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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 (*Nb*SWP5, SWP26 and SWP32). In the current study, we identified a novel endosporal protein *Nb*SWP12 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, *Nb*SWP12.

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

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(Becnel and Andreadis, 1999). Even now, it is still epizootic and causing heavy economic losses in silk-producing countries such as China.

Although microsporidia have no active stages outside their host cells and can survive only by living inside cells, all microsporidia produce environmentally resistant spores with a unique extrusion apparatus that contains a coiled polar tube ending in an anchoring disc at the apical part of the spore (Vavra et al., 1999). Under appropriate conditions inside a suitable host, the polar tube is discharged through the thin anterior end of the spore thereby penetrating a new host cell and inoculating the infective sporoplasm through the hollow tube into the new host cell (Bigliardi and Sacchi, 2001). The sporoplasms released into host cells then enter a proliferative phase, undergoes the sporogony, sporonts, and the sporoblasts stages and then differentiates into mature spores protected by a thick wall. The spore wall, which consists of an electron-dense, proteinaceous outer layer (exospore), an electron-lucent inner layer (endospore) composed of chitin and protein, and a plasma membrane (Vavra et al., 1999), provides structural rigidity and protects the mature spore from the outer environment. The spore wall proteins (SWPs) of microsporidia may play a role in recognition by the host during the invasion process (Hayman et al., 2005; Southern et al., 2006), and the interactions between the SWPs and the host plasma membrane mediate the microsporidian spores being phagocytized by the host cell (Couzinet et al., 2000; Franzen et al., 2005).

Due to little sequence similarity between the known microsporidial spore wall proteins with any other eukaryotic proteins, it is very difficult to identify a new spore wall protein. To date, only a total of seven spore wall proteins have been reported in *E. cuniculi* (Bohne et al., 2000; Brosson et al., 2005; Peuvel-Fanget et al., 2006; Southern et al., 2007; Xu et al., 2006) and *Encephalitozoon intestinalis* (Southern et al., 2007; Hayman et al., 2001), and four spore wall proteins from *N. bombycis* including SWP25, SWP30, SWP26 and SWP32 were addressed on the location and function (Wu et al., 2008; 2009; Li et al., 2009). In our previous study, we identified an exosporal protein *Nb*SWP5 that can protect spores from phagocytic uptaking by cultured insect cells (Cai et al., 2011).

In the current study, we identified another endosporal protein *Nb*SWP12 which was found in mature spores of *N. bombycis*. Its location and function were elucidated through matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique, gene cloning and protein expression, polyclonal antibody production, indirect immunofluorescence antibody (IFA) test, and immunoelectron microscopy(IEM) analysis. These results propose reliable experimenta data for the interaction of endosporal protein *Nb*SWP12 with *N. bombycis vitro* and *vivo*，and provide foundation for further study of the mechanism of microsporidia of *N. bombycis*.

MATERIALS AND METHODS

Production and purification of *Nosema 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 (10^8 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, 10^9 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 *Nb*SWP12 and polyclonal antibody production

The *Nb*SWP12 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′-GCGGATCCATGAAAGATTTTAAAAAG-3′ and reverse primer: 5′-TCAAGCTTACTTAGTCCTCTCTAATGC-3′; the forward primer and reverse primers contained *Bam*H I 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 *Nb*SWP12 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 *BamH*I and *Hind*□ and inserted into a *Bam*HI/*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 *Nb*SWP12 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 *Nb*SWP12. Mono specific polyclonal antiserum against the purified recombinant *Nb*SWP12 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-*Nb*SWP12 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 were allowed to grow at 27°C for 16 h. The purified *N. bombycis* spores (10^7 cells) were added into the cell culture medium and host cells were incubated for 24 h at 28°C. The host cells including some *N. bombycis* spores were fixed with 100% methanol for 10 min at 4°C, and then permeabilised with 0.5% Triton X-100 for 30 min. Incubated with polyclonal antisera or a negative serum, the slides were kept in a moist chamber for 60 min at 37°C. Bound antibodies were detected with 1:64 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma). DNA was stained with 1 mg/ml DAPI for 20 min. The spores were examined with an OLYMPUS BX50 immunofluorescence microscope (excitation wave WL of FITC-IgG: 495 nm; DAPI: 359 nm; magnification, 1000×).

Ultrastructural localization by immunoelectron microscopy (IEM)

N. bombycis was prepared as described above, and IEM was performed as previously described (Wu et al., 2008). Briefly, the mature spores were fixed with 3.0% (v/v) paraformaldehyde and 1.0% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, and then rinsed five times (15 min each time) in PBS buffer (pH 7.2) at 4°C. To enable samples to be imbedded and photopolymerised in K4M resin, the fixed spores were dehydrated with graded ethanols and sequentially permeabilised in K4M at -20°C. Ultrathin sections were placed on 300-mesh nickel grids coated with Formvar and carbon. In order to immunostaining, these grids were transferred into a blocking solution, obtaining 1% bovine serum albumin (BSA), 0.02% polyethyleneglycol mol. wt. 20,000 (PEG 20,000) and 0.1 M NaCl, 1% NaN3, for 30 min, and then incubated with a 1:200 dilution of the primary anti-*Nb*SWP12 polyclonal antibodies, or a negative serum or polyclonal antibodies against total proteins of *N. bombycis*, in blocking solution for 1 h at 25°C, followed by incubation with a 1:100 dilution of the gold conjugated anti-rabbit IgG (Sigma, 10 nm ID) for 1 h at 25°C. Grids were rinsed five times in PBS buffer (pH 7.2), stained with uranyl acetate and lead citrate, and then dried, examined, and photographed with a JEM-1230 transmission electron microscope (JEOL).

Spore adherence assays and infectivity assays

BmN cells were used as host cells to measure adherence of *N. bombycis* spores and infection. Adherence of *N. bombycis* spores and infection were measured as previously described (Hayman et al., 2005; Wu et al., 2009). 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 Nb*SWP12

The band for protein *Nb*SWP12 with calculated molecular mass of 25.56 kDa and pI 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 *Nb*SWP12 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 *Nb*SWP12 of *Nosema bombycis.*

Protein ID	pI/MM (kDa)	Cov (%)	Mmobs (Da)	Mmcalc (Da)	Mmdiff (Da)	Position	Sequence
SWP12	6.49/25.56	12.72	812.3641	812.3347	-0.0294	189-194	TIEMMR
			910.3835	910.3457	-0.0378	125-131	FNEQCGR
			1167.5211	1167.4718	-0.0493	123-131	EKFNEQCGR
			1323.6539	1323.5967	-0.0572	178-188	LSELFENSQTR
			1423.6998	1423.6396	-0.0602	63-75	IYHGLSMVSSASR
			1458.7223	1458.6606	-0.0617	211-222	DLNIEFHQESVK
			1461.7697	1461.7043	-0.0654	195-208	DFIGADGLQGVLTR
			1798.7740	1798.6978	-0.0762	76-90	MNYFSDADIFEGFAR

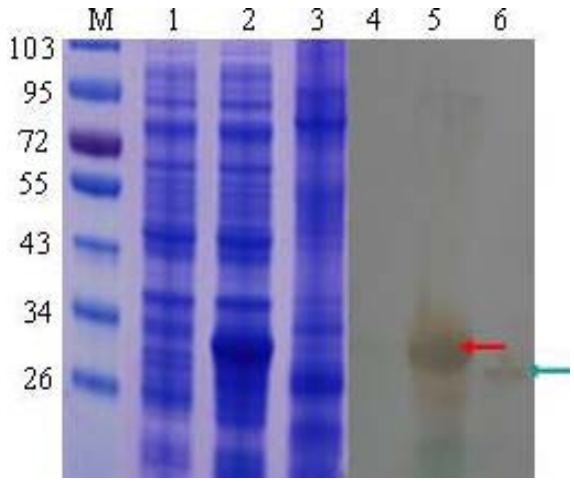

Figure 1. SDS-PAGE and western blot analysis of spore protein SWP12 of *Nosema bombycis*. *N.bombycis* spore proteins were run on a 12% SDS-PAGE gel and detected with anti-*Nb*SWP12 polyclonal antibody. M, molecular mass standards; lane 1 to lane 3, SDS-PAGE analysis; lane 1, crude extract from *E. coli* transetta (DE3) with pET30 vector induced by 1 mmol/L IPTG; lane 2, crude extract from *E. coli* transetta (DE3) with pGT30-SWP12 induced by 1mmol/L IPTG; lane 3, crude extract of *N.bombycis* spore proteins; lane 4 to lane 6, western blot analysis, lane 4, no band of normal rabbit sera (1:200 dilution) as negative control of the lane1; lane 5, apparent fractions (˜31.95 kDa) of the anti-recombinant *Nb*SWP12 sera (1:200 dilution) after His-Binding affinity chromatography; lane 6, only tenuous band (˜26kDa) of *N. bombycis* spore proteins (1:200 dilution) as positive control of the lane 3. The blotting bands are indicated by red arrowhead and blue arrowhead, respectively.

neither *O*-glycosylation sites nor transmembrane domain are predicted (http://www.cbs.dtu.dk). *Nb*SWP12 is predicted to be an extracellular (for example, cell wall) protein (http://www.psort.org/) by in silico analysis, but it possesses no amino-acid signal peptides (www.cbs.dtu.dk/services/SignalP/).

Expression of *Nb*SWP12 fusion proteins and western blot analysis

To further characterise *Nb*SWP12, we constructed the recombinant expression plasmid pET30-*Nb*SWP12 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 *Nb*SWP12-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 *Nb*SWP12.

Expression of *Nb*SWP12 in *Nosema bombycis* spores

The antiserum to *Nb*SWP12 was used in an immunofluorescence assay with purified mature spores of *N. bombycis* added in BmN cells (Figure 2A_1). Little to no fluorescence signal was detected in the control (Figure 2B_1). Rabbit anti-*Nb*SWP12 sera were shown to bind to *N. bombycis* spores (Figure 2 A_1, A_3). 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 A_2, B_2).

Location of *Nb*SWP12 in the *N. bombycis* parasite

IEM was employed to determine the cellular location of the *Nb*SWP12 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

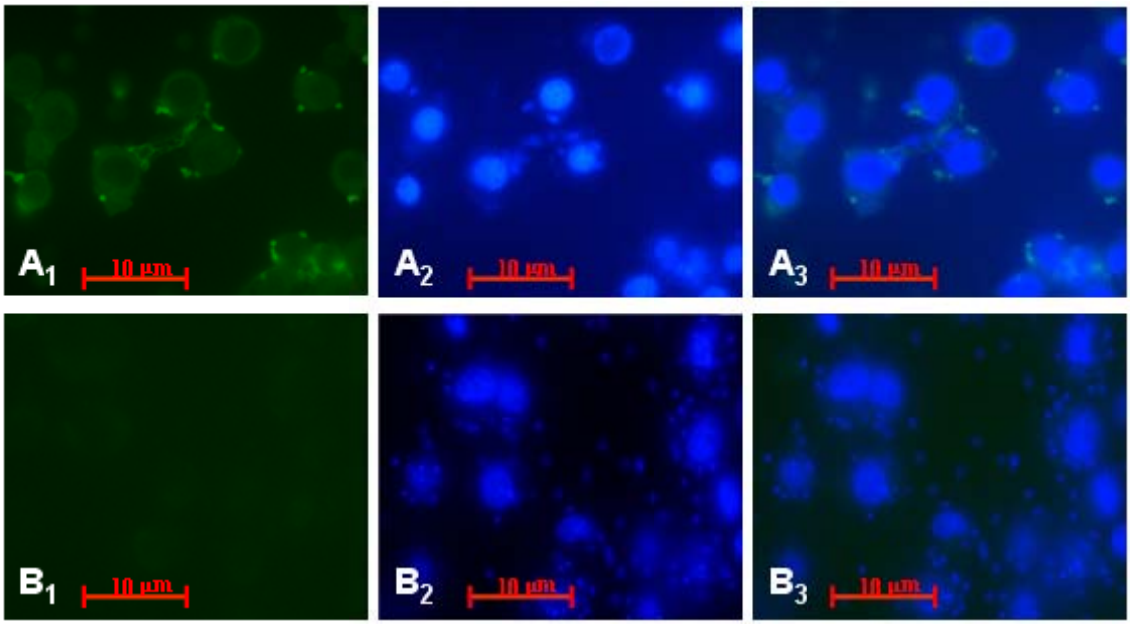

Figure 2. Indirect immunofluorescence assay with rabbit anti-*Nb*SWP12 showing labeling of the intracellular parasite and the purified mature spores. *N. bombycis* spores infected BmN cells and purified mature spores (A) are visualized with a fluorescence microscope, after incubation with the primary antibodies against *Nb*SWP12 (A_1). Image B_1 is the negative control, image A_2 and B_2 are DAPI staining. A_3 and B_3 are the merged images of A_1 and A_2, B_1 and B_2, respectively. Anti-*Nb*SWP12 was used at a 1:100 dilution. The secondary antibody was FITC-conjugated goat anti rabbit IgG(Sigma) used at a 1:64 dilution. All images are magnified 1000×. The scale bar represents 10 μm.

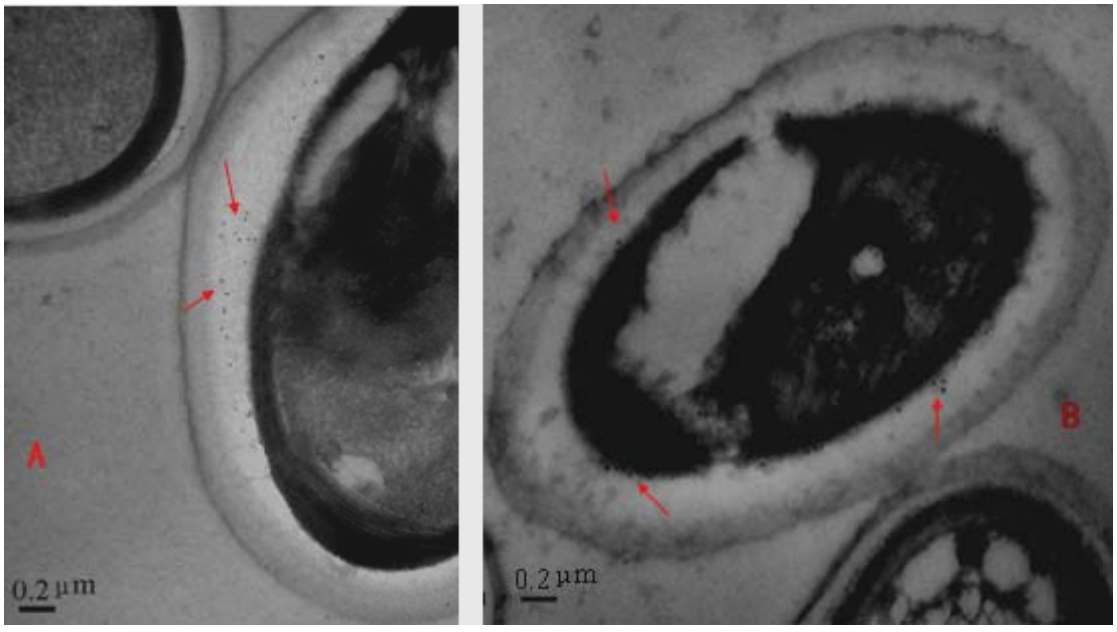

Figure 3. Immunogold electron microscopy of *N. bombycis* using the anti-*Nb*SWP12 polyclonal antibodies and secondary antibodies conjugated with 10nm colloidal gold (Sigma). Immunogold labeling of *Nb*SWP12 demonstrated the location of *Nb*SWP12 in the endospore of *N. bombycis*. The colloidal gold particles are marked by red arrowheads. The scale bar represents 200 nm.

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 *Nb*SWP12 may be located in the endospore

Table 2. Statistics of the spore adherence and host cell infection assay with exogenous recombinant *Nb*SWP12 protein.

Exogenous *Nb*SWP12 protein (μg/mL)	0	0.5	1.0	2.0	5.0
Adherence spores/host cell (average)	15.67	14.56	15.67	14.66	14.55
Percentage of infected cells (%)	25.33	24.61	25.33	24.66	24.52

region of the *N. bombycis* spore.

The effect of exogenous *Nb*SWP12 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 (*Nb*SWP5, 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 *Nb*SWP12 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 *Nb*SWP12 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 *Nb*SWP12, 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 *Nb*SWP12 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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