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Comparative Proteomic Studies of Midguts from *Bombyx mori* Inoculated Orally with either *Nosema bombycis* or *Nosema antheraeae* or Water

Lin-Ling Wang[1,2], Cui-Ming Tang[3], Ze-Yang Zhou[1] and Ke-Ping Chen[2]*

[1]College of Life Sciences, Chongqing Normal University, Chongqing - 400047, China.
[2]Institute of Life Sciences, Jiangsu University, Zhenjiang - 212013, China.
[3]Sericultural & Agri-Food Research Institute of Guangdong Academy of Agricultural Sciences, Guangzhou 510610, China.

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To identify microsporidian infection-related proteins, comparative proteomic studies were performed of midguts from *Bombyx mori* (*B. mori*) inoculated orally with either *Nosema bombycis* (*N. bombycis* group) or *Nosema antheraeae* (*N. antheraeae* group) or water (the control group) at 24 h and 72 h post-inoculation (pi). At 24 h pi, there were 478, 485 and 432 protein spots in the two-dimensional electrophoresis gel images for *N. bombycis* group,. *antheraeae* group and the control group, respectively. However, at 72 h pi, only 428 protein spots were detected in *N. bombycis* group, whereas 481 and 498 protein spots were detected in *N. antheraeae* group and the control group, respectively. A total of 63 differentially-expressed protein spots were excised from gels and analyzed by mass spectrometry, and 21 protein spots were identified. These proteins include: (i) enzymes involved in carbohydrate metabolism such as enolase, (ii) enzymes involved in protein and amino acid metabolism such as proteasome alpha 3 subunit, (iii) proteins involved in immune responses such as thiol peroxiredoxin, and (iv) some other proteins such as vacuolar ATP synthase catalytic subunit A. Particularly, in *N. bombycis* group, the expression of actin-depolymerizing factor 4 was absent at the two time points tested. Possible causes for the differential expression are discussed.

Key words: *Bombyx mori*; Comparative proteomics; Midgut; *Nosema antheraeae*; *Nosema bombycis*.

Currently, for over 30 million families in the world, sericulture is their most important income source. However, pebrine disease, caused by microsporidia, seriously affects its development. In China, the two main microsporidia are *Nosema bombycis* (*N. bombycis*), whose host is *Bombyx mori* (*B. mori*), and *Nosema antheraeae* (*N. antheraeae*), whose host is *Antheraea pernyi* (*A. pernyi*) (Ding and Su, 1998).

* To whom all correspondence should be addressed.
Tel: 86-0511-8791923; Fax: 86-0511-8791923;
E-mail: kpchen@ujs.edu.cn

These two microsporidia have significantly similar life cycles (Zhang and Ding, 1996; Ding and Su, 1998; Su and Ding, 2003). According to molecular phylogenetic analyses, *N. antheraeae* is closely related to *N. bombycis* (Wang et al. 2006). However, using *N. antheraeae* spore suspension, Zhang et al. 1996 have showed that *N. antheraeae* is unable to infect *B. mori*.

Unexpectedly, many other microsporidian species can infect *B. mori*, including the big spore SCM6 (*Nosema* sp), the small spore SCM7 (*Endoreticulatus bombycis* sp nov) and *Vairimorpha ceraces* sp.nov, all of which are less closely related to *N. bombycis* than is *N. antheraea* (Wan 2005). The mechanisms involved remain unknown.

Because midgut is the first organ to be infected when *B. mori* is orally inoculated, we performed comparative proteomic studies of midguts from *B. mori* inoculated orally with either *N. bombycis* or *N. antheraeae* or water to identify microsporidian infection-related proteins.

MATERIALS AND METHODS

Animals

Disease-free eggs of *B. mori* strain C108 were maintained in the *B. mori* germplasm of Jiangsu University, and larvae reared with mulberry leaves under standard conditions (26°C, 80% relative humidity). Prior to feeding, newly-molted fifth instar larvae were orally inoculated with 5¼l of *N. bombycis* (*N. bombycid* group) or *N. antheraeae* (*N. antheraeae* group) spore suspension (10^8 spores/ml) or water (the control group). At 24 and 72 h pi, midguts were collected from *B. mori* larvae (20 each inoculation group) and stored at -80°C for future use.

Two-dimensional Electrophoresis and Gel Visualization

Isoelectric focusing (IEF) was performed according to manufacturer's instructions. Briefly, midguts were mixed with lysis solution containing 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris base, 2% IPG buffer, and 30 mM DTE. The mixture was fully lysed for 1 h in an ice bath and sonicated for 3 min, then centrifuged twice at 15 000 *g* for 15 min at 4°C. The supernatant was mixed further with rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer (pH 3-10), 0.4% DTT, and 0.002% bromophenol blue. Rehydration and IEF were carried out automatically on the IPGphor platform at 20°C. After IEF separation, the gels were equilibrated for 15 min in an equilibration buffer (50 mM Tris base, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 1% DTT and equilibrated subsequently in the same buffer for another 15 min except that DTT was replaced with 2.5% iodoacetamide. The equilibrated gel strip was subjected to 12.5% SDS-PAGE and sealed with 0.5% agarose. The separated proteins were visualized by silver staining.

Two-dimensional Protein Image Acquisition and Analysis

All the two-dimensional images were scanned at an optical resolution of 300 dpi by a high-resolution image scanner. The images were analyzed using the PD-QUEST software supplied by the manufacturer.

In-Gel Digestion and MALDI-TOF-MS Analysis

The protein spots of interest were excised manually from the silver-stained gels with a clean scalpel blade. Each sample was washed twice in milli-Q water, then destained by washing with a 1:1 solution of 30 mM potassium ferrocyanide and 100 mM sodium thiosulfate, and equilibrated to pH 8.0 in 100 mM ammonium bicarbonate for 20 min. After washing twice in milli-Q water, the gels were dehydrated by addition of acetonitrile, and then dried in a SpeedVac (Thermo Savant) for 15 min. Subsequently, the gel spots were rehydrated in trypsin (Sigma, St. Louis, MO) solution and incubated at 37°C overnight. Peptides were first extracted by 50% acetonitrile for 15 min, and then extracted by 5% trifluoroacetic acid for 15 min. Extracted peptides dried in a SpeedVac were dissolved in 0.1% trifluoroacetic acid prior to loading to the sample plate. Peptide mixture was mixed with an equal volume of 10 mg/mL R-cyano-4-hydroxycinnamic acid (Sigma) saturated with 50% acetonitrile in 0.1% trifluoroacetic acid, and analyzed by MALDI-TOF-MS(Bruker).

Protein Identification and Database Searching

Protein identification using peptide mass fingerprinting (PMF) was performed by the MASCOT search engine (http:// www.matrixscience. com) against the NCBI protein database.

RESULTS

Two-dimensional Electrophoresis of Midgut Proteins

At 24 h pi (Fig.1) and 72 h pi (Fig.2), midgut proteins from *N. bombycis* groupÿ *N. antheraeae* group and the control group were collected and separated by two-dimensional gel electrophoresis, and the obtained gel images were then analyzed by using Bio-rad's PDQuest software.

At 24 h pi (Fig.1), there were 478, 485 and 432 protein spots in the two-dimensional electrophoresis gel images of midgut proteins from *N. bombycis* group, *N. antheraeae* group and the control group, respectively.

However, at 72 h pi (Fig.2), only 428 protein spots were detected in *N. bombycis* group,

Table 1. Differentially-expressed proteins in midguts of *B. mori* at 24 h pi

Spot No.	Putative protein with accession no. (*species*)	Theoretic mass(KDa)/PI	Mascot Score	Differential protein expression		
				Group Inoculated orally with *N. bombycis*	Group Inoculated orally with *N. antheraeae*	Group Inoculated orally with water
1	actin-depolymerizing factor 4(*Bombyx mori*)gi|95102548	17/6.4	172	undetectable	detectable	detectable
3	vacuolar ATP synthase catalytic subunit A (*Bombyx mori*)gi|119220834	68.3/5.2	375	low	high	high
4	thiol peroxiredoxin(*Bombyx mori*)gi|112982996	21.9/6.35	121	high	high	low
7	proteasome alpha 3 subunit (*Bombyx mori*)gi|114051245	28.3/5.3	94	detectable	undetectable	undetectable
14	exodeoxyribonuclease VII large subunit(*Bordetella parapertussis* 12822)gi|33597151	50.3/11.2	84	detectable	undetectable	undetectable
16	vacuolar ATP synthase catalytic subunit A(*Bombyx mori*)gi|119220834	68.3/5.2	218	low	high	high
17	fumarylacetoacetate hydrolase (*Bombyx mori*)gi|114051481	47.4/6.2	96	detectable	undetectable	undetectable
19	Enolase(*Bombyx mori*)gi|119381542	47/5.7	161	undetectable	detectable	detectable
20	hydroxypyruvate isomerase(*Bombyx mori*)gi|114052328	29.2/6.4	171	undetectable	detectable	detectable
22	putative ATP-dependent RNA helicase (*Encephalitozoon cuniculi* GB-M1)gi|19073987	55.8/9.3	75	detectable	undetectable	undetectable
23	actin (*Spodoptera exigua*)gi|40218738	41.6/5.1	115	undetectable	detectable	detectable

Table 2. Differentially-expressed proteins in midguts of B. mori at 72 h pi

Spot No.	Putative protein with accession no. (*species*)	Theoretic mass(KDa)/PI	Mascot Score	Differential protein expression		
				Group Inoculated orally with *N. bombycis*	Group Inoculated orally with *N. antheraeae*	Group Inoculated orally with water
1	actin-depolymerizing factor 4(*Bombyx mori*)gi|95102548	17/6.4	142	undetectable	detectable	detectable
11	PREDICTED: similar to LB1(*Bos taurus*)gi|119920792	85.5/9.3	98	detectable	undetectable	undetectable
12	chaperonin containing TCP-1 epsilon subunit(*Physarum polycephalum*) gi|20149215	58.5/6.4	80	detectable	undetectable	undetectable
24	probable microtubule associated protein(*Pneumocystis carinii*)gi|14599402	93.3/8.2	79	high	low	low
27	thiol peroxiredoxin(*Bombyx mori*)gi|112982996	21.9/6.4	81	low	high	high
28	antichymotrypsin precursor (*Bombyx mori*)gi|112983471	44.6/5.2	101	high	low	low
30	heat shock cognate protein (*Bombyx mori*)gi|112982828	71.2/5.3	84	undetectable	detectable	detectable

whereas 481 and 498 protein spots were detected in *N. antheraeae* group and the control group, respectively.

Analysis of Differential Protein Expression in Midguts at 24 h pi

A total of 25 spots were analyzed through MALDI-TOF-MSÿwith 10 spots having a mascot score higher than 79 (Table 1).

Of the 10 protein spots, 7 protein spots were identified to be the actin-depolymerizing factor 4, vacuolar ATP synthase catalytic subunit A, thiol peroxiredoxin, proteasome alpha 3 subunit, fumarylacetoacetate hydrolase, enolase and hydroxypyruvate isomerase, all of which were proteins of *B. mori* registered in NCBI Database; spots 3 and 16 are believed to be the same protein displaying different molecular sizes and different isoelectric points (pI); and the remaining 3 spots were identified to be homologous proteins of actin from *Spodoptera exigua*, hypothetical protein DEHA0C15796g from *Debaryomyces hansenii*, and exodeoxyribonuclease VII large subunit from *Bordetella parapertussis*.

With a mascot score of 75, protein spot 22 was identified to be a homologous protein of an ATP-dependent RNA helicase from another microsporidium, *Encephalitozoon cuniculi* (*E. cuniculi*). Expression of the ATP-dependent RNA helicase was detected in midguts from *N. bombycis* group but not in midguts from *N. antheraeae* group or the control group.

Midguts from *N. bombycis* group had no detectable expression of actin-depolymerizing factor 4 (Fig.3) and low expression of V-ATPase, whereas those from *N.antheraeae* group and the control group had high expression of both actin-depolymerizing factor 4 (Fig.3) and V-ATPase.

Moreover, the expression of many enzymes involved in carbohydrate metabolism such as enolase and hydroxypyruvate isomerase was down-regulated in *N.bombycis* group relative to *N. antheraeae* group and the control group. However, for many enzymes involved in protein and amino acid metabolism, the opposite was observed.

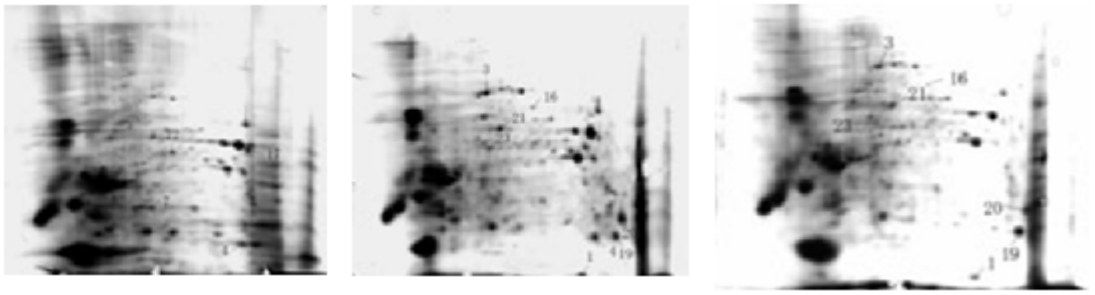
Notably, compared with the control group, the expression of thiol peroxiredoxin, an antioxidase involved in immune response (Nappi and Christensen, 2005; Choi *et al*, 2005), was up-regulated in both *N. bombycis* group and *N.*

antheraeae group (Fig.4).

Analysis of Differential Protein expression in Midguts at 72 h pi

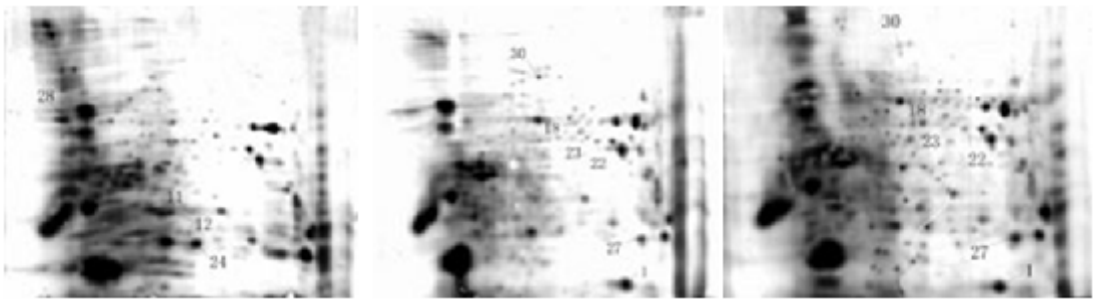
A total of 38 spots were analyzed through MALDI-TOF-MS, with 7 spots having a mascot score higher than 79 (Table 2).

At 72 h pi, expression of actin-depolymerizing factor 4 was still undetectable in *N. bombycis* group but remained well detectable in *N. antheraeae* group and the control group (Fig.3). *N. bombycis* group had decreased expression of thiol peroxiredoxin compared with *N. antheraeae*



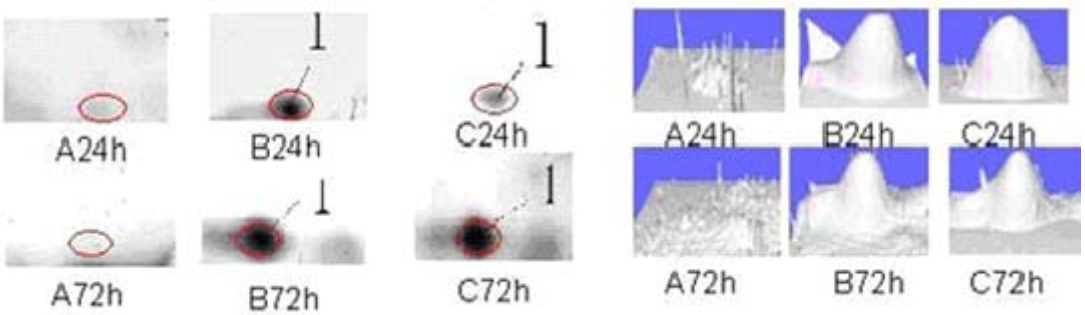
A) *N. bombycis* group B) *N. antheraeae* group C) The control group

Fig. 1. Silver nitrate-stained two-dimensional electrophoresis gel images of midgut proteins from *N. bombycis* group or *N. antheraeae* group or the control group at 24 h pi, with pH values increasing from left to right and molecular weights decreasing from top to bottom



A) *N. bombycis* group B) *N. antheraeae* group C) The control group

Fig. 2. Silver nitrate-stained two-dimensional electrophoresis gel images of midgut proteins from *N. bombycis* group or *N. antheraeae* group or the control group at 72 h pi, with pH values increasing from left to right and molecular weights decreasing from top to bottom



A) *N. bombycis* group B) *N. antheraeae* group C) The control group

Fig 3. Actin-depolymerizing factor 4 on two-dimensional gels and its three-dimensional images created using PDQuest software (BioRad) at 24 h and 72 h pi

group or the control group (Fig.4).

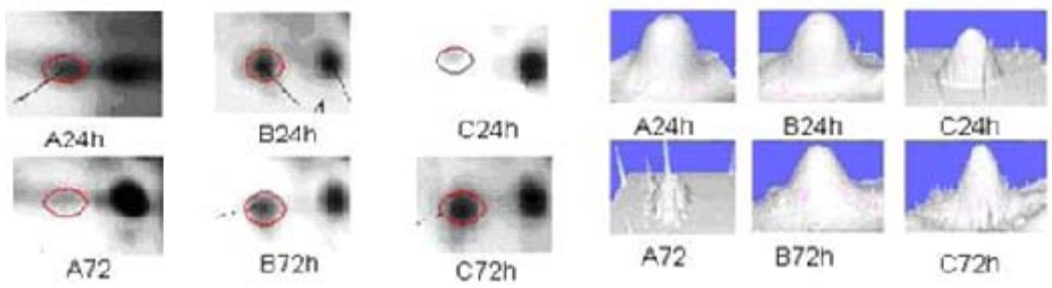
Expression of heat shock cognate protein was not detected in *N. bombycis* group, whereas its expression was observed in *N. antheraeae* group and the control group. However, sharply increased expression of antichymotrypsin precursor was detected in *N. bombycis* group (Fig.5).

qPCR analysis of differentially-expressed proteins

To verify results obtained from two-dimensional gel electrophoresis, qPCR was used to analyze the expression of *Thiol peroxiredoxin* and vacuolar *ATP synthase catalytic subunit A*.

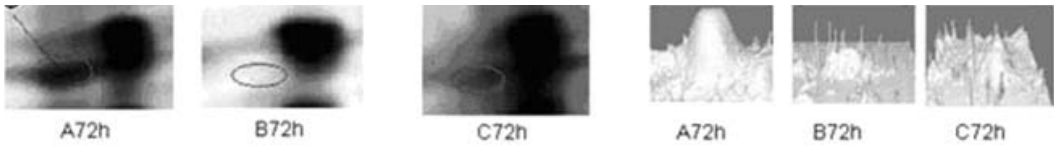
At 24 h pi, the expression of *Thiol peroxiredoxin* was up-regulated approximately 5-fold in *N. bombycis* group relative to the control group. However, at 72 h pi, the level of expression was slightly higher in the control group than in the *N. bombycis* group (Fig.6).

At 24 h pi, the expression of vacuolar *ATP*



A) *N. bombycis* group B) *N. antheraeae* group C) The control group

Fig. 4. Thiol peroxiredoxin on two-dimensional gels and its three-dimensional images created using PDQuest software (BioRad) at 24 h and 72 h pi



A) *N. bombycis* group B) *N. antheraeae* group C) The control group

Fig. 5. Antichymotrypsin precursor on two-dimensional gels and its three-dimensional images created using PDQuest software (BioRad) at 72 h pi

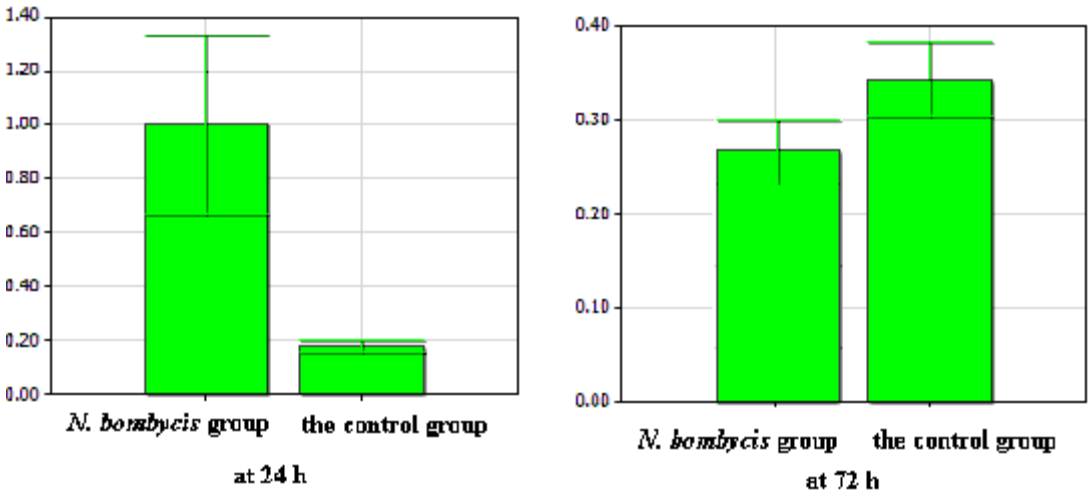


Fig. 6. Expression of *Thiol peroxiredoxin* was analyzed using qPCR at 24 h and 72 h pi

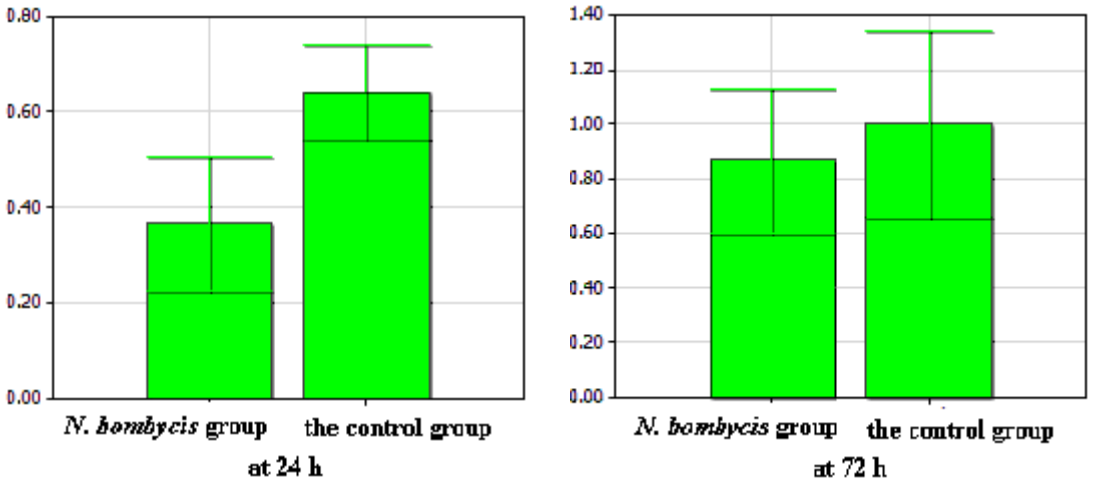


Fig. 7. Expression of vacuolar *ATP synthase catalytic subunit A* was analyzed using qPCR at 24 h and 72 h pi

synthase catalytic subunit A was down-regulated nearly 2-fold in *N. bombycis* group relative to the control group. Despite increased expression in *N. bombycis* group at 72 h pi, the level of expression was still lower in *N. bombycis* group than in the control group (Fig.7).

DISCUSSION

There are many varieties of *B. mori*. However, most varieties available are hybrids, which differ greatly among individuals. Therefore, to minimize individual differences, we used a pure breed C108 in our study.

To avoid silkworms being infected by other pathogens, a thorough sterilization was performed in the rearing room, rearing tools were sterilized by high temperature, and mulberry leaves were soaked in chloramphenicol solution and then washed.

To identify microsporidian infection-related proteins, we performed for the first time comparative proteomic studies of midguts from *B. mori* inoculated orally with either *N. bombycis* or *N. antheraeae* or water at 24 h and 72 h pi. The results showed that, there were more protein spots in the images for *N. bombycis* group and *N. antheraeae* group than for the control group at 24 h pi (Fig.1), whereas *N. bombycis* group had less protein spots than *N. antheraeae* group and the control group at 72 h pi (Fig.2). These results might be explained as follow. Microsporidian intrusion might trigger cellular immune response involving many proteins, thus increasing numbers of proteins. In addition, microsporidia might have immediate early genes, which could also contribute to increased numbers of protein spots. However, at 72 h pi, *N. bombycis* might have successfully established its infection and thus shut down or reduce the expression of some host proteins to the extent that they could not be detected in two-dimensional images. Because *N. antheraea* is unable to infect *B. mori*, as mentioned in the introduction, *N. antheraea* group will, after early immune response, return to normal and develop normally as the control group, resulting in similar numbers of protein spots between *N. antheraea* group and the control group at 72 h pi, as demonstrated in the present study.

At 24 h pi, a differentially-expressed protein was identified to be a homologous protein of a putative ATP-dependent RNA helicase from microsporidium *E. cuniculi*. Although the genome sequence of *N. bombycis* has not been published yet and there are a limited number of protein sequences in databases, given the fact that *Encephalitozoon cunicul* is also a microsporidium and a relatively high mascot score, the identification is considered to be reliable. ATP-dependent RNA helicase is an important enzyme involved in a range of biological activities, including transcription, pre-RNA splicing and ribosome biogenesis. The observation that the putative ATP-dependent RNA helicase was highly expressed at 24 h pi in the *N. bombycis* group implies that the microsporidian transcription was

highly activated.

Franzen *et al*. showed that microsporidium *E. cuniculi* can enter its host cells before the extrusion of the polar tube by phagocytosis, a process forming phagosomes that mature into lysosomes, where some spores can extrude their polar tube and infect host cells before being digested. Interestingly, this type of phagocytosis can be inhibited by cytochalasin D, suggesting that this process is mediated by actin (Franzen 1998, 1999, 2004, 2005a, 2005b). In our study, two differentially expressed proteins support this mechanism. One protein was vacuolar ATP synthase catalytic subunit A, which is a subunit of V-ATPase. Because V-ATPases contribute to maintaining low pH in phagosomes and lysosomes (Nishi and Forgac 2002), the reduced expression of vacuolar ATP synthase catalytic subunit A in *N. bombycis* group would result in increased pH in phagosomes and lysosomes, which gives spores more time to extrude its polar tube due to reduced digestion, thus facilitating infection. This is consistent with the fact that alkaline environment is an environmental factor that induces spores to extrude its polar tube and is used to induce in vitro germination of *N. bombycis* spores(Qian 1996). The other protein was actin-depolymerizing factor 4, which is a widespread actin-binding protein in eukaryotes that plays a key role in the depolymerization and polymerization of actin filament(Maciver 2002). Given its role, the absence of actin-depolymerizing factor 4 in *N. bombycis*-inoculated group at both 24 h and 72 h pi (Fig.3) implies that actin is involved in the infection, consistent with the result of Franzen *et al.*, and that phagocytosis is inhibited in *N. bombycis*-infected silkworm cells in later stages of infection.

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