Deletion of the *Autographa californica* nucleopolyhedrovirus chitinase KDEL motif and *in vitro* and *in vivo* analysis of the modified virus

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Infection of insect larvae with *Autographa californica* nucleopolyhedrovirus (AcMNPV) results in the liquefaction of the host, a process involving the action of virus-encoded chitinase and cathepsin gene products. Chitinase is localized in the endoplasmic reticulum (ER) during infection because of the presence of a C-terminal ER retrieval motif (KDEL). In this study, the KDEL coding region was removed from the chitinase gene so that expression of the modified chitinase remained under the control of its own gene promoter, at its native locus. The deletion of KDEL resulted in the redistribution of chitinase within the cell during virus infection. Chitinase lacking the KDEL motif was detectable at the plasma membrane and was also evident in the culture medium of virus-infected cells from as early as 12 h post-infection (p.i.). Secretion of chitinase from the cell continued up to 72 h p.i., until cytolysis. The biological activity of the recombinant virus in *Trichoplusia ni* larvae was enhanced, with a significant reduction in the lethal dose and lethal time associated with infection. Furthermore, a reduction in feeding damage caused by infected larvae was observed compared to AcMNPV-infected individuals.

INTRODUCTION

*Autographa californica* nucleopolyhedrovirus (AcMNPV) causes post-mortem liquefaction of the infected larval host by expressing chitinase (*chiA*; Ayres et al., 1994; Hawtin et al., 1995) and cathepsin (*cath*; Rawlings et al., 1992; Slack et al., 1995) genes. Recombinant viruses lacking either gene were unable to liquefy insects whereas co-infection with these single gene deletion mutants restored the process (Hawtin et al., 1997; Thomas et al., 1998, 2000). Disintegration of the virus-infected host is probably advantageous in maximizing dissemination of polyhedra in the environment.

In the AcMNPV genome, *chiA* is located between *lef-7* and *cath* (Rawlings et al., 1992; Ayres et al., 1994; Hawtin et al., 1995). The mature enzyme possesses high levels of exo- and endo-chitinase activity detectable from 12 h post-infection (p.i.; Hawtin et al., 1995; Thomas et al., 1998). Chitinase enters the secretory pathway of virus-infected cells with concomitant cleavage of a eukaryotic signal peptide (Thomas et al., 1998) but is ultimately retained in the endoplasmic reticulum (ER) until virus-induced cell lysis results in its release (Thomas et al., 1998). This is consistent with the presence of a C-terminal KDEL motif (Thomas et al., 1998) known to function as an ER retrieval signal in plant and animal systems (Lewis & Pelham, 1992; Hawes et al., 1999). Proteins possessing a conserved CXXEL motif are restored to the ER from the Golgi apparatus via a retrograde pathway, after leaving the ER as a result of forward vesicular transport. Proteins without these motifs are presumably permitted to continue on through the Golgi apparatus to their final destination in the cell or beyond.

In an earlier study we showed that deleting the AcMNPV chitinase KDEL and expressing the modified gene under the control of the polyhedrin promoter, in an occlusion-deficient virus, resulted in accumulation of chitinase in the culture medium of infected cells (Saville et al., 2002). In this study, we generated an occlusion-positive recombinant AcMNPV that produced a KDEL-deficient chitinase at its natural locus. The enzymic activity and distribution of chitinase within infected cells was investigated, together with the potential for improving the insecticidal efficacy of the virus, using *Trichoplusia ni* as a test species.

METHODS

**Viruses and cells.** Recombinant viruses were propagated in *Spodoptera frugiperda* (Sf9) cells maintained in Sf900II serum-free medium (King & Possee, 1992). Plaques containing viruses encoding *lacZ* were identified by the addition of 2% X-Gal to the culture medium.

**Production of plasmid transfer vectors.** The transfer vector plef-7*cath* was produced by inserting portions of the coding
sequences of the AcMNPV lef-7 and cath (Fig. 1a) into the multiple cloning site (MCS) of pGEM-3Zf (Promega). A 475 bp fragment of lef-7 was amplified by PCR using a reverse primer, LEF-7(R), 5'-CGATGAATTCCGTTCTGTTATCGA-3' [104807-104826 nt; relative to the AcMNPV genome (Ayres et al., 1994), non-virus sequences in italics] containing an EcoRI site (bold) and a forward primer, LEF-7(F), 5'-TGAAAGTCAGCAATATATATTGAGTAGCATTTAG-3' (105256-105281 nt) containing a BglII site (bold).

![Diagram of AcMNPV and recombinant baculoviruses](image)

Fig. 1. Genomic organization of AcMNPV and recombinant baculoviruses. (a) Region of AcMNPV genome showing locations and orientation of lef-7, chiA and cath genes. The positions of the PCR primers used to amplify portions of each gene are indicated. (b) Upstream and downstream regions from chiA amplified using PCR (see a) and ligated to produce plef-7/cath. (c) Replacement of chiA with lacZ (not to scale) under chiA promoter control to derive AcΔchiA.lacZ recombinant virus. (d) Region of AcChiA^KDEL recombinant virus genome showing modified chiA lacking nucleotides encoding the C-terminal KDEL motif at the chiA locus of AcMNPV. The unmodified AcMNPV sequence is shown for comparison.
The product was digested with EcoRI and BglII. A 400 bp fragment of cath was produced by using PCR with two additional oligonucleotides. The forward primer, CATH(F), 5'-TGAAGATGTTT-TAATTTATCATTTTAACTTTAGTTG-3' (106938–106962 nt), which spanned the chIA promoter (antisense TAAG motif underlined) and contained a BglII site (bold). The reverse primer, CATH(R), 5'-CACAAGGCTTTGATTTTTACGCTAGGAC-3' (107352–107370 nt) with a HindIII site (bold). The product was digested with BglII and HindIII. The MCS of the vector pGEM-3Zf was co-digested with EcoRI and HindIII, and the amplified portions of lef-7 and cath ligated simultaneously into the purified vector to derive plef-7/cath (Fig. 1b).

The recombinant virus AcChIA/lacZ was produced by inserting the coding region of lacZ into the chitinase locus of AcMNPV (Fig. 1c). This sequence was isolated from pCH110-BglII (Possee & Howard, 1987) using BamHI and BglII. It was then inserted into plef-7/cath, digested with BglII, to produce plef-7/lacZ/cath. This vector was co-transfected into SF9 cells with circular AcMNPV/6C DNA (Possee, 1986) using Lipofectin (Invitrogen). The recombinant AcChIA/lacZ was purified from AcMNPV by selecting for β-galactosidase production after addition of X-Gal to plaque assay medium. Thereafter, AcChIA/lacZ was purified to homogeneity through 12 rounds of plaque assay, amplified and virus DNA purified (King & Possee, 1992).

A recombinant virus producing KDEL-deficient chitinase at the chIA locus was derived using AcChIA/lacZ as a parental virus. Initially, modified chIA was constructed by using PCR amplification of the coding region of chIA in which the sequences encoding the KDEL motif had been deleted. The PCR amplification of the coding sequence of chIA was achieved using a forward primer, chIA(F), 5'-ATAAGGATCCATGTTGACTAAATTTGTTAACC-3' (109616–109637 nt) containing a BamHI site (bold) and a reverse primer, chIA(R), 5'-ACACCGGATCCCTAGTTGACGTTGTTTGCA-3' (105294–105320 nt) with a BamHI site (bold). The amplified sequence was cloned into the BglII site of plef-7/ lacZ to derive plef-7/chIA KDEL/cath. All transfer vectors were sequenced to confirm flanking region integrity, relevant inserts and deletion of the sequences encoding the KDEL tetrapeptide.

The recombinant virus AcChIA KDEL (Fig. 1d) was produced by co-transfecting SF9 cells with Bsu36I-digested AcChIA/lacZ viral DNA and plef-7/chIA KDEL/cath. The recombinant virus AcChIA KDEL was isolated from AcChIA/lacZ by selection of white plaques from a plaque assay containing X-Gal, and purified to homogeneity through five further rounds of plaque purification.

SDS-PAGE and Western blotting. SF9 cells (1 x 10⁶) were infected with either AcChIA KDEL or AcMNPV at a m.o.i. of 5 p.f.u. per cell, or mock-infected with medium. Cells were harvested at appropriate times p.i., pelleted (3000 g, 5 min), and the medium removed to a fresh tube. Cell pellets were washed three times in PBS, resuspended in 100 µl of the same buffer before analysing 15 µl using 10% SDS-PAGE gel and Western blots as described previously (King & Possee, 1992; Thomas et al., 2000). Membranes developed for chitinase were incubated with primary anti-chitinase antisera (1:1000; Hawtin et al., 1995), and a secondary anti-guinea pig IgG antibody conjugated to alkaline phosphatase (1:10000). Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as described previously (Hawtin et al., 1997; Thomas et al., 1998).

Enzyme assays. SF9 cells (1 x 10⁶) were infected with a virus as described above and harvested at appropriate times p.i. Cells were pelleted, freeze–thawed three times and assayed for chitinolytic activity using the microtitre plate assay of McCreaath & Gooday (1992), as described by Saville et al. (2002).

Confocal microscopy. The distribution and localization of chitinase in virus-infected cells was examined using confocal laser scanning microscopy (CLSM). Sterile coverslips (13 mm) were seeded with SF9 cells and infected with AcMNPV, AcChIA KDEL (m.o.i. = 5 p.f.u. per cell), or mock-infected with medium. Cells were incubated at 28°C, harvested and fixed with paraformaldehyde at the desired time-points. Immunostaining was performed for chitinase, microtubules or ER. Chitinase staining was carried out using a primary anti-chitinase antibody (1:500; Hawtin et al., 1995), and secondary FITC conjugated antibody (1:64; Sigma). Staining for the ER was carried out using a primary anti-KDEL antibody (1:200; Molecular Probes) and secondary antibody conjugated to Texas red (1:1000; Molecular Probes). Coverslips were washed between antibody applications with 1% BSA/PBS and rinsed with ultra-pure water before mounting on Citifluor antifadant and sealing with clear lacquer. Samples were examined on a Zeiss Axiosvert 510 CLSM microscope using appropriate filter sets. The multitracking facility on the microscope was used to avoid cross talk.

Preparation of purified polyhedra. Third instar T. ni larvae were infected with cell culture-derived virus (1 x 10⁶ p.f.u. per larva) via micro-injection into the haemolymph at the first posterior proleg (Saville et al., 2002). Larvae were housed separately on semi-synthetic diet (Hunter et al., 1984) and monitored daily. Infected larvae were harvested at 5 days p.i., and polyhedra purified by sucrose gradient centrifugation (King & Possee, 1992). These were used to infect T. ni larvae per os and polyhedra purified as described above. The identity of the virus was confirmed by examining genomic DNA with restriction endonucleases.

Larval bioassays. The effects of AcMNPV or AcChIA KDEL infection upon larval liquefaction was examined by feeding 10⁵ polyhedra to third instar T. ni larvae. Plugs of semi-synthetic diet were inoculated with polyhedra (10⁶ µl⁻¹), and those individual larvae which had consumed the entire plug after 12 h were reared on semi-synthetic diet and monitored until death or pupation. Larval condition at death and liquefaction was recorded for AcChIA KDEL, AcChIA/lacZ and AcMNPV-infected larvae.

Lethal dose analysis. The lethal dose 50 (LD₅₀) of AcMNPV or AcChIA KDEL was assayed by infecting second instar T. ni larvae (n=30) with defined doses of purified polyhedra. Early second instar larvae from a single hatch were fed dilutions of polyhedra, in 1 µl drops, on plugs of sterile semi-synthetic diet (Hunter et al., 1984). Polyhedra were administered in doubling or log dilutions. Larvae were monitored until death or pupation and were tested for viral infection after death by Giemsa staining of polyhedra. The LD₅₀ of AcChIA KDEL and AcMNPV were calculated by using an appropriate statistical program (GLIM 3.77).

Lethal time analysis. The mean lethal time to death of AcChIA KDEL- and AcMNPV-infected larvae was assayed using neonate T. ni larvae from a single hatch infected with 10⁵ polyhedra mixed with blue food dye, via a droplet feeding method (King & Possee, 1992). Those larvae which had consumed the entire plug after 12 h were reared on semi-synthetic diet and monitored until death or pupation (n=35). The assay was repeated twice and the mean time to death was calculated.

Feeding damage assays. Early third instar T. ni larvae from a single hatch were infected with a virus dose sufficient to produce 100% mortality (10⁶ polyhedra), via inoculation of a 1 µl drop of polyhedra suspension on a plug of semi-synthetic diet. Those larvae fully consuming the diet plug were housed individually in Petri dishes and fed on fresh cabbage (Huspi F1) in a leaf disc-feeding

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assay. Larvae were given fresh cabbage discs of a known area and weight at 24 h intervals. Humidity was maintained by damp Whatman filter paper in each dish. The weight (adjusted against desiccation) and surface area of cabbage consumed for each individual larva \( (n=30) \) was calculated and the cumulative damage assessed. AcChiA\(^{AKDEL} \) and AcMNPV-infected larvae were compared directly to mock-infected larvae \( (n=30) \).

**RESULTS**

Production of Ac\(\Delta\chi A.lacZ\) and Ac\(\chi A^{AKDEL}\)

Recombinant viruses were produced expressing either lacZ or a modified \( \chi A \) from the chitinase promoter of AcMNPV. Firstly, regions flanking \( \chi A \) (lef-7 and cath; Fig. 1a) were amplified and cloned to construct plef-7/cath (Fig. 1b) into which the lacZ coding region was inserted to derive plef-7/lacZ/cath. This vector was co-transfected with AcMNPV DNA to produce Ac\(\chi A.lacZ\) (Fig. 1c). Secondly, \( \chi A \) with a deletion of the KDEL coding region was generated by PCR (Fig. 1a) and cloned into plef-7/cath. This vector was used to co-transfect Sf9 cells with Bsu36I-linearized Ac\(\chi A.lacZ\) DNA to produce Ac\(\chi A^{AKDEL}\) (Fig. 1d), which was anticipated to express a mutated \( \chi A \) from the chitinase locus of AcMNPV.

Assessment of chitinase production by AcMNPV, Ac\(\chi A.lacZ\) and Ac\(\chi A^{AKDEL}\)

Cells were either mock-infected or infected with AcMNPV, Ac\(\chi A.lacZ\) or Ac\(\chi A^{AKDEL}\). Samples harvested from 0 to 72 h p.i. were assayed for the presence of chitinase by Western blot. Fig. 2(a) illustrates that in Ac\(\chi A.lacZ\)–infected cells, chitinase was undetectable in any of the samples. This was consistent with the substitution of lacZ for \( \chi A \). The recombinant virus Ac\(\chi A^{AKDEL}\) was shown to express a 58 kDa chitinase-specific product at 24 h p.i. (Fig. 2b). The presence of cathepsin in the intracellular fraction of AcMNPV–, Ac\(\chi A.lacZ\)– and Ac\(\chi A^{AKDEL}\)–infected cells was detected by Western blot analysis (data not shown).

A detailed analysis of the temporal production of chitinase in AcMNPV– and Ac\(\chi A^{AKDEL}\)–infected cells was performed (Fig. 3). Chitinase was first detected in the intracellular fraction of AcMNPV– (Fig. 3a) and Ac\(\chi A^{AKDEL}\)–infected cells (Fig. 3c) from 12 h p.i. and 18 h p.i., respectively.

In culture medium from Ac\(\chi A^{AKDEL}\)–infected cells, a chitinase-specific band of 58 kDa was identified from 12 h p.i. (Fig. 3d) that was not detectable in mock-infected cells. This band was not observed in AcMNPV–infected cells until the terminal stages of infection from 48 to 72 h p.i.
(Fig. 3b), which was accompanied by cellular lysis as observed under the light microscope.

Detection of chitinolytic activity in AcMNPV- and AcChiA<sub>KDEL</sub>-infected cells

The N-acetylglucosaminidase, exo- and endo-chitinase activities in virus- and mock-infected Sf9 cells were compared between 0 and 72 h p.i. Low levels of N-acetylglucosaminidase activity were detectable throughout this period. High levels of exo- and endo-chitinase activity were detected in AcMNPV- and AcChiA<sub>KDEL</sub>-infected cells from 12 h p.i (Fig. 4). Thereafter, subtle differences in chitinase activity were observed between the two viruses. AcMNPV-infected cells (Fig. 4a) exhibited almost the same levels of chitinase throughout the time-course, with slightly higher endo- compared to exo-chitinase activity. The chitinase activity in AcChiA<sub>KDEL</sub>-infected cells increased from 12 h p.i. and reached a maximum by 27 h p.i. (Fig. 4b). Until 15 h p.i., endo-chitinase activity was higher than exo-chitinase. However, this was reversed from 18 h p.i. onwards. Levels of chitinase activity in AcChiA<sub>KDEL</sub>-infected cells were generally higher than those associated with AcMNPV infection throughout the experiment. Levels of exo- and endo-chitinolytic activity in AcΔChiA.lacZ- and mock-infected cells were not readily detectable at 48 h p.i., which was in agreement with the lack of chitinase expression by these viruses (Fig. 4a, b).

Examining the localization of chitinase in virus-infected cells using CLSM

The localization of chitinase during AcMNPV or AcChiA<sub>KDEL</sub> infection of Sf9 cells was examined using CLSM. Cells were infected with virus or mock-infected and harvested at 48 h p.i., before dual immunostaining for chitinase and either the ER or microtubules, by addition of anti-KDEL or anti-α-tubulin primary antibody, respectively.

Fig. 5 illustrates the results of CLSM with mock-, AcMNPV- or AcChiA<sub>KDEL</sub>-infected cells. The distribution of the ER in mock-infected Sf9 cells at 48 h p.i. (Fig. 5a), detected using anti-KDEL, illustrates the perinuclear location of the ER in insect cells. Cortical microtubules emanated throughout the cytoplasm in non-infected Sf9 cells, outlining the area of the nucleus and reaching underneath the plasma membrane (Fig. 5b). In AcMNPV-infected cells this network of microtubules appeared to retract underneath the plasma membrane (Fig. 5e). This characteristic of AcMNPV infection is responsible for the rounding of virus-infected cells (Volkman & Zaal, 1990). This change in microtubule localization was employed as a marker to differentiate periplasmic from ER staining. The localization of chitinase in AcMNPV-infected cells (Fig. 5c) was shown to be perinuclear, by co-localization with anti-KDEL and anti-chitinase antibodies at 48 h p.i. This conclusion was reinforced by the association of the two peaks for each signal (Fig. 5c1). In AcChiA<sub>KDEL</sub>-infected cells (Fig. 5d), co-staining of chitinase and ER at 48 h p.i. revealed only partial co-localization with the ER, with most chitinase being detected in the periplasmic region, as confirmed in Fig. 5d1.

When the distribution of chitinase relative to the plasma membrane and microtubules was examined, a distinct immunostaining pattern was detected. In AcMNPV-infected cells, two separate regions of staining were observed corresponding to chitinase and microtubules (Fig. 5e). This indicated that at 48 h p.i., chitinase was localized in the ER of infected cells and did not reach as far as the plasma membrane. This was further confirmed by the shift in peaks of the associated graph (Fig. 5e1), demonstrating lack of co-localization of the two stains. The staining pattern observed for AcChiA<sub>KDEL</sub>-infected cells (Fig. 5f)
**Fig. 5.** CLSM of AcMNPV- and AcChiA<sup>AKDEL</sup>-infected cells. Cells (S/9) were infected with AcMNPV or AcChiA<sup>AKDEL</sup> and harvested at 48 h p.i. Cells were immunostained for chitinase using a primary anti-chitinase antibody (1 : 1000) and secondary FITC-conjugated antibody (1 : 64). ER was stained using a primary anti-KDEL antibody (1 : 100) and secondary antibody conjugated to Texas red (1 : 1000). Microtubules were stained by addition of anti-α-tubulin antibody (1 : 20) and secondary Texas red-conjugated antibody (1 : 1000). Cells were visualized on a Zeiss Axiovert 510 CLSM microscope using appropriate filter sets. Bar, 5 μm. (a) Mock-infected cell stained with anti-KDEL. (b) Mock-infected cell stained with anti-α-tubulin. (c) AcMNPV-infected cell co-stained with anti-chitinase and anti-KDEL. (d) AcChiA<sup>AKDEL</sup>-infected cell co-stained with anti-chitinase and anti-KDEL. (e) AcMNPV-infected cell co-stained with anti-chitinase and anti-α-tubulin. (f) AcChiA<sup>AKDEL</sup>-infected cell co-stained with anti-chitinase and anti-α-tubulin.
was found to be markedly different from that of AcMNPV-infected cells (Fig. 5e), with high levels of co-localization occurring beneath the plasma membrane at 48 h.p.i. (Fig. 5f). The presence of chitinase at the plasma membrane was highlighted by the co-localization of the associated peaks on the graph (Fig. 5f).

Examining the effects of AcMNPV or AcChiA<sup>AKDEL</sup> infection upon larval liquefaction

The host liquefaction associated with acute AcMNPV infection was examined using <i>T. ni</i> larvae. Third instar larvae from a single hatch were fed purified polyhedra on plugs of semi-synthetic diet and housed individually until death or pupation. The condition of larvae was monitored at 8 h intervals and recorded. Mock-infected (PBS) larvae showed no signs of infection and pupated approximately 8 days later. Those larvae infected with AcMNPV or AcChiA<sup>AKDEL</sup> showed characteristic signs of infection from 4 to 5 days p.i., including reduced larval movement, suspension of feeding activity and a creamy cuticle appearance, with larval death and liquefaction occurring from 7 days p.i. This indicated that the terminal liquefaction associated with AcMNPV-infected larvae was present in AcChiA<sup>AKDEL</sup>-infected larvae.

Lethal dose analysis of AcMNPV and AcChiA<sup>AKDEL</sup>

The LD<sub>50</sub> of AcChiA<sup>AKDEL</sup> was compared to AcMNPV. Second instar <i>T. ni</i> larvae (n = 30) from a single hatch were purified polyhedra on a plug of semi-synthetic diet and monitored until death or pupation. The number of larvae succumbing to virus infection was recorded, and the LD<sub>50</sub> of the viruses was calculated (Table 1). The LD<sub>50</sub> analysis of AcChiA<sup>AKDEL</sup> indicated that the virus required fewer polyhedra to initiate a lethal infection in <i>T. ni</i> larvae. A reduction of over 50% in LD<sub>50</sub> was observed for AcChiA<sup>AKDEL</sup>, with 26 polyhedra required to cause 50% mortality of a defined population, whereas AcMNPV required 59 polyhedra to cause the same level of mortality. These data indicated that the virus AcChiA<sup>AKDEL</sup> conferred a higher efficacy to second instar <i>T. ni</i> than AcMNPV, which was confirmed by statistical analysis (χ<sup>2</sup> = 491.9, 7 d.f., P &lt; 0.001) and the lack of overlap of the calculated 95% confidence limits.

**Lethal time analysis**

The mean time to death of populations of <i>T. ni</i> was calculated for individuals infected with AcMNPV or AcChiA<sup>AKDEL</sup>. The lethal time 50 (LT<sub>50</sub>) was examined by infecting individual larvae (neonate), with sufficient polyhedra (10<sup>3</sup>) to induce 100% mortality, using a droplet feeding method. Dilutions of purified polyhedra were mixed with blue food dye, and those larvae visibly consuming the droplet of virus suspension were transferred to individual pots and monitored at regular intervals (8 h) until death. Fig. 6 illustrates the survival time of larvae infected with AcMNPV or AcChiA<sup>AKDEL</sup>. The time-point at which 50% of the population succumbed to virus infection was calculated from the means of two individual experiments. The AcMNPV-infected individuals were found to have an LT<sub>50</sub> of 69 h p.i., whilst AcChiA<sup>AKDEL</sup>-infected larvae had an LT<sub>50</sub> of 61 h p.i. The two values were significantly different (F = 10.97, P &lt; 0.01).

**Feeding damage assays**

The damage caused by feeding larvae was examined over a period of 8 days. Larvae (<i>n</i> = 30) from a single hatch were fed 10<sup>9</sup> polyhedra and maintained on cabbage until death or pupation. Larvae were housed individually in Petri dishes with damp Whatman paper and given a fresh disc of cabbage of known surface area and weight at 24 h intervals. The damage caused by feeding larvae was examined over a period of 8 days.
96 h experimental period, and the cumulative damage per larva assessed at the end. Fig. 7(a) illustrates that mock-infected larvae consumed a significantly greater surface area of cabbage ($F=218.2; P<0.001$) compared to AcMNPV- or AcChiA$_{KDEL}$-infected larvae. AcMNPV-infected larvae appeared to consume a greater surface area of cabbage than AcChiA$_{KDEL}$-infected larvae, but no significant difference was observed ($F=1.91; P>0.05$).

When the weight of cabbage consumed by larvae was examined (Fig. 7b), mock-infected larvae were again shown to consume significantly more than either virus-infected larvae ($F=279.2; P<0.001$). AcMNPV-infected individuals were also shown to consume a small but significantly greater weight of cabbage than AcChiA$_{KDEL}$-infected larvae ($F=2.99; P<0.05$).

**DISCUSSION**

Deletion of the AcMNPV C-terminal chiA ER retrieval (KDEL) motif (Thomas *et al.*, 1998) or its substitution with other sequences was sufficient to alter the localization of chitinase during virus infection (Saville *et al.*, 2002). Furthermore, the modified viruses liquefied *T. ni* larvae 24 h earlier than AcMNPV. The use of a polyhedrin-negative phenotype, however, meant that infection was carried out by infection of the budded virus. Here, a recombinant AcMNPV lacking the chiA KDEL was constructed so that the efficacy of the virus and pathology could be compared with unmodified AcMNPV after infection per os. In contrast with the viruses described by Saville *et al.* (2002), which expressed modified versions of chiA using a polyhedrin gene-based vector, this study utilized a virus (AcChiA$_{KDEL}$) producing a KDEL-less chitinase from its native location in the AcMNPV genome. Chitinase was synthesized in the late phase of virus gene expression, providing more appropriate comparison with chiA expression by the wild-type virus.

Chitinase was detected in the intracellular fraction of cells infected with the recombinant virus AcChiA$_{KDEL}$ or AcMNPV, but was not evident in AcΔchiA.lacZ- or mock-infected cells. Monitoring chitinase production throughout virus infection showed that it was present in the culture medium of AcChiA$_{KDEL}$-infected cells from 12 h p.i. and accumulated until 72 h p.i. Chitinase was only detectable in extracellular medium in AcMNPV-infected cells from 48 h p.i. and accompanied the onset of cell lysis. This indicated that its release was a cell lysis-induced process, in contrast with constitutive secretion by AcChiA$_{KDEL}$-infected cells. Secretion of chitinase lacking the KDEL motif was reported by Saville *et al.* (2002), but after expression from the polyhedrin gene promoter. In this study the modified chiA was expressed by its native promoter. These data indicated that deletion of KDEL from chiA was sufficient to alter the localization of the encoded enzyme in virus-infected cells, probably as a result of trafficking of chitinase through the secretory pathway.

The enzymic activity of chitinase produced by AcChiA$_{KDEL}$ was compared with that made by AcMNPV. The assay used differentiates between the characteristic exo- and endo-chitinolytic activities displayed by AcMNPV chitinase (Hawtin *et al.*, 1995). Low levels of N-acetylglucosaminidase were observed in virus-infected cells, which was in agreement with previous studies (Hawtin *et al.*, 1995; Saville *et al.*, 2002). High levels of exo- and endo-chitinase were detected in the intracellular fraction of AcMNPV- and AcChiA$_{KDEL}$-infected samples from 12 h p.i.
Levels of chitinase activity associated with AcChiA\textsuperscript{AKDEL}-infected cells were higher than those for AcMNPV throughout the time-course. We suggest that this is a result of reduced accumulation of chitinase within the ER of the infected cell, as a consequence of the KDEL deletion and secretion of chitinase. Aggregation of the enzyme within the ER may reduce its activity or simply make it less accessible for assay. The reasons for the higher levels of exochitinase observed in AcChiA\textsuperscript{AKDEL}-infected cells in relation to endo-chitinase are unknown. It may be a consequence of passage beyond the ER, where the protein could be exposed to various post-translational modifications. We were unable to demonstrate chitinase activity in culture medium of AcChiA\textsuperscript{AKDEL}-infected samples, as the current assay provided very high levels of background fluorescence in the samples, which masked activity in this fraction. It would be very interesting to compare the activity of secreted chitinase with intracellular material.

The distribution of chitinase in AcMNPV-infected cells was perinuclear and associated with the ER, as shown by co-localization with the anti-KDEL antibody. In AcMNPV-infected cells, there was a separation of antibody labels between the ER and plasma membrane region for chitinase and \( \alpha \)-tubulin, respectively. Chitinase was distributed in a perinuclear manner that did not co-localize with the microtubule stain at the plasma membrane region of the cell. In AcChiA\textsuperscript{AKDEL}-infected cells, however, the two antibodies reacted with proteins beneath the plasma membrane, suggesting that chitinase without a KDEL motif had moved through the secretory pathway. Some chitinase in AcChiA\textsuperscript{AKDEL}-infected cells remained associated with the ER, as shown by co-localization with the anti-KDEL antibody in dual staining experiments.

The presence of intracellular chitinase in AcChiA\textsuperscript{AKDEL}-infected cells suggested that removing the KDEL motif from the enzyme did not result in complete secretion of the modified product. This is consistent with the presence of the enzyme as it moves through the ER and the Golgi prior to secretion. Quantitative studies to compare the amount of chitinase produced by AcMNPV- or AcChiA\textsuperscript{AKDEL}-infected cells and to determine relative amounts of secreted or intracellular enzyme were not undertaken. Chitinase would have to be isolated from extracellular medium to remove the background activity that masks the virus-induced product in enzyme assays. The Western blots used to assess chitinase production in virus-infected cells showed slightly higher amounts of protein in AcMNPV-infected cells. Conversely, more chitinase was seen in AcChiA\textsuperscript{AKDEL}-infected cell culture medium. Chitinase in AcMNPV-infected cells normally resides in the ER and may be poorly adapted for passage through the secretory pathway.

Chitinase genes have been identified in many baculoviruses to date. Examples include: AcMNPV (Ayres \textit{et al}, 1994), \textit{Orgyia pseudotsugata} (Op) MNPV (Ahrens \textit{et al}, 1997), \textit{Cydia pomonella} ( Cp) GV (Kang \textit{et al}, 1998; Luque \textit{et al}, 2001), Spodoptera exigua (Se) NPV (Ikel \textit{et al}, 1999), \textit{Antheraea pernyi} NPV (accession no. AB072731), \textit{Hyphantria cunea} NPV (AF121457), Bombyx mori (Bm) NPV (Gomi \textit{et al}, 1999), \textit{Lymantria dispar} (Ld) MNPV (Kuzio \textit{et al}, 1999), \textit{Xestia c-nigrum} (Xecn) GV (Hayakawa \textit{et al}, 1999), \textit{S. litura} (Split) NPV (Pang \textit{et al}, 2001), \textit{Helicoverpa armigera} (Ha) MNPV (Chen \textit{et al}, 2001), \textit{Epiphya postvittana} (Eppo) NPV (Hyink \textit{et al}, 2002), \textit{H. zeas} (Hz) SNPV (Chen \textit{et al}, 2002), \textit{Mamestra configurata} (Maco) NPV (Li \textit{et al}, 2002), \textit{Chloristoneura fumiferana} (Cf) MNPV (NC_004778) and \textit{Rachiplusia oleracea} (Ro) MNPV (Harrison \& Bonning, 2003). Chitinase is present as a truncated sequence in \textit{Cryptophlebia leucotreta} GV (Lange \& Jehle, 2003). It is absent from \textit{Plutella xylostella} GV (Hashimoto \textit{et al}, 2000), \textit{Culex nigripalpus} NPV (Alfonso \textit{et al}, 2001), \textit{Pithiorimae operculla} GV (accession no. NC_004062), \textit{Adoxophyse homnai} NPV (NC_004690) and \textit{Adoxophyes orana} (Ador) GV (Wormleaton \textit{et al}, 2003). O’Reilly (1997) defined \textit{chiA} as an auxiliary gene of baculoviruses. Such genes are not essential for baculovirus replication in insects, but may serve a role that makes the process more efficient, enhances virus production or aids dissemination. Whether or not a baculovirus has a chitinase gene may be one of the slow GVs (Winstanley \& O’Reilly, 1999) that facilitates horizontal transmission. For example, AdorGV is defined as an auxiliary gene of baculoviruses. Such genes are not essential for baculovirus replication in insects, but may serve a role that makes the process more efficient, enhances virus production or aids dissemination. Whether or not a baculovirus has a chitinase gene may be reflected in subtle differences in the pathology associated with infection of insect larvae or how well occlusion bodies facilitate horizontal transmission. For example, AdorGV is one of the slow GVs (Winstanley \& O’Reilly, 1999) that kills the host in the final instar, irrespective of the stage at which it was infected and its genome lacks chitinase (Wormleaton \textit{et al}, 2003).

Not all of the baculoviruses encoding \textit{chiA}s have a C-terminal KDEL retrieval motif. It is present in AcMNPV (Ayres \textit{et al}, 1994), OpMNPV (Ahrens \textit{et al}, 1997) and SeMNPV (Ikel \textit{et al}, 1999), but is found as an RDEL variant in BmNPV (Gomi \textit{et al}, 1999), EppoNPV (Hyink \textit{et al}, 2002) and CMNPV (accession no. NC_004778), RVEL in LdMNPV (Kuzio \textit{et al}, 1999), HNEL in HaMNPV, HaSNPV and HzSNPV (Chen \textit{et al}, 2001, 2002), KTEL in MacoNPV (Li \textit{et al}, 2002) and HSEL in SpltNPV (Pang \textit{et al}, 2001). None of the above tetrapeptide sequences are found in CpGV (Kang \textit{et al}, 1998; Luque \textit{et al}, 2001) or XecnGV (Hayakawa \textit{et al}, 1999). It would be interesting to determine if the \textit{chiA}s encoded by these two viruses are expressed and where the mature proteins are localized within the virus-infected cells. The absence of an ER retrieval motif suggests that the putative XecnGV and CpGV chitinases might progress through the ER and be secreted from the cell. The availability of cell culture systems for \textit{C. pomonella}, which support the replication of CpGV...
in vitro (Winstanley &Crook, 1993), makes this a relatively easy question to answer.

The biological activity of AcChiA<sup>KDEL</sup> was tested in vivo using *T. ni* larvae, to elucidate if the redistribution of chitinase associated with the deletion of KDEL would affect the efficacy of the virus. Those larvae infected with AcMNPV or AcChiA<sup>KDEL</sup>-polyhedra per os showed signs of infection from 4 to 5 days p.i. which resulted in death and liquefaction from 7 days p.i. This indicated that the modification of chiA in AcChiA<sup>KDEL</sup> had not affected the biological activity of chitinase or cathepsin and the liquefaction characteristic of AcMNPV infection was retained (Hawtin et al., 1997). The LD<sub>50</sub> of AcChiA<sup>KDEL</sup> was found to be significantly lower than that of AcMNPV, indicating that AcChiA<sup>KDEL</sup> had a higher efficacy to *T. ni* than AcMNPV.

An LT<sub>50</sub> of 69 h p.i. was recorded for AcMNPV-infected larvae, whilst a significantly lower LT<sub>50</sub> of 61 h p.i. was determined for AcChiA<sup>KDEL</sup> (F = 10.97; P < 0.01). These data coupled with the reduced LD<sub>50</sub> of the virus AcChiA<sup>KDEL</sup> indicated that the deletion of KDEL and redistribution of chitinase in infected cells may have increased the effectiveness of the virus to *T. ni* compared with AcMNPV. Furthermore, the feeding damage caused by AcChiA<sup>KDEL</sup>-infected larvae was significantly lower than that caused by AcMNPV-infected larvae when comparing the weight of food plant consumed. A similar result was not observed when surface area was compared, but this discrepancy might be explained by the fact that leaf discs of identical surface area did not always weigh the same because of fluctuating leaf thickness.

The improvements in biological activity of the recombinant AcChiA<sup>KDEL</sup> were marginal. In other studies where insect-specific scorpion neurotoxins (Maeda et al., 1991; McCutchen et al., 1991; Stewart et al., 1991), mito neurotoxins (Tomalski & Miller, 1991; Popham et al., 1997) or juvenile hormone esterase (Hammock et al., 1990; Bonning et al., 1997, 1999) were expressed using baculoviruses, greater reductions in LD<sub>50</sub>, LT<sub>50</sub> or feeding damage were determined for AcMNPV-infected insects (Hawtin et al., 2002). The virus producing a modified chitinase-induced liquefaction of the infected hosts 24 h earlier than AcMNPV. The current study employed the native chiA promoter. The modified virus only accelerated larval liquefaction by 8 h. It may be necessary to re-engineer the AcChiA<sup>KDEL</sup> virus so that the modified chitinase is expressed by the polyhedrin gene promoter at the chiA locus. This should enable higher levels of chitinase production to be achieved and thus improve the biological activity of the recombinant virus. However, it might help public acceptance of a genetically modified virus insecticide if modulating levels of gene expression and minor deletions to the coding region of a gene prevent the need to add foreign sequences to the virus genome.

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**References**


