Full Length Research Paper

Expression and characterization of a novel spore wall protein from *Nosema bombycis*

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Microsporidia are obligate intracellular, eukaryotic, spore-forming parasites. The environmentally resistant spores, which harbor a rigid cell wall, are critical for their survival outside their host cells and host-to-host transmission. The spore wall comprises two major layers: the exospore and the endospore. In *Nosema bombycis*, five spore wall proteins have been identified which contain two endosporal proteins (SWP25 and SWP30) and three exosporal proteins (NbSWP5, SWP26 and SWP32). In the current study, we identified a novel endosporal protein NbSWP12 with calculated molecular mass of 25.56 kDa and pI of 6.69 using SDS-PAGE and MALDI-TOF MS technique. Followed by gene cloning and protein expression, polyclonal antibody production, indirect immunofluorescence antibody test, and immunoelectron microscopy analysis, the results indicate that this protein is localized to the endospore and has no obvious enhancement on adherence to host cells. The characterization of this novel spore wall protein from *N. bombycis* may facilitate our further investigation of the relationship between *N. bombycis* and its host, *Bombyx mori*.

Key words: Microsporidia, *Nosema bombycis*, Spore wall protein, NbSWP12.

INTRODUCTION

Microsporidia, which are unicellular eukaryotes and obligate intracellular parasites, have long been recognized as pathogenic agents in sericulture, apiculture, and mammals (Wittner, 1999). Microsporidia were previously divided into primitive eukaryotes, however, more and more molecular evidences based on the recent phylogenetic analyses of rDNA sequences, conserved proteins and the complete genome sequences of microsporidia *Encephalitozoon cuniculi* have demonstrated that these organisms are phylogenetically related to the fungi with remnant mitochondrial organelles (Hirt et al., 1999; Keeling et al., 2000; Fabienne et al., 2004; James et al., 2006; Goldberg et al., 2008; Lee et al., 2008). To date, the identified microsporidia have approximately 160 genera and 1300 described species (Corradi et al., 2008). Of these, at least 13 species are reported to infect humans (Dider et al., 2008), and five microsporidian genera have been found to infect the silkworm *Bombyx mori* (Bhat et al., 2009). *Nosema bombycis*, as the causative agent of silkworm pebrine (Naegli, 1857), ravaged the silkworm industry of Europe, especially in Italy and France during the mid-19th century...
These results propose reliable experimental data for the gene cloning and protein expression, polyclonal antibody production, and provide foundation for further study of the mechanism of microsporidia of *N. bombycis*.

**MATERIALS AND METHODS**

**Production and purification of *Nozema bombycis***

*N. bombycis* was originally isolated from infected silkworms in Zhejiang, China. Spores were propagated and purified as previously described (Zhang et al., 2007). Briefly, spores were harvested from infected moths. The fifth molted silkworm larvae were challenged by feeding on mulberry leaves artificially polluted by *N. bombycis* spores (10⁸ spores/200 larvae). The moths developed from the infected fifth larvae were dissected, homogenized, and centrifuged. Spores were purified on Percoll and centrifuged at 21,000 g for 90 min (Canning et al., 1999). Purified spores were stored in deionized water supplemented with antibiotics (Sigma, 100 mg/ml streptomycin, 100 U/ml penicillin) at 4°C for later use (Gatehouse and Malone, 1998).

**Spore wall proteins extraction and MALDI-TOF MS analysis**

Spore wall proteins of *N. bombycis* were extracted as described previously (Wu et al., 2008) with slight modifications. Briefly, 10⁸ spores were disrupted in a lysis buffer (Takara, 0.1M DTT, 4% CHAPS and 0.2% SDS) adding acid-washed glass beads (Sigma, diameter: 425 - 600 μm) with a FastPrep-24 (MP BIO). Following, proteins were incubated in an extraction buffer containing 2 M thiourea, 7 M urea, 0.1 M DTT, 4% CHAPS and 0.2% SDS for 6 h at room temperature. After centrifugation for 10 min at 20,000 g, the supernatant was collected and the samples were stored at -80°C for later use.

After quantification with Plus-One 2D Quant kit (Amersham), the proteins samples were analyzed by standard SDS-PAGE on 12.5% polyacrylamide gels and stained with Coomassie Blue. Protein bands were then excised for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis as described previously (Cai et al., 2011). The generated data were used to search the UniprotKB/SwissProt database using the software GPS Explorer (version 3.6, Applied Biosystems) and MASCOT (version 2.1, Matrix Science) with the following parameters: trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance set to 100 ppm, fragment tolerance set to ± 0.3 Da, and minimum ion score confidence interval for MS/MS data set to 95%.

**Recombinant protein expression of spore wall protein NbSWP12 and polyclonal antibody production**

The NbSWP12 protein was expressed with prokaryotic expression vector pET-30a (Novagen). Based on the genomic DNA sequences of *N. bombycis* (GenBank: EF683112.1), polymerase chain reaction primers were designed using Primer 5.0 software (Premier Inc.) as forward primer: 5'-GCCGGAATCCATGAAAGATTTTAAAAAG-3' and reverse primer: 5'-TCGAGCTTACATTAGTCCCTCTTATGC-3'; the forward primer and reverse primers contained BamHI and Hind III restriction site (underlined) respectively. These primers amplified a 687-bp genomic DNA fragment corresponding to the amino acid regions 1-228 of the 228-amino acid *N. bombycis* protein.

PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 5 min, 35 amplification cycles (denaturing at 95°C for 45 s, annealing temperature at 52.6°C for 45 s, and extension at 72°C for 1 min), and a final
extension step at 72°C for 10 min. The PCR products were analyzed on 1% agarose gels with ethidium bromide staining, the primer-specific product band with the expected size was excised from the gel and recovered with QiAquick PCR purification kit (Qiagen) following the protocol of the manufacturer.

The amplified products were digested with BamHI and Hind III and inserted into a BamHI/Hind III-digested prokaryotic expression vector pET-30a. The resultant recombinant plasmids were transformed into Escherichia coli Transetta (DE3) competent cells and expression of recombinant NbSWP12 was induced for growth for 6 h at 37°C in the presence of 1 mM isopropyl-b-thio-galactopyranoside (IPTG). The expressed fusion proteins were then purified with His-Bind Purification Kit (Novagen) from recombinant protein expression of spore wall protein NbSWP12. Mono specific polyclonal antiserum against the purified recombinant NbSWP12 was produced by immunization of native rabbit using the standard 56-day antibody production protocol. Meanwhile, a rabbit was injected with PBS, its sera were collected and stored at -20°C and used as a negative control. The animal house and experimental staff were approved by Chinese veterinary services, and experiments were conducted in accordance with ethical guidelines.

Immunoblotting analysis

N. bombycis proteins were extracted according to the procedure as previously described (Wang et al., 2007). Briefly, N. bombycis spores (10^9 cells) were suspended in a lysis buffer, containing 100 mM DTT, 4% CHAPS and 0.5% Triton X-100 to protect the protein from disruption, and 0.5 g acid-washed glass beads (0.425-0.600 microns, Sigma) were added in a 1.5-mL Eppendorf tube, followed by vigorously shaking for 2-3 min at maximum speed on the Fast-Prep24. The homogenate was transferred to a fresh Eppendorf tube, containing 200 μL extraction buffer (0.2% (w/v) SDS, 4% (v/v) CHAPS, 100 mM DTT, 2M thiourea, 7 M urea), and incubated at room temperature for 6 h. After centrifugation at 3,000 g for 5 min, the supernatant was collected as protein samples and stored at -80°C for later use.

For immunoblotting analysis, protein samples were subjected to SDS-PAGE on 12.5% polyacrylamide gels. Electrophoresis, transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore) and blocking were performed under standard conditions. Anti-NbSWP12 sera (1:200 dilution) was used as the primary antibody. The secondary antibody, a goat anti- rabbit IgG-IgM antiserum (Sigma) labelled with peroxidase, was detected by addition of the substrate tetrahydrochloride (DAB).

Indirect immunofluorescence assay analysis (IFAs)

For indirect immunofluorescence assay analysis, BmN cell line derived from the ovary of B. mori was cultured in TC-100 insect cell culture medium (Sigma) in glass bottom culture dishes (GBD-35-20, Nest Biotechnology Co.) at 27°C, supplemented with 10% fetal bovine serum(GibcoBRL Life Technologies) and 50 μg/mL of gentamycin (Takara). Approximately 1 × 10^6 cells with 2 ml growth medium in each dish were allowed to grow at 27°C for 16 h. Recombinant proteins (0.1-10 μg/ml), as well as the control sample (negative serum), were incubated with 5.0×10^6 N. bombycis spores on monolayers for 4 h at 28°C. The culture dishes were thoroughly washed with PBS to remove unbound spores. To identify host cell infection, fresh cell culture medium was added and incubated at 28°C for additional 48 h. An immunofluorescent assay was performed to treat these samples and the average numbers of spores bound to per host cell were calculated by counting microsporidia in at least 30 host cells of magnification. The results were shown as the inhibition percentage of adherent spores relative to the control samples at least 30 host cells of 400× magnification. The significance of the differences between the control and experimental assays were measured using the two-tailed Student's t-test in the Statistical Package for Social Science (version 12.0, SPSS). P values of 0.001 or less were considered statistically significant. The aforementioned experiments were repeated three times with similar results.

RESULTS

Identification of a spore wall of N. bombycis NbSWP12

The band for protein NbSWP12 with calculated molecular mass of 25.56 kDa and pl of 6.69 was excised and analyzed by MALDI-TOF-MS. It matched a 228-amino acid protein (Table 1) which was correspondent with that previously named as NbSWP12 under GenBank Accession number EF683112 (Wu et al., 2008). Analyses indicated that the protein possesses one predicted N-glycosylation site and 16 phosphorylation sites, but
Table 1. MALDI-TOF MS analysis of the spore wall protein NbSWP12 of Nosema bombycis.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>pI/MM (kDa)</th>
<th>Cov (%)</th>
<th>Mmobs (Da)</th>
<th>Mmcalc (Da)</th>
<th>Mmdiff (Da)</th>
<th>Position</th>
<th>Sequence</th>
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<tr>
<td>SWP12</td>
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<td>12.72</td>
<td>812.3641</td>
<td>812.3347</td>
<td>-0.0294</td>
<td>189-194</td>
<td>TIEMMR</td>
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<td>910.3835</td>
<td>125-131</td>
<td>FNEQCGR</td>
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<tr>
<td></td>
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<td>1167.4718</td>
<td>-0.0493</td>
<td>123-131</td>
<td>EKPNNEQCGR</td>
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<tr>
<td></td>
<td>1323.6539</td>
<td>1323.5967</td>
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<td>MNYFSDADIFEGFAR</td>
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</tr>
</tbody>
</table>

Expression of NbSWP12 fusion proteins and western blot analysis

To further characterise NbSWP12, we constructed the recombinant expression plasmid pET30-NbSWP12 and transformed the recombinant plasmids into the E. coli transetta (DE3) strain. The heterologously expressed proteins with about 31.95 kDa were purified by affinity chromatography using a His-Bind Purification Kit (Novagen). The NbSWP12-specific polyclonal antibody was generated by immunising rabbit with the purified fusion protein and used in western blot analyses. A single 26-kDa band was detected from the N. bombycis spore protein lysates (Figure 1), which is in agreement with the size calculated from the sequence. The result clearly demonstrated that the antiserum was successfully produced in rabbit and had strong reactivity to NbSWP12.

Expression of NbSWP12 in Nosema bombycis spores

The antiserum to NbSWP12 was used in an immunofluorescence assay with purified mature spores of N. bombycis added in BmN cells (Figure 2A1). Little to no fluorescence signal was detected in the control (Figure 2B1). Rabbit anti-NbSWP12 sera were shown to bind to N. bombycis spores (Figure 2 A1, A3). The fluorescence patterns were consistent with these proteins reacting specially with the spore walls, showing strong immunofluorescence in the vitro-infected host cells, and also in the purified mature spores, which display a bright signal and are readily recognized at 1,000× magnification. The existence of spores was confirmed by DAPI staining (Figure 2 A2, B2).

Location of NbSWP12 in the N. bombycis parasite

IEM was employed to determine the cellular location of the NbSWP12 protein. The mature spores were postfixed with 1% osmium tetroxide, and then were treated using immunogold. As shown in Figure 3, a number of gold particles were distributed along the endospore regions of
the spore wall but few in the exospore regions (Figure 3). No gold particles were detected in the control sample using control sera (data not shown). These results implied that NbSWP12 may be located in the endospore.
The effect of exogenous NbSWP12 protein on N. bombycis attachment and host cell invasion

A host cell invasion experiment was performed to determine whether SWP12 functions in the spore attachment process, and the generated data are shown in Table 2. Statistical analysis shows that there were no statistically significant differences in spore adherences or host cells infection compared with that of control samples.

DISCUSSION

As a group of eukaryotic intracellular parasites, microsporidia infect almost all vertebrates and invertebrates (Didier et al., 1998). As composition of rigid spore wall, spore wall proteins play an important role in microsporidian invasion (Southern et al., 2007; Frixione et al., 1992). However, little is currently known about the components of the spore wall. Only seven spore wall proteins in E. cuniculi and E. intestinalis were identified by monoclonal or polyclonal antibodies (Bohne et al., 2000; Brosson et al., 2005; Peuvel-Fanget et al., 2006; Southern et al., 2007; Xu et al., 2006; Hayman et al., 2001). Among fourteen hypothetical spore wall proteins from N. Bombycis deposited in GenBank data, only five complete spore wall proteins have been identified, which contain two endosporal proteins (SWP25 and SWP30) and three exosporal proteins (NbSWP5, SWP26, and SWP32) (Wu et al., 2008; Wu et al., 2009; Li et al., 2009; Cai et al., 2011).

In the current study, based on protein technique, MALDI-TOF MS analysis, and on the GenBank data, we have identified a new spore wall protein named as NbSWP12 in the silkworm parasite N. Bombycis. Sequence analysis demonstrated the protein to have a calculated molecular mass of about 25.56-kDa that is distinct from the previously reported spore wall proteins. IEM data implied NbSWP12 was located into endospore, even so there is much work to be done in order to attain an accurate positioning of this protein. Only the function of spore wall proteins has been illuminated, can we execute some related downstream research?

Little reduction of spore adherence or host cells infection compared with that of control samples was implied by the following spore adherence and host cell infection assays. It may be presumed that NbSWP12, as a supposed endospore wall protein, may not be a surface adherence ligand or not the main adherence factor, and that this protein is more likely to be involved with spore wall construction/maintenance than the infection process. Further studies on the molecular function of NbSWP12 on parasite-host interactions are currently carried out in our laboratory, which will help us to clarify the infection mechanism of N. Bombycis.

In summary, studies of the spore wall proteins should facilitate our further investigation of the relationship between these ubiquitous pathogens and their hosts, B. mori, which is beneficial for us to control the silkworm, B. mori pebrine disease in sericulture.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES


