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Proteomic analysis of nucleopolyhedrovirus infection resistance in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

Xiaoyong Liu [a], Qin Yao [a], Yong Wang [b], Keping Chen [a,*]

[a] Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, People's Republic of China
[b] Department of Biological Technology, Jiangsu University, Zhenjiang 212013, People's Republic of China

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ABSTRACT

Silkworm hemolymph is an important defense tissue to resist bacteria and virus infections. To study the response of silkworm hemolymph in the resistance of *Bombyx mori* L. nucleopolyhedrovirus (BmNPV), we constructed a near-isogenic silkworm line with BmNPV resistance using highly resistant and highly susceptible parental strains. In this paper, two-dimensional gel electrophoresis (2-DE) and Matrix-Assisted Laser Desorption/Ionization (MALDI)-mass spectrometry were employed to investigate the differences of protein patterns in the hemolymph of the highly resistant, highly susceptible and near-isogenic silkworm strains after BmNPV was administrated to the larvae. A comparison between the proteomes of these three silkworm strains led us to identify two differentially expressed proteins, beta-*N*-acetylglucosaminidase 2 and aminoacylase. The expression levels of these proteins were higher in the BmNPV resistant strains.

1. Introduction

Bombyx mori is a commercially important insect for production of silk and recombinant proteins, and is also a good model lepidopteran (Goldsmith et al., 2005; Nagaraju and Goldsmith, 2002). Infection of *B. mori* larvae with *Bombyx mori* nucleopolyhedrovirus (BmNPV) reduces silk production, resulting in economic damage (Chen et al., 2003). Increased understanding of BmNPV resistance in the silkworm could reduce the loss in cocoon production, and facilitate the development of new strategies for pest control (Xue, 2005). Following *per os* inoculation of baculovirus, the midgut cells are infected, and the infection spreads from those initial foci to tracheoblasts, and thus to the haemocoel resulting in a fatal infection (Washburn et al., 1995, 2001). A number of studies have been conducted on insect resistance to baculoviruses (Sparks et al., 2008). However the mechanism of resistance to BmNPV is not fully elucidated.

Insects lack adaptive immune responses (Hoffmann, 2003), but many host factors interact to determine the particular insect response to virus infection (Cory and Myers, 2003; Sparks et al., 2008). Those factors could be grouped into the following six categories. The first category is the pH and substrate of the gut. A few antiviral proteins have been purified from the digestive juice of silkworm larvae, such as lipase, serine proteases (Ponnuvel et al., 2003; Nakazawa et al., 2004), and NADPH oxidoreductase (Selot et al., 2007). The second category is physical and physiological barriers. The third category is subcellular immune mechanisms. Apoptosis or programmed cell death is an important antiviral defense mechanism used by Lepidoptera (Narayan, 2004). Larvae resist baculovirus infection by selective apoptosis of infected midgut epithelial cells and by sloughing off infected cells from the midgut before they release virions into the haemocoel (Federici and Hice, 1997; Clem, 2001). The fourth category is cellular immune responses. Hemocytes in the haemocoel are recruited to the foci of infection, phagocytosing smaller microbes and encapsulating larger invaders, yet the mechanism is poorly understood (Popham et al., 2004; Lavine and Strand, 2002). In addition, immunosuppression of pestiferous moth larvae has revealed the likelihood of a cell-mediated antiviral immune response (Washburn et al., 2000). The fifth category is humoral immunity. Antiviral activity from insect hemolymph has been described (Chernysh et al., 2002). The plasma phenoloxidase of *Heliothis virescens* exhibits antiviral activity against several vertebrate viruses *in vitro* (Ourth and Renis, 1993). *In vitro* incubation of *Helicoverpa zea* single capsid nucleopolyhedrovirus (*Hz*SNPV) with plasma from *H. virescens* reduced the infectivity of the virus, and the enzyme phenoloxidase may act as an innate antiviral reagent (Popham et al., 2004). In addition, baculovirus infection increased hemolin expression, and hemolin might associate with virus in the hemolymph thereby slowing the progression of virus infection (Hirai et al., 2004). The sixth category consists of developmental, environmental and genetic factors (Watanabe, 2002; Engelhard and Volkman, 1995; Granados and Federici, 1986).

* Corresponding author. Fax: +86 511 88791923.
E-mail address: kpchen@ujs.edu.cn (K. Chen).

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Silkworm resistance to BmNPV was defined by the differences in 50% lethal dosage (LD_{50}) values among silkworm populations (Chen et al., 2003). Watanabe discovered a silkworm strain with a BmNPV LD_{50} that was 800-fold higher than other silkworm strains (Watanabe, 1966). Previously we reported a BmNPV resistant silkworm strain NB and BmNPV susceptible strain 306 with LD_{50} difference up to 1000-fold (Chen et al., 2003). Based on their different genetic backgrounds, we established one near-isogenic line (NIL) with resistance to BmNPV. Near-isogenic lines have been established in many species through introgression (Busov et al., 2005), which is accomplished by repeatedly backcrossing a line carrying a gene of interest (donor parent) to a line having other properties (recurrent parent). After each cross, progeny that possess the phenotype of the target gene are selected. This process results in a line that carries a small portion of genomic sequences from the donor parent. Thus, the genetic background of the near-isogenic strain is similar to the recurrent parental strain. In the present study, the ninth near-isogenic line generation strain was established and compared to the resistant and susceptible parental strains (NB and 306, respectively).

To investigate the character and mechanism of inherited resistance to BmNPV, we employed proteomic analysis to study the hemolymph proteins of a near-isogenic strain of silkworm with the susceptible, recurrent parent and the donor, non-isogenic resistant parent. Two proteins were differentially expressed in the resistant silkworm strains which may contribute to the observed difference in resistance to BmNPV.

2. Materials and methods

2.1. Silkworm parental strains

BmNPV resistant silkworm strain NB ($LD_{50} = 2.5 \times 10^8$ polyhedral/larvae) and BmNPV-susceptible silkworm strain 306 ($LD_{50} = 3.4 \times 10^5$ polyhedral/larvae) were used in this study. These strains were preserved and reared in our laboratory. The LD_{50} of virus (BmNPV) was determined with fifth instar larvae by oral inoculation with polyhedra. One hundred larvae were used for each infection and the experiments were repeated three times. The mortality of the infected larvae was observed within 10 days, and virus infection confirmed in the hemolymph with a microscope. The LD_{50} values were calculated by comparison of means analysis (SPSS Inc.) and further compared using an Independent-Samples *T*-Test.

2.2. Preparation of near-isogenic line

The near-isogenic line was prepared in accordance with the method reported (Chen et al., 2003). To generate the BC_9 line ($LD_{50} = 2 \times 10^8$ polyhedra/larva), females of the susceptible strain 306 were crossed with males of the resistant strain NB. The offspring were selected for resistance to BmNPV administrated at 5×10^6 polyhedra/larva *per os*. Then the selected crossing progeny of NB × 306 backcrossed with strain 306. The backcrosses were conducted for nine generations followed by two generations of self-crossing.

2.3. Sample preparation

Silkworm larvae were raised with mulberry leaves under a 12 h light/12 h dark photoperiod. Then, BmNPV (5×10^6 viruses/larva) was administrated to the fifth instar larvae *per os*. Because BmNPV occlusion bodies introduced *per os* enter the hemolymph at approximately 12–24 h post inoculation (hpi) (Yao et al., 2005), hemolymph samples were collected at 22 hpi ($n = 15$ larvae per treatment) in the presence of protease inhibitor (Protease Inhibitor Cocktail, Amresco) and phenylthiourea (final concentration of 2 μg/ml). The hemolymph was also collected from silkworms without infection of BmNPV as control. Hemolymph samples from each one of 15 larvae were collected and pooled for each strain, and triplicate samples were used for analysis.

2.4. Two-dimensional gel electrophoresis (2-DE)

Hemolymph containing hemocytes (0.8 mL) was combined with one-fourth volume of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% Bio-Lyte pH 3–10 (Bio-Rad), 1 mM PMSF, 2 mM EDTA, and 65 mM dithiothreitol, pH 8.5). The solutions were sonicated for 3 min followed by centrifugation at 35,000*g* for 30 min at 4 °C. The protein concentration was determined by Bradford assay (Bradford, 1976).

Isoelectric focusing electrophoresis was carried out with 17 cm (pH 3–10) IPG strips at 20 °C according to the manufacturer's instructions (Bio-Rad). Briefly, the strips were rehydrated under 50 V for 13 h. and isoelectric focusing was programmed at a gradient mode. The IPG strips were focused for 1 h at progressively increasing voltages, 250 V, 1000 V, 4000 V and 10,000 V, respectively, then continued at 10,000 V until reaching a total of 60 kVh. The focused strips were equilibrated in buffer with 6 M urea, 0.375 M Tris–HCl, 20% glycerol, 2% SDS and a trace amount of bromophenol blue, and subsequently treated by DTT and iodoacetamide. The treated strips were transferred onto 12% SDS–polyacrylamide gels running at 5 W per gel for 15 min and 15 W per gel until the bromophenol blue dye reached the bottom of the gel. The gels were stained with Coomassie blue. The gels were scanned using an Image scanner and the image analysis was conducted with PDQuest licensed by Bio-Rad. Approximately 0.8 mg protein was loaded onto gels and each pooled sample was run three times to achieve reproducibility. Student's *t*-tests were used to determine significant differences.

2.5. In-gel digestion

Protein spots were excised manually using a hand-held pipette with a trimmed polypropylene tip and digested by trypsin according to Mirza et al., 2000. Briefly, excised spots were destained and cleaned by shaking the gels with water, 50 mM ammonium bicarbonate, 50% acetonitrile, and 100% acetonitrile, respectively. After reduction with dithiothreitol and derivatization with iodoacetamide, the gel pieces were treated with freshly prepared trypsin (Promega) solution (20 μg/ml in 40 mM ammonium bicarbonate/10% acetonitrile) and incubated at 37 °C overnight. The gels were extracted with 5% formic acid in 1:1 (v/v) water/acetonitrile.

2.6. Mass spectrometry analysis

Peptide mixture (1 μl) was mixed with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) saturated with 50% acetonitrile in 0.1% trifluoroacetic acid, and analyzed by Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight (MALDI-TOF) Mass Spectrometer (Bruker, Ultraflex tof/tof), to acquire spectra with a mass range from 1000 to 4000 Da. External calibration was performed with standard peptides. The matrix and the autolytic peaks of trypsin served as internal standards for mass calibration.

2.7. Protein identification

Proteins were identified by comparing the masses of peptides to theoretical tryptic peptides of the NCBI protein database and a self-constructed silkworm EST translated database. Peptide mass