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Improved insecticidal efficacy of a recombinant baculovirus expressing mutated JH esterase from *Manduca sexta*

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ABSTRACT

Juvenile hormone esterase (JHE), a member of the carboxylesterase family (EC 3.1.1.1), metabolizes JH that is found in juvenile insects. A highly conserved amphipathic alpha helix is found on the surface of known JHEs. This helix is implicated in receptor-mediated binding and endocytosis of JHE by the pericardial cells resulting in the clearance of JHE activity from the hemolymph. In this study, Lys-204 and Arg-208 of the amphipathic alpha helix of the JHE of *Manduca sexta* (MsJHE) were mutated to histidine residues generating MsJHE-HH. Pharmacokinetic studies following the injection of MsJHE-HH into the hemocoel of larval *M. sexta*, *Heliothis virescens*, and *Agrotis ipsilon* indicated that MsJHE-HH and wild type MsJHE are cleared at similar rates. The infectivity (lethal concentration and lethal time) of a recombinant baculovirus, AcMsJHE-HH, expressing MsJHE-HH was not significantly different than that of a recombinant baculovirus, AcMsJHE, expressing MsJHE in first instars of *H. virescens* and *A. ipsilon*. However, the mass of AcMsJHE-HH-infected larvae was 40–50% lower than that of larvae infected with AcMsJHE, and 70–90% lower than that of wild type AcMNPV-infected larvae.

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1. Introduction

Juvenile hormone (JH) is a critical insect hormone that regulates development, reproduction, and a number of other key biological events in insects. There are at least six forms of JH; all are sesquiterpenoids with a methyl ester at one end and an epoxide at the other (Goodman and Granger, 2005). The hemolymph titer of JH is determined by a combination of its synthesis and release by the *corpora allata* (Slade and Zibitt, 1971) as well as other factors such as the concentration, catalytic activity, and/or binding affinity of JH degrading and/or JH binding proteins (Hammock, 1985; Jones et al., 1996; Nijhout and Reed, 2008; Roe et al., 1993). JH is metabolized by two hydrolytic enzymes in the α/β-hydrolase fold family known as JH esterase (JHE) and JH epoxide hydrolase (JHEH). Metabolism of JH by JHE and JHEH generate JH acid and JH diol, respectively, that can be further metabolized to JH acid diol. JHE is primarily found in the hemolymph and tissues, whereas JHEH is tissue bound. JHEs show high specificity for JH but only a moderate turnover rate (reviewed in Kamita and Hammock, 2010). JHE efficiently hydrolyzes JH under biological conditions, where total JH concentrations are in the low nM range in caterpillars (Baker et al., 1987; Hidayat and Goodman, 1994). Two remarkable morphological effects demonstrate the crucial role that JHE plays in insect metamorphosis. Firstly, when JHE is inhibited in caterpillars, JH titer remains stable, the larvae continue to feed, and giant larvae are the result (Sparks and Hammock, 1980). Secondly, when JHE is injected into a caterpillar or expressed *in vivo* by a recombinant baculovirus vector, an anti-JH effect is seen that is manifested by a dose-dependent blackening and decrease in feeding in the early larval instars (Hammock et al., 1990; Philpott and Hammock, 1990).

Baculoviruses are arthropod-specific viruses that are known pathogens in numerous lepidopteran pests; and they are a major factor in the natural regulation of population densities in many pest species. Baculoviruses are commonly used in research laboratories as protein expression vectors. Baculoviruses are also used as effective biological control agents for the protection of forest and field crops. Perhaps the most successful application of a baculovirus biopesticide has been the use of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgNPV) for the protection of multiple millions of hectares of soybean in Brazil (Moscardi, 1999). Unfortunately, the general use of natural baculoviruses such as AgNPV in augmented control strategies for crop protection has been limited primarily because of slow speed of kill in comparison to synthetic chemical insecticides. Several approaches to genetically modify the baculovirus for improved insecticidal efficacy have been investigated

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(reviewed in Kamita et al., 2010). One elegant approach involves the baculovirus expression of a protein that can alter the normal physiology of the host caterpillar. Genetically-modified or recombinant baculoviruses expressing, for example, diuretic hormone (Maeda, 1989), eclosion hormone (Eldridge et al., 1991), prothoracicotropic hormone (O'Reilly et al., 1995), and pheromone biosynthesis activating neuropeptide (Ma et al., 1998) have been generated. These constructs, however, show only modest or no improvement in insecticidal efficacy. In a related approach, a recombinant *Autographa californica* NPV (AcMNPV) has been generated that expresses a juvenile hormone (JH) hydrolyzing esterase (Hammock et al., 1990). The premature overexpression of JHE in pest insect larvae is an attractive approach for insect control because JHE has a very high catalytic efficiency (k_{cat}/K_M ratio) and the resulting precocious decrease in JH titer will decrease feeding and disrupt normal development. First instar larvae of *Trichoplusia ni* that are infected with the recombinant AcMNPV that expresses JH esterase of *Heliothis virescens* (HvJHE) show profoundly reduced feeding and growth in comparison to wild type AcMNPV-infected controls. This improvement in insecticidal efficacy, however, is not seen in later instars supposedly because the level of recombinant JHE activity in the hemolymph is not sufficient (Hammock et al., 1990).

Although an extraordinarily stable protein to many denaturing condition including heat and organic solvents, JHE is rapidly cleared from hemolymph following injection. JHE is cleared more rapidly in fact than many foreign proteins suggesting a selective mechanism for its recognition and removal (Booth et al., 1992; Ichinose et al., 1992a). The rapid removal of endogenous JHE from the hemolymph is critical for normal development in lepidopteran larvae and involves receptor-mediated endocytosis by pericardial cells followed by degradation in lysosomes (Bonning et al., 1997a; Booth et al., 1992; Ichinose et al., 1992a,b). A highly conserved amphipathic alpha helix that is located on or near the surface of JHEs has been implicated in the receptor-mediated endocytosis of JHEs of lepidopteran, coleopteran, and dipteran origin (Thomas et al., 1999; Wogulis et al., 2006). Three amino acid residues (generally arginine or lysine) are conserved on the "hemolymph side" of this amphipathic alpha helix (Kamita and Hammock, 2010). In the case of the JHE of *Manduca sexta* (MsJHE), these conserved residues are Arg-197, Lys-204, and Arg-208 (Fig. 1A). Following endocytosis, the JHE associates with one or more binding proteins that are involved in sorting within the early endosome and/or transport of JHE following release from the early endosome to a lysosome (Bonning et al., 1997a; Shanmugavelu et al., 2001). One cytosolic JHE binding protein called P29 has been well studied in *M. sexta* (Shanmugavelu et al., 2000) and *Drosophila melanogaster* (Liu et al., 2007). Site-directed mutagenesis experiments indicate that P29 interacts with Lys-29 and Lys-524 of HvJHE (Bonning et al., 1997b; Shanmugavelu et al., 2000). Mutation of both of these lysine residues results in a mutant protein (JHE-KK) that shows reduced efficiency of lysosomal targeting. Furthermore, neonates of *H. virescens* infected with AcJHE-KK, a recombinant AcMNPV expressing JHE-KK, show a dramatic reduction in feeding damage in comparison to larvae infected with the wild type AcMNPV.

In this study, we test the hypothesis that a recombinant baculovirus expressing JHE with a mutated amphipathic alpha helix will show improved insecticidal activity in comparison to a recombinant baculovirus expressing wild type JHE. Specifically, a double histidine mutant of the amphipathic alpha helix of MsJHE (MsJHE-HH) was generated and expressed by a recombinant AcMNPV (AcMsJHE-HH). The mutations were generated so that MsJHE-HH could potentially chelate Ni^{2+} and form a homodimer complex (i.e., Ni^{2+} sandwiched between two MsJHE-HH proteins). Our expectation was that the mutations would reduce or block clearance of MsJHE-HH from the hemolymph by reducing or blocking its binding with the JHE receptor of pericardial cells. The hemolymph stability of MsJHE-HH following injection and insecticidal efficacy of AcMsJHE-HH were determined in larvae of *M. sexta*, *H. virescens*, and *Agrotis ipsilon*.

2. Materials and methods

2.1. Molecular modeling

Molecular modeling was performed using PyMOL software (DeLano Scientific, Palo Alto, CA). The Protein Data Bank identification number of MsJHE is 2fj0.

2.2. Construction of MsJHE-HH

Site-directed mutagenesis was performed in order to generate MsJHE-HH, a mutant MsJHE in which Lys-204 and Arg-208 were mutated to histidine residues (Fig. 1B). Site-directed mutagenesis was performed with the wild type *jhe* gene of *M. sexta* (Genbank Accession No. AF327882) in the baculovirus transfer vector pAcUW21 (Weyer et al., 1990) using the primers MsJHE-HHfor

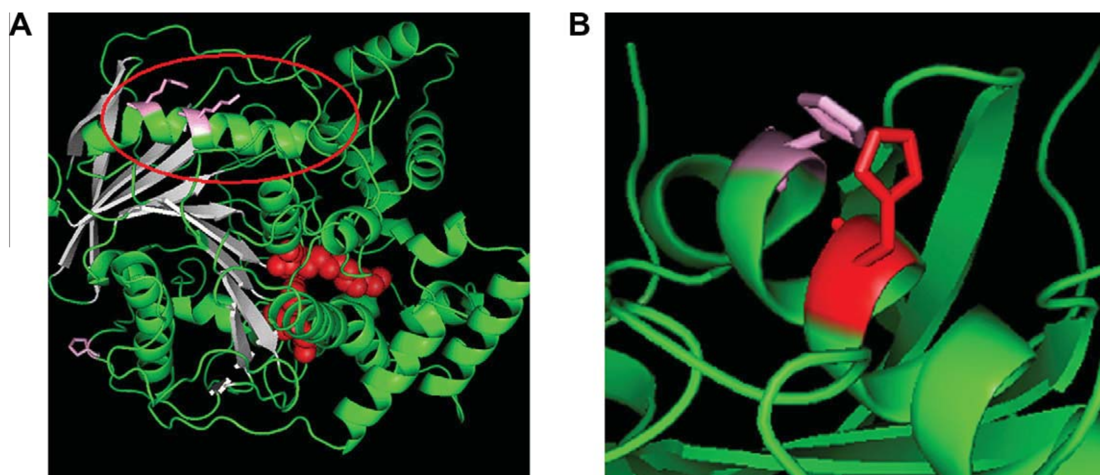

Fig. 1. Crystal structure of MsJHE showing the conserved amphipathic alpha helix (A) and refined model of the corresponding amphipathic alpha helix of MsJHE-HH showing the imidazoles of the K204H and R208H substitutions (B).

(5′ TGACGCTCCTCCATTGGGTGCAGCATAACGCGCAT-3′) and MsJHE-HHrev (5′-ATGCGCGTTATGCTGCACCCAATGGAGGAGCGTC A-3′). The presence of the mutations in the resulting baculovirus transfer vector, pAcUW21-MsJHE-HH, was verified by DNA sequencing. AcMsJHE-HH, a recombinant AcMNPV expressing MsJHE-HH was generated by transfecting Sf21 cells with pAcUW21-MsJHE-HH and linearized BacPAK6 viral DNA (Clontech, Mountain View, CA) using Cellfectin Transfection Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. AcMsJHE-HH was isolated by two rounds of plaque assay on Sf21 cells cultured on ExCell 420 medium (SAFC Biosciences, Lenexa, KS) supplemented with 2.5% fetal bovine serum following standard procedures (Merrington et al., 1999). The presence of contaminating parental virus (i.e., BacPAK6) was detected by the addition of 0.2 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) in the final plaque assay overlay solution.

2.3. Protein expression and purification, and determination of catalytic activity

MsJHE-HH and wild type MsJHE were produced in insect High Five cells (Invitrogen) inoculated with AcMsJHE-HH or AcMsJHE, respectively, following standard procedures (Merrington et al., 1999). The High Five cells were cultured in serum free medium (ESF 921, Expression Systems, Woodland, CA). At 65–70 h p.i., the virus-infected cell culture medium was harvested and centrifuged at 2000*g* for 20 min at 4 °C in order to remove cells and cell debris. The supernatant was then applied to a Centricon-30 (Millipore, Bedford, MA) filtration unit and subjected to multiple rounds of centrifugation following the manufacturer's protocol in order to desalt (into 50 mM sodium phosphate buffer, pH 7.4) and concentrate the sample. The protein concentrations of the samples were determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (Sigma–Aldrich, St. Louis, MO) as a standard. JH III hydrolysis was assayed as described previously (Hammock and Sparks, 1977) using tritiated JH III (17 Ci $mmol^{-1}$, PerkinElmer, Boston, MA). The labeled JH III was diluted with unlabeled JH III (Sigma–Aldrich) and brought to 0.5 mM in ethanol. All enzyme assays were done with a proper dilution of the enzyme so that a linear rate of product formation was observed within the time frame of the assay.

2.4. SDS–PAGE, IEF, and western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF) were performed with precast 10% NuPAGE Bis–Tris (Invitrogen) and Novex pH 3–7 IEF (Invitrogen) gels, respectively, using running buffers recommended by the manufacturer. The separated proteins were stained with SimplyBlue SafeStain (Invitrogen) following the manufacturer's protocol. Following IEF gel electrophoresis, esterase activity was detected by naphthyl acetate staining by soaking the gel in 50 mM sodium phosphate buffer, pH 6.8, containing 0.05% Fast Blue RR salt (Sigma–Aldrich), 0.02% α-naphthyl acetate (Sigma–Aldrich), and 0.02% β-naphthyl acetate (Sigma–Aldrich) at 35 °C. In addition, following IEF gel electrophoresis lanes containing MsJHE-HH and MsJHE were cut into 5 mm-long sections and placed into 50 mM phosphate buffer, pH 7.4, and the proteins were eluted at 4 °C for 24 h. Following elution JHE activity was measured as described above.

Western blot analysis was performed following SDS–PAGE separation of proteins and electroblotting to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Following protein transfer, the membrane was blocked with nonfat dry milk (50 mg ml^{-1} in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h, then incubated for 1 h with a 1:5000 dilution of primary antibody (a polyclonal rabbit antibody, antibody #643, that was raised against affinity purified MsJHE (Abdel-Aal et al., 1988)) in TBS. The membrane was then washed with TBS containing 0.02% Tween 20 (TBS-T) and incubated for 1 h with a 1:8000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphate (Sigma–Aldrich) in TBS. Finally, the membrane was washed with TBS-T for 20 min and the blots were developed with ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ).

2.5. Binding to Ni–NTA and Ni^{2+}

The ability of MsJHE-HH to chelate Ni^{2+} was initially determined using Ni–NTA His bind resin (Novagen, Madison, WI). The Ni–NTA (25 μl) was washed with 50 mM sodium phosphate buffer, pH 8.0, then allowed to bind with 100 μl of the concentrated protein (total JH hydrolytic activity of 2.3 or 20 nmol JH III acid min^{-1} for MsJHE-HH and MsJHE, respectively) diluted with 400 μl sodium phosphate buffer, pH 8.0, in a batch format on a rotator for 1 h at 5 °C. Following the 1 h binding on the rotator, the Ni–NTA-protein complex was allowed to settle by placing the Ni–NTA-protein mixture on ice for 10 min. JHE activity in the supernatant (i.e., unbound JHE) was determined as described above.

The ability of two MsJHE-HH molecules to form a homodimer by binding Ni^{2+} (i.e., Ni^{2+} sandwiched between two MsJHE-HH molecules) was determined by gel filtration following the incubation (at 4 °C for at least 12 h) of a 0.11 nmol ml^{-1} solution of MsJHE-HH with $NiCl_2$ (final concentration of 1.1, 0.11, or 0.011 μM). The molar ratios of Ni^{2+} to MsJHE-HH were 10:1, 1:1, and 1:10. Gel filtration was performed using an open column (82.3 cm long by 1.0 cm in diameter) of Sephacryl S-200 High Resolution (Pharmacia Biotech, Uppsala, Sweden). The void volume (54 ml) of the column was determined using Blue Dextran 2000 (Pharmacia Biotech). Calibration standards (aldolase (158,000 Da) and albumin (67,000 Da)) were purchased from Pharmacia Biotech. The running buffer 50 mM sodium phosphate, pH 7.4, contained the same concentration of $NiCl_2$ (i.e., 1.1, 0.11, and 0.011 μM) as in the protein sample. Following separation, JHE activity in each fraction was determined by the JH partition assay described above.

2.6. Insect larvae and generation of polyhedra

Eggs of *M. sexta* were obtained from Carolina Biological Supply (Burlington, NC) and Educational Science (League City, TX). Eggs of *H. virescens* and *A. ipsilon* were obtained from Benzon Research (Carlisle, PA). All of the larvae were reared at 27 °C on a 12:12, light:dark cycle, 60% relative humidity, on hornworm diet (Carolina Biological Supply).

In order to generate polyhedra, fourth instar larvae of *H. virescens* were injected with 10^4 budded virus of AcMsJHE-HH, AcMsJHE (a recombinant baculovirus carrying the wild type MsJHE (Kamita et al., 2003), or AcMNPV (a wild type baculovirus). The inoculated larvae were reared as described above until death, and polyhedra were purified from dead larvae and quantified as described previously (Kamita et al., 2003).

2.7. Pharmacokinetic analysis

Fourth instars of *M. sexta, H. virescens*, and *A. ipsilon* were partially anesthetized by cold temperature and injected with wild type MsJHE, MsJHE-HH, or MsJHE-HH equilibrated with Ni^{2+} (1:1 M ratio). The injections were performed as described previously (Ichinose et al., 1992a) except that a 50 μl Hamilton syringe fitted with a 33 gauge needle was used for injection. The specific activities of the solutions of MsJHE, MsJHE-HH, and MsJHE-HH equilibrated with Ni^{2+} (MsJHE-HH-Ni) that were used for injection were 119, 98.6, and 98.6 nmol of JH III acid formed min^{-1} mg^{-1},

respectively. The *M. sexta* were injected with 0.25 nmol min^{-1} of MsJHE or 0.19 nmol min^{-1} of MsJHE-HH or MsJHE-HH-Ni per 100 mg of larval body weight. The *H. virescens* and *A. ipsilon* were injected with 0.25 nmol min^{-1} of MsJHE or 0.19 nmol min^{-1} of MsJHE-HH or MsJHE-HH-Ni per 15 mg of larval body weight. At 15, 30, 90, and 150 min post injection, an equal volume of hemolymph was collected from at least 3 larvae per treatment and collected into a single tube. Subsequently, the hemolymph was diluted in 50 mM sodium phosphate buffer, pH 7.4, and JHE activity was determined in triplicate as described above. The hemolymph volumes of *M. sexta*, *H. virescens*, and *A. ipsilon* were assumed to be 400, 15, and 15 μl, respectively. Half-life was determined by plotting the natural log of the percentage of total JHE activity remaining at each time point. In a separate experiment, third instars of *M. sexta* were injected with MsJHE-HH (0.07 nmol min^{-1} $larva^{-1}$) or MsJHE (1.0 nmol min^{-1} $larva^{-1}$) as described above. At 30, 90, and 180 min post injection an equal volume of hemolymph was collected from 3 larvae per treatment and collected into a single tube. Two microliters of this hemolymph mixture was diluted 5-fold with phosphate balanced salt buffer and subjected to Western blot analysis as described above.

2.8. Bioassay

Bioassays to determine lethal concentration and lethal time were performed using first instar larvae within 6 h of emergence. The first instars of *H. virescens* and *A. ipsilon* were inoculated with polyhedra at five concentrations ranging from 1×10^5 to 2×10^6 polyhedra per ml for lethal concentration determination and at LC_{90} for time-mortality assays. A modified droplet feeding method (Hughes et al., 1986) was used to perform the inoculations. In brief, the procedure involved exposing groups of 4–50 larvae to 1 μl droplets of virus solution (each containing 10% w:v sucrose and 5% w:v blue food coloring). Larvae were allowed to consume the virus for 10–15 min then transferred individually into bioassay trays (1 larva per well) containing fresh diet. The time of transfer was noted as time zero and mortality was determined at 12 h intervals. Larvae that died during the initial 12 h period were considered injured by the treatment and discarded. The experiments were stopped at 12 days post inoculation (p.i.) or earlier if all of the larvae had died.

Assays to determine weight gain and feeding damage were performed with first instar *M. sexta* that were inoculated with 1×10^3 polyhedra per insect by droplet feeding as described above. The experiments were performed in triplicate with 8–15 larvae per treatment. Following inoculation, the larvae were placed individually on artificial diet or into a 20 ml glass tube containing a single tomato leaf (ca. 10 cm^2) that was stabbed into 2 ml of a 1% agar solution as a water supply. The larvae were reared at 26 °C on a 12:12, light:dark cycle, 60% relative humidity. After 4 days, the larvae were weighed and the leaves were scanned on a desktop scanner.

2.9. Statistical analysis

The LC_{50} and corresponding 95% confidence limits (CL) of each virus were calculated using Polo-PC Plus version 3.1 statistical software (LeOra Software, Berkeley, CA). Differences in lethal concentrations were considered significant when the respective 95% CLs did not overlap. The LT_{50} and standard error of the mean (SEM) were determined using the ViStat 2.1 program (Hughes, 1990). Analysis of variance (ANOVA) of the half life of JHE activity following injection into the hemocoel and mean mass of larvae following the various treatment regimes was determined using SAS statistical software version 7.0 (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Expression and purification of MsJHE-HH and MsJHE

Recombinant MsJHE-HH and MsJHE activities were found in the cell culture supernatant of High Five cells infected with AcMsJHE-HH or AcMsJHE, respectively. The specific activity of MsJHE-HH in the cell culture supernatant of High Five cells at 70 h p.i. was 6.5 nmol of JH III acid formed min^{-1} mg^{-1}. In comparison, the specific activity of MsJHE was nearly 10-fold higher at 61.3 nmol of JH III acid formed min^{-1} mg^{-1}. This 10-fold difference in specific activity was found following repeated protein expression experiments. Western blot analysis following SDS–PAGE separation of the same amount of total protein suggested that the expression and release of MsJHE-HH and MsJHE into the supernatant occurred at similar levels (Fig. 2). This suggested that the K204H and/or R208H mutations, although apparently far-removed from the catalytic site of the enzyme, induced allosteric effects that led to a reduction in specific activity. We have previously shown that mutation of an aspartic acid residue that is found within the amphipathic alpha helix of the JHE of *H. virescens* (corresponding to D198 of MsJHE) to an asparagine residue removes all detectable JHE activity (Ward et al., 1992).

Multiple α/β-naphthyl acetate-staining proteins were identified in the supernatant of High Five cells infected with AcMsJHE-HH or AcMsJHE following IEF gel electrophoresis (Fig. 3). When the IEF gel was cut into fourteen 5 mm-long slices, the major α/β-naphthyl acetate-staining bands also showed JH-specific esterase activity (Fig. 3). The pH of the gel slices that contained JHE activity ranged from 4.4 to 5.6. Approximately 66% and 72% of the JHE activity of MsJHE-HH and MsJHE, respectively, that was applied to the IEF gel was recovered.

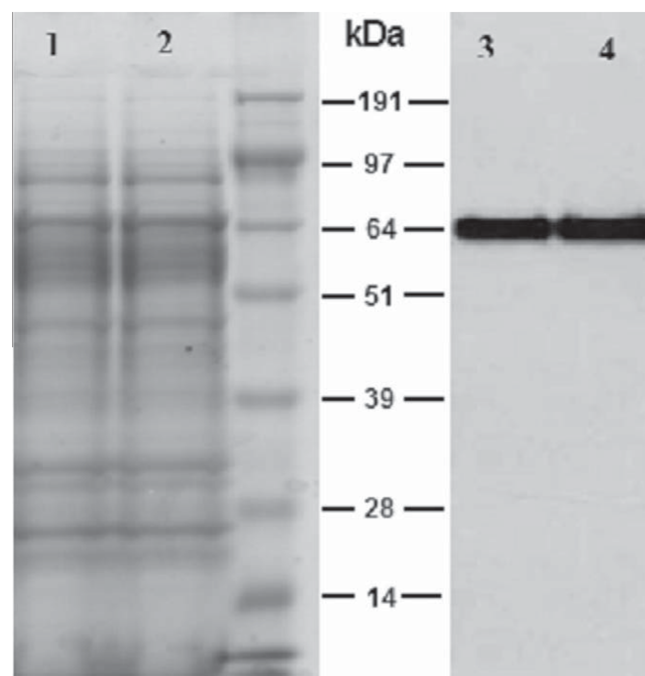

Fig. 2. Coomassie brilliant blue staining and western blot analysis of proteins in the cell culture supernatant of High Five cells that were infected with AcMsJHE-HH (lanes 1 and 3) or AcMsJHE (lanes 2 and 4). The proteins (2.1–2.4 μg per lane) were collected at 70 h p.i., separated by SDS-(10%) PAGE, and subjected to Coomassie brilliant blue staining (lanes 1 and 2) or western blot analysis (lanes 3 and 4) with polyclonal rabbit anti-MsJHE antibody. The mass of size standards are indicated in kilo-Daltons (kDa).

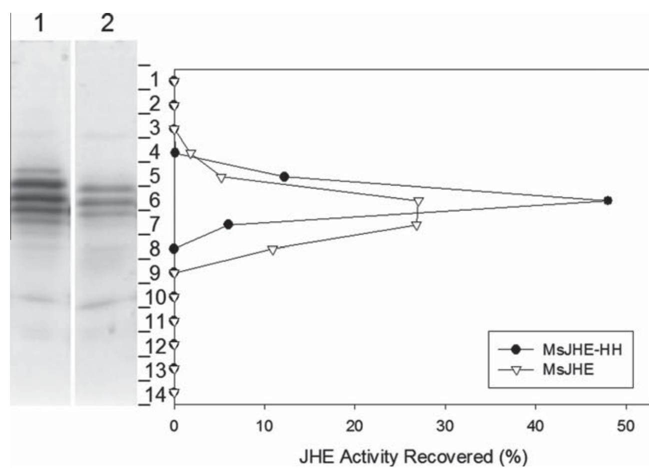

Fig. 3. IEF gel separation of semi-purified MsJHE-HH (lane 1) and MsJHE (lane 2). Each protein sample (3 μg) was separated in duplicate on a Novex narrow range (pH 3–7) isoelectric focusing gel. The separated proteins were subjected to α/β-naphthyl acetate-Fast Blue RR staining or JH-selective esterase activity assays. For the JHE activity assays, each lane was cut into 5 mm-long slices from which JHE activity was measured as described in Section 2. The graph to the right shows the percentage of total JHE activity that was recovered from each of the 14 gel slices (indicated to the right of lane 2). Gel slices 4, 5, 6, 7, and 8 showed JHE activity. The measured pH of these gel slices was 4.4, 4.7, 5.0, 5.3, and 5.6, respectively. Approximately, 66% and 72% of the JHE activity of MsJHE-HH and MsJHE, respectively, that was applied to the gel was recovered.

3.2. Binding of MsJHE-HH and MsJHE to Ni–NTA and chelation of Ni^{2+}

The crystal structure of MsJHE (Wogulis et al., 2006) shows an amphipathic alpha helix with a line of positively charged amino acid residues (Arg-197, Lys-204, and Arg-208) that is located on or near the surface of the protein and away from the entrance to the catalytic pocket (Fig. 1A). MsJHE-HH was generated by mutating Lys-204 and Arg-208 of the amphipathic alpha helix to histidine residues (Fig. 1B). The K204H and R208H mutations of MsJHE-HH were predicted to result in histidine residues that would be in the appropriate conformation to form a strong bidentate chelate with bivalent cation such as Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} that are found in the hemolymph of caterpillars. In order to test this hypothesis, MsJHE-HH was incubated with Ni–NTA His bind resin for 1 h at 5 °C. Following this incubation, about 83% of the MsJHE-HH activity was bound to Ni–NTA. In parallel experiments, about 20% of wild type MsJHE activity was bound to Ni–NTA. These results indicated that the histidine residues of MsJHE-HH were in the appropriate orientation to form a chelate complex with the Ni^{2+} ion of Ni–NTA.

We next tested whether MsJHE-HH could form a homodimer complex in which two MsJHE-HH molecules bind a single Ni^{2+} (i.e., Ni^{2+} sandwiched between two MsJHE-HH molecules). Should such a homodimer form, we hypothesized that the amphipathic alpha helix of MsJHE-HH will be blocked and unavailable to bind to a putative JHE receptor that is found in the clathrin coated pits of the pericardial cell complex. In these experiments, MsJHE-HH was incubated with $NiCl_2$ so that the Ni^{2+} to MsJHE-HH molar ratio was 10:1, 1:1, or 1:10 (final $NiCl_2$ concentration of 1.1, 0.11, or 0.011 μM, respectively). These three concentrations were used in an attempt to find an equivalence point for dimer formation. After overnight incubation at 5 °C, 3.8 μg of protein containing 0.24 nmol min^{-1} of JHE activity was separated by gel filtration chromatography. When MsJHE-HH was incubated with Ni^{2+} at a molar ratio of 10:1, 1:1, 1:10, or without Ni^{2+}, peak activity was found at an elution volume of 76.0, 76.2, 77.6, and 75.7 ml, respectively. No JHE activity was detectable before an elution volume of 71 ml under any of the separation conditions. The peak elution volume of the albumin standard (67 kDa) was 72.6 ml, whereas that of the aldolase (158 kDa) was 65.4 ml under the same separation conditions. These results suggested that the formation of an MsJHE-HH homodimer (predicted MW of 124.2 kDa) did not occur under the conditions tested. This finding was puzzling at first because MsJHE-HH was able to chelate Ni^{2+} bound to NTA. Analysis of the crystal structure of MsJHE (Wogulis et al., 2006), however, indicated that the amphipathic alpha helix of MsJHE (and putatively MsJHE-HH) is found within a slight valley on the surface of the protein. Homodimer formation may thus be unachievable because of steric hindrance.

3.3. Pharmacokinetic analysis

A significant difference in the clearance of JH hydrolysis activity was not found following the injection of fourth instars of *M. sexta* with MsJHE-HH, MsJHE-HH equilibrated with Ni^{2+} (1:1 M ratio), or MsJHE (Table 1). Similarly, there were no statistically significant differences in the clearance of JH hydrolysis activity in fourth instars of *H. virescens* and fourth instars of *A. ipsilon*. However, the calculated half-life of MsJHE-HH or MsJHE-HH equilibrated with Ni^{2+} was about 10% longer, respectively, than that of MsJHE in these species. In a previous study from our laboratory (Ichinose et al., 1992a) we showed that the half-life of recombinant JHE from *H. virescens* that was injected into second instars of *M. sexta* or *H. virescens* was about 70 min. In all three species, total endogenous JHE activity was less than 1% of the total JHE activity that was injected. Western blot

Table 1
Pharmacokinetic clearance of MsJHE-HH and MsJHE following injection into fourth instar hemocoel.

Insect	JHE[a]	Total activity injected[b] (pmol min^{-1})	Activity remaining at indicated time post injection (pmol min^{-1} ± s.d.)				Half life[d] (min)
			15 min	30 min	90 min	150 min	
M. sexta	MsJHE-HH	1500	1410 ± 350 [94][c]	670 ± 130 [45]	260 ± 13 [18]	130 ± 22 [9]	35[b]
	MsJHE-HH + Ni^{2+}	1400	1130 ± 48 [81]	780 ± 95 [55]	310 ± 18 [22]	140 ± 29 [10]	38[b]
	MsJHE	2000	1840 ± 280 [92]	1280 ± 390 [64]	520 ± 23 [26]	170 ± 49 [9]	41[b]
H. virescens	MsJHE-HH	560	370 ± 21 [66]	300 ± 21 [54]	140 ± 2 [24]	130 ± 10 [23]	70[a]
	MsJHE-HH + Ni^{2+}	540	320 ± 13 [59]	300 ± 17 [55]	210 ± 32 [38]	110 ± 4 [20]	69[a]
	MsJHE	750	290 ± 4 [39]	250 ± 17 [33]	150 ± 6 [21]	150 ± 7 [20]	63[a]
A. ipsilon	MsJHE-HH	560	350 ± 7 [63]	170 ± 3 [31]	100 ± 3 [18]	56 ± 4 [11]	43[b]
	MsJHE-HH + Ni^{2+}	540	210 ± 1 [40]	160 ± 7 [31]	80 ± 5 [15]	70 ± 6 [14]	38[b]
	MsJHE	750	340 ± 3 [46]	260 ± 4 [35]	120 ± 3 [16]	40 ± 4 [6]	35[b]

[a] A 1:1 M ratio of MsJHE-HH to Ni^{2+} was used when Ni^{2+} was added to the injection solution.
[b] At least three larvae were injected per treatment.
[c] Values in brackets are percent of activity remaining post injection at the indicated time.
[d] Half life values that show the same letter are not significantly different ($p < 0.001$) as tested by ANOVA.

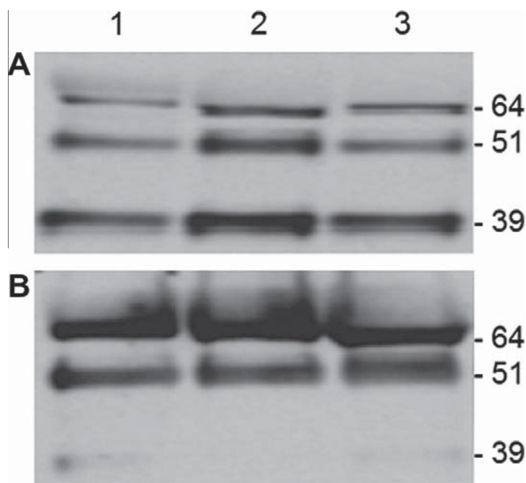

Fig. 4. Western blot analysis of MsJHE-HH (A) or MsJHE (B) following injection into the hemolymph of third instar larvae of *M. sexta*. The hemolymph was collected at 30 (lane 1), 90 (lane 2), and 180 (lane 3) min post injection from each of three insects, pooled, diluted 5-fold, and then a volume equivalent to 2 μl of hemolymph was separated by SDS–(10%)PAGE. The approximate masses in kilo-Daltons of proteins that reacted with the anti-JHE antibody are indicated to the right. The 64 kDa bands were predicted to correspond to full-length JHEs whereas the 51 kDa bands were predicted to correspond to C-terminal portion of JHEs following cleavage of a disordered loop (amino acid residues 128–135) that has been identified on the surface of MsJHE (Wogulis et al., 2006).

analysis identified three major immunoreactive bands of approximately 64, 51, and 39 kDa, and two major bands of approximately 64 and 51 kDa in the hemolymph of MsJHE-HH- and MsJHE-injected insects, respectively (Fig. 4). There appeared to be no significant difference in the rate of degradation between MsJHE-HH and MsJHE at 30, 60, and 90 min post injection.

3.4. Insecticidal efficacy of AcMsJHE-HH in comparison to AcMsJHE and AcMNPV

Bioassays were performed with first instars of *H. virescens* and *A. ipsilon* to determine the effect of the expression of MsJHE-HH on the insecticidal efficacy of AcMNPV. In comparison to wild type AcMNPV, the median lethal concentration (LC_{50}) of AcMsJHE-HH was 3.2-fold lower in *H. virescens*, but it was not significantly different in *A. ipsilon* (Table 2). A statistically significant difference in LC_{50} between AcMsJHE-HH and AcMsJHE was not found in *H. virescens*, however, the LC_{50} of AcMsJHE-HH was 1.9-fold higher than that of AcMsJHE in *A. ipsilon* (Table 2). In addition, the LC_{50} of AcMsJHE-HH in *H. virescens* was 3.5-fold lower than that in *A. ipsilon*. The median lethal time (LT_{50}) of first instars of *H. virescens* and *A. ipsilon* inoculated with AcMsJHE-HH was about 4.8 and 5.3 days, respectively (Table 3). There was no statistically significant difference in the LT_{50} of AcMsJHE-HH, AcMsJHE, and AcMNPV in *H. virescens* or between AcMsJHE-HH and AcMNPV in *A. ipsilon* (Table 3).

Table 3
Time-mortality of neonate *H. virescens* and *A. ipsilon* following inoculation with AcMsJHE-HH, AcMsJHE or AcMNPV.

Insect	Virus[a]	LT_{50} ± SEM (h p.i.)
H. virescens	AcMsJHE-HH	122 ± 3.1
	AcMsJHE	122 ± 2.7
	AcMNPV	120 ± 2.9
A. ipsilon	AcMsJHE-HH	137 ± 3.0
	AcMsJHE	145 ± 5.6
	AcMNPV	135 ± 4.9

[a] An LC_{90} concentration of each virus as indicated in Table 2 was used to inoculate each larva. Mortality was scored at 12 h intervals.

Table 4
Effect of virus exposure on the mass of larval *M. sexta* reared on artificial diet or tomato leaf for 4 days post inoculation.

Diet	Virus	Mean mass* ±s.d. (mg)
Artificial diet	AcMsJHE-HH	6.2 ± 2.3[c]
	AcMsJHE	10.2 ± 2.0[b]
	AcMNPV	12.0 ± 2.1[b]
	Mock	20.4 ± 4.3[a]
Tomato leaf	AcMsJHE-HH	3.8 ± 0.4[d]
	AcMsJHE	7.6 ± 0.4[c]
	AcMNPV	10.6 ± 2.7[b]
	Mock	37.0 ± 4.0[a]

* For each type of diet, mean mass values that show the same letter designation are not significantly different ($p < 0.001$) as tested by ANOVA.

3.5. Larval feeding assay and larval mass

Feeding assays were performed using first instars of *M. sexta*. The larvae were inoculated with 1×10^3 polyhedra of AcMsJHE-HH, AcMsJHE, or AcMNPV then allowed to feed for 4 days on artificial diet or a tomato leaf. In comparison to larvae that were mock-infected, all of the virus-infected larvae showed dramatically lower (41–90%) mass at 4 days post inoculation (Table 4). Of the virus-infected larvae, AcMsJHE-HH-infected larvae showed the lowest mass followed by larvae that were infected with AcMsJHE and then larvae infected with wild type AcMNPV regardless of whether they were allowed to feed on artificial diet or tomato leaves (Table 4). Consistent with their reduced mass, the virus-infected larvae appeared to consume significantly smaller amounts of artificial diet (data not shown) and tomato leaf (Fig. 5) in comparison to mock-infected larvae.

4. Conclusions

Numerous transgenes have been used successfully to improve the natural insecticidal efficacy of the baculovirus. Genes encoding

Table 2
Lethal concentrations of AcMsJHE-HH, AcMsJHE, and AcMNPV in neonate *H. virescens* and *A. ipsilon*.

Insect	Virus	LC_{50} ($\times 10^5$)[a] (95% CL)	LC_{90} ($\times 10^5$)[a] (95% CL)	Slope ± SEM	Heterogeneity (χ^2/n)	*g* value[b]	Potency ratio[c]
H. virescens	AcMsJHE-HH	1.8 (1.0–2.6)	14 (8.6–35)	1.44 ± 0.26	2.45	0.194	3.22
	AcMsJHE	2.7 (1.8–3.8)	17 (11–38)	1.62 ± 0.27	3.34	0.213	2.15
	AcMNPV	5.8 (4.3–7.8)	24 (16–47)	2.08 ± 0.31	4.32	0.198	1.00
A. ipsilon	AcMsJHE-HH	6.3 (3.6–13)	130 (41–390)	0.96 ± 0.24	0.06	0.200	1.24
	AcMsJHE	3.3 (2.3–4.6)	18 (11–42)	1.73 ± 0.29	0.35	0.195	2.36
	AcMNPV	7.8 (5.0–15)	72 (30–360)	1.33 ± 0.29	3.20	0.226	1.00

[a] LC_{50} and LC_{90} values (polyhedra ml^{-1}) were obtained using Polo-PC Plus version 3.1 and shown with 95% confidence limits (CL).
[b] If $g < 0.5$, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is invalid (Finney, 1971).
[c] Potency ratio = LC_{50} of AcMNPV divided by the LC_{50} of AcMsJHE-HH or AcMsJHE for each insect (Robertson and Preisler, 1992).

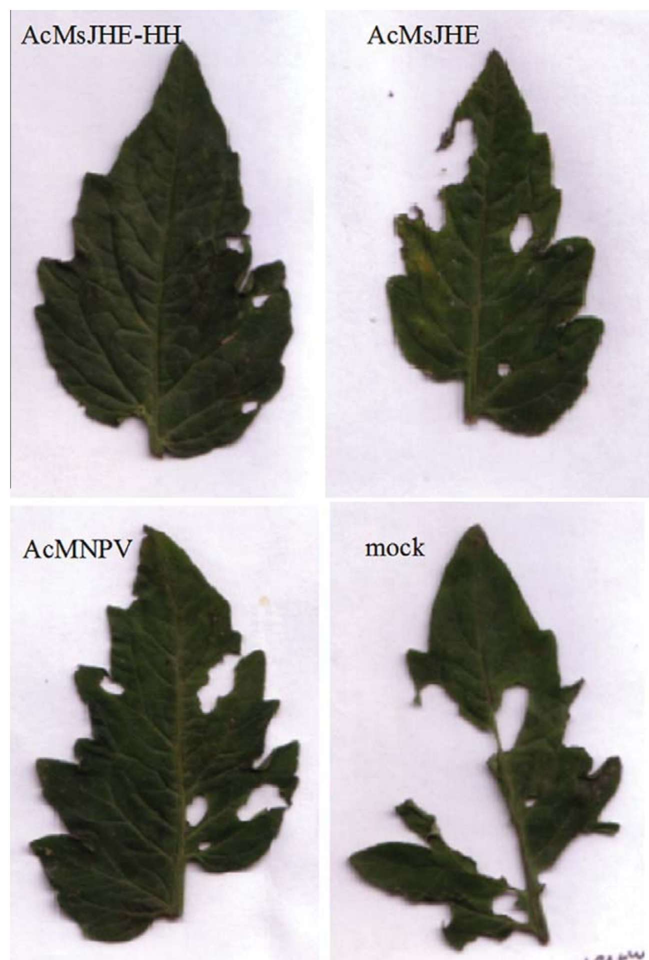

Fig. 5. Typical example of feeding damage following exposure of tomato leaves to first instars of *M. sexta* that were inoculated with AcMsJHE-HH, AcMsJHE, or AcMNPV, or mock-infected. The larvae were allowed to feed on the tomato leaves for 4 days post inoculation. The amount of leaf consumption was estimated by scanning to be 1%, 15%, 15%, and 45% following feeding by larvae infected with AcMsJHE-HH, AcMsJHE, AcMNPV, or mock-infected, respectively.

scorpion toxins that show exceptionally high insect selectivity are among the most effective in this regard. In field tests, genetically modified or recombinant baculoviruses prove to be cost effective green biopesticides that preserve biocontrol organisms and other non-targets. In this study, we experimentally validate the original ideas of Keeley and Hayes (1987), Menn and Borkovec (1989), and Maeda (1989) to use proteins and peptides of insect origin to improve insecticidal efficacy of the baculovirus. We show that the mass of larvae infected with a recombinant baculovirus expressing JHE with a mutation in its amphipathic alpha helix was dramatically lower than that of larvae infected with a recombinant baculovirus expressing wild type JHE or the wild type baculovirus. Significantly reduced leaf damage was also observed. Our results are promising from an insect control standpoint, but they leave a mystery. The altered amphipathic alpha helix clearly can form a bidentate chelate with Ni^{2+} (i.e., with Ni–NTA). However, MsJHE-HH did not form a detectable dimer with Ni^{2+} in solution as we hypothesized nor was there a dramatic increase in the stability of MsJHE-HH *in vivo*. Although we did not unequivocally show that mutation of the amphipathic alpha helix results in improved stability of JHE, the minor mutation was sufficient for improving insecticidal efficacy. Our findings also indicated that a small change in the physiology of the insect can result in a dramatic reduction in feeding damage. Possibly other alteration in JHE that can significantly increase *in vivo* stability while retaining high catalytic activity on JH will further improve efficacy of a recombinant baculovirus expressing such a protein. Our study once again demonstrates that the efficacy of a recombinant baculovirus carrying a transgene of insect origin has significant potential as an effective biological insecticide.

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