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Research article

Expression and purification of the transcription factor StMsn2 from *Setosphaeria turcica* in *Escherichia coli*

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ABSTRACT

Background: In *Saccharomyces cerevisiae*, Msn2, which acts as a key transcription factor downstream the MAPK-HOG cascade pathway, also regulates the expression of genes related to stress responses. However, little is known about the regulation mechanisms of the transcription factor in *Setosphaeria turcica*.
Results: In this study, a zinc finger DNA-binding protein, designated as *StMSN2*, was cloned from *S. turcica*. Sequencing results showed that *StMSN2* had a 1752 bp open reading frame (ORF), which was interrupted by an intron (135 bp) and encoded a putative 538-amino acid protein. Phylogenetic analysis further revealed that StMsn2 was more closely related to Msn2 of *Aspergillus parasiticus*. *StMSN2* was cloned into the pET-28a vector with His (Histidine) tags and induced with 1 mM IPTG (isopropyl-β-D-thiogalactoside) at 37°C. The recombinant His-tagged StMsn2 was purified, and a band of size approximately 58.8 kDa was obtained. The high specificity of the polyclonal antibody Msn2-2 was detected with the StMsn2 protein from *S. turcica* and prokaryotic expression system, respectively.
Conclusions: A new gene, named *StMSN2*, with 1617 bp ORF was cloned from *S. turcica* and characterized using bioinformatics methods. StMsn2 was expressed and purified in a prokaryotic system. A polyclonal antibody, named Msn2-2, against StMsn2 with high specificity was identified.
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1. Introduction

Setosphaeria turcica (anamorph *Exserohilum turcicum* and formerly known as *Helminthosporium turcicum*) is a plant fungal pathogen that causes the northern corn leaf blight (NCLB) in maize. It is a severe and ubiquitous foliar disease that is increasingly becoming a worldwide problem [1,2], and the pathogen *S. turcica* was characterized by frequent variation and significant physiological differentiation [3]. These will directly impact corn production in the future once the crop loses its resistance to the disease [4,5].

Previous studies showed that the growth, development, and pathogenicity of the plant pathogenic fungi were regulated by extracellular signal transduction pathways [6]. Specifically, three main signaling pathways may exist in the pathogenic fungi, namely, Ca^{2+}, cyclic adenosine monophosphate (cAMP), and mitogen-activated protein kinase (MAPK) pathways. In recent years, studies on MAPK signal transduction pathway, which regulates growth, development, pathogenicity, and stress response in plant fungal pathogens, have become the focus in the field of cell signal transduction research [7,8,9,10]. A variety of extracellular stimuli including neurotransmitters, hormones, growth factors, cytokine, osmotic pressure, and radiation injury are thought to activate the above-mentioned pathways, in which the MAPK pathway triggers the transcription factors downstream and successively regulates the expression of specific genes [11,12,13]. Recent studies have reported that the HOG-MAPK, FUS3/KSS1-MAPK, and CWI-MAPK cascade pathways, which share high homology with the corresponding MAPK form in *Saccharomyces cerevisiae*, are also identified in the plant pathogenic fungi [13,14,15,16]. In particular, the HOG-MAPK cascade pathway, which is composed of Hog1 (MAPK), Pbs2 (MAPK kinase), and Ste11 and Ssk2/Ssk22 (MAPK kinase kinase), is mainly involved in the regulation of hypertonic and oxygen stress reaction [17,18,19]. Under hyperosmotic stress condition, the kinases in the HOG-MAPK cascade pathway are activated, thereby resulting in the accumulation of osmotic protectants and finally retention of the cell membrane osmotic pressure in yeast cell [20,21]. Four transcription factors, namely, Hot1, Msn2/Msn4, and Sko1, are downstream of the HOG-

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MAPK cascade pathway. Among them, Msn2/Msn4 acts as a key downstream transcription factor and regulates many metabolic processes including stress response [22,23,24,25,26,27].

Previous research revealed that Msn2 was involved in the regulation of osmotic and oxidative stress response, cell wall construction, and pathogenetic processes in some pathogenic fungi [28]. However, little is known about StMsn2 in *S. turcica*.

In the present study, *StMSN2* from *S. turcica* was cloned, expressed, and purified in *E. coli* BL21 (DE3). Further, polyclonal antibodies against StMsn2 were prepared for identifying StMsn2 in *S. turcica*. This study will not only provide new insights to understand the regulation mechanisms of StMsn2 but also lay the foundations for discovering new drug targets and developing effective approaches to prevent fungal disease.

2. Materials and methods

2.1. Materials, vectors, and strain culture conditions

The fungal strain used in this study was *S. turcica* wild-type (WT) strain 01–23, which was cultured on PDA medium (Potato Dextrose Agar, 20% potato, 2% dextrose, and 1.5% agar) and incubated at 25°C for 7 days [29]. Total RNA from *S. turcica* was extracted using the UNIQ-10 column Trizol total RNA Extraction Kit (Tiangen, China) following the manufacturer's instructions. The PrimeScript™ RT reagent Kit with gDNA Eraser (Tiangen, China) was used for the reverse-transcription of the first cDNA. Competent *E. coli* DH5α and BL21(DE3) (Transgene, China) were prepared for vector construction and protein expression. *E. coli* strains containing the plasmid pET-28a and reconstructed plasmid pET-28a-*StMSN2* were grown in LB medium (Luria-Bertani, 1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride) with 100 μg ml^{-1} kanamycin at 37°C. Isopropyl-β-D-thiogalactoside (IPTG) and bovine serum albumin (BSA) were purchased from Tiangen, China, while anti-His monoclonal antibody and goat antimouse IgG were purchased from ZSGB-BIO, China. All other chemicals were of analytical grade or higher.

2.2. Cloning of the full-length cDNA of StMSN2

The gene *StMSN2* was deposited in GenBank under accession number XP-008031569.1. The cDNA sequence of *StMSN2* was amplified using the following primer pairs: F: GAATTCATGCAGTCACCATTCTTCTACT and R: AAGCTTCTACTCGTTCCTCTTGCGCT, which were designed with Primer 5.0. *Eco*RI and *Hind*III restriction sites (underlined above) designed for gene cloning, were included in the primer sequences. PCR amplification of the ORF of *StMSN2* was carried out in 25 μl reaction mixtures containing 1 μl template (100 ng/μl); primer F (100 μM) 1 μl; primer R (100 μM) 1 μl; 0.25 μl LA Taq enzyme (5 U/μl); 2.5 μl 10× LA Taq enzyme Buffer (Mg2 + Plus); 2.5 μl dNTP mixture; and 16.5 μl ddH_2O. PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 2 min, with a final extension at 72°C for 10 min. The amplified fragment was inserted into the vector pMD-19 and transformed into DH5α before sequencing. The ORF of *StMSN2*, which was inserted in the reconstructed vector pMD-T-*StMSN2*, was digested by *Eco*RI and *Hind*III and then cloned to the expression vector pET-28a(+). The resulting expression vector pET-28a(+)-*StMSN2* was transformed into *E. coli* BL21(DE3) cultured on LB with 50 μg ml^{-1} kanamycin. The recombinant vector was identified by digesting with *Eco*RI/*Hind*III and target fragment PCR detection.

2.3. Bioinformatics analysis of StMSN2

The diagram of *StMSN2* structure was generated using the online tool GSDS. The conserved domains of StMsn2 were predicted with InterProScan and SMART. The tertiary structure of the StMsn2 protein was analyzed using online software SWISS-MODLE. Amino acid sequences of other related fungi were obtained in NCBI-NR database using BLASTP, which were then aligned using ClustalX. The phylogenetic tree was drawn in MEGA5 using the Neighbor-Joining method.

2.4. Prokaryotic expression and purification of the recombinant StMsn2

The recombinant plasmid pET-28a-*StMSN2* with 6 × His-Tag was transformed into *E. coli* BL21 (DE3) cells. The transformant were inoculated in 20 ml LB medium with 100 μg ml^{-1} kanamycin and grown at 37°C in a shaker at 220 rpm for 2–3 h until the OD_{600} of the culture was up to 0.6–0.8. Then, 1 mM IPTG was added to the culture to induce the expression of the target protein. The culture samples were collected after 10, 20, 30, 40, 50, and 60 min and after 1, 2, 3, 4, 5, and 6 h of induction, respectively. The cell pellets were subsequently harvested by centrifugation at 12,000 rpm for 10 min at 4°C. The recombinant protein produced in *E. coli* BL21(DE3) was purified using Ni-Charged MagBeads according to the manufacturer's instructions (GenScript, China). The culture of *E. coli* BL21(DE3) was concentrated to 50 ml and later centrifuged at 12,000 rpm for 1 min at 4°C. The cell pellets were re-suspended in 5 ml of binding buffer (0.1 M Tris–HCl, pH 8.0), added to 50 μl lysozyme (final concentration of 100 μg/ml), and incubated on ice for another 30 min. Cells were disrupted by sonication (20% of power treatment for 30 s at 4°C) and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant and cell pellets were, respectively, collected and detected using 12% SDS-PAGE to identify the expression of StMsn2 in *E. coli*.

Soluble StMsn2 was purified by adding NaCl to the supernatant to the final concentration of 0.5 M and was injected in 1 ml of Ni-Charged MagBeads, which then was balanced with both 25 ml buffer A (10 mM Tris–HCl, pH 8.0) and 25 ml buffer B (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0) in a rocker for 30 min. The mixture was further agitated for 1 h at 60 rpm 4°C in a rocker before centrifugation at 12,000 rpm 4°C for 10 min. The pellet was washed 2–3 times with 25 ml buffer B. After this, freshly prepared 1 ml elution buffer (300 mM imidazole 0.5 M NaCl, 10 mM Tris–HCl, pH 8.0) was added in the homogenizer and rotated for 1 h at 4°C. The beads with His-tagged StMsn2 was eluted and collected 2–3 times. The effluent was collected for both 12% SDS-PAGE and western blotting.

2.5. Western blot analysis for the recombinant His-tagged StMsn2

The supernatant and the purified His-tagged StMsn2 protein were first separated by SDS-PAGE using a vertical gel electrophoretic system, with 5% (*w*/*v*) stacking gel and 12% (w/v) separation gel at 120 V for 60 min [30]. After SDS-PAGE, all proteins were electro-transferred onto a PVDF (polyvinylidene fluoride) membrane (Millipore, USA) at 4°C, 200 V for 90 min using the Bio-Rad Trans-Blot apparatus (Bio-Rad, Hercules, USA). The membrane was incubated with a blocking solution, 1% (*w*/*v*) bovine serum albumin in TBST-buffered saline (24.7 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.5% Tween-20, pH 7.5) for 1 h at room temperature (20°C) before incubating with mouse anti-His monoclonal antibody (ZSGB-BIO, China) (1:2000) in 3% albumin bovine V at 4°C for 1 h. After washing with TBST six times, the membrane was incubated with a goat antimouse polyclonal antibody labeled with alkaline phosphatase (BioDev-Tech, Beijing, China) (1:5000) for immune-detection of His-antibody for 1 h at room temperature. Then, the membrane was again washed with TBST and the protein bands were detected using a color substrate solution (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, 0.01 mM NBT, 0.01 mM BCIP, pH 9.5) to observe a color reaction (NBT/BCIP; Roche).

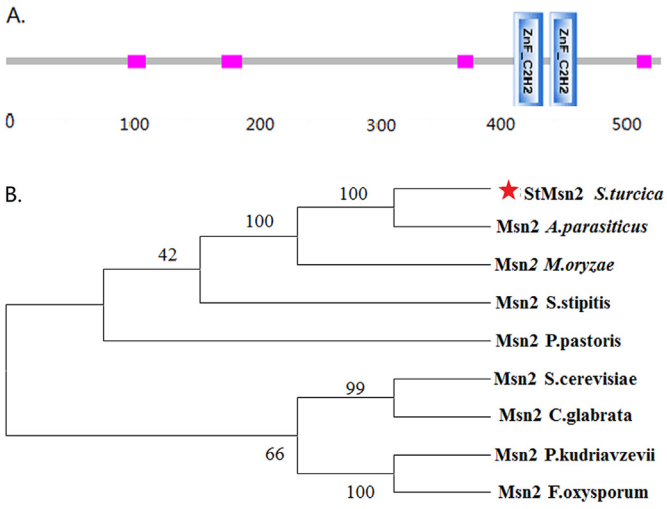

Fig. 1. Structure prediction and phylogenetic analysis of StMsn2. (A) The model of StMsn2 proteins showing the conservative domain ZnF-C_2H_2. (B) Phylogenetic tree showing evolutionary relationships of fungal Msn2 proteins. Phylogenies were estimated using a PHYLIP-based program (DNAMAN version 6.0.3.99) to create an unrooted phylogenetic tree from alignments of the full MAPK amino acid sequences using the Neighbor-Joining (NJ) method in MEGA5.0.

2.6. Prediction of B-cell antigen epitope for StMsn2 and preparation of polyclonal antibody

All procedures involving animals were approved by the Institutional Animal Care and Project of Beijing Municipal Science & Technology Commission, License 034, China.

The StMsn2 antigen epitope was analyzed using DNAstar software and referencing Jameson-Worf comprehensive prediction scheme. Three peptides (Msn2-1, 82-95 AA; Msn2-2, 230-243 AA; and Msn2-3, 433-449 AA) with strong hydrophilicity and high antigen index were selected for further analyses. The three polypeptides were synthesized and immunized in New Zealand white rabbits, which was carried out by Beijing Huada Protein Research & Development.

The three peptides of StMsn2 mentioned above were used to immunize rabbits at the same time, and the detailed protocol is as follows. First, we took serum from rabbit's ear vein as negative control before immunization. Then, the three synthetic peptides were generally cross-linked with bovine serum albumin (BSA). The 1 mg of cross-linked materials were dissolved in 1 ml of phosphate-buffered saline solution (PBS), and the solution above mentioned was emulsified with the same volume of Freund's adjuvant. In the first immunization, one milliliter of the emulsion was inoculated under the skin of the rabbit. after two weeks, enhanced immunization with the same dose of vaccine or control solution was administered twice, the interval is 14 day. Then, 1 ml of blood was collected from the ear vein to detect the titer of the antibody. When the titer was up to 20,000, blood was collected from the artery of the rabbit's neck. The corresponding antibody was purified by protein A/G and then stored at -80°C. After the latest immunization, 1 ml of blood was collected from the ear vein to detect the titer of the antibody. When the titer was up to 20,000, blood was collected from the artery of the rabbit's neck. Finally, the corresponding antibody was purified by protein A/G and then stored at -80°C.

3. Results

3.1. Characterization of StMSN2

The cDNA sequence of *StMSN2*, which contained a 1617 bp ORF and encoded a putative 538 amino acid protein, was cloned from WT *S. turcica*. In addition, a 1752-bp genomic DNA sequence of *StMSN2* was obtained, and sequence analysis showed that an intron of length 135 bp was present in the genomic DNA sequence. Conserved domain analysis further showed that the StMsn2 had two conserved domains of zinc finger structure (ZnF-C_2H_2) at the N-terminal located at amino acid residues 418–441 and 447–469, separately (Fig. 1A).

Twelve Msn2 sequences were further selected from typical fungi for phylogenetic analysis, including *S. turcica* (StMsn2), *Aspergillus parasiticus* (ACV03836.1), *Magnaporthe oryzae* (XP003718560.1), *Scheffersomyces stipitis* (XP001386050.2), *Pichia pastoris* (XP002491652.1), *Saccharomyces cerevisiae* (KZV08884.1), *Candida glabrata* (XP446232.1), *Pichia kudriavzevii* (OXA48662.1), and *Fusarium oxysporum* (SCO83385.1). The results revealed that the Msn2 proteins from different species mainly clustered into two clades, in which StMsn2 was more closely related to the Msn2 protein of *A. parasiticus* (Fig. 1B).

3.2. Construction and identification of the recombinant vector pET-28a-StMSN2

The cDNA sequence of *StMSN2* was amplified using the first-strand cDNA of *S. turcica* as the template. The amplified *StMSN2* was then cloned into the vector pMD19 and sequenced. The 1617-bp *StMSN2* was inserted into the vector pET-28a (Fig. 2A). The recombinant vector, named pET-28a-*StMSN2*, was digested by *Eco*RI/*Hind*III. Two fragments, 1617 bp and 5243 bp, were detected (Fig. 2B), revealing that *StMSN2* was successfully inserted into the pET-28a vector.

3.3. Expression of StMSN2 in E. coli BL21 (DE3)

An expected band of size approximately 58.8 kDa was detected in the experiment, and its expression level was found to be enhanced

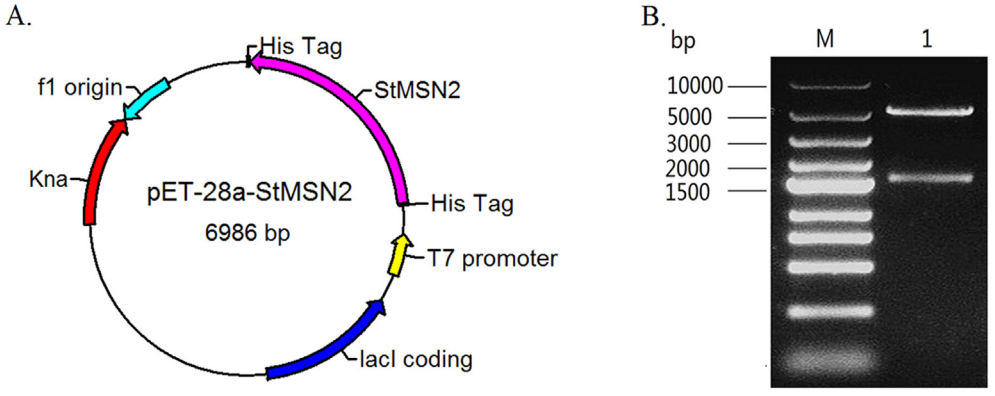

Fig. 2. Construction of the vector pET-28a-*StMSN2*. (A) Schematic diagram of the vector pET-sample-*StMSN2*; (B) Gel image confirming the recombinant vector pET-28a-*StMSN2*, digested with *Eco*RI and *Hind*III. Lane 1 represents sample, and M indicates DL2503 DNA marker.

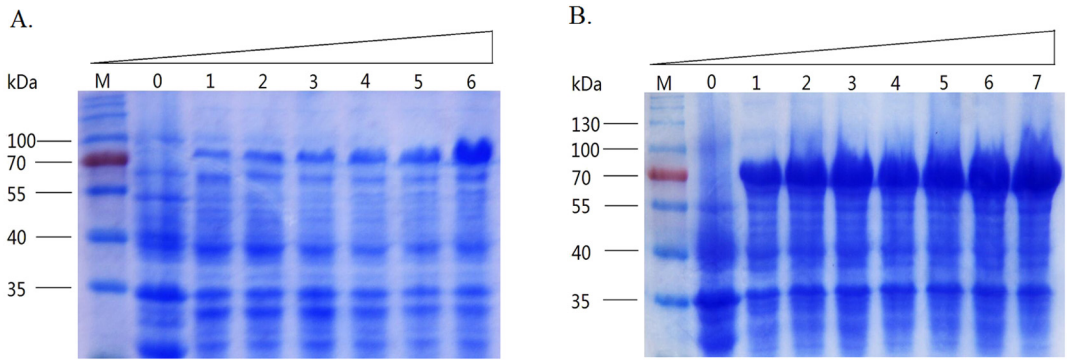

Fig. 3. Gel bands confirming the optimum expression time of *StMSN2* by SDS-PAGE technique. Lane M, the standard protein marker (9–235 kDa); (A) Lanes 0–6 indicating induction with IPTG from 0 min to 60 min; (B) Lanes 0–7 indicating induction with IPTG from 0 to 7 h.

from 10 min to 60 min (Fig. 3A). Further investigations then focused on exploring the relationship between the expression level of the recombinant protein and the induction time from 1 h to 7 h. The results showed that the expression level of StMsn2 gradually enhanced as the time increased. Moreover, the expression level of StMsn2 reached the maximum abundance at 7 h during the induction time from 1 to 7 h (Fig. 3B). These results indicated that StMsn2 was successfully expressed in *E. coli* BL21 (DE3), and the expression level was increased as the induction time increased.

3.4. Purification and verification of the recombinant protein

To verify the relative distribution of the recombinant His-tagged StMsn2 in *E. coli* BL21 (DE3), both the supernatant and pellet of the lysate from the induced cells were examined by 12% SDS-PAGE. We observed that StMsn2 occurred not only in the supernatant but also in the pellet of the lysate. Moreover, the expression level of the protein in the supernatant was much more than that in the pellet (Fig. 4A). Thus, the soluble supernatant from *E. coli* BL21 (DE3) was extracted to purify the recombinant StMsn2 in the downstream analysis. Further, proteins in the supernatant from the strain induced by IPTG for 4 h were purified by nickel column affinity chromatography, as the N-terminal of the fusion target protein was expressed in the T7 promoter system of *E. coli* BL21 (DE3) containing His-tag. These results showed that the recombinant protein was successfully expressed by the presence of a specific band of size approximately 58.8 kDa as we expected (Fig. 4B). To further verify if StMsn2 was expressed in the strain, Western blotting analysis with mouse anti-His mAb was conducted. The result showed that the anti-His mAb reacted with both His-tagged StMsn2 proteins from the induced cells and with the purified His-tagged StMsn2 protein (Fig. 4C). In a word, *StMSN2*, a transcription factor gene from *S. turcica*, can be expressed in *E. coli* BL21 (DE3) in soluble form.

3.5. Analysis of antigen epitopes and identification of polyclonal antibody of StMsn2

Based on hydrophilicity, flexibility, antigenic index, and accessibility, three segments of peptides were selected as the epitopes of the StMsn2 protein, Msn2-1 (PQDSPYLHPIDTDY), Msn2-2 (EEHKVVLKGETAKA), and Msn2-3 (LKRHYRSLHTHDKPFE) (Fig. S1). After these peptides were synthesized and immunized in the rabbits, three polyclonal antibodies, designated as Msn2-1, Msn2-2, and Msn2-3, were obtained. The secondary antibody used was the IgG-FITC from HRP-labeled goat antirabbit IgG for Western blotting, while the serum was used as the negative control. The specificity of the antigens to StMsn2 in *S. turcica* was validated by Western blotting. The results showed that the primary antibody Msn2-2 produced two bands of size approximately 58.8 kDa and 70 kDa, respectively (Fig. 5A), while no bands were detected in Msn2-1 and Msn2-3 (data not shown). The molecular weight prediction of StMsn2 suggested that the 58.8 kDa band was the targeted band. Another 70 kDa band detected was probably a phosphorylated StMsn2, which was approximately 10 kDa larger than the target band. To further determine the specificity of the Msn2-2, another Western blotting experiment was performed for the antigen StMsn2, which was expressed and purified in the prokaryotic expression system obtained above. Only a single band was detected, suggesting that StMsn2 could be combined with the antibody Msn2-2 (Fig. 5B).

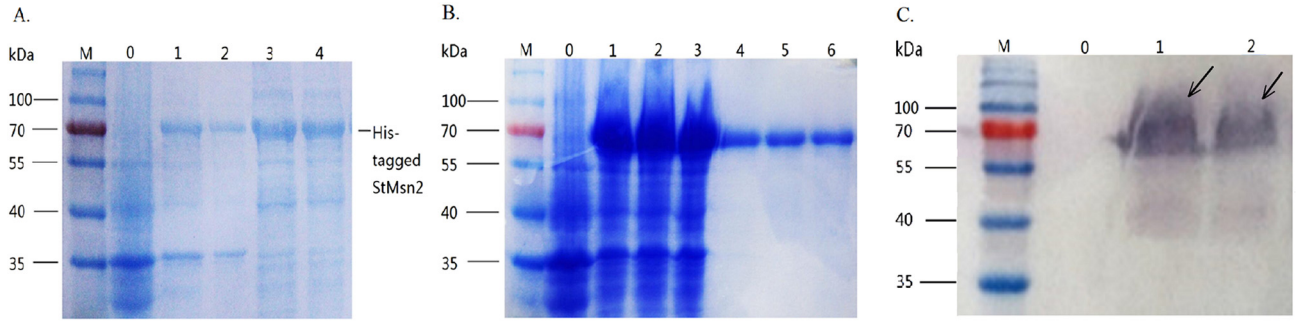

Fig. 4. Identification of StMsn2 expressed in *E. coli* BL21(DE3). (A) Relative distribution of StMsn2 in *E. coli* BL21(DE3). The protein sample was separated on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane M, protein molecular mass markers (kDa); Lane 0: total crude extracts of *E. coli* BL21 containing the His-tagged StMsn2 without IPTG induction; Lanes 1 and 2: proteins from insoluble cell debris; Lanes 3 and 4: proteins from the supernatant. (B) Purification of StMsn2 expressed in the supernatant of *E. coli* BL21 (DE3) by SDS-PAGE. The protein sample was separated on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane M: protein molecular mass markers (kDa); Lanes 1–3: supernatant proteins from BL21 (DE3) induced by IPTG for 4 h; Lanes 4–6: proteins purified by nickel column affinity chromatography. (C) Identification of StMsn2 by Western blotting. Analysis for expression of the recombinant His-tagged StMsn2 protein using His-tag antibody. Lane M: protein molecular mass markers (kDa); Lane 0: total crude extracts of *E. coli* BL21 containing the His-tagged StMsn2 without IPTG induction; Lane 1: total crude extracts of *E. coli* BL21 containing the His-tagged StMsn2 induced by IPTG for 4 h. Lane 2: purified protein StMsn2 with His-tagged.

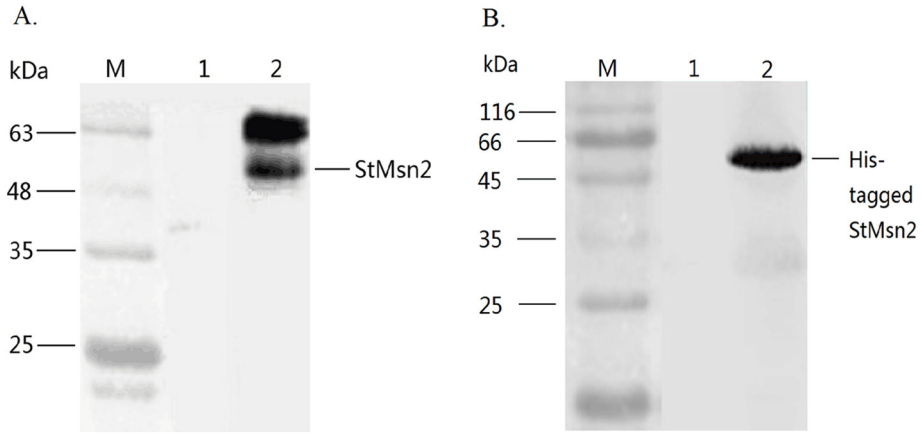

Fig. 5. Specificity confirmation of the polyclonal antibody against StMsn2. (A) The expression level detection of endogenous StMsn2 in *S. turcica* by Western blotting. Lane M: protein molecular mass markers; Lane 1: nonimmune serum (1:2000 dilution) reacting with the cell lysates derived from *S. turcica* cells; Lane 2. (B) Polyclonal antibodies bound exogenous StMsn2 protein, which was expressed in *E. coli* BL21(DE3). Lane1: Nonimmune serum reacting with the cell lysates derived from *E. coli* BL21(DE3) cells; Lane 2: the immune serum (antibody Msn2–2, 1:100 dilution) reacting with the cell lysates derived from *E. coli* BL21(DE3) cells.

4. Discussion

Prokaryotic expression system, which is advantageous owing to its simple and fast manipulation, low cost, and high production of proteins, has been widely used in expressing heterologous genes. However, it also has two major disadvantages. First, it has a very highly reductive environment in the cytosol of *E. coli*, which makes it unable to perform eukaryotic post-translational modification and usually results in the formation of insoluble proteins [31,32]. Second, the conversion of the protein from inclusion body to soluble protein is a very difficult and cumbersome process. Therefore, many factors should be considered before heterologous proteins are expressed in the prokaryotic system [33,34]. In the present study, we successfully obtained StMsn2 in the prokaryotic system, and its expression level was increased with increase in time using the conventional method. Under induced conditions of 1 mM IPTG, at 37°C, the target protein was detected after 10 min, and a large amount of StMsn2 was detected after 4 h, which can meet the needs of the subsequent experiments. The most important factor taken into consideration when we optimize the expression of heterologous gene in the prokaryotic system is the pre-expression analysis including transcription initiation site, GC content, size of protein, hydrophobicity, and secondary structure [31]. Then, this is followed by codon optimization to the target gene, which ensures that soluble protein is expressed in *E. coli*. Obtaining a large amount of the StMsn2 protein is crucial for further studying its function and mechanisms, as the research of StMsn2 in *S. turcica* is still in the initial stage. Further, the target gene fused with tag heterologously expresses in *E. coli* is the most preferred method for detecting and purifying the protein.

Antibody preparation technique, which is an important tool in modern life science research, plays an indispensable role in the study of gene function and the immunological diagnosis in humans, animals, and plants. Whole-protein antigens or synthetic peptides are usually used to immunize animals to obtain antibodies using the traditional methods, which is time-consuming and laborious [35]. However, the immune cells usually do not recognize the entire antigen molecule and only distinguish a specific part of the antigen, which is called the epitope antigenic determinant [36]. In the present study, StMsn2 polyclonal antibodies were prepared using the antigen epitope method, which was more effective, convenient, and time-saving. The antibody can be used as an important material in other experiments such as chromatin immunoprecipitation, to determine the downstream target genes regulated by StMsn2. The exogenous StMsn2 protein expressed in the prokaryotic cells can also be used as an important material for gel retardation experiment to verify the target genes regulated by StMsn2 in vitro. Therefore, the heterologous protein expressed in the prokaryotic expression system can be widely used, especially in revealing the functions and the mechanisms of transcription factors in eukaryotes.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary material

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