry. Overall 8 peptides (FLFDGTR, LMDAFCER, LMDAFCER, KLMDAFCER, KLMDAFCER, VTDNNNEVFFK, VRPEDTPDTLEMADGDTEVHQEIGG, VRPEDTPDTLEMADGDTEVH–QEQIGG) was obtained, and the two peptides as in (Figure 3A/B) showed single domain polypeptides. The target protein’s molar mass was 10296 Da. The score was 12.84 emPAI (Exponentially modified protein abundance index). The identified peptides were matched with the creative sequence of SUMO from NCBI, which represented, the protein identification was accurate. The theoretical molar mass of recombinant protein was 10245.52 Da by Expasy's ProtParam prediction server, and experimentally mass spectrometry showed 10296 Da, which confirmed that the molar mass was very closely related. In the previous study to identify conjugation sites of ubiquitin-like protein by MS methods [26], but we analysis the mass spectrometry, to achieved the molar mass, and peptides matched the creative sequence from NCBI, representing the identification of the protein.
Figure 3. SUMO peptide determination by mass spectrometry (A) Determination of peptide by MS-MS spectrum FLFDGTR (B) Analysis of peptide by MS-MS spectrum VRPEDTPDTLEMADGDTLEVHQEQIGG m/z = ratio of mass to charge

3.4. Immunization and antiserum analysis
The mice were injected with purified SUMO protein, and booster injections were assumed intervals at fourteen days. After three times injection, the anti-serum was collected from the tails of the two mice, and the anti-serum titer of SUMO injected mice was tested through indirect noncompetitive ELISA. In the previous study higher antiserum titer was 1:8000, which indicated that the immunized mice had high Interferon gamma antibody titer [22]. Our result showed that two mice showed high titer, and mice 1 confirmed the highest anti-serum titer (1:64000). The result of the data was processed using Microsoft Excel to make a graph (Figure 4). Which indicated that immunogenic response was fully induced by SUMO protein, and the anti-SUMO antibody was successfully obtained.
Figure 4. Detection of Sumo antiserum titer by ELISA, Blue colour mice 1, Pink mice 2 antiserum titer and the black is for control mice.

3.5. Antibody detection by western blott

To further confirm the identity of the above anti-SUMO antibody, western blot experiments were implemented by anti-SUMO antibody. In the previous study SUMO-1 proteins analyzed by western blotting by C21 antibody [27]. Our result in (Figure 5), (Lane 2) showed that the SUMO protein was successfully recognized by this anti-SUMO antibody from western blot, while the negative control protein (Lane 1) was not bind to the anti-SUMO antibody. This result indicated that this anti-SUMO antibody bind to SUMO protein with high specificity.

Figure 5. Detection of antiSUMO antibody by western blot. M: marker, Lane 1 control, Lane 2 the untagged SUMO protein.

3.6. Predicted superposition of SUMO
SUMO protein from the PDB has the closest structural similarities, and the maximum TM-score to the I-TASSER predicted model. The comparison of predicted model and known protein model (Figure 6). 3D structure of the template (known protein) showed in pink colour. The predicted model at N-terminus contains the long loop. Protein loops take on specific conformations that are important to protein function [28]. The C-score was usually in the range of -5, 2. When a C-score showed a higher value that signifies a model among high confidence and C Score was 0.53. When a C-score shows a higher value that means a model with higher certainty. To measure the similarity between template and the structural query of the protein represent 0.625 TM score.

Figure 6. Superposition of SUMO protein. Superposition model pink colour showed known protein and blue colour demonstrated the predicted model consist of an extra loop nearby N-terminus (black circle).

4. Conclusions
The sumoylation of proteins has numerous consequences aimed at the target substrates, which depend on the function. In this study, to understand the SUMO biological function in the SUMOylated and non-SUMOylated prospective, the SUMO gene was cloned then expressed in the E. coli BL21 (DE3), and the protein was purified through nickel column affinity chromatography and analyzed by mass spectrometry. The BALB/c mice was immunized by SUMO protein, and anti-SUMO antibody was successfully prepared with high specificity. The prediction of superposition model of SUMO protein was analyzed by ITASEER server. It could be an available tool by using antibody produced from this study to determine the SUMOylation of A. flavus.

Conflicts of Interest
We state that, there is no conflict of interest between any authors.

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