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Identification of a Soluble NADPH Oxidoreductase (BmNOX) with Antiviral Activites in the Gut Juice of *Bombyx mori*

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Silkworms show high variability in silk quality and disease resistance. Attempts are on to combine the disease tolerance of multivoltine races and the silk quality of bivoltine races to generate new races with desirable phenotypic traits. We report the identification of a 26.5-kDa protein that is overexpressed in the gut juice of disease-resistant multivoltine races and that has anti-BmNPV activity. We have characterized this protein as a soluble NADH-oxidoreductase-like protein (BmNOX). Treatment of live BmNPV particles with BmNOX inhibited the capability of the viral particles to infect BmN cells *in vitro*.

Key words: silkworm; anti-viral; NOX; virus resistance

Silk produced by multivoltine races of silkworm is inferior to that produced by bivoltine races. But bivoltine sericulture poses special problems under tropical conditions, mainly due to the lack of adaptable technology for rearing and disease prevention. Grasserie, a silkworm disease caused by baculovirus (BmNPV) infection, is one major hurdle in trying to establish bivoltine sericulture in India, and it is estimated that 40–50% crop loss occurs in sericulture due to this disease.[1–3] High temperature with high humidity in tropical regions is conducive to the proliferation of viral diseases.[4] The intensity of infection and loss in yield vary depending upon the breed and also several environmental conditions of rearing.[5] It is generally noticed that bivoltine breeds are more susceptible to Grasserie than multivoltine breeds[3,6] which is a main constraint to the adoption of bivoltine sericulture in India.

Insects can rapidly clear microbial infections by producing a variety of immune-induced molecules including antibacterial and/or antifungal peptides/polypeptides[7] collectively known as antimicrobial peptides (AMPs). In *Drosophila*, seven groups of inducible AMPs have been identified. On the basis of the silkworm genome sequence and expressed sequence tags, 35 AMP genes have been identified, mostly belonging to the cecropin, moricin, and gloverin gene families.[8] Regulatory motifs such as the kappaB-like and GATA sequences have been identified in *Bombyx mori* antibacterial proteins.[9] The core promoters required for gene transcription and the *cis*-regulatory elements for NF-kappaB/Rel and GATA transcription factors have been predicted.[8] Lepidopteran larvae resist baculovirus infection by selective apoptosis of infected midgut epithelial cells and by sloughing off infected cells from the midgut. Once the infection breaches the midgut epithelial barrier and propagates from infective foci to the hemocoel, however, there are few mechanisms known to account for the resistance and clearance of infection observed in some virus-host combinations.[10] The literature also suggests that insects have evolved digestive enzymes with antiviral properties, as in the case of BmSP-1, BmSP-2,[11] and Bmlipase-1,[12] that check viral propagation at the initial site of viral infection.

The baculoviruses make up a large, diverse family of DNA viruses that have evolved a number of fascinating mechanisms to manipulate their insect hosts. One of these is the ability to inhibit host-cell apoptosis during infection. The anti-apoptotic activity of baculoviruses in manifested in the expression of proteins that can inhibit caspase activation and/or activity, including the caspase inhibitor P35 and its relatives, and the inhibitor of apoptosis (IAP) proteins.[13,14] In Sindbis virus-host cell interactions, a viral nonstructural protein, nsP2, which is a component of the replicative enzyme complex required for replication and transcription of viral RNAs, plays a role in suppressing the antiviral response in Sindbis virus-infected cells.[15]

In this study, we report the identification, purification, and characterization of a 26.5-kDa gut-juice protein showing antiviral activities. The expression of this protein in larvae from seven multivoltine and six bivoltine strains of *B. mori* was also evaluated to draw

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correlations between the presence/activity of antiviral proteins in the gut juice of silkworm larvae and their resistance to baculovirus infection.

Materials and Methods

Insects. Silkworm larvae of the multivoltine (Nistari, TX, BL67, Sarupat, Moria, Cnichi, and PM) and bivoltine (CSR5, CSR6, CSR2, CSR3, NB4D2, and KA) races were reared on mulberry leaves at $27 \pm 1\,^{\circ}C$ under aseptic conditions.

Collection of gut juice. Digestive juice was collected from 3rd-day 5th instar larvae by induced vomiting using mild electric shock or by chloroform vapors. The gut juice was centrifuged at $3{,}130 \times g$ for 30 min at $4\,^{\circ}C$ to remove undigested mulberry leaves. The supernatant was collected and neutralized to pH 7.0 with 2 M acetic acid.

Purification of the 26.5 kDa protein. For preparation of antiviral protein, the procedure of Uchida *et al.*[16] was adopted, with some modifications. Gut juice from the Nistari race was used for the purification of 26.5-kDa protein. After neutralizing the gut juice as detailed above, solid ammonium sulphate was added to it to give 40% saturation, and this was mixed for 2 h and 30 min at $4\,^{\circ}C$. After mixing, the precipitate was collected by centrifugation at $14{,}000 \times g$ for 30 min at $4\,^{\circ}C$ and suspended in 1 ml of 40 mM sodium phosphate buffer, pH 7.4. The suspension was mixed with an equal volume of *n*-butanol and kept overnight for incubation, after which it was centrifuged at $14{,}000 \times g$ for 30 min at $4\,^{\circ}C$. The lower aqueous layer was collected and mixed with cold acetone. After 30 min, the precipitate was collected by centrifugation and dissolved in 40 mM sodium phosphate buffer (pH 7.4) and dialyzed for 4 h against the same buffer with two changes. The dialyzed fraction was centrifuged at $14{,}000 \times g$ for 30 min to remove insoluble debris. The supernatant (1 ml) was applied to a Sephadex G-75 column (1.2×35 cm) previously equilibrated with the same buffer. The initial flow-through of 10 ml from the column was discarded. Thereafter, 40 fractions of 1 ml each were collected. All the fractions were loaded into a centricon-10 centrifugal filtration device (Millipore, Boston, MA) and concentrated at $3{,}000 \times g$ centrifugal force. All procedures were carried out at $4\,^{\circ}C$. The concentrated fractions (100 µl each) were run on 10% SDS–PAGE, and the purity of the fractions was analyzed (results not presented). Since fractions 30–40 contained the protein of interest, these fractions were pooled and designated as purified 26.5-kDa protein. The molecular mass and purity of the pooled protein were checked using MALDI-TOF mass spectroscopy. The protein concentration was measured using BCA protein assay reagent, and the final concentration was adjusted to 70 µg/ml.

Protein microsequencing. The purified protein sample and separated on SDS–PAGE and electrophoretically transferred onto a sequencing grade PVDF membrane (0.2 µm, GE Healthcare Limited, Buckinghamshire) in 10 mM CAPS buffer at 50 volts for 1 h for N-terminal micro sequencing. After transfer of the protein, the membrane was stained with Coomassie Brilliant Blue R 250 and destained with 50% methanol (v/v). The band of interest was excised with a sterile blade, dried in air, and subjected to N-terminal microsequencing on a Shimadzu PPSQ 21 protein sequencer following Edman degradation.

Antisera production. The purified protein was mixed with TiterMax Gold Adjuvant (Sigma, Milwaukee, WI) and used for the generation of polyclonal antibodies by immunizing one rabbit. Conventional ELISA was applied to monitor the antibody production.

Western blot analysis. Gut-juice proteins from the larvae of the various races of silkworm included in this study were separated on SDS–PAGE, as described earlier. After electrophoresis, the proteins were electroblotted on PVDF membrane (0.2 µm; BioRad, Hercules, CA) in presence of 40% v/v methanol, 25 mM Tris pH 8.2, 190 mM glycine at 30 mA for 12–16 h using Mini Trans Blot cell (BioRad) as described in Ref. 18.

For development of the blots, the membranes were pre-wet in methanol and incubated in 5% non-fat skim milk in Tris buffered saline containing 0.1% Tween 20 (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) overnight, followed by extensive washing in Tris buffered Saline containing 0.1% Tween 20 (TBS-T) at room temperature. The blots were incubated for 2 h at $20\,^{\circ}C$ in 1:2,000 dilution of anti-26.5-kDa antiserum. The blots were then incubated in anti-rabbit IgG-peroxidase at a dilution of 1:2,000 for 1 h at room temperature. They were washed extensively, as described above. The immunoblots were developed by dipping the blots in 0.05% diaminobenzidine and 0.1% H_2O_2/0.04% nickel chloride in 50 mM Tris–HCl, pH 7.5, until the desired contrast was obtained. They were photographed on a gel documentation system. A negative control was prepared by skipping the incubation of membranes in the primary antibody and processing the membrane as in the case of the experimental blot.

SDS–PAGE. The sample for loading was prepared by adding 5 µl of gut juice to SDS sample buffer (100 mM Tris–Cl, pH 6.8, 20% v/v glycerol, 4% w/v SDS, 200 mM dithiothreitol, and 0.2% bromophenol blue), and boiled for 5 min. Samples were loaded in 10% SDS–PAGE and run at 60 volts in stacking gel, followed by 120 volts in separating gel in Tris, Glycine, SDS (25 mM Tris, 192 mM glycine and 0.1% SDS) buffer by the method of Laemmli[17] on a Mini Cell (Bio-Rad) electrophoresis system. The gel was stained with Coomassie Blue R-250 for visualization of proteins.

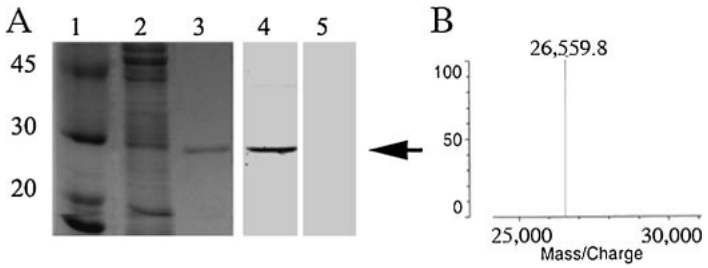

Fig. 1. Characterization of BmNOX.

A, SDS–PAGE analysis of gut juice from *B. mori* (Nistari) (lane 2), and the protein purified as detailed in "Materials and Methods" (lane 3). Lane 1 shows molecular weight markers. Lane 4 is the western blot detection of BmNOX in gut-juice extracts from *B. mori* (Nistari), and a control blot probed with secondary antibody alone in the absence of anti-BmNOX antiserum. B, MALDI-TOF analysis of purified BmNOX for molecular weight determination.

Measurement of NADPH oxidase activity. The NADPH oxidase activity of the 26.5-kDa protein purified from the gut juice of the Nistari race of silkworm included in this study, following the protocol mentioned above, was measured using the Cytochrome c Reductase (NADPH) Assay Kit (Sigma, Milwaukee, WI) following the manufacturer's instructions. The assay was standardized using NADPH-cytochrome c reductase from rabbit liver (Sigma). The reduction of cytochrome c was monitored by measuring the time-dependent change in absorbance at 550 nm.

Neutralized gut juices from the larvae of other races of silkworm were processed as described in the previous section detailing the procedure for the purification of the 26.5 kDa protein. However, the gel filtration was excluded and the NADPH oxidase activity of the dialysate prepared from the gut juice was measured. The experiment was repeated three times with fresh larvae, and the results obtained were averaged.

Antiviral activity of protein. A BmN cell line derived from the *B. mori* ovary was maintained at 27 °C in TC-100 insect medium (Sigma), supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD) and 50 µg/ml of gentamycin (Sigma). The virus was collected from the Silkworm Pathology Section of Central Sericultural Research and Training Institute, Srirampura, Mysore, India, and the virus stock was maintained and the titer value was measured by end point dilution, and the TCID 50 was calculated (8.3×10^8 pft/ml). For budded virus purification, BmNPV-infected cell culture supernatant was centrifuged at 100,000 × *g* for 90 min at 4 °C, and the virus pellet was suspended in 100 µl of 40 mM phosphate buffer, pH 7.4. We prepared four aliquots each of 2 MOI, 3 MOI, and 5 MOI virus suspensions in eppendrof tubes. The first aliquot from each of the three viral titers served as a negative control and thus did not receive any antiviral protein. The second set of aliquots of viral particles was mixed with 2.94 µg of purified 26.5-kDa protein. The third and the fourth sets of aliquots of viral particles received 4.41 µg and 7.35 µg of purified 26.5 kDa protein respectively. All the suspensions were adjusted to a final volume of 200 µl by the addition of 40 mM phosphate buffer (pH 7.4), and were incubated at room temperature with intermittent mixing for 1 h. After incubation, the viral suspensions were centrifuged at 100,000 × *g* and the supernatants were discarded. The pellets were reconstituted in 10 µl phosphate buffer and added to individual wells of a 96-well plate containing cells (2.3×10^4 cells/well) without media. The plates were rocked gently and intermittently. After 1 h of infection, the wells were drained, and washed with PBS to remove any unadsorbed virus, and 100 µl of complete media was again added to the cells. The plates were incubated for 5 d at 27 °C, and the cells were observed under an inverted microscope to evaluate the extent of infection. The experiment was repeated three times and the plates were photographed.

Results

Purification of the 26.5 kDa antiviral protein

SDS–PAGE of gut juice extracted from *B. mori* (TX) is shown in Fig. 1A, lane 2. The 26.5-kDa protein was purified as detailed in "Materials and Methods." The purified protein fraction showed a single band at about 27 kDa as shown in Fig. 1A, lane 3. A western blot analysis of the gut juice extract separated on SDS–PAGE yielded a single band at 27 kDa, which also corresponded to the purified protein fraction (Fig. 1A, lane 4), while secondary antibody alone did not produce any band (Fig. 1A, lane 5) showing the specificity of the primary antibody used. MALDI-TOF analysis of the purified protein produced a peak at a molecular mass of 26,559.8 Da (Fig. 1B).

Expression of the 26.5-kDa protein in several B. mori strains

The expression of the 26.5-kDa protein was assayed in seven multivoltine and six bivoltine strains of *B. mori*, which are known to show varying degrees of resistance to BmNPV, as summarized in Table 1. While protein profiles in the 27-kDa region of a 1D gel did not show remarkable differences, the western blot analysis

Table 1. NADPH Oxidase Activity in the Gut-Juice of Selected Races Employed in This Study

Race	NADPH Oxidase activity (units/μg protein)	Tolerance level[28]
Nistari (purified protein)	285.4 ± 1.24	
Nistari	24.5 ± 0.25	High
TX	19.3 ± 0.26	High
BL67	13.0 ± 0.47	High
Sarupat	11.5 ± 0.31	Moderate
Moria	8.3 ± 0.72	Moderate
Cnichi	6.6 ± 0.45	Weak
PM	6.6 ± 0.80	Weak
CSR5	19.5 ± 0.67	High
CSR6	10.5 ± 0.41	Moderate
CSR2	10.7 ± 0.50	Weak
CSR3	5.7 ± 0.11	Weak
NB4D2	5.3 ± 0.96	Weak
KA	7.7 ± 0.17	Weak

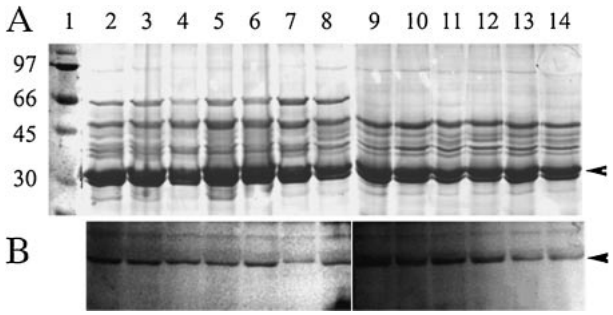

Fig. 2. BmNOX in Various Silkworm Races.

A, Protein profile in gut-juice extracts from multivoltine (lanes 2–8, in the order Nistari, TX, BL67, sarupat, moria, cnichi, and PM) and bivoltine (lanes 9–14, in the order CSR5, CSR6, CSR2, CSR3, NB4D2, and KA) races of *B. mori*. Panel B shows the corresponding western blot showing the immunopositive band detected by anti-BmNOX in gut-juice proteins.

showed an interesting pattern. Thus the Bm-NPV-resistant strains from both the multivoltine (Fig. 2B, lanes 2 and 3), and the bivoltine (Fig. 2B, lanes 9–11) groups showed significantly high levels of the 26.5-kDa protein, while the moderately resistant and susceptible strains showed significantly lower levels of expression of this protein. This observation suggests a correlation between the amount of 26.5-kDa protein present in the digestive juice and the virus resistance of various strains included in this study.

Characterization of the 26.5 kDa protein

The purified protein was subjected to automated N-terminal sequencing, which yielded the sequence SMIGGVMSKG (accession no. P 84517), which showed 87% identity and 100% positive with NADH dehydrogenase subunit 6 of *Ancylostoma duodenale* (accession no. GI 19073879).

Antiviral property of the 26.5 kDa protein

The antiviral activity of the purified protein was evaluated for BmNPV budded virus in BmN cells under different concentrations. The cells were observed microscopically on the 5th d of infection. Control incubation was also performed. The control was not inoculated with the virus (Fig. 3A). Cells infected with 2 MOI, 3 MOI, and 5 MOI of viral particles are shown in Fig. 3(B–D) with profuse polyhedra formation, signifying viral replication. In presence of the 26.5-kDa protein in the medium, viral infection of the BmN cells was significantly reduced in a dose-dependent fashion (Fig. 3, E–M) in all the cultures.

NADPH oxidase activity of the 26.5-kDa protein

The 26.5 kDa protein purified from Nistari larvae showed a specific activity of 285.4 ± 1.24 units/μg protein. NADPH-dependent superoxide production in the gut juice from various strains of *B. mori* included in this study is presented in Table 1. It matched well with the intensities of the 26.5-kDa band from these samples on western blots (Fig. 2).

Discussion

In a previous study, it was found that a protein with an approximate molecular weight of 28-kDa in silkwork gut juice possessed anti-BmNPV activity.[16] Reports on the presence of proteins with antiviral activity displaying protease and lipase activity are also available.[11,12,16] Though the molecular size of the protein identified in our laboratory falls close to the previously reported antiviral proteins, the establishment of the molecular identity of the protein detected by us as an NADPH-oxidoreductase like protein signifies that it is different from the previously reported molecules. Further, the levels of this antiviral protein in the gut juices collected from a selected set of silkworm races (multivoltine and bivoltine) indicates that the races which are tolerant to BmNPV infection had relatively higher quantities this protein in their gut juices (Fig. 3).

We identified and purified a protein from the gut juice of silkworm larvae which displayed antiviral activity against BmNPV budded virus particles in BmN cell-lines. We were also successful in partially sequencing the gene encoding this protein, which shows homology with NADPH oxidoreductase and xanthine dehydrogenase proteins. Since this protein showed NADPH oxidase activity, we named it BmNOX. NADPH oxidase and NO synthase pathways have been observed in the self-defense system of *Mytilus galloprovincialis*,[18] and production of ROS has also been detected in hemocytes; evidence of both $O_2^{\bullet -}$ [19] and its dismutation product,

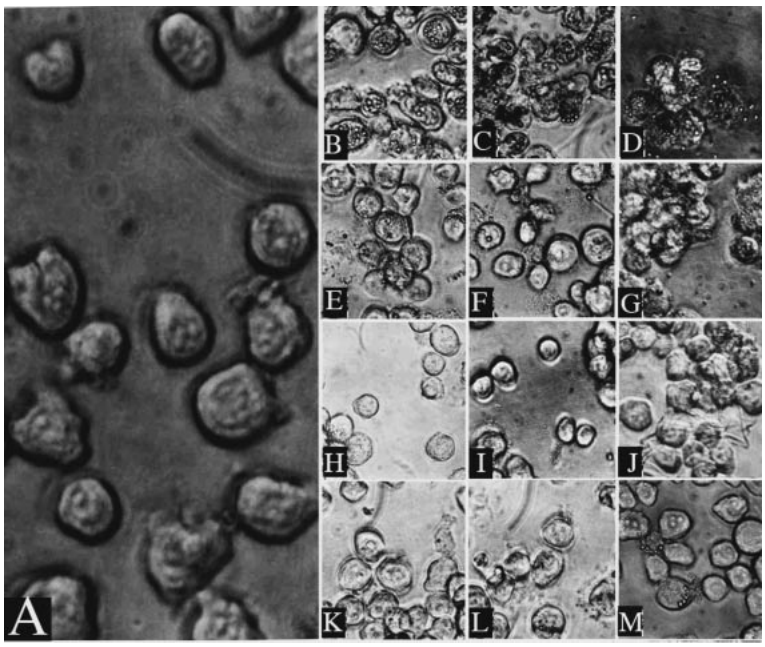

Fig. 3. Antiviral Activity of BmNOX *in Vitro*.

BmN cells were maintained as described in "Materials and Methods," and an uninfected culture is shown in A. The cultures were infected with 2 MOI (B, E, H, and K), 3 MOI (C, F, I, and L), and 5 MOI (D, G, J, and M) viral particles respectively. Untreated viral particles (B, C, and D) and viral particles pretreated with 2.94 µg (E, F, and G), 4.41 µg (H, I, and J) and 7.35 µg (K, L, and M) BmNOX protein were used for inoculations to evaluate the effect of this protein on viral infectivity.

H_2O_2, has been found in plasmatocytes of *Drosophila melanogaster* larvae[20] and *Galleria melonella*.[21] This protein might play an important role in the defense system of the silkworm against BmNPV. It is quite possible that it might function to destroy the virus in other insects or other animals. It has been found that adult *Drosophila* in which the NADPH oxidase system is silenced show marked increase in mortality rate, indicating that gut dDuox (dual oxidase) is indispensable for gut antimicrobial activities.[22] Hemocytes of *G. mellonella* were capable of phagocytosing bacterial and fungal cells and killing them by a mechanism similar to the mechanism used by human neutrophils *via* the production of superoxide. Proteins homologous to a number of proteins essential for superoxide production in human neutrophils were identified and it was found that significant regions of the 67-kDa and 47-kDa insect proteins are identical to regions of the p67phox and p47phox proteins of neutrophils.[23] Translocation of homologous hemocyte proteins 67 and 47 kDa from the cytosol to the plasma membrane upon phorbol 12-myristate 13 acetate (PMA) activation further ratifies the similarity between neutrophil and hemocyte duox systems.[24] This observation strongly supports arguments that the immune system of insects exhibits structural and functional similarity to the innate immune system of mammals.[25–27] Thus the BmNOX in the immune cells of *B. mori* larvae might help them inactivate the occluded virus involved in oral NPV infection *in vivo*. However, BmNOX appears to be capable of functioning independently of other possible regulatory subunits. It is quite likely that BmNOX can initiate cell death pathways in target cells or organisms in a reactive oxygen-dependent manner. Nonetheless, at present, we do not know whether BmNOX activity is modulated by p67 and/or p47-like molecules *in vivo*.

We have identified and partially characterized a *B. mori* gene that codes for an antiviral protein showing homology with and an activity of NADH oxidoreductase. The strong association between the activity levels of this protein and the BmNPV resistance of the silkworm raises the possibility of overexpression of this protein in silkworms to raise the disease-tolerant phenotype in *B. mori*.

Acknowledgments

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