Journal of General Virology (2004), **85**, 2255–2261 DOI 10.1099/vir.0.79965-0

Potent virucidal activity in larval *Heliothis virescens* plasma against *Helicoverpa zea* single capsid nucleopolyhedrovirus

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Received 8 January 2004
Accepted 6 May 2004

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 haemocoel, however, there are few mechanisms known to account for the resistance and clearance of infection observed in some virus–host combinations. The hypothesis that factors present in the plasma of infected pest larvae act to limit the spread of virus from initial infective foci within the haemocoel was tested. An *in vitro* bioassay was developed in which *Helicoverpa zea* single capsid nucleopolyhedrovirus (*Hz*SNPV) was incubated with plasma collected from uninfected *Heliothis virescens* larvae. Infectious *Hz*SNPV particles were then titrated on *Hz*AM1 cells. Diluted plasma from larval *Heliothis virescens* exhibited a virucidal effect against *Hz*SNPV *in vitro*, reducing the $TCID_{50}$ ml^{-1} by more than 64-fold (from $4{\cdot}3 \pm 3{\cdot}6 \times 10^5$ to $6{\cdot}7 \pm 0{\cdot}6 \times 10^3$). The antiviral activity was heat-labile but was unaffected by freezing. In addition, protease inhibitors and specific chemical inhibitors of phenol oxidase or prophenol oxidase activation added to diluted plasma eliminated the virucidal activity. Thus, in the plasma of larval lepidopterans, the enzyme phenol oxidase may act as a constitutive, humoral innate antiviral immune response.

INTRODUCTION

The panoply of vertebrate innate immune responses against virus infection has been the subject of considerable productive investigation for decades (Guidotti & Chisari, 2001). In contrast to vertebrate immunobiology, there is a remarkably limited understanding of the mechanisms by which insects resist virus infections, recognize infected cells, recruit immune cells to the infective foci or clear infected cells. Insects appear to lack any adaptive immune responses that operate analogously to the well-documented antibody or histocompatibility adaptive immune responses of vertebrates (Hoffmann, 2003). Mucosal innate immune responses are well known in vertebrates but, for the most part, are unknown in insects. Epithelial barriers, such as the peritrophic membrane and the midgut cells lining the lumen of the digestive tract, are the primary determinant in resistance of lepidopteran larvae to baculovirus infections (Cory & Myers, 2003). Following *per os* inoculation, one or more midgut cells become infected, and the infection appears to spread from these initial foci to tracheoblasts, and thus obtains access to the haemocoel resulting in a fulminating, fatal infection (Washburn *et al.*, 1995, 2001; Engelhard & Volkman, 1995; Kirkpatrick *et al.*, 1998). Lepidopteran larvae resist baculovirus infection by selective apoptosis of and sloughing off infected cells from the midgut (Federici & Hice, 1997; Clem, 2001), and a number of baculoviruses encode genes that disrupt this host apoptotic response (Clem, 2001). Once the baculovirus infection has breached the midgut barrier, however, there are few mechanisms known to account for the resistance and clearance of infection observed in some virus–host combinations (Cory & Myers, 2003).

The immune response of insects to bacterial or fungal infection and to filarial or parasitoid infestation is well documented (Gillespie *et al.*, 1997; Carton & Nappi, 1997; Hultmark, 2003; Kumar *et al.*, 2003; Leulier *et al.*, 2003). A suite of antimicrobial peptides, enzymes and metabolites that limit the proliferation of microbes within the host are induced on detection of pathogen-associated biopolymers, and secreted into the haemolymph. In addition, haemocytes present in the haemocoel are recruited to the foci of infection, phagocytosing smaller microbes and encapsulating larger invaders. Acting in concert, these humoral and cellular responses halt microbial infection and clear, or wall off, the resulting debris from the haemocoel by wrapping it in a toxic shroud of cross-linked protein, melanin and melanized haemocytes (Gillespie *et al.*, 1997; Carton & Nappi, 1997). Recently, immunosuppression of pestiferous moth larvae by chemical or biological means has revealed tantalizing hints of a cell-mediated antiviral

immune response (Washburn *et al.*, 1996, 2000). A generalized immunosuppression caused by parasitoid wasp oviposition (and their symbiotic polydnaviruses) prevents the clearance of an otherwise non-fatal baculovirus infection (Washburn *et al.*, 1996; Coudron *et al.*, 1997, 1999; Shelby & Webb, 1999; Escribano *et al.*, 2000; Trudeau *et al.*, 2001).

Antiviral activities from insect haemolymph (Chernysh *et al.*, 2002) or cultured insect cells (Ridel & Brown, 1979) have been described. Conversely, baculovirus promoting factors have been isolated from larval silkworm plasma (Kanaya & Kobayashi, 2000). The plasma enzyme phenol oxidase (PO; L-DOPA : oxygen oxidoreductase; EC 1.14.18.1) of the tobacco budworm, *Heliothis virescens*, exhibits antiviral activity against several vertebrate viruses (Ourth & Renis, 1993). Inhibition of the host melanization response by parasitoids, filarial parasites and bacteria, as well as by entomopathogenic fungi, is abetted via reduced PO activity (Shelby & Webb, 1999; Shelby *et al.*, 2000). We examined the possibility of *Heliothis virescens* haemolymph having antiviral activity against an insect virus. Our results extend and confirm the results of Ourth & Renis (1993) by demonstrating that brief *in vitro* incubation of *Helicoverpa zea* single capsid nucleopolyhedrovirus (*Hz*SNPV) with plasma from *Heliothis virescens* reduces the infectivity of the virus.

METHODS

Insects, cells and virus. *Heliothis virescens* (F.) eggs were received from the North Carolina State University Department of Entomology Insectary from a colony established from field insects in July 2002. Larvae were reared individually on an artificial wheat germ-based diet under a photoperiod of 14 : 10 h (light : dark) at 55 % relative humidity and 28 °C to the appropriate assay instar (Ignoffo & Boening, 1970). A *Helicoverpa zea* cell line, *Hz*AM1 (McIntosh & Ignoffo, 1981), was provided by Art McIntosh (USDA ARS BCIRL, Columbia, MO, USA) and maintained as monolayers at 28 °C in Excel 401 medium (JRH Biosciences) supplemented with 10 % fetal bovine serum (Integen). A wild-type *Hz*SNPV isolate (Ignoffo & Heimpel, 1965) was used and amplified in *Hz*AM1 cells.

Collection and processing of plasma. Early fourth-instar larvae were surface-sterilized in ethanol, rinsed with sterile water and anaesthetized on ice before bleeding. Haemolymph was gently extruded from an anterior proleg through a small puncture wound made with a sterile 26-gauge needle, and collected directly into a chilled 1·5 ml microcentrifuge tube containing ice-cold, sterile PBS (Shelby *et al.*, 2000). Haemolymph was adjusted to a final dilution of 1 : 10 by addition of cold PBS, after which haemocytes were removed by microcentrifugation at 5900 ***g*** for 3 min. The plasma supernatant was sterilized by centrifugation at 3200 ***g*** for 3 min through a 0·65 μm Millipore Ultrafree-MC centrifugal filter. All plasma samples were collected during the morning.

Plasma *in vitro* virucidal assay. *Heliothis virescens* plasma dilutions were combined with *Hz*SNPV at a ratio of 3 : 1 (v/v), gently mixed and incubated at 20 °C for 1 h. PBS was used as a control in the absence of plasma. Virus titres of these incubations were determined by end-point dilution assay (Summers & Smith, 1987; Slavicek *et al.*, 2001). *Hz*AM1 cells were seeded at 5×10^4 cells ml^{-1} in 96-well plates (BD Falcon) and allowed to attach for 1 h. The cells were infected with dilutions of virus/plasma or virus/PBS at dilutions of 10^{-1} to 10^{-6} and plates were incubated for 1 week at 28 °C. The plate wells were then scored positive for virus infection if polyhedra were visible within two or more cells, or negative for viral infection, and the results were used to calculate the virus titre as the $TCID_{50}$ (ml inoculum)$^{-1}$ (Slavicek *et al.*, 2001). Statistical comparisons were done with SigmaStat 3.0 or SigmaPlot 8.0 (SPSS). The Student–Newman–Keuls procedure was used for multiple comparisons when significant variation of the means was found when one-way ANOVA was performed ($P < 0{\cdot}001$).

Cell viability assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) dye reduction assay in which viable cells convert MTS into a coloured formazan product (A_{492}) (Cory *et al.*, 1991). *Hz*AM1 cells (5×10^4 ml^{-1}) were plated in 96-well microtitre plates and incubated with 1 : 10 serial dilutions of plasma and virus for 7 days. Following the manufacturer's instructions, 20 μl MTS (CellTitre 96 AQueous One; Promega) was added to 100 μl of culture medium, incubated for 1–4 h at 20 °C and the A_{492} was determined with a Perkin-Elmer HTS 7000 Plus plate reader. Results reported are means of eight wells ± SD, and each experiment was repeated at least three times.

Plasma treatments. Heat-inactivation experiments were done by incubation in a water-bath at 65 or 55 °C for 20 min. Samples were cooled before addition of virus. Plasma samples stored at −85 °C for 1 week were thawed on ice before incubation with *Hz*SNPV.

Plasma was subjected to limited inactivating proteolysis with proteinase K by incubating with proteinase K conjugated to polystyrene beads (#P0803; Sigma). Polystyrene beads (400 μl) were combined with an equal volume of 1 : 10-diluted plasma for 30 min at 20 °C with occasional agitation. The mixture was then filter-sterilized by centrifugation at 4000 ***g*** for 3 min. A cocktail of protease inhibitors with broad specificity for inhibition of serine, cysteine, aspartic and metalloproteases (#P2714; Sigma) was added at a final concentration of 10 μg ml^{-1} during collection of plasma.

In some experiments PO activation during plasma collection and subsequent incubation was inhibited by addition of the copper chelator 1-phenyl-2-thiourea (PTU) to plasma as larvae were being bled into PBS. The final concentration of PTU was 100 μM in 1 : 10-diluted plasma. To activate prophenol oxidase fully, freshly collected plasma was incubated with 1 μg lipopolysaccharide (LPS) ml^{-1} for 30 min at 20 °C to activate the melanization cascade (Kanost *et al.*, 2001). All experiments were repeated at least three times and representative experiments are presented.

RESULTS

Incubation of 1 : 10-diluted *Heliothis virescens* larval plasma with *Hz*SNPV *in vitro* at 20 °C for 1 h dramatically reduced the $TCID_{50}$ ml^{-1} when dilutions were subsequently assayed on *Hz*AM1 cells (Fig. 1). The virus titre declined significantly from $4{\cdot}3 \pm 3{\cdot}6 \times 10^5$ to $6{\cdot}7 \pm 0{\cdot}6 \times 10^3$ $TCID_{50}$ ml^{-1} ranging from 33- to 146-fold lower over five replicates (one-tailed paired *t*-test, $n = 5$, $P = 0{\cdot}0284$), indicating the presence of a factor(s) in larval plasma with virucidal activity against *Hz*SNPV.

A dilution series of *Heliothis virescens* larval plasma with PBS demonstrated that the virucidal activity was still detectable at a 1×10^{-3} dilution (Fig. 2). The virucidal activity declined in a linear fashion from the 1 : 10 dilution,

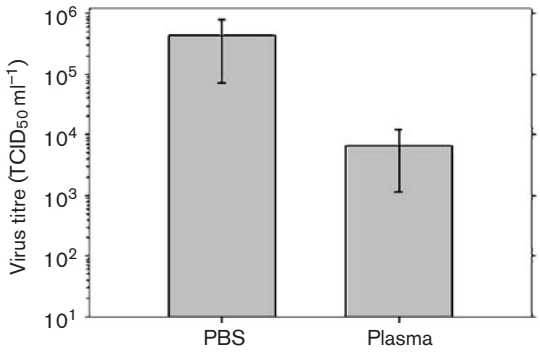

Fig. 1. *In vitro* virucidal activity in *Heliothis virescens* plasma. Freshly collected plasma was diluted and incubated with budded virus of PBS-diluted *Hz*SNPV or PBS alone at room temperature for 1 h. Virus titre ($TCID_{50}$ ml^{-1}) was calculated several days post-infection ($n=5$, mean ± SD).

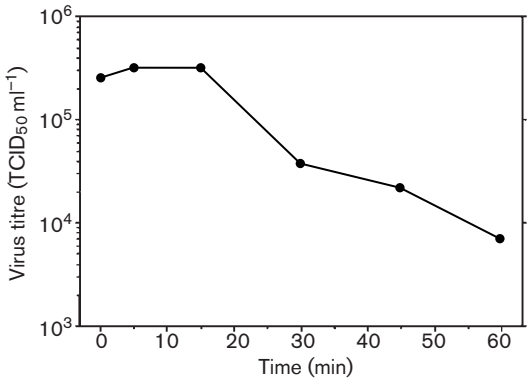

Fig. 3. Extent of *Hz*SNPV inactivation by 1 : 10-diluted *Heliothis virescens* plasma increases with incubation time. *Hz*SNPV was added at time zero and samples were removed at the indicated intervals for determination of virus titre.

becoming undetectable at a dilution of 1×10^{-4} ($R^2=0{\cdot}962$). At the 1 : 10 dilution, inactivation of *Hz*SNPV was evident within 30 min, and continued at a linear rate to 1 h (time 15–60 min, $R^2=0{\cdot}938$) (Fig. 3). Additional assays indicated that the rate did not change between 1 and 3 h (data not shown), suggesting that the antiviral activity was enzymic.

For subsequent bioassays of antiviral activity, the most favourable conditions determined empirically (as shown in Figs 1–3) were used. All *in vitro* assays were done at a plasma dilution of 1 : 10 for 1 h at 20 °C. Greater levels of activity might have been detected at lower dilutions of plasma; however, we adopted the 1 : 10 dilution ratio in order to minimize significant melanization (blackening and precipitate formation) during the incubation period and subsequent exposure of *Hz*Am1 cells to cytotoxic melanization products. The possibility that inhibitory or cytotoxic activity in plasma would interfere with the *in vitro* assay by affecting the viability of *Hz*AM1 cells during subsequent incubation with plasma-treated *Hz*SNPV dilutions was examined by using the Promega AQueous One Cell viability kit. Reduction of MTS by healthy *Hz*AM1 cells was monitored at 3 and 7 days after addition of serial dilutions of PBS, virus, plasma, and diluted plasma with virus. The assay was linear to well over 2 absorbance units (data not shown). No significant differences in cell viability were detected with plasma from the other treatments by the seventh day of the assay, except for PBS alone (Fig. 4). Surprisingly, diluted plasma seemed to have a salutary effect on *Hz*SNPV-infected cells compared with PBS controls, as determined by a higher absorbance value at the 1×10^{-2} dilution. However, all combinations inhibited cell viability

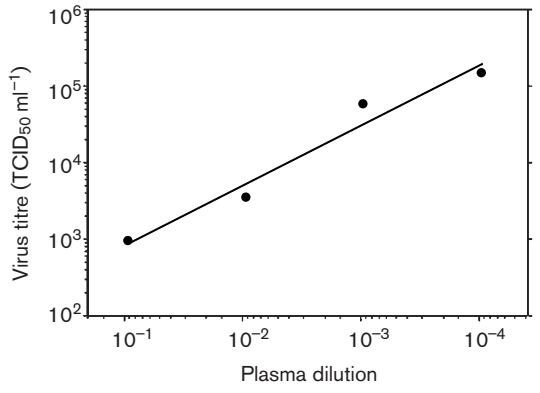

Fig. 2. Virucidal activity of *Heliothis virescens* larval plasma is rapidly diminished by serial dilution in PBS. Freshly collected plasma was prepared at four serial dilutions before addition of *Hz*SNPV. The mixture was incubated at 20 °C for 1 h before samples were taken for measurement of virus titre.

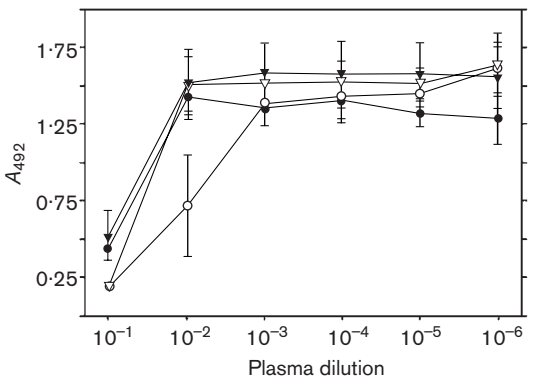

Fig. 4. Effect of plasma dilution factor on cell viability determined by the MTS assay. The number of viable cells was determined in the presence of diluted plasma, virus and PBS ($n=8$, mean ± SD). Plasma and virus were diluted with PBS. ●, PBS; ○, PBS + virus; ▼, plasma; ▽, plasma + virus.

at the initial dilution. Titres for all virucidal activity assays were taken within the 10^{-3} to 10^{-6} range, well beyond any dilution that was detrimental to cell growth.

Freezing diluted plasma at −85 °C preserved the antiviral activity (Fig. 5a). The mean value for fresh plasma was not significantly different from that for frozen plasma, although both were significantly lower than for PBS alone (Student–Newman–Keuls multiple comparison procedure; $P<0{\cdot}05$). This greatly eased the collection and storage of plasma for subsequent experiments. Incubation of diluted plasma at 55 °C for 20 min partially inactivated the virucidal activity, whereas incubation at 65 °C for 20 min completely eliminated it (Fig. 5b). The plasma mean was significantly lower than all other treatments including the 55 °C incubation, which was significantly lower than both the PBS and the 65 °C treatments. This is consistent with the inactivation pattern of an enzyme of higher molecular mass, and with commonly accepted haemolymph collection methods, which are known to prevent haemolymph melanization of *Heliothis virescens* larvae.

Pre-incubation of diluted plasma for 20 min with proteinase K conjugated to polystyrene beads, followed by removal of the beads during filter-sterilization, completely eliminated virucidal activity (Fig. 6). Inclusion of a cocktail of protease inhibitors to block the zymogen-mediated proteolytic activation of plasma prophenol oxidase activity during collection of haemolymph also drastically reduced the level of observed virucidal activity (Fig. 6). In this figure, only the plasma-alone mean was significantly lower than any other mean. Collection of larval *Heliothis virescens* plasma in the presence or absence of a well-characterized inhibitor of PO, the copper chelator PTU, prevented the inactivation of *Hz*SNPV seen in the plasma control (Fig. 7). The mean value for plasma alone was significantly lower

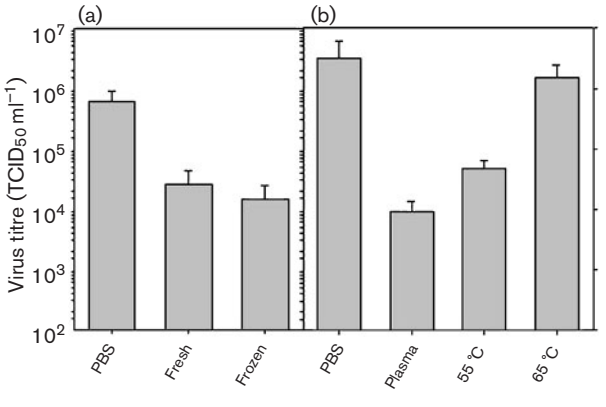

Fig. 5. Effect of freezing and heating on virucidal activity. (a) Virucidal activity of plasma was retained after diluted plasma was stored at −85 °C and subsequently thawed for determination of activity. (b) Incubation of diluted plasma at 55 °C partially inactivated the virucidal activity whereas incubation at 65 °C completely inactivated virucidal activity ($n=4$, mean ± SD).

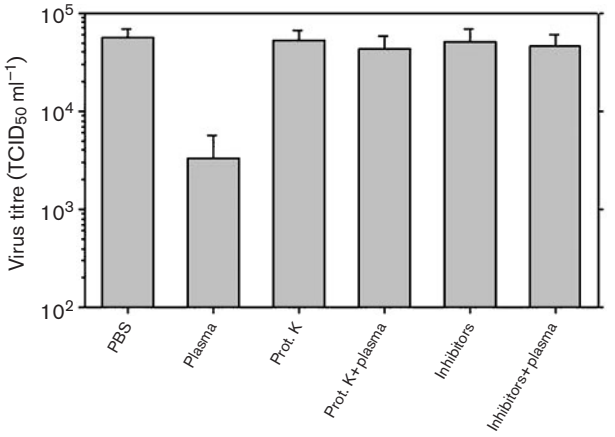

Fig. 6. Reduction in virucidal activity following addition of either proteinase K (prot. K) or a cocktail of protease inhibitors to plasma ($n=4$, mean ± SD).

than in all other treatments. Taken together, these data indicate that PO may be the activity responsible for the virucidal activity observed in larval plasma. LPS is a known activator of the prophenol oxidase activation pathway in insect plasma (Kanost *et al.*, 2001). If PO is responsible for the virucidal activity, then treatment of *Heliothis virescens* plasma with LPS should elevate observed PO activity, and consequently virucidal activity. However, pre-incubation of diluted plasma with LPS for 30 min did not induce further virucidal activity compared with non-induced control plasma (data not shown). Control plasma to which 100 μM PTU had been added prior to LPS to cancel any activation of PO activity showed activity comparable with the control to which PTU alone had been added (data not shown). The lack of any increased virucidal activity after LPS treatment may be explained by the observation that PO in these samples may already be maximally activated, since the haemolymph was extruded from larvae and

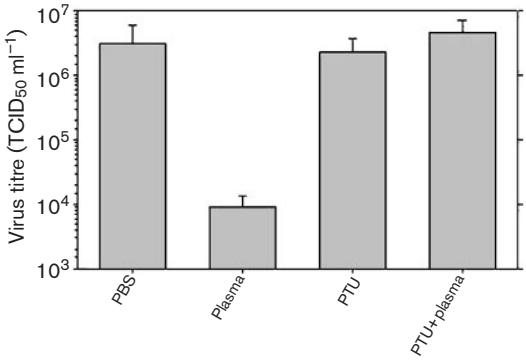

Fig. 7. Effect of addition of PTU to larval plasma. PTU added to plasma inhibited the virucidal activity and restored the virus titre to non-plasma levels ($n=4$, mean ± SD).

processed through several steps, including centrifugal filtration, with no inhibitors present.

DISCUSSION

Induction of antimicrobial immune responses against bacterial, fungal, filarial and parasitoid infestations of insects has been extensively documented; however, antiviral defences, and any interactions between antiviral and other antimicrobial defence, remain unexplored. This is despite the enthusiasm with which members of the *Baculoviridae* (double-stranded DNA viruses) have been adopted for the biological control of lepidopteran insect pests throughout the world (Bonning & Hammock, 1996). Insects have none of the well-characterized mechanisms documented in vertebrates for recognizing viruses or virus-infected cells. Yet this does not apparently prevent lepidopteran larval haemocytes from recognizing and initiating an encapsulation/melanization response against tracheoblasts infected with *Autographa californica* multicapsid nucleopolyhedrovirus (Washburn *et al.*, 1996, 2000). This cell-mediated encapsulation response against baculovirus-infected cells was revealed by the chemical- and parasitoid-mediated immunosuppression of *Manduca sexta* (Washburn *et al.*, 2000) and *Heliothis virescens* larvae (Washburn *et al.*, 1996). Factors injected into the host by the female parasitic wasp during oviposition, i.e. venoms and symbiotic polydnaviruses, inhibit the humoral and cell-mediated immune responses against the wasp eggs and larvae – including melanization catalysed by PO (Shelby *et al.*, 1998, 2000; Shelby & Webb, 1999). Parasitized larvae are subsequently more susceptible to opportunistic microbial and baculoviral infections (Coudron *et al.*, 1995, 1999; Escribano *et al.*, 2000), which supports the hypothesis that PO plays an important role in resistance to baculoviral infection.

As in other insects, microbial challenge of *Heliothis virescens* larvae induces expression of a suite of antibacterial and antifungal proteins such as cecropins A, B and C and attacins (Ourth *et al.*, 1994), lysozyme (Lockey & Ourth, 1996; Shelby *et al.*, 1998; Chung & Ourth, 2002), heliocin, heliomicin (Lamberty *et al.*, 1999), virescein and viresin (Chung & Ourth, 2000). Insect prophenol oxidase exists in plasma as a zymogen, in multimeric complexes with other proteins, and is secreted by the fat body, haemocytes, cuticular epidermis and other tissues (Asano & Ashida, 2001). It is activated to PO by the presence of fungal 1,3-β-glucans, LPS from Gram-negative bacteria and peptidoglycans of Gram-positive bacteria (Kanost *et al.*, 2001). The plasma PO of *Heliothis virescens* exhibits antiviral activity against several vertebrate viruses *in vitro* (herpes simplex virus types 1 and 2, vesicular stomatitis virus, human parainfluenzavirus 3, coxsackievirus B3 and Sindbis virus) (Ourth & Renis, 1993). Of these, vesicular stomatitis virus and Sindbis virus were the only viruses tested which are known to infect insects or insect cells (Lewis *et al.*, 1999). Activity against known insect viruses such as baculoviruses was not tested, and is the subject of this report. The antiviral activity was present constitutively in plasma, i.e. immunization by bacterial injection did not increase activity over naïve controls. The authors attributed the observed antiviral activity in *Heliothis virescens* plasma to PO because: (i) it was inhibited by PTU and addition of EDTA/citrate to plasma; (ii) the antiviral effect could be restored to a plasma chromatographic fraction containing PO activity; (iii) by addition of known PO substrates; and (iv) the antiviral effect could be mimicked *in vitro* by incubation with mushroom tyrosinase and the same substrates (Ourth & Renis, 1993). With a similar assay we have found that, although *Hz*SNPV was substantially inactivated by *Heliothis virescens* plasma, the level of inhibition was much lower than that seen against vertebrate viruses, for which *Heliothis virescens* is not a host.

The mode of action against *Hz*SNPV appears to be direct, as it occurs *in vitro*, before addition to *Hz*AM1 cells. Addition of plasma and virus directly to *Hz*AM1 cells, bypassing the 20 °C *in vitro* incubation, did not yield significant differences in the $TCID_{50}$. Pre-incubation of *Hz*AM1 cells for 2 h with 1 : 10-diluted plasma before addition of *Hz*SNPV did not affect the subsequent course of infection; $TCID_{50}$ values were not affected. This would seem to militate against mechanisms whereby the uncharacterized factor interferes with the infective process by blocking a required receptor, or by activating *Hz*AM1 cells to render them more resistant to initial infection. The known inhibitory action of PTU, a copper chelator, against the *Heliothis virescens* plasma PO, provides a strong argument for involvement of this enzyme in the virucidal activity against *Hz*SNPV detailed above.

High molecular mass melanogenic and sclerotinogenic multimeric protein complexes (multimers of prophenol oxidase, dopachrome tautomerase, interleukin 1-like molecule and other enzymes) are present at high concentration in insect plasma (Beck *et al.*, 1996), and are thought to participate in the recognition and 'opsinization' of invading pathogens, and in the recruitment of haemocytes, which phagocytose smaller pathogens (Marmaras *et al.*, 1996; Sugumaran, 2002). The density-dependent prophylaxis against a wide variety of entomopathogens observed in lepidopteran larvae is directly correlated with elevated cuticular and plasma melanizing capability, i.e. PO activity (Reeson *et al.*, 1998; Wilson *et al.*, 2001). Conversely, reduction of plasma melanizing activity, by targeting prophenol oxidase expression in the mosquito *Armigeres subalbatus* with a Sindbis virus encoding an antisense transcript, eliminated melanization of *Dirofilaria immitis* microfilaria (Shiao *et al.*, 2001). Generation of cytotoxic free radicals (e.g. quinones, quinone methides, semiquinones, superoxide, hydrogen peroxide, nitric oxide, peroxynitrite) in the haemolymph of insects by these soluble enzymic activities and by LPS-stimulated haemocytes is a well-documented response to microbial infection, and to filarial and parasitoid infestation (Luckhart *et al.*, 1998; Nappi & Ottaviani, 2000; Kumar *et al.*, 2003; Leulier *et al.*, 2003). The possible

virucidal activity of these free radicals in infected insects has not yet been adequately explored.

In conclusion, we have documented the presence of an antiviral activity, present in the plasma of susceptible larvae, which is active against baculoviruses *in vitro*. Inhibitor and substrate studies indicate that the virucidal activity is coincident with activity of the plasma enzyme PO. Isolation and further characterization of this virucidal activity will be the subject of a future report. The mechanism by which this factor can distinguish the invading virus from host proteins and tissues residing within the haemocoel remains an extremely intriguing question, which we intend to pursue, as is the possibility that this activity may be involved in vector competence of arthropods that transmit viruses to vertebrates or plants.

ACKNOWLEDGEMENTS

We thank Mark Garriot and John Willenberg for technical assistance. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. All programmes and services of the US Department of Agriculture are offered on a non-discriminatory basis without regard to race, colour, national origin, religion, sex, age, marital status or handicap.

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