

Expression of Hornet Genes Encoding Venom Allergen Antigen 5 in Insects

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Antigen 5, also known as *Dol m V*, is a major allergen found in the venom of the baldfaced hornet, *Dolichovespula maculata*. We have inserted the f10 and f17 cDNAs, which encode hornet antigen 5 (HA5) forms 2 (*Dol m*; VB) and 3 (*Dol m* VA), respectively, into the genome of the baculovirus, AcMNPV, to produce the recombinant baculovirus gene expression vectors, vEV-HA5f10 and vEV-HA5f17. Insect cells infected with either vEV-HA5f10 or vEV-HA5f17 produce and secrete a novel protein with an electrophoretic mobility which is similar if not identical to authentic mature *Dol m V*. The gene products also react specifically with a polyclonal antiserum raised to *Dol m* VB as expected. *Dol m V* gene products were not acutely toxic when injected into insect larvae. However, infection of fifth instar larvae with vEV-HA5f17 resulted in premature melanization of the larvae and lower weight gain than infection with control virus. Thus, the *Dol m V* gene product has a subtle, possibly cytotoxic or biochemical effect on insects. The expression systems may prove useful in further structural and functional characterization of these proteins. © 1993 Wiley-Liss, Inc.

Key words: baculovirus, *Autographa californica* nuclear polyhedrosis virus, insect toxin, protein production, gene expression, hornet venom

INTRODUCTION

Antigen 5 is one of three major proteins found in the venoms of hornets, wasps, and yellowjackets [1,2] and appears to be the most active allergen in vespid-allergic patients [1]. Three chromatographic forms of *Dol m V** desig-

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nated as *Dol m* VC (form 1), VB (form 2), and VA (form 3) [3] have been isolated from venom sac extracts of the baldfaced hornet, *Dolichovespula maculata* (also referred to as the white-faced hornet) [1,4]. The cDNAs which encode *Dol m* VB and VA have been isolated and characterized [3,5]. The predicted amino acid sequences indicate that mature *Dol m* VB and VA have 204 and 205 amino acids, respectively, and share 67% amino acid sequence identity, which is consistent with their antigenic similarity. The presence of a 23 amino acid hydrophobic N-terminal domain, which was not found in the protein sequence of *Dol m* VB isolated from venom sac extracts, indicates that antigen 5 is synthesized as a precursor protein [5].

No enzymatic activity has been found to be associated with purified *Dol m* V and its biochemical function has not been established [1,2]. The physicochemical properties and amino acid composition of *Dol m* V are similar to a presynaptic neurotoxin from the hornet *Vespa mandarinia* which is active at the neuromuscular junctions of crustaceans [6]. Limited amino acid sequence similarity has been noted with scorpion neurotoxins, pathogenesis-related proteins from plants, and a mammalian sperm-coating protein [3,4]. The similarity to scorpion venom neurotoxins suggested the possibility that *Dol m* V might have neurotoxic activity and was of interest to study further.

We have inserted *Dol m* V cDNAs into the genome of AcMNPV to construct baculovirus vectors for *Dol m* VB and VA production. Such gene expression vectors were expected to provide a convenient source of *Dol m* V for further biochemical, toxicological, and allergenicity studies. Based on the possible relationship of *Dol m* V to neurotoxins, it was also considered possible that expression of *Dol m* V during the course of baculovirus infection might increase the rate at which the viruses kill their insect hosts. Baculoviruses are considered to have excellent potential as biological pesticides, but they normally require several days to kill their hosts [7]. Enhancement of the rate of morbidity might provide for improvement of baculoviruses as biological pest control agents.

We report that both *Dol m* VB and VA are produced at sufficiently high levels in recombinant AcMNPV-infected cells for this expression system to be useful as a source of *Dol m* V proteins. Neither form of purified *Dol m* V had detectable toxicity in insect larvae upon injection. However, infection of larvae with a recombinant virus expressing *Dol m* V resulted in premature melanization and an effect on insect growth during infection was noted.

MATERIALS AND METHODS

Cells and Viruses

Spodoptera frugiperda (fall armyworm) IPLB-SF21 (SF21) cells [8] were maintained in TC-100 medium (GIBCO Laboratories, Grand Island, NY) supple-

*Abbreviations used: AcMNPV = Autographa californica nuclear polyhedrosis virus; *Dol m* V = *Dolichovespula maculata* venom form 3; *Dol m* VB = *Dolichovespula maculata* venom antigen form 2; *Dol m* VC = *Dolichovespula maculata* venom antigen form 1; DTT = dithiathreitol; pfu = plaque forming units; p.i. = post infection; SDS = sodium dodecyl sulfate; SF-21 = the IPLB-SF21 line derived from *Spodoptera frugiperda*; TC-100 = cell culture media; vEH-HA5f10 = an AcMNPV-based vector for expression of *Dol m* VB; vEV-HA5f17 = an AcMNPV-based vector for expression of *Dol m* V; wt = wild type.



mented with 10% fetal bovine serum, 0.25% tryptose broth, and antibiotics. Stocks of wt virus, AcMNPV L-1 [9], and recombinant viruses were propagated and titered in this cell line as previously described [10].

In order to generate recombinant viruses which could direct the expression of *Dol m* VB and VA in infected insect cells, cDNAs encoding these proteins [5] were cloned into the transplacement vector pEV-55 [10,11]. The *Dol m* VB and VA cDNAs (f10 and f17, respectively) were first excised from the plasmids pUC8-f10 and pUC8-f17 [3,5] by EcoRI cleavage and then inserted into the EcoRI site of pEV-55 downstream of the AcMNPV polyhedrin promoter (Fig. 1). The DNA sequence of the pEV-55/cDNA junctions in the resulting plasmids, pEV-HA5f10 and pEV-HA5f17, were verified by double-stranded sequencing of plasmid DNA [12] using a primer corresponding to residues -26 to -42 in the polyhedrin leader sequence.

Recombinant viruses expressing these genes were generated by the allelic replacement of the AcMNPV polyhedrin coding sequences with the *Dol m V* coding sequences. Insect cells were cotransfected with a mixture of wt AcMNPV DNA and either pEV-HA5f10 or pEV-HA5f17 DNA [10]. The plaque-purified recombinant viruses, vEV-HA5f10 and vEV-HA5f17, were identified on the basis of their occlusion negative phenotype [10] and characterized by restriction endonuclease analysis and Southern blotting.

Analysis of Proteins Synthesized in Infected Insect Cells

Proteins, which were synthesized in insect cells infected with wt or recombinant viruses, were analyzed as previously described [13]. Briefly, Sf21 cells (10^6 cells/35 mm dish) were infected with wt or recombinant virus at a multiplicity of infection of approximately 10 pfu/cell. At various times p.i., the medium was removed and the cells incubated in methionine-deficient medium for 1 h followed by labeling for 1 h with [^{35} S]methionine. The cells were rinsed of excess label, and then harvested, or overlaid with media containing no serum for another 2 h for experiments monitoring protein secretion. The unsupplemented media was collected separately and the cells were disrupted in lysis buffer containing 1% Nonidet P-40 and proteinase inhibitors phenylmethylsulfonyl fluoride (1 mM, Fluka, Ronkonkoma, NY), pepstatin (40 μ M, Fluka), and leupeptin (20 μ M, Fluka). Some samples were also incubated with polyclonal antiserum specific for *Dol m* VB [5] and cognate proteins were immunoprecipitated using a previously described protocol [13]. Proteins were separated on 12% polyacrylamide gels (SDS-PAGE) [14] and then visualized by Coomassie blue staining and autoradiography.

Larval Toxicity Assays

Authentic *Dol m* VB purified from venom sacs and the baculovirus-derived *Dol m* V gene products were assayed for toxicity to insect larvae. Late fourth instar larvae of *Trichoplusia ni* (approximately 40–50 mg each) were injected with up to 900 ng of authentic *Dol m* VB per insect or 2–5 μ L of wt-AcMNPV, vEV-HA5f10, or vEV-HA5f17 infected cell extracts equivalent to 24,000 cells per μ L of extract. Extracts were prepared from cells 48 h p.i. by pelleting the cells, resuspending them in phosphate-buffered saline containing the proteinase inhibitors, and then sonicating them.

Larval Weight Gain Analyses

The effects of in vivo expression of *Dol m* VA were studied by injecting first day fifth instar larvae with 400,000 pfu of vEV-HA5f17, vEV55, or TC-100. The latter virus is a recombinant AcMNPV carrying a deletion in the polyhedrin gene; this recombinant was constructed by allelic replacement using pEV55 [11]. Thirty larvae were used for each of the virus treatments as well as for a mock-infected control in which TC-100 tissue culture fluid was injected instead of virus. Larvae were placed on semi-synthetic alfalfa-based diet and examined each day for weight gain, melanization, paralysis, or death.

RESULTS

Construction of Recombinant Viruses

cDNAs encoding *Dol m* VB and VA were cloned into the transplacement plasmid pEV-55 to produce pEV-HA5f10 and pEV-HA5f17, respectively (Fig. 1). In pEV-HA5f10, the junction of polyhedrin and *Dol m* VB leader sequence is at position –16 relative to the *Dol m* VB ATG (+1, +2, +3) while in pEV-HA5f17, the junction occurs at position –5 relative to the *Dol m* VA ATG. Recombinant viruses vEV-HA5f10 and vEV-HA5f17 were constructed by

cotransfection of wt-AcMNPV DNA with pEV-HA5f10 and pEV-HA5f17, respectively. This allows for the allelic replacement of the wt-polyhedrin sequences with the *Dol m V* gene which is flanked by viral sequences. Recombinant viruses were first identified by screening for an occlusion-negative phenotype since the polyhedrin gene is replaced in these viruses. We further confirmed the position of the *Dol m V* cDNAs in the AcMNPV genome by Southern blot analysis of recombinant viral DNA using radiolabeled *Dol m* VB or VA fragments as probes.

Analysis of *Dol m* VB and VA Expression

The expression of *Dol m* VB and VA cDNAs was examined by SDS-PAGE of extracts of SF21 cells pulse-labeled with [³⁵S]methionine at various times after infection with wt or recombinant viruses. In the Coomassie blue stained gel presented in Figure 2A, a novel M_r 24,000 protein, which comigrates with authentic *Dol m* VB, is visible in the lysates of cells infected with recombinant viruses at 36 and 48 h p.i., but not in mock- or wt-infected cells. The M_r 24,000 polypeptide was not detected by Coomassie blue staining in the extracellular fluids from any of the cell preparations (the data shown are only for 36 h p.i.). The steady-state levels of the *Dol m* VA gene product appear to be slightly higher than that of *Dol m* VB. Polyhedrin, which accumulates to high levels in wt-infected cells, is not visible in Figure 2A because the crystalline polyhedrin (occluded virus particles) was removed from the cell lysates by centrifugation before denaturation for SDS-PAGE.

The temporally regulated synthesis of the *Dol m* VA and VB gene products is observed in an autoradiogram (Fig. 2B) of the gel shown in Figure 2A. Although the level of *Dol m V* production is less than that of polyhedrin (data not shown), the regulation of expression is similar. The M_r 24,000 polypeptide is synthesized from 24–48 h p.i. only in cells infected with recombinant virus, whereas the synthesis of polyhedrin (Polh) (which is present here in residual amounts) is detected only in wt-infected cells. We also observe a faint, novel M_r 27,000 polypeptide in the lysates of recombinant virus-infected cells at 36 h p.i. that may correspond to a precursor form. Host cell protein synthesis is inhibited at late times p.i. and, by 36–48 h p.i., the only proteins being actively synthesized are very late viral proteins and the *Dol m V* gene products. A M_r 24,000 polypeptide was also present in the extracellular fluids of both vEV-HA5f10 and vEV-HA5f17 infected cells at 24–48 h p.i. but not in wt-infected cells (Fig. 2B; the data shown are for 36 h p.i. only) indicating that this protein could be secreted from the cells.

To confirm that the M_r 24,000 polypeptides correspond to the expression of *Dol m* VB and VA, we used a polyclonal antibody obtained from mice immunized with *Dol m* VB [5] to immunoprecipitate proteins from wt or recombinant virus-infected cells. The antibody reacts specifically with the M_r 24,000 polypeptide which is present in both vEV-HA5f10- and vEV-HA5f17-infected cells at 24 and 48 h p.i., but not to any proteins produced in either mock- or wt-infected cells (Fig. 3). Therefore, the expression of each *Dol m V* cDNA produces a *Dol m V*-related polypeptide. Laying the autoradiogram over the Coomassie blue stained gel, in which authentic unlabeled *Dol m* VB was included for comparison, showed that the immunoprecipitated proteins

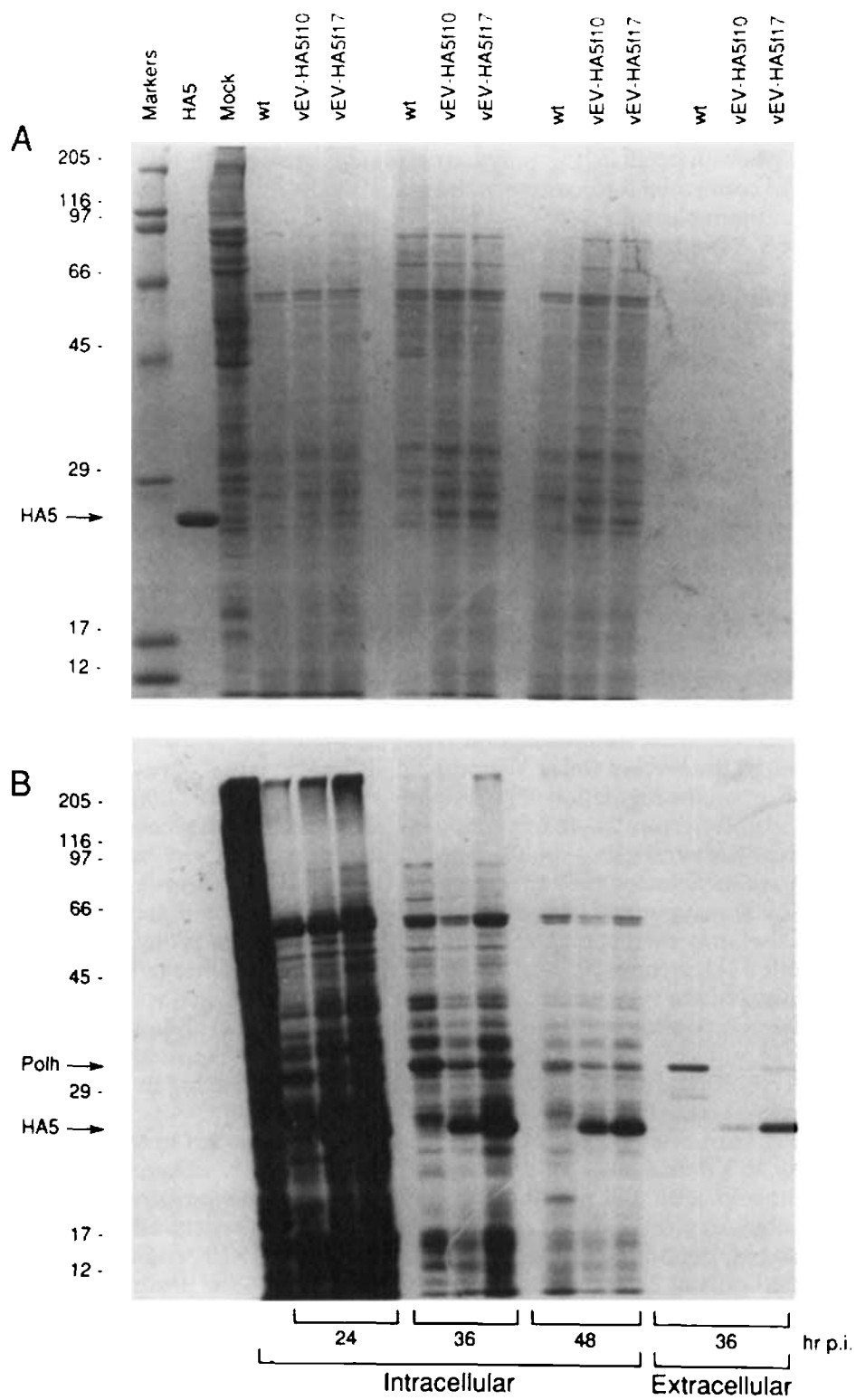


Fig. 2.

comigrate with the authentic *Dol m* VB (overlay not shown). Although there is a slight difference in the apparent molecular weights of the *Dol m* V polypeptides in this figure, there is no observable mobility difference between these proteins purified from venom or in the gel in Figure 2.

Larval Toxicity

Authentic *Dol m* VB did not produce any detectable toxic effects in *Trichoplusia ni* larvae when administered at the highest dose of 900 ng per larvae. Toxic activity was not observed upon injection of larvae with 48 h infected cell extracts or with media of vEV-HA5f10- or vEV-HA5f17-infected cells. We estimate that each larva received approximately 100 ng of *Dol m* V gene products. Larvae injected with media derived from wt or recombinant virus-infected cells died of baculovirus infection at similar times p.i. However, in the course of these experiments, we noted that larvae injected with the HA5-recombinant viruses melanized sooner than wt-infected larvae. *Trichoplusia ni* larvae are normally pale green but become almost white during infection with wt AcMNPV. Late in infection, they develop localized dark brown spots on the cuticle and melanize completely shortly after death.

To further pursue the observation of premature melanization and to more accurately assess the possible toxicological impact of *Dol m* V expression, the coloration of larvae and their weight were monitored during infection with vEV-HA5f17. For a synchronous infection, fifth instar larvae were injected with 400,000 pfu of budded virus and monitored as described in Materials and Methods. A polyhedrin deletion mutant, vEV55, was used as a control for virus infection in the absence of *Dol m* VA and polyhedrin gene expression while mock-infected insects served as a comparative control for virus infection. Melanization of the majority of vEV-HA5f17-infected insects occurs within two days post-injection, whereas melanization of vEV55-infected insects is not observed until 4 days post-injection (Fig. 4, bar graph) when significant mortality occurs. All injected insects died of virus infection between days 4 and 5, whereas all mock-infected insects pupated by day 2. Average cumulative weight gains of vEV-HA5f17-infected insects were slightly but significantly lower than those observed for vEV55-infected insects (Fig. 4, line graph). Pupated mock-infected insects have lower weight gains than vEV55-infected insects owing, in part, to the pupation process.

Fig. 2. Expression of *Dol m* V genes in recombinant virus-infected insect cells. SF21 cells were infected with vEV-HA5f10, vEV-HA5f17, or wt-AcMNPV and pulse-labeled with [³⁵S]methionine for 1 h. At 24, 36, and 48 h p.i., cells were briefly washed 3 × with phosphate-buffered saline to remove excess radioisotope, overlaid with 250 μL of serum-deficient media, and allowed to incubate for another 2 h. The extracellular media was collected separately and the cells were disrupted in 50 μL of lysis buffer. Extracts were clarified by centrifugation before cell-equivalent volumes of cell lysate (5 μL) and extracellular fluid (25 μL) were denatured in buffer containing DTT and SDS, and analyzed by SDS-PAGE. Proteins were visualized by Coomassie blue staining (A) and autoradiography (B). As a standard, 3 μg of venom-purified *Dol m* VB (HA5) was included. The positions of *Dol m* VB and polyhedrin (Polh) are noted.

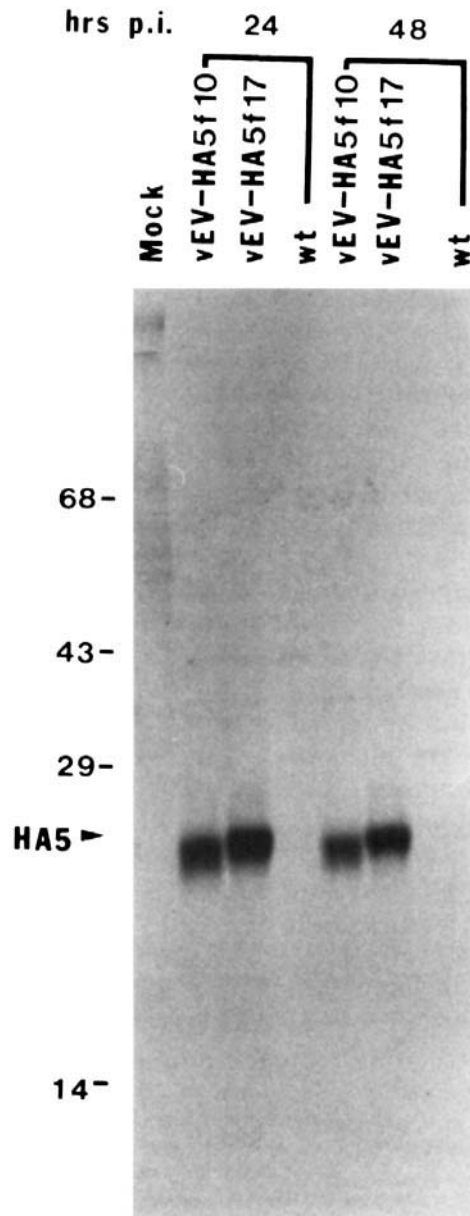


Fig. 3. Mock-, vEV-HA5f10-, vEV-HA5f17-, and wt-AcMNPV-infected cell lysates from cells pulse-labeled at 24 and 48 h p.i. (see legend to Fig. 2) were immunoprecipitated with mouse antiserum specific for *Dol m* VB [5] and analyzed by SDS-PAGE and autoradiography. Molecular size markers are in kilodaltons at the left. The position of venom-purified *Dol m* V (HA5) is indicated.

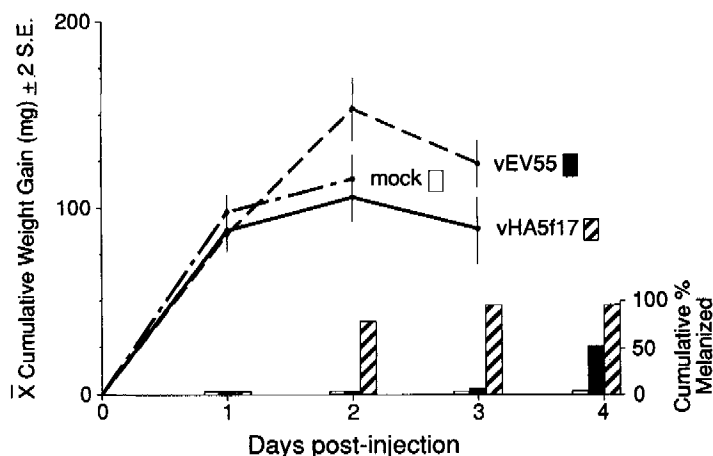


Fig. 4. Average cumulative weight gains of larvae and cumulative percent of melanized larvae during infection with recombinant viruses. Fifth instar *Trichoplusia ni* larvae were infected by injection with vEV-HA5f17, vEV55, or TC-100 (mock). All mock-infected insects pupated by day 2. The bar graph shows the percent of larvae exhibiting a brown coloration (melanization) at each day after injection. The line graph shows the average cumulative weight gains of the larvae during infection.

DISCUSSION

In this study, we constructed the expression vectors vEV-HA5f10 and vEV-HA5f17 which contain the hornet venom allergen genes *Dol m* VB and VA, respectively, under the control of the polyhedrin gene promoter. Insect cells infected with these recombinant viruses produce *Dol m* VB and VA at levels of approximately $1 \mu\text{g}/10^6$ cells as estimated by Coomassie blue staining intensity compared to known quantities of purified *Dol m* VB.

Our experiments also indicate that the baculovirus-produced *Dol m* Vs are efficiently processed since essentially all the protein is the same size as *Dol m* VB purified from venom. Low levels of a putative M_r 27,000 precursor were observed in pulse-labeling and it is likely that the *Dol m* Vs are synthesized as 226 or 227 amino acid polypeptide precursors of approximately M_r 27,000 and then cleaved to generate the 204 or 205 amino acid mature forms of approximately M_r 24,000. The data are consistent with those of Fang et al. [5] who found that the predicted amino acid sequence of the *Dol m* VB cDNA contains a 23-residue hydrophobic N-terminal segment which is missing in the empirically derived protein sequence.

In insect bioassays, neither authentic *Dol m* VB nor *Dol m* V gene products elicited any detectable toxicological effects. In a previous study of baldfaced hornet venom, Schmidt and Blum [15] reported an LD_{50} of $17.2 \mu\text{g}$ dry whole venom/100 mg *Galleria mellonella* larvae. If 2.9% of the venom dry weight is *Dol m* V, this LD_{50} corresponds to approximately $0.5 \mu\text{g}$ *Dol m* V/100 mg larvae. The LD_{50} of the venom to insects of other orders (i.e., Orthoptera and Diptera) were similar [15]. Thus, the doses administered in our bioassay (up to $1.8 \mu\text{g}$ of purified *Dol m* VB/100 mg larvae) should have been sufficient to

elicit this toxicological effect if *Dol m* VB were solely responsible for the lethal effects reported by Schmidt and Blum [15].

We have observed, however, an effect of *Dol m* V gene expression during infection of insect larvae. *Dol m* V production results in a premature melanization of the infected insect larvae and a slight but significant reduction in larval weight gains during infection. These results may be due to a cytotoxic or enzymatic effect of the *Dol m* V gene product. No neurotoxic effects were observed. We conclude that the *Dol m* V gene product alone is not highly toxic to insects although it does exert a subtle effect during continuous exposure and/or during virus infection. The conclusion is consistent with the view that hornet venom is adapted to be used primarily as a defense against vertebrates rather than for subduing invertebrate prey [15]. Indeed, hornets do not usually envenom other insects but physically attack them, using their mandibles to advantage.

The baculovirus expression vectors described herein may prove to be convenient sources of *Dol m* V for biochemical, toxicological, and allergenic studies. The observation of premature melanization may provide a bioassay for further *Dol m* V functional analysis.

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