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Reduced infectivity and environmental resistance of microsporidian *Nosema bombycis* after spore wall proteins-removal treatment

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***Nosema bombycis* is a highly virulent causative agent of the pebrine disease in sericulture. The only infective stage of the parasite is the environmentally resistant spore, on which the protein-rich spore wall supposedly contributes to their infection and resistance. In this study, we explore the potential roles of spore wall proteins (SWPs) of mature *N. bombycis* spores by evaluation of their performances on staining, infectivity, and resistance, following SWPs-removal treatment. Although no significant structural change is discerned by ultrastructural micrographs, the treated spores showed enhanced eosin B staining with strong shades of reddish purple or violet. Moreover, their infectivity to silkworms was significantly affected, resulting in 38.89% compared to 61.11% for the untreated control. Further infectivity bioassays show clearly that they are more susceptible to sodium chloride and heating pretreatment. In all, the enhanced permeability, decreased infectivity and environmental resistance of the SWPs-removal spores indicated that SWPs play multiple roles in the infection and resistance for *N. bombycis*. These results have significant implication for controlling the pebrine disease in sericultrue.**

Key words: *Nosema bombycis*, microsporidia, spore wall proteins, infectivity, resistance.

INTRODUCTION

Microsporidia are obligate intracellular fungal parasites parasitizing a broad spectrum of hosts form insects to most mammals, including human being (Keeling and Fast, 2002; Corradi and Keeling, 2009). Since the first description of the microsporidia, *Nosema bombycis*, in the mid-1850s as the causative agent of the pebrine disease which devastated the European silk industry, these pathogens demonstrate their great medical and economic importance. To date, over 1300 species (in 160 genera) have been identified (Corradi and Keeling, 2009). At least 14 species are reported as opportunistic pathogens particular in AIDS patients (Didier et al., 1998), and more than 150 species, including *N. bombycis*, are known to infect 12 orders of insect. They are considered as major threat to beekeeping, aquaculture, and sericulture (Texier et al., 2010).

The only infective stage of microsporidia is the environmentally resistant spore. The thick protein-rich two-layered spore wall enables them to survive under adversity outside of the hosts (Vávra and Larsson, 1999), thereby increasing the chance of infection. *N. heliothidis*, *Vairimorpha necatrix*, and *Encephalitozoon cuniculi* spores have been proved to survive direct sunlight, ultraviolet radiation (Brooks, 1988), high temperature (Maddox, 1977), acidic and alkaline condition treatment (Shadduck and Polley, 1978; Waller, 1979; Koudela et al., 1999) for a few hours or weeks. Several Encephalitozoon species remain infective for months to years outside their hosts (Shadduck and Polley, 1978; Waller, 1979). As for *N. bombycis*, in sterile water suspensions at 2 to 5°C, they were maintained to be viable for 10 years (Ohshima, 1964). Moreover, no obvious loss of infectivity was found when we used *N. bombycis* spores, stored at 4°C for five

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to six years, to infect silkworms (unpublished data). Although discovered for 160 years and increasing knowledge available, *N. bombycis* still remain ravaging silkworm industry, especially to China of about 70% breeding scale in world (Wan and Zeng, 2002). Further understanding of their resistance and weakness are necessary and meaningful for pebrine control. However, little evidences of environmental resistance of spores referring to proteins of spore wall were documented.

Proteins on spore wall were the first encounter for diverse environmental condition, and their existence and integrity are of great significance for spore viability. To address the significance, we compared the performance of staining and infectivity of *N. bombycis* spores with or without spore wall proteins (SWPs)-removal treatment. We found that the loss of SWPs not only affected the spore permeability, also decreased the infectivity and environmental resistance. This research facilitates us to further find antimicrosporidial treatment focusing on SWPs for conquering pebrine disease.

MATERIALS AND METHODS

Production and purification of *N. bombycis* spores

N. bombycis spores were propagated in laboratory-reared silkworm larvae. Following 14 days postinfection, spores were harvested and purified from the infected silk glands of the fifth instar larvae by a routine method as previously described (Weidner, 1976). Mature spore pellet was achieved through centrifugalization at 20,000 g at 4°C for 20 min on discontinuous Percoll (Pharmacia, Sweden) gradient (25, 50, 75, and 100%, v/v) (Canning et al., 1999), and then rinsed with physiological salt solution and stored in distilled water at 4°C until use.

SWPs-removal treatment

Proteins on spore wall (SWPs) of *N. bombycis* spores were removed by a modification of previously described methods (Irby et al., 1986; Wicher et al., 1991). Briefly, spore pellet (10^9 cells) was suspended in 100 μl 10% SDS (w/v) and 100 μl extraction buffer containing 20% sucrose, 4.6% SDS, 10% β-mercaptoethanol (v/v) and 0.125 mol l-1 Tris, followed by EDTA addition with final concentration of 100 mM and incubation in 30°C water bath for 30min. Then, the mixture was centrifuged at 1,000 g at 4°C for 10 min. The supernatant was collected and stored at -80°C, and the treated spore pellet was washed 6 times with distilled water and adjusted final concentration to 10^7 spores/ml for use.

Transmission electron microscopy (TEM) analysis

TEM analysis was employed to examine the changes of ultrastructure of *N. bombycis* spores before and after the SWPs-removal treatment. Spores were precipitated by centrifuging at 1000 g for 15 min. The resulting pellet was then prefixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) solution for 2 h. The prefixed sample was washed with 0.1 M PBS, and postfixed in 1.0% osmium tetroxide in cacodylate buffer for 2 h, followed by routine ethanol dehydration and sequential Epon-Araldite 812 (Epon 812) infiltration. Then the sample was embedded with pure Epon 812. Ultrathin sections were obtained using glass knife on

Leica ultracut cut A-1170 and collected on Formvar-coated nickel grids. The sections were then stained with uranyl acetate and lead citrate, and examined with HITACHI H-7500 transmission electron microscope at an accelerating voltage of 80 KV.

Eosin B staining

SWPs-removal spore sample was spread on the clean microscope slides. After air-dried, spores on the slide were flooded with 1% eosin B solution at 25°C for 10 min, followed by gentle washing to remove dye molecules that yet enter the spore, air-dried, and then processed by a Panasonic WV-CP230 digital camera system under 640 magnification (40×16) for microscope examination. The intact spores treated in the same way served as control.

Infection of *N. bombycis* to silkworm

Silkworm larvae were fed with fresh mulberry leaves at room temperature. On the first day of second instar, each group of larvae was fed with air-dried leaves smeared by 5 ml of spore suspension (10^7/ml). 8 h later, the larvae were fed with fresh leaves as routine procedures. Those fed only with *N. bombycis*-free leaves were set as the negative control. Each assay was performed in triplicate with thirty silkworm larvae. Morphological and anatomical observations were to help identify larval infected by *N. bombycis*. Mortality in each group was recorded daily.

To assess performance of SWPs-removal *N. bombycis* spores to environmental stress, NaCl solutions and temperatures mimicking the adverse conditions were used to incubate the spores. Briefly, spores were incubated with 1, 2 and 4% NaCl solutions for 1 h at room temperature, respectively, and wash them down extensively. In temperature pretreatment, spore sample was put into chamber at 35, 40 and 45°C for 2 h. After the treatment, both the spores was washed six times, and a final concentration of 10^7/ml was adjusted for infection assay as described earlier.

RESULTS

Ultrastructural change of the spores

Ultrastructural examinations of the SWP-removal treated and untreated *N. bombycis* spores indicated that the structure of spore was unaffected (Figure 1). However, the SWPs-removal spores demonstrated thinner exspore (Figure 1d-f), as compared to the intact spores (Figure 1a-c). Moreover, unknown substances were easily to enter and aggregate in the endospore (EN) of SWPs-removal spores, as representative in Figure 1f.

Evaluation of spore stain performance

Eosin B is one of the most widely used acid dyes for histological staining. In the case of SWPs-removal *N. bombycis* spores staining (Figure 2b), the color of mature spores turned to be darkly stained with various shades of reddish purple or violet, whereas in the case of SWPs-intact spores (Figure 2a), slight staining with faint blue to light red was readily seen, and even their original color of the spores were retained. The obvious difference
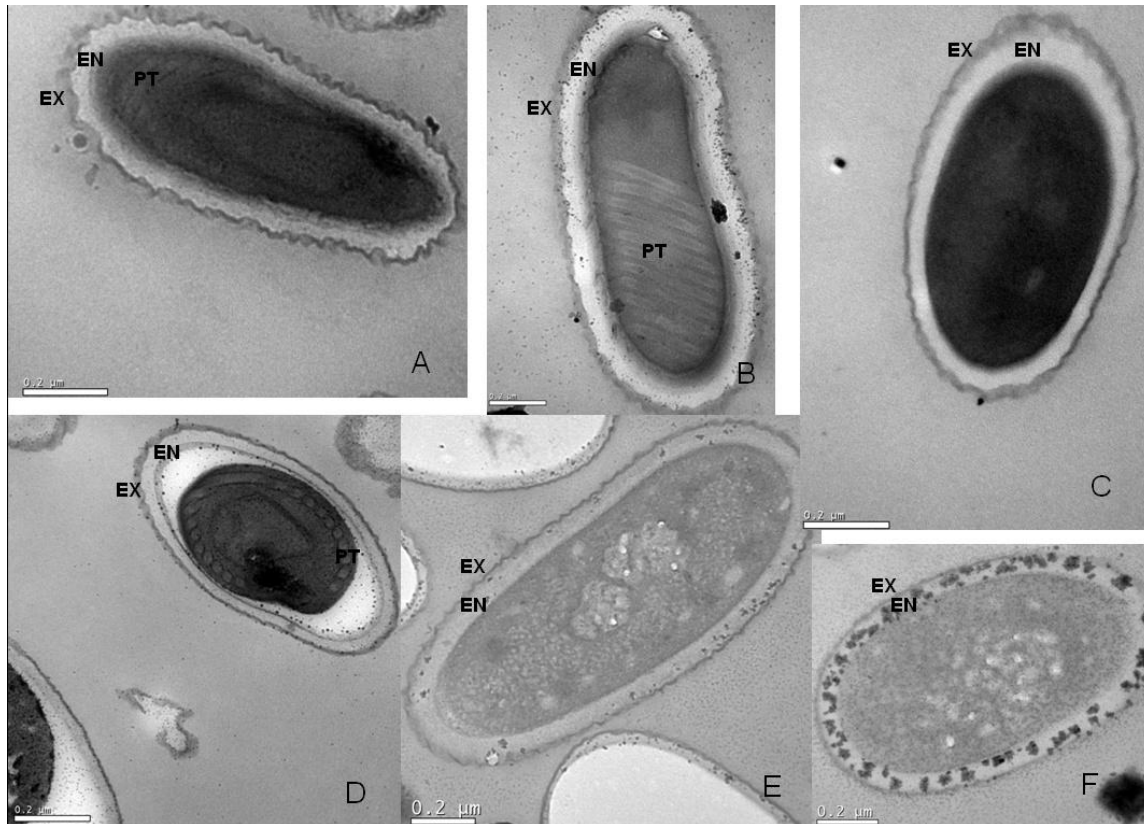
Figure 1. Ultrastructural morphological characteristics of purified *N. bombycis* spores with SWPs-intact (a-c), and SWPs-removal (d-f) generated by transmission electron microscopy. After SWPs-removal treatment, the treated spores show thinner exosore, but remain structurally unimpaired (d-f). EN, endospore; EX, exospore; PT, polar tube. Bar=200 nm.

indicated that loss of SWPs resulted in increase in spore permeability, and facilitated dye ion influx to spores.

Infectivity of SWPs-removal *N. bombycis* spores

Bioassay of silkworms with SWPs-removal spores in comparison with intact spores was given in Figure 3. The cumulative mortality rate generated from SWPs-removal spores was much lower, resulting in 38.89% compared to 61.11% for the intact spores, resulting a 22.22% reduction. In addition, the primary tissues such as midgut and silk gland were confirmed to be infected with fewer spores by microscopic examination (data not shown) and a delayed onset of the disease was observed when the SWPs-removal spores were used in the bioassay. Those showed loss of SWPs resulted in reduced infectivity.

Resistance of SWPs-removal *N. bombycis* spores

To examine their resistance to adverse conditions, we pretreated SWPs-removal spores with NaCl solutions and temperatures as mimic environment press prior to infectivity assay. As could be seen from the mortality, the cumulative mortality rate decreased in the two cases with dose-dependent pattern but differed between different pretreatment (Figure 4). Mortality rate of silkworm infected with SWPs-removal spores with lower NaCl solution preincubation was significantly higher than that with higher concentration of NaCl solution. The rate was 37.78% for 1%, 32.33% for 2%, and 16.67% for 4% NaCl pretreatment, respectively (Figure 4). Interestingly, despite of the varying concentration pretreatment, the rate of intact spores remained stable around 60%, approaching that of intact spores suffering from no treatment before the assay (Figure 3), and even high concentration NaCl treatment, if any, could have slight effect on them. In the assay with heating pretreatment, the SWPs-removal spores are more susceptible to temperature with infectivity reduced by 22.22, 21.11, and 29.89% at 35, 40 and 45°C, respectively, as compared to the intact spore assay (Figure 5). As for the intact spores, they are susceptible with the heating treatment too, although the cumulative rate remains stable around 40%. Those dataset demonstrate that spores with intact SWPs
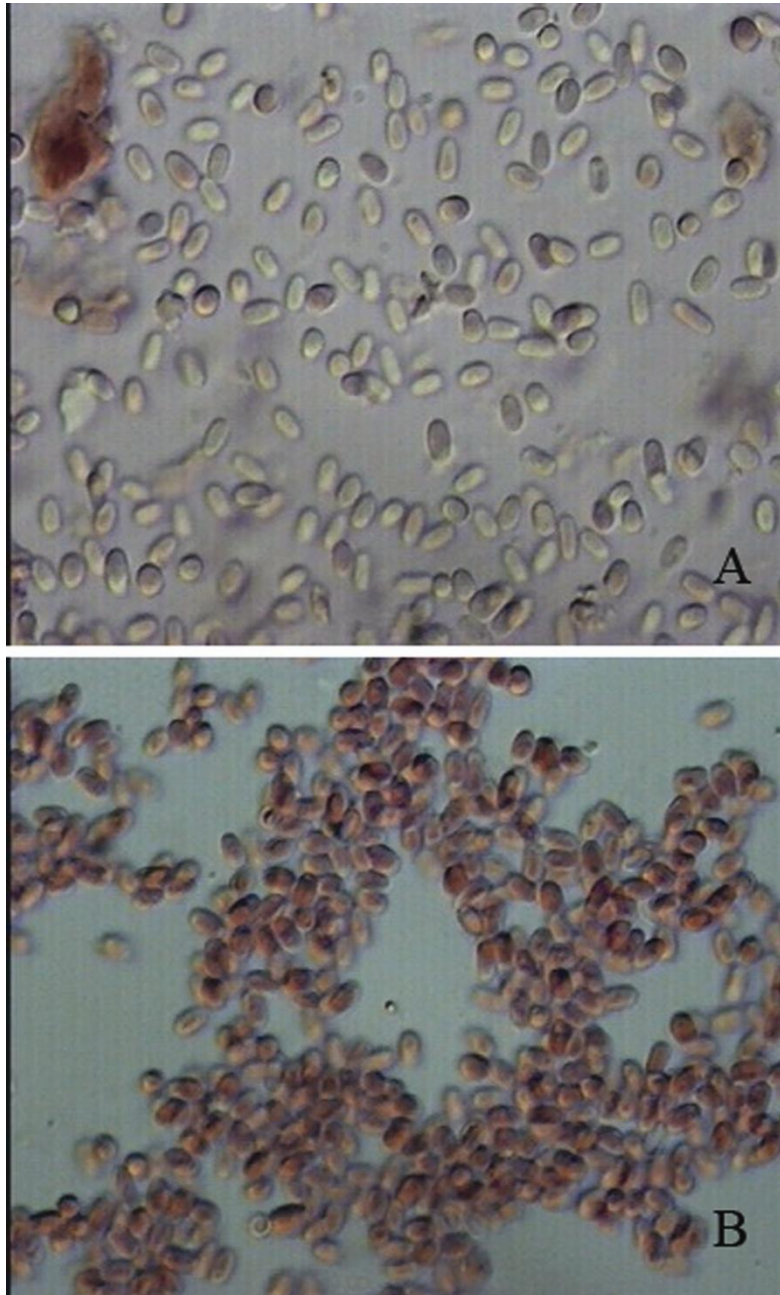
Figure 2. Staining performance evaluation. Stained with 1% eosin B, the untreated *N. bombycis* spores showed faint coloration, and some retained their original color (a), while SWPs-removal ones represented a strong staining with various shades of reddish purple or violet (b).

are more resistant to NaCl and temperature treatment than impaired ones.

DISCUSSION

Production of silkworm seed is an opening system in many silkworm-breeding countries. Several genus of microsporidia, such as Nosema (Nägeli, 1857), Thelohania, Vairimorpha (Fujiwara, 1980), Pleistophora (Fujiwara, 1984) and Endoreticulatus (Wan et al., 1995), are pathogenic to the silkworm, among which, *N. bombycis* is the most destructive for the silkworm seed production due to their 100% transovarial transmission.

The structure of different microsporidia is highly similar, including the thick bilayer spore wall, comprising the
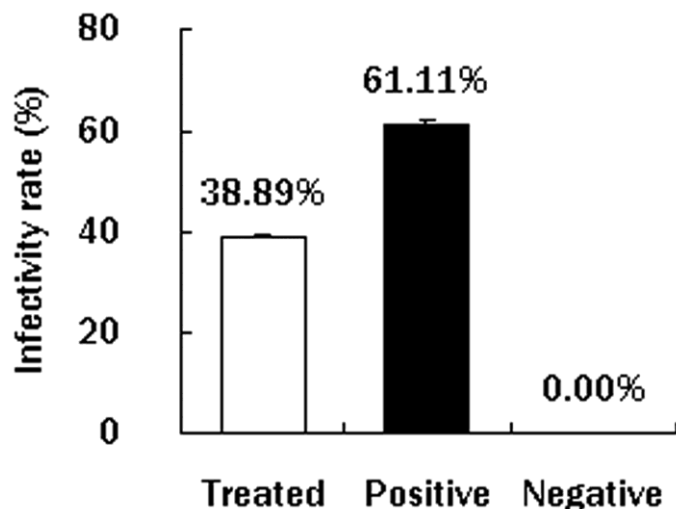
Figure 3. Removal of the SWPs causing decreased infectivity. Spore infectivity to silkworms was significantly affected for removal of SWPs (Treated), comparing to that of intact spores (Positive), and double distilled water as a negative control (Negative). The data shown above bars were mean of three separate infectivity assays with similar results.
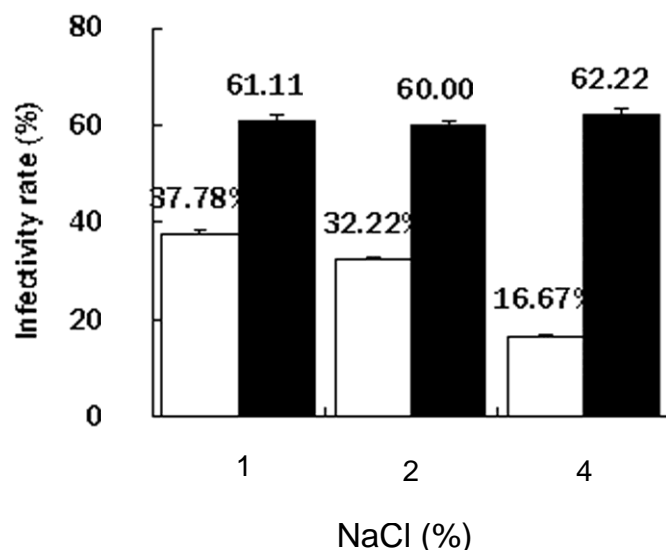

Figure 4. Effects of sodium chloride (NaCl) pretreatment on infectivity of SWPs-removal *N. bombycis* spores (white bars) and -intact spores (black bars).
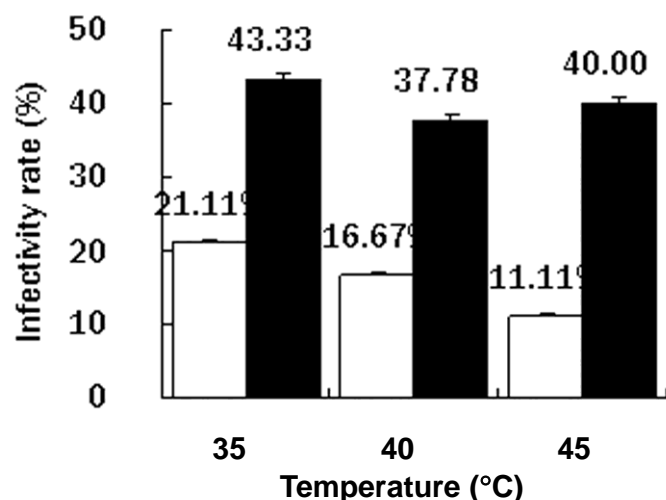

Figure 5. Effects of heating pretreatment on infectivity of SWPs-removal (white bars) and -intact spores (black bars).

proteinaceous exospore and the chitinous endospore (Vávra and Larsson, 1999), which provides mechanical properties and resistance to environmental pressure. Therefore, better understanding of the biological roles the proteins on the spore wall play is of great prevention and treatment importance for pebrine disease.

In this study, we removed the proteins of spore wall (SWPs), and explored how they could affect the *N. bombycis* spores. Results indicated (i) that integral SWPs were advantageous for operation of the dye ion influx into

protoplasm; (ii) that loss of SWPs resulted in decreased infectivity of *N. bombycis* spores to silkworms; (iii) that SWPs-removal spores displayed higher susceptibility to environmental stress than intact ones. Briefly, besides the structural importance reported elsewhere, SWPs of *N. bombycis* spores are endowed with significant roles involved in permeability, infection and resistance. Those results are indicative to study chemical and physical treatment targeting the SWPs of *N. bombycis* spores.

Ultrastructural examinations indicated that SWPs-removal treatment affected the spore wall, mainly on exospore. Exospore of microsporidia is assumed to provide protection and resistance to environmental stresses (Vávra and Larsson, 1999; Keeling and Fast, 2002). *N. bombycis* spores retain viable in water for about ten years, representing the most resistant spores we know. The absence of certain proteins on *N. bombycis* spores wall results in increased Eosin B dye ion influx into protoplasm (Garcia, 2002) and thus staining enhancement. Together with the inversely correlation of infectivity of SWPs-removal spores to the higher NaCl concentration, we could conclude that some of the removed SWPs could command ion influx such as dye ion and Na^+ to retain inner stability, indicating the existence of more ion channels than one calcium channel ever identified from *Spraguea lophii* (Pleshinger and Weidner, 1985).

Spore wall are involved in the infection. When an ingested spore comes in close proximity to host cell in a gastrointestinal tract, proteins on their exospore might readily sense the surroundings for activation and polar filament extrusion prior to infection (Cali and Takvorian, 1999). Evidences from *E. intestinalis* show that spore adheres to host cell surface before activation and

infection, and certain divalent cation salts such as magnesium or manganese chloride can facilitate the spore adherence (Hayman et al., 2005; Southern et al., 2006a). A potential adherence-related protein ligand has been characterized in both endospore and exospore (Southern et al., 2006b), and augmented adherence serves to enhanced infectivity (Hayman et al., 2005; Southern et al., 2006a, b). The involvement of spore wall in spore activation and infection has ever been demon-strated by the varying architecture (phosphorylation and disassembly) of spore wall in *Spraguea lophii* (Weidner, 1992) and *Thelohania* sp. during spore activation (Weidner and Halonen, 1993). In our study, removal of SWPs is correlated to the decreased infectivity of SWPs-removal *N. bombycis* spores. Moreover, a delayed onset of the disease and fewer spores in midgut and silk gland were observed. It is likely that microsporidia routine infection course including spore adherence and activation was held back due to the loss of proteins on the spore wall. But whether the impaired proteins involved can be recovered remains further clarified.

In the study, we present a direct experimental correlation between SWPs and permeability, infection and environmental resistance of *N. bombycis* spores, by addressing the SWPs as whole research target. Detailed analysis of proteins involved and how to effectively destroy them is needed, and might direct to effective treatment.

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