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Recombinant, Catalytically Inactive Juvenile Hormone Esterase Enhances Efficacy of Baculovirus Insecticides

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The insecticidal efficacy of baculoviruses can be enhanced by engineering the viral genome to express proteins that disrupt the physiology of the host insect. Here we describe the development of a genetically engineered *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) which expresses a modified form of juvenile hormone esterase (JHE). Previously, two viruses expressing different modified JHEs were found to have a greater insecticidal effect on larvae of *Trichoplusia ni* and *Heliothis virescens* than a virus expressing wild-type JHE. To study a possible synergistic effect, the distinct mutations in the modified JHEs were combined in a new JHE construct. Two lysine residues were replaced with arginine residues to reduce the efficiency of lysosomal targeting (JHE-KK) and the catalytic serine was replaced with glycine, which eliminated catalytic activity (JHE-SG). The modified JHE, JHE-KSK, was expressed in a recombinant baculovirus, AcJHE-KSK. Larvae of *H. virescens* infected with this recombinant virus caused 44% less feeding damage to lettuce than larvae infected with the wild-type AcMNPV. However, AcJHE-KSK did not have significantly improved insecticidal properties over the parent viruses AcJHE-KK and AcJHE-SG, suggesting that the separate mutations have no major synergistic effect. Infection with a control recombinant baculovirus expressing JHE with the same lysine to arginine conversions and in which a catalytic histidine was converted to lysine (AcJHE-KHK) did not reduce feeding damage compared with that caused by larvae infected with AcMNPV. © 2000 Academic Press

***Key Words: Autographa californica* nucleopolyhedrovirus; AcMNPV; *Heliothis virescens;* tobacco budworm; juvenile hormone esterase; baculovirus; insecticide.**

INTRODUCTION

Baculoviruses are used in crop protection but are not yet competitive with classical chemical insecticides in terms of speed of action. After viral infection of lepidopteran larvae, feeding continues for several days to weeks until death of the host insect. However, recent developments in genetic engineering of baculoviruses have increased the potential for development of fast-acting recombinant viral insecticides. Several baculoviruses that express insect-specific toxins or components of the insect's own physiological make up have been constructed (Vlak, 1993; Bonning and Hammock, 1996; Black *et al.*, 1997; Hammock, 1998, 1999; Harrison and Bonning, 2000). Among the latter are viruses that express modified forms of juvenile hormone esterase (JHE; EC 3.1.1.1). This enzyme hydrolyzes juvenile hormone (JH) into the corresponding carboxylic acid that is less active in morphogenetic assays (Hammock, 1985). JH is a key hormone involved in larval development, and a decrease in the JH titer signals the insect larva to stop feeding in preparation for molting. To exploit this observation a recombinant *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) was constructed which expressed JHE derived from *Heliothis virescens* Fabricius (Hammock *et al.*, 1990). This recombinant virus in comparison to wild-type virus showed only slightly improved insecticidal effects in first instar larvae of *Trichoplusia ni* Hübner. In subsequent studies, several modified forms of JHE were constructed and two of these showed improved insecticidal activity when expressed via AcMNPV (Ward *et al.*, 1992; Bonning *et al.*, 1995, 1997a, 1999). Infection of larvae of *H. virescens* with these recombinant viruses resulted in a dramatic reduction of feeding damage compared with wild-type AcMNPV-infected larvae.

The first improved virus expressed a modified JHE that was produced as a result of a site-directed mu-

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tagenesis study on the catalytic mechanism of JHE. JHE is a serine esterase with a catalytic tetrad composed of Ser_{203}-Ser_{229}-His_{448}-Glu_{334} (Hanzlik *et al.*, 1989; Ward *et al.*, 1992; Thomas *et al.*, 1999). The separate mutation of the nucleophilic Ser_{203} to Gly, Glu_{334} to Gln, or His_{448} to Lys reduced the enzyme activity at least 10^6-fold compared to that of wild-type JHE (Ward *et al.*, 1992). Recombinant AcMNPV expressing JHE, where Ser_{203} was mutated into Gly (AcJHE-SG), surprisingly was found to kill larvae more rapidly than its catalytically active JHE counterpart (Bonning *et al.*, 1995). Symptoms exhibited by infected larvae suggested that JHE-SG might act by disruption of events during insect molting, since 27% of the larvae died at the molt with extreme cuticular blackening. The remaining larvae were similar in appearance to larvae infected with AcJHE. Some of the latter larvae exhibited contractile paralysis with only partial or no response to stimulation. Infection of larvae of *H. virescens* with AcJHE-SG resulted in a reduction of feeding damage on lettuce of up to 66%. The mechanism of the enhanced insecticidal effect of JHE-SG, however, is unknown. Expression of the catalytically inactive His_{448} (JHE-HK) and Glu_{334} (JHE-DN)-modified JHEs by recombinant AcMNPV did not improve the insecticidal activity of the viruses relative to wild-type AcMNPV (Bonning *et al.*, 1995).

In a second study, two lysine residues, Lys_{29} and Lys_{524}, which appear to be involved in lysosomal targeting, were converted to arginine (Bonning *et al.*, 1997a, 1999). From pharmacokinetic and histological studies, it was clear that the chemically stable JHE is removed rapidly from the hemolymph by the pericardial cells (Ichinose *et al.*, 1992, 1993; Booth *et al.*, 1992; Bonning *et al.*, 1997b). However, modification of residues Lys_{29} (JHE-29) or Lys_{524} (JHE-524) or its combination (JHE-KK) to Arg did not decrease the rate of clearance from the hemolymph in *Manduca sexta* L. (Bonning *et al.*, 1997a). Nevertheless, within the pericardial cells, JHE-KK was fivefold less concentrated in the lysosomes than JHE, JHE-29, or JHE-524, indicating that intracellular processing of JHE-KK had been affected. The altered intracellular processing of JHE-KK correlated with higher insecticidal efficacy (Bonning *et al.*, 1999) and with reduced binding to a putative JHE binding protein (Shanmugavelu *et al.*, 2000). Infection of larvae of *H. virescens* with AcJHE-KK resulted in up to 50% decrease in feeding damage on lettuce in comparison with wild-type virus, virus expressing wild-type JHE (AcJHE), JHE-29 (AcJHE-29), or JHE-524 (AcJHE-524). Larvae infected with AcJHE-KK exhibited contraction paralysis in 17% of the cases (Bonning *et al.*, 1999).

The modes of action of JHE-KK and JHE-SG are probably distinct because there are differences in symptomology of larvae infected with viruses expressing these modified JHE forms. No putative residues involved in the degradation of the enzyme were altered in catalytically inactive JHE-SG, and yet AcJHE-SG was more effective than AcJHE. The Ser_{203} to Gly mutation is at the catalytic site deep within the enzyme (Thomas *et al.*, 1999). To study a possible synergistic effect between the mutations in JHE-KK and JHE-SG, a new modified JHE was generated in which the mutations at Lys_{29}, Ser_{203}, and Lys_{524} were combined to result in the recombinant virus AcJHE-KSK. This modified JHE (JHE-KSK) is catalytically inactive due to the Ser_{203} to Gly mutation, and lysosomal targeting is most likely to be compromised through the Lys_{29} and Lys_{524} to Arg mutations. To assess the insecticidal effect of the Lys_{29} and Lys_{524} mutations alone, in absence of the insecticidal effect due to the Ser_{203} mutation and in the absence of catalytic activity, a control virus AcJHE-KHK was constructed. In this virus the mutations at Lys_{29} and Lys_{524} were combined with a mutation at the catalytic base of the proton relay system, His_{448} (conversion to Lys) (Ward *et al.*, 1992).

MATERIALS AND METHODS

Combination of the different mutations in JHE. The cDNA clone of JHE from *H. virescens* (clone 3hv16B; Hammock *et al.*, 1990) was modified by inversion of the clone as described in Ward *et al.* (1992) to generate JHE cDNA clone 16BglRev. This clone contains the JHE coding sequence flanked by *Eco*RI and *Bgl*II restriction endonuclease sites. Site-directed mutagenesis was employed to generate three separate clones: $K_{29}R$ (Lys_{29} converted to Arg), $S_{203}G$ (Ser_{203} converted to Gly), and $K_{524}R$ (Lys_{524} converted to Arg). The generation of these mutants is described in Ward *et al.* (1992). To generate the triple mutant JHE-KSK containing all of these mutations, the procedure outlined in Fig. 1 was employed. The region of JHE clone $K_{29}R$ containing the $K_{29}R$ mutation was gel purified as a *Mlu*I–*Nco*I fragment from an agarose gel using a Qiaex gel extraction kit (Qiagen Inc., Valencia, CA). The region of JHE clone $S_{203}G$ containing the $S_{203}G$ mutation was isolated as a *Nco*I–*Sph*I fragment by gel purification. The JHE-$K_{524}R$ cDNA clone was digested with the restriction enzymes *Mlu*I and *Sph*I and the digest was treated with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN). A three-way ligation was performed using the JHE-$K_{524}R$ *Mlu*I–*Sph*I clone, with the gel purified *Mlu*I–*Nco*I $K_{29}R$ fragment and the *Nco*I–*Sph*I $S_{203}G$ fragment. Sequencing of the JHE-KSK in pBluescript was carried out using the dideoxy chain termination method (Sequenase kit; USB Corp., Cleveland, OH) to confirm that the three mutations were correct. The JHE-KSK cDNA was then gel purified as an *Eco*RI fragment and cloned into the *Eco*RI restriction endonuclease site of the baculovirus transfer vector pAcUW21 (Weyer *et al.*, 1990; Bishop, 1992). In this vector the polyhedrin gene is maintained, facil-

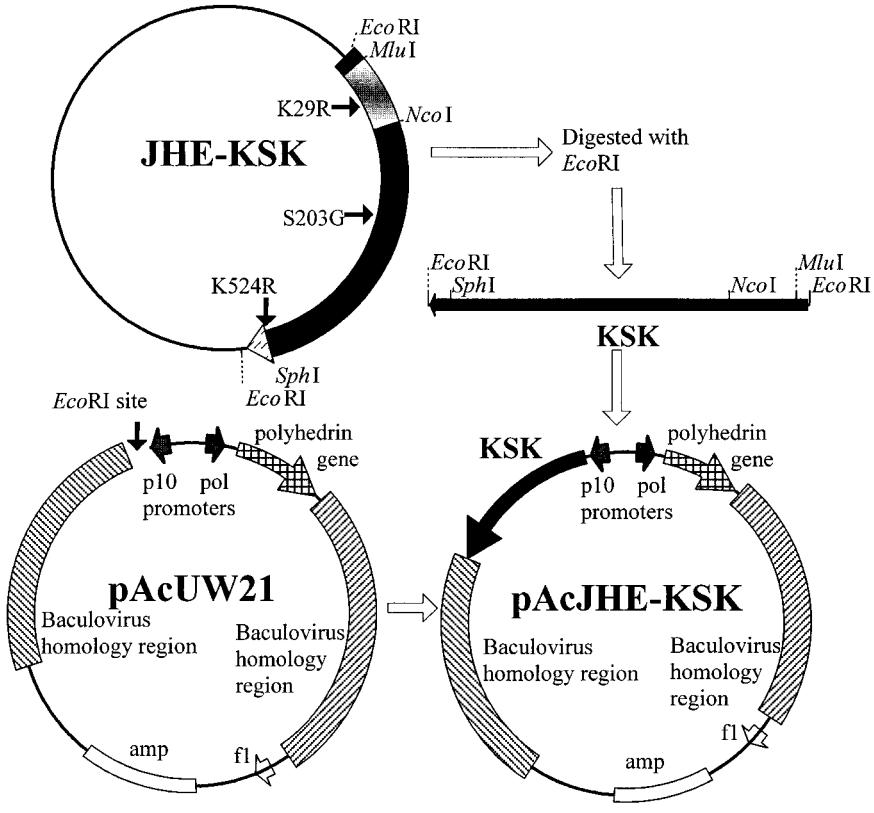

FIG. 1. Construction of the transfer vector pAcJHE-KSK. The modified JHE sequence, JHE-KSK, was extracted from pBluescript and ligated into the *Eco*RI cloning site of pAcUW21, resulting in the transfer vector pAcJHE-KSK.

itating field stability of the resulting virus. The correct insertion of the sequence coding for JHE-KSK was confirmed using restriction enzyme analysis. The JHE-KHK construct was produced similarly by replacing the *Nco*I–*Sph*I His_{448} region of JHE-KK with the *Nco*I–*Sph*I fragment containing the $H_{448}K$ (His_{448} converted to Lys) mutation from JHE-HK (Ward *et al.*, 1992).

Isolation and purification of AcJHE-KSK and AcJHE-KHK. Cells of *Spodoptera frugiperda* (Sf21; Vaughn *et al.*, 1977) maintained at 28°C in ExCell 401 medium (JRH Biosciences, Woodland, CA) with 3% fetal calf serum (FCS: Intergen, Purchase, NY) were cotransfected with the transfer vector DNA of pAcUW21.JHE-KSK or pAcUW21. JHE-KHK and AcRP6-SC (Kitts *et al.*, 1990; King and Possee, 1992). The AcRP6-SC DNA was linearized with the restriction enzyme *Bsu*36I to reduce background levels of nonrecombinant virus (Kitts *et al.*, 1990). Recombinant viruses, identified by the presence of polyhedra against a polyhedrin-negative background, were purified and amplified using standard techniques (King and Possee, 1992; O'Reilly *et al.*, 1992).

Western blotting of recombinant protein. Recombinant JHEs were produced following infection of Sf21 cells in 80-cm^2 T-flasks in a minimum volume of medium (15 ml). Cells were infected with five plaque-forming units per cell and maintained at 28°C. Medium was harvested 4 days after infection. Uninfected cells and cells infected with the recombinant virus expressing wild-type JHE or AcJHE (Bonning *et al.*, 1992) were used as controls under the same conditions. SDS–PAGE was performed using a BioRad Mini Protean II electrophoresis unit, a 4% stacking gel, pH 6.8, and 10% resolving gel, pH 8.3 (Laemmli, 1970). Aliquots of 40 μl of cell culture medium from AcJHE-, AcJHE-KSK-, and AcJHE-KHK-infected cells or uninfected cells were run in the gel along with molecular weight markers (Rainbow; BioRad, Hercules, CA). Proteins were transferred to nitrocellulose. Western blotting was carried out using polyclonal rabbit anti-JHE antiserum raised to affinity-purified JHE from *H. virescens.* Immunoreactive proteins were detected using the Enhanced Chemi-Luminescence (ECL) detection system (Amersham International plc, Buckinghamshire, UK.).

Analysis of catalytic activity. Cell culture media from Sf21 cells infected with AcJHE, AcJHE-KK, AcJHE-KSK, or AcJHE-KHK were assayed for JHE activity by employing a highly sensitive radiochemical assay using [^{3}H]JH III as substrate as described previously (Hammock and Sparks, 1977). Esterase activity was assayed by the conversion of radiolabeled JH to

JH acid and subsequent partitioning of the substrate and product by solvent extraction. Conditions that gave linear dependence with both time and protein concentration were used. Assays were performed in triplicate. Each assay produced hydrolysis levels within 10% of the average percentage hydrolysis. For assay of the KSK and KHK mutants, a more sensitive assay was used with a [^{3}H]JH III concentration of 1×10^{-8} M and an increased assay time of 1000 min (Ward *et al.*, 1992). For determination of catalytic parameters, wild-type and mutant JHE were purified by DEAE chromatography (Ichinose *et al.*, 1992). The recombinant enzyme in serum-free medium (ExCell 401) was diluted fourfold with distilled water and the pH was adjusted to 8.5 with Tris–HCl to a final concentration of 2.5 mM. The diluted samples were applied to DEAE–Sepharose equilibrated with 10 mM Tris–HCl, pH 8.5, containing 50 mM NaCl. JHE bound to the column was eluted with the same buffer with a 50 to 200 mM NaCl gradient. Fractions containing JHEs were tested for catalytic activity by radiochemical assay and catalytically inactive mutant JHEs were detected by dot blot analysis. Mutant and wild-type JHEs were concentrated using Centricon-30 microconcentrators (Millipore Corp., Bedford, MA). Following concentration, samples were equilibrated in 0.05 M sodium phosphate buffer, pH 8.5. The K_m, V_{max}, and k_{cat} were determined using [^{3}H]JH III as substrate, with cell culture medium and bovine serum albumin as control samples, as described previously (Ward *et al.*, 1992).

Bioassay of recombinant baculoviruses. Bioassays were carried out to compare the lethal doses (LD) and median survival times (ST) of the recombinant viruses AcJHE-KSK and AcJHE-KHK with the parental virus (AcMNPV clone C6), the recombinant virus expressing wild-type JHE (AcJHE), and the recombinant viruses AcJHE-HK, AcJHE-SG, and AcJHE-KK expressing modified JHEs. The recombinant virus expressing an insect-selective scorpion toxin, AcAaIT (McCutchen *et al.*, 1991; Stewart *et al.*, 1991), was also included for comparative purposes. Larvae of *H. virescens* were maintained at 26°C on a semisynthetic diet (BioServ, Frenchtown, NJ) (Hunter *et al.*, 1984).

For determination of lethal doses, neonate larvae of *H. virescens* were placed individually into the wells of 24-well Falcon plates (Becton-Dickinson, San Diego, CA) with diet for 5 days at 26°C. Larvae that had reached the third instar were infected on a diet plug as described previously (Bonning *et al.*, 1992). Larvae were infected with virus at a dose of 37, 111, 333, 1000, or 3000 occlusion bodies (OBs) by contamination of a diet plug (1 mm^3). Thirty-five larvae were infected per dose and bioassays were replicated three times. Only larvae that had completely consumed the diet plug after 24 h were transferred to cups of diet and maintained at 26°C. Mortality was scored 10 days later.

TABLE 1

Characteristics of Modified Forms of Juvenile Hormone Esterase

Enzyme	Mutations		
JHE			
JHE-29	K29R		
JHE-524			K524R
JHE-KK	K29R		K524R
JHE-SG		S203G	
JHE-HK		H448K	
JHE-KSK	K29R	S203G	K524R
JHE-KHK	K29R	H448K	K524R

Note. The first letter indicates the amino acid changed followed by its position number in the JHE sequence (Hanzlik *et al.*, 1989). The second letter represents the residue with which the amino acid was replaced.

Lethal doses were determined using a probit analysis program (Russell *et al.*, 1977). Comparison of lethal doses was carried out using the lethal dose ratio comparison method (Robertson and Preisler, 1992). For determination of median ST, larvae were infected as neonates with approximately 15 to 20 OBs/larva (Hughes *et al.*, 1986). Larvae were transferred to cups of diet, and mortality was monitored every 4, 6, or 8 h according to the mortality rate. Thirty larvae were infected per bioassay for each virus, and bioassays were replicated at least three times. Larvae were monitored for unusual symptoms. Median ST values and 95% confidence intervals were obtained using the Kaplan Meier Estimator to give nonparametric estimates of survival functions for the insects undergoing each treatment (Kalbfleisch and Prentice, 1980; Collett, 1994). The survival time for larvae was taken as the midpoint of the interval in which they died. Survivors were excluded from the analyses. Survival time ratios (STR) were calculated from the median ST data (Bonning and Hammock, 1993).

Feeding damage was assessed on lettuce (variety Salinas). Thirty neonate larvae were infected per virus by droplet feeding with 2000 OBs/μl. Larvae were transferred individually to inverted petri dishes containing damp filter paper and a piece of leaf. Dishes were sealed with parafilm and maintained at 26°C. Leaves were changed every 48 h and the area was scanned before and after feeding using a CI-202 area meter (CID Inc., Vancouver, WA). Data were analyzed by one-way ANOVA followed by the Scheffe means separation F test (Steel and Torrie, 1980).

RESULTS

Construction of recombinant baculoviruses. The presence of the correct mutations in JHE-KSK and JHE-KHK, produced by combining the different mutations in one clone (Table 1), was confirmed by sequence

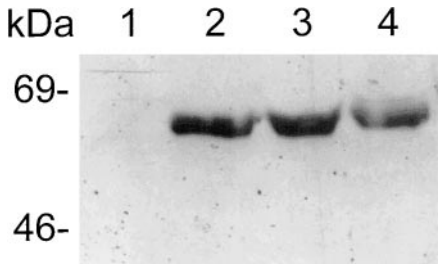

FIG. 2. Western blot of proteins from cell culture medium from uninfected cells (lane 1) and cells infected with AcJHE (lane 2), AcJHE-KSK (lane 3), and AcJHE-KHK (lane 4). Proteins in cell culture medium (40 μl) were separated by SDS–PAGE (10% gel) and transferred to nitrocellulose. Recombinant JHE or mutant JHE were detected using polyclonal rabbit anti-JHE antiserum and an ECL detection kit. The molecular weights were estimated from the migration of protein standards (BioRad, Hercules, CA).

analysis (Ward *et al.*, 1992). The modified sequences JHE-KSK and JHE-KHK were ligated into the baculovirus transfer vector pAcUW21 (Bishop, 1992) downstream of the p10 promoter and upstream of the polyhedrin gene. The resulting recombinant plasmids pAcUW21.JHE-KSK (Fig. 1) and pAcUW21.JHE-KHK (not shown) were digested with restriction endonucleases to confirm that the inserts were in the correct orientation. Cotransfection of Sf21 cells with these plasmid DNA's and linearized viral DNA (AcRP6-SC; Kitts *et al.*, 1990) resulted in production of the recombinant, polyhedrin-positive baculoviruses AcJHE-KSK and AcJHE-KHK, which were purified by plaque assay. Construction of the other recombinant viruses AcJHE-SG, AcJHE-HK, and AcJHE-KK has been described previously (Ward *et al.*, 1992; Bonning *et al.*, 1997a).

Confirmation of expression of modified JHE. SDS–PAGE and Western blot analysis of infected cell culture medium were used to confirm the expression of JHE-KSK and JHE-KHK by the recombinant viruses (Fig. 2). Western blotting was carried out using a rabbit polyclonal anti-JHE antiserum. The level of expression of JHE-KHK and JHE-KSK was comparable to the levels of expression of wild-type JHE or JHE-HK and JHE-SG (Ward *et al.*, 1992). The molecular weights of JHE-KSK and JHE-KHK were the same as that of wild-type JHE (66 kD; Hammock *et al.*, 1990; Fig. 2) and no differences were seen among the expressed proteins in terms of p*I* or native gel electrophoresis. As expected, no catalytic activity was detected on analysis of samples of JHE-KSK or JHE-KHK in cell culture medium using the standard or even the more sensitive assay for JHE (Ward *et al.*, 1992; Table 2). Catalytic activity in cell culture medium from the flask infected with AcJHE and AcJHE-KK was 35 and 22 nmol [^{3}H]JH III hydrolyzed/min/ml, respectively. Activity in medium taken from uninfected cells was not above background levels for either assay.

Bioassay of the recombinant baculoviruses. Bioassays were carried out to evaluate the insecticidal efficacy of the recombinant baculoviruses AcJHE-KSK and AcJHE-KHK on larvae of *H. virescens*. The recombinant viruses AcJHE (which expresses wild-type JHE; Table 1), AcJHE-KK, AcJHE-SG, and AcJHE-HK were also bioassayed along with the wild-type AcMNPV C6 (Ayres *et al.*, 1994) and served as controls. Median ST and STR values are shown in Table 3. The likelihood ratio test (equal slopes and intercepts C.I. 0.95) showed a significant difference between the median ST for AcJHE-KK and AcAaIT and the median ST for the wild-type virus AcMNPV C6. These viruses killed larvae 20 to 30% more rapidly than the wild-type virus. The median ST values for the viruses AcJHE-SG, AcJHE-KSK, AcJHE, AcJHE-HK, and AcJHE-KHK were not significantly different from the ST values of wild-type virus.

Larvae infected with AcJHE-KSK exhibited symptoms similar to those of larvae infected with AcJHE-SG, with about 25% of those infected (n = 93) dying during the molt (Fig. 3). These symptoms were distinct from those observed for larvae infected with AcJHE-KK, in which a proportion of larvae (15%) exhibited contraction paralysis as described previously (Bonning *et al.*, 1999). No unusual symptoms were observed for larvae infected with the control viruses AcMNPV C6, AcJHE, AcJHE-HK, or AcJHE-KHK. Lethal doses were determined for the recombinant baculoviruses (Table 3) using a probit analysis program (Russell *et al.*, 1977). Based on the lethal dose ratio comparison method (Robertson and Preisler, 1992), none of the LD_{50} values were significantly different from that of AcMNPV C6, indicating that engineering of the virus and modification of the expressed JHE did not significantly affect the infectivity of the virus.

TABLE 2

Kinetic Parameters for Wild-Type and Mutant JHE[a]

Enzyme	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (min^{-1})
JHE	0.03 ± 0.01	1.57 ± 0.06	104
JHE-KK	0.02 ± 0.001	2.01 ± 0.02	133
JHE-SG[b]	No detectable activity		
JHE-HK[b]	No detectable activity		
JHE-KSK	No detectable activity		
JHE-KHK	No detectable activity		

[a] Kinetic parameters were determined using [^{3}H]JH III as described under Materials and Methods. Substrate concentrations were 20, 40, 80, 160, 320, and 640 nM. Substrate hydrolysis was kept below 30%, which is within the linear range for this enzyme, by adjustment of assay times and JHE concentrations. Samples were diluted in 0.05 M sodium phosphate buffer containing 10% sucrose and 0.01% bovine serum albumin as carriers. Wild-type and mutant JHE were purified by DEAE chromatography as described under Materials and Methods.

[b] Ward *et al.*, 1992.

TABLE 3

Median Survival Time (ST), Lethal Doses (LD) with 95% Confidence Limits (CL), and Feeding Damage Caused by Larvae of *Heliothis virescens* Infected with Wild-Type and Recombinant Baculoviruses Expressing Modified JHEs

Virus	Median ST(h)[a] (95% CL)	STR[b]	LD_{50} (OBs)[c] (95% CL)	Mean feeding damage (cm^2)[d]	Standard deviation
AcMNPV C6	117 (107–121)	1.00	107 (73–156)	1.45	1.50
AcJHE	116 (106–120)	0.99	53 (25–82)	1.53	0.23
AcJHE-KK	90* (82–98)	0.77	97 (14–233)	0.73*	0.87
AcJHE-SG	81 (81–115)	0.69	80 (16–170)	0.49*	0.64
AcJHE-HK	117 (113–138)	1.00	47 (22–73)	1.60	0.34
AcJHE-KSK	97 (83–111)	0.83	39 (1–82)	0.81*	0.28
AcJHE-KHK	113 (109–139)	0.97	47 (18–80)	1.81	0.27
AcAaIT[e]	84* (77–94)	0.72	45 (15–79)	0.39*	0.57
Uninfected	—	—	—	2.88	1.62

[a] The median survival time was determined for first instar larvae of *H. virescens* (Kaplan Meier Estimator: Kalbfleisch and Prentice, 1980; Collett, 1994).

[b] Survival time ratio (STR) = median ST of test virus/median ST of wild-type virus.

[c] The dose resulting in 50% mortality (LD_{50}) was determined for third instar larvae of *H. virescens* (probit analysis: Russell *et al.*, 1977).

[d] Feeding damage on lettuce caused by larvae of *H. virescens* infected during the first instar. (One-way ANOVA followed by the Scheffe means separation *F* test: Steel and Torrie, 1980).

[e] Recombinant baculovirus expressing an insect-selective scorpion toxin (Stewart *et al.*, 1991; McCutchen *et al.*, 1991).

* Significantly different from AcMNPV C6 ($P < 0.05$).

The feeding damage caused by larvae infected with the different viruses was assessed on lettuce. Larvae of *H. virescens* were infected as neonates and the degree of feeding damage caused was measured. Data for AcJHE-KSK, AcJHE-KHK, AcJHE, AcJHE-HK, AcJHE-KK, AcJHE-SG, and wild-type virus are shown in Table 3. Significantly less feeding damage was caused by larvae infected with AcJHE-KK ($F = 0.32$, $df = 2$, $P < 0.05$), AcJHE-SG ($F = 0.27$, $df = 2$, $P < 0.05$), or AcJHE-KSK ($F = 3.62$, $df = 2$, $P < 0.05$) compared with the wild-type virus (AcMNPV C6). A 44 to 66% reduction in damage was seen for these viruses compared to damage caused by larvae infected with the wild-type virus AcMNPV C6. Feeding damage caused by larvae infected with AcJHE, AcJHE-HK, or AcJHE-KHK was not significantly different from damage caused by larvae infected with wild-type AcMNPV C6. Fifty percent less damage was caused by larvae infected with AcMNPV C6 compared to damage caused by uninfected larvae. Under these conditions, the recombinant virus expressing an insect-selective scorpion toxin, AcAaIT (McCutchen *et al.*, 1991; Stewart *et al.*, 1991), gave a 73% reduction in feeding damage. This reduction in feeding damage is not significantly different from the damage caused by larvae infected with AcJHE-KK ($F = 0.32$, $P > 0.05$), AcJHE-SG ($F = 0.03$, $P > 0.05$), or AcJHE-KSK ($F = 1.71$, $P > 0.05$).

DISCUSSION

Wild-type baculoviruses have been successfully used in controlling a number of insect pest species (Entwistle and Evans, 1985) but are not yet competitive with classical chemical insecticides in terms of speed of action. With the advent of recombinant DNA technology, baculoviruses were exploited as eukaryotic expression vectors of foreign genes (O'Reilly *et al.*, 1992; King and Possee, 1992), and the insecticidal efficacy of these viruses was improved. DNA sequences, encoding proteins that exert toxicity on larvae, were introduced into the genome of AcMNPV (Vlak, 1993; Bonning and Hammock, 1996; Harrison and Bonning, 2000). Successful examples include insect-selective toxins such as a mite neurotoxin (Tomalski and Miller, 1991), a scorpion toxin (McCutchen *et al.*, 1991; Stewart *et al.*, 1991; Hammock, 1999), and hormones/enzymes (diuretic hormone, Maeda, 1989; juvenile hormone esterase, Hammock *et al.*, 1990; chitinase, Golpalakrishnan *et al.*, 1995). For a review of approaches see Harrison and Bonning (2000).

Among the most effective of the recombinant baculovirus insecticides are those which express modified forms of JHE (Bonning *et al.*, 1995, 1997a, 1999). One hypothesis for the insecticidal action of JHE-KK is that this enzyme disrupts the normal regulation of JH in

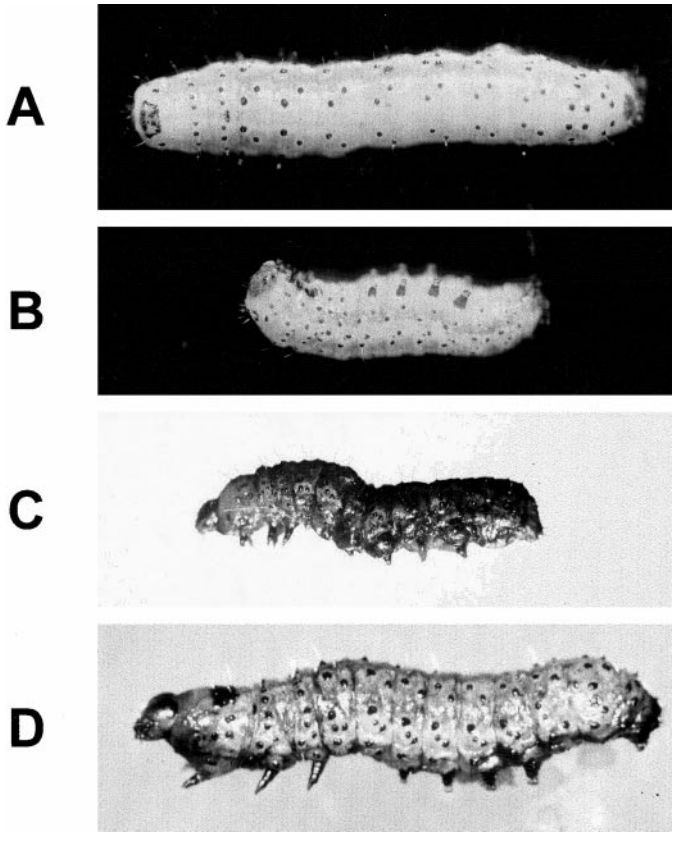

FIG. 3. Symptoms of *Heliothis virescens* infected as neonate larvae with (A) AcJHE, (B) AcJHE-KK, (C) AcJHE-SG, and (D) AcJHE-KSK. Larvae infected with AcJHE-KHK were indistinguishable from larvae infected with the wild-type virus or AcJHE (A) in terms of both symptoms and median ST. Larvae infected with AcJHE-SG (C) were indistinguishable from those infected with AcJHE-KSK (D). About one quarter of larvae infected with AcJHE-KSK died at the molt. The larvae shown in (C) and (D) are trapped within the old cuticle that has failed to split. Contraction of larvae infected with AcJHE-KK (B) was seen in 15% of cases.

the pericardial cells (Bonning *et al.*, 1997a). Approximately 15% of the larvae infected with AcJHE-KK exhibit symptoms of contraction paralysis (Bonning *et al.*, 1999). In contrast, JHE-SG appears to disrupt events at the molt because a considerable proportion of the larvae infected with AcJHE-SG die at the molt with extreme cuticular blackening (Bonning *et al.*, 1995). The symptoms noted above for AcJHE-SG and AcJHE-KK are not seen in insects infected with wild-type AcMNPV C6. The mechanism of action of JHE-SG is not known, but it does not act as a JH-binding protein and its activity is not reversed by juvenoid application.

The modifications made in JHE-SG and JHE-KK were combined to produce JHE-KSK. JHE-KSK and its control JHE-KHK were used to assess whether a recombinant baculovirus with even greater insecticidal efficacy than AcJHE-KK and AcJHE-SG could be generated by combination of the three mutations. These enzymes were also used to investigate whether catalytic activity is necessary for the toxic effect of JHE-KK by combining the Lys_{29} to Arg and Lys_{524} to Arg mutations, which apparently disrupt lysosomal targeting, with mutation of the catalytic His (JHE-KHK). Because expression of JHE-KK in AcMNPV has improved insecticidal properties, it was unclear whether this was due to the reduced efficacy of lysosomal targeting alone or that in conjunction with the catalytic activity of JHE-KK. If catalytic activity is necessary for the enhanced toxicity of AcJHE-KK, removal of histidine from the catalytic site would remove the insecticidal action and would result in a recombinant baculovirus (AcJHE-KHK) with a median ST comparable to that of the wild-type virus.

Biochemical analysis of JHE-KHK and JHE-KSK produced in insect cell culture indicates that mutation of the catalytic site serine in JHE-KSK or histidine in JHE-KHK removes all JHE catalytic activity. This confirms the previous observation that the mutations at the nucleophilic Ser_{203} or general base His_{448}, which are part of the serine esterase catalytic tetrad, result in complete loss of catalytic activity (Ward *et al.*, 1992). It is expected that this is due to loss of catalytic residues rather than large structural changes since there were no changes detected in electrophoretic properties of the mutants.

Bioassays with larvae of *H. virescens* showed significant differences in median ST for AcJHE-KK and AcAaIT in comparison to AcJHE-SG, AcJHE-KSK, and the control viruses AcJHE, AcJHE-HK, AcJHE-KHK, or AcMNPV C6. The enhanced insecticidal efficacy (median ST) seen for JHE-KK was removed by replacement of the catalytic His_{448} in JHE-KHK. Larvae infected with AcJHE-KHK were indistinguishable in pathology from larvae infected with AcJHE-HK, AcJHE, or wild-type virus. These larvae appear to be pale and swollen and eventually disintegrate as result of the virus infection. The symptomology of larvae infected with AcJHE-KSK was similar to that of AcJHE-SG, with disruption of the molting process and unsuccessful molts in about 25% of cases. As discussed by Bonning *et al.* (1995) the mechanism of insecticidal action of JHE-SG remains to be established.

The feeding damage results on lettuce, which are more relevant from a practical standpoint than ST data, show more significant differences between the test viruses than the ST data. Significantly less feeding damage is caused by larvae infected with AcJHE-KK (50%), AcJHE-SG (66%), or AcJHE-KSK (44%) compared to that caused by larvae infected with the wild-type virus (Table 3). Larvae infected with AcJHE or JHE-KHK or wild-type virus showed similar mean feeding damage. These data indicate that expression of JHE without the mutations at Lys_{29} and Lys_{524} does not result in a reduction of feeding damage and that for

JHE-KK catalytic activity is essential for enhanced insecticidal effect (lower median ST and a reduction in feeding damage).

Why the modifications of Lys_{29} and Lys_{524} to Arg do not enhance the insecticidal activity of viruses expressing the catalytically inactive JHEs (AcJHE-KSK and AcJHE-KHK) is unclear. Bioassays with AcJHE-29 and AcJHE-524 revealed that LD_{50} and ST values were not different from those of AcJHE, indicating that both mutations at Lys_{29} and Lys_{524} are necessary for a statistically significant toxic effect (Bonning *et al.*, 1997a, 1999). In JHE-KHK a new Lys residue at His_{448} was introduced so that it could have properties similar to those of JHE-29 or JHE-524. However, according to a 3-D computer-predicted structure (Thomas *et al.*, 1997, 1999), JHE is folded in such a way that the newly introduced Lys residue at 448 would not be exposed on the surface of the molecule and hence is not likely involved in lysosomal targeting. Why no increased activity was found for JHE-KSK relative to JHE-SG and JHE-KK is unclear, but the symptoms and bioassay results were similar to those of JHE-SG, suggesting that modification of Ser_{203} to Gly is the main factor conferring enhanced toxicity. The symptoms and bioassay data for AcJHE-KSK and AcJHE-KHK support the hypothesis that JHE-KK has a local anti-JH effect in the pericardial cells that could affect protein synthesis (Bonning *et al.*, 1997a).

Expression of modified JHE can improve the insecticidal properties of AcMNPV. Although the symptomology suggests that the modes of action of JHE-SG and JHE-KK are different, combining these mutations in JHE-KSK did not further enhance the speed of kill within the limits of sensitivity of our bioassay. Bioassay results with AcJHE-KHK also revealed that catalytic activity is required for the enhanced biological activity of AcJHE-KK. Replacement of Lys_{29} and Lys_{524} to Arg in the catalytically inactive JHEs JHE-KSK and JHE-KHK did not enhance the insecticidal action of these proteins. Since the catalytic site of JHE is in a groove and all residues are away from the protein surface, it is unlikely that these residues have anything to do with lysosomal targeting. Histological studies of JHE-KHK and JHE-KSK are needed to determine whether lysosomal targeting of these proteins is disrupted in a manner similar to that of JHE-KK.

Because significantly less feeding damage is caused by larvae infected with AcJHE-KK, AcJHE-SG, or AcJHE-KSK, these viruses have potential for use for field applications. Further enhancement of these viruses may be achieved by deletion of the ecdysteroid UDP–glucosyl transferase (*egt*) gene (O'Reilly and Miller, 1989) or further modification of JHE, such as removal of the putative pericardial cell targeting amphipathic helix (Thomas *et al.*, 1999).

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