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Liquefaction of *Autographa californica* Nucleopolyhedrovirus-Infected Insects Is Dependent on the Integrity of Virus-Encoded Chitinase and Cathepsin Genes

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We examined the role of the *Autographa californica* nucleopolyhedrovirus (AcMNPV)-encoded chitinase in virus pathogenesis in *Trichoplusia ni* larvae. In conjunction with the AcMNPV-encoded cathepsin, it promotes liquefaction of the host in the latter stages of infection. Insects infected with virus mutants lacking either the chitinase A gene (*chiA*) or cathepsin gene (*cath*) remained intact several days after death. However, if both viruses were used to infect insects, liquefaction of the host was restored. Chitinase was readily detected in AcMNPV-infected insects using a chitinase-specific antibody, but it was absent from insects infected with a *chiA* deletion mutant (Ac*chiA*$^-$). The chitinase was also detected in polyhedra purified from AcMNPV-infected insects but not in those from Ac*chiA*$^-$. However, polyhedra derived from a virus lacking an intact *chiA* were no less effective in initiating an infection in second instar *T. ni* larvae than those of the unmodified AcMNPV. It was also demonstrated that the virus chitinase retained high levels of activity between pH 3.0 and 10.0. In contrast, chitinases isolated from *Serratia marcescens,* although active under acidic conditions, rapidly lost activity above pH 7.0 illustrating that despite 57% sequence identity, the two proteins have distinct enzymic activities.

INTRODUCTION

The *Autographa californica* nucleopolyhedrovirus (AcMNPV; Murphy *et al.,* 1995) is the prototype member of the nucleopolyhedrovirus genus of the *Baculoviridae.* It has a covalently closed, circular, double-stranded DNA genome of 133,894 base pairs (bp), which has been sequenced (Ayres *et al.,* 1994). Rod-shaped virus particles, containing DNA, are occluded within a proteinaceous matrix to form polyhedra. The virus can be propagated in the larvae of a number of different lepidopteran species, including *Trichoplusia ni.* After ingestion, the virus replication process in insect larvae initiates with the dissolution of polyhedra in the alkaline environment of the midgut. Virus particles, containing variable numbers of nucleocapsids, are released into the midgut lumen. The virus particles traverse the chitinous peritrophic membrane before fusing with columnar epithelial cells. Virus-infected cells produce nonoccluded virus which buds from the plasma membrane and spreads infection throughout the host. While there is still considerable debate as to how the virus is disseminated within the insect host, at the time of death the larva is packed with polyhedra. Shortly afterward, the virus-infected insect host liquefies into an "amorphous puddle" (Volkman and Keddie, 1990) and polyhedra are released, providing fresh inoculum to infect other insects. This process probably plays an important role in ensuring the efficient dissemination of virus by physical forces such as wind and rain splash. A virus-encoded cathepsin has been implicated in the liquefaction process in *Bombyx mori* NPV-infected insect larvae (Ohkawa *et al.,* 1994). A closely related cathepsin gene (*cath*) in AcMNPV, with the same function in virus-infected *T. ni,* has also been identified (Rawlings *et al.,* 1992; Slack *et al.,* 1995). It has also been suggested that the antiapoptotic P35 protein may also aid liquefaction, although this could simply be a consequence of retarded virus replication in the host insect (Clem and Miller, 1993).

We have identified a chitinase gene (*chiA*) within the AcMNPV genome (Ayres *et al.,* 1994) and the production of a functional protein within virus-infected cells (Hawtin *et al.,* 1995). The AcMNPV chitinase protein sequence predicted from the gene is 57% identical to that of the *Serratia marcescens* chitinase A (Hawtin *et al.,* 1995) which indicates that the genes share a common ancestor. Several independent studies suggested that the AcMNPV chitinase might have a role in virus pathogenesis. A strain of *S. marcescens* lacking proteases and chitinases was less virulent than wild-type bacteria in *Drosophila melanogaster* (Flyg and Boman, 1988). *Lymantria dispar* second instar larvae died more rapidly when fed a combination of NPV and an unspecified chitinase, instead of virus alone (Shapiro *et al.,* 1987). These two studies indicate that the presence of chitinase in

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the insect gut might aid the dissolution of the chitinous peritrophic membrane. In a baculovirus infection, this would enable virus particles liberated from polyhedra to reach the midgut epithelial cells more efficiently. Finally, a recombinant AcMNPV containing a *Manduca sexta* chitinase gene required less time to kill *Spodoptera frugiperda* fourth instar larvae when injected into the hemocoel (Gopalakrishnan *et al.,* 1995). This suggests that a virus-encoded chitinase, expressed in the insect host, might have a role in the latter stages of infection.

In this study, a *chiA*-deficient virus was constructed and its infectivity in insect larvae was compared with that of unmodified AcMNPV and a *cath*$^-$ mutant. We examined the role of the AcMNPV chitinase in the early stages of infection by determining if the enzyme was associated with purified polyhedra since its presence in the very late stage of infection (Hawtin *et al.,* 1995) suggested that it might become packaged within these structures. We also monitored the activity of the chitinase under a wide range of pH conditions, including highly alkaline solutions to mimic the pH of the insect larval midgut. Finally, the disease process in the latter stages of infection was examined using insects infected with unmodified, *chiA*-deficient or *cath*-deficient viruses to establish the involvement of the chitinase in the liquefaction of the host.

METHODS

Cells and virus

The AcMNPV C6 clone (Possee, 1986; Possee *et al.,* 1991; Ayres *et al.,* 1994) was propagated in *S. frugiperda* cells (IPLB-SF21; Vaughn *et al.,* 1977). Recombinant viruses containing the *lac*Z coding region under the control of baculovirus gene promoters were amplified in a similar manner except that X-gal was added to plaque titrations to identify β-galactosidase production (Possee and Howard, 1987). The virus mutant lacking a functional cysteine protease gene (AcDVC1 or *cath*$^-$) has been described previously (Slack *et al.,* 1995).

Production of virus deletion mutants

AcchiA$^-$*/cath*$^-$*.lacZ.* The AcMNPV *Pst*I M fragment (105,164–107,943 nucleotides), containing the complete chitinase gene was inserted into pUC119 digested with *Pst*I to derive pUC119-M. Site-directed mutagenesis (Kunkel, 1985) was employed to introduce a unique *Age*I restriction site 300 bp downstream of the *chiA* translation initiation codon, producing pAge. The oligonucleotide used for this purpose was 5′ GTGAGCGGGCAATTTAACCGGTGTGTCAAACTTTGCAATGAGGACGGC 3′ (the *Age*I site is underlined). The mutagenized sequence was disrupted by the insertion of a 3.5-kb DNA fragment containing the polyhedrin gene promoter linked with the *Escherichia coli lac*Z coding region and SV40 transcription termination signals. This fragment was isolated from pAcRP23+8.*lac*Z (Possee and Howard, 1987) by digestion with *Bam*HI and *Bgl*II, annealed with *Xma*I linker adaptors (5′ GATCCCCGGGCGCG 3′, *Xma*I site underlined; complementary strand, 5′ CGCGCCCGGG 3′) in the presence of T4 DNA ligase and then digested with *Xma*I. This provided the *lac*Z cassette with ends which were compatible with *Age*I. It was ligated with pAge digested with *Age*I to derive pAge-*lac.* Subsequent sequencing of this plasmid revealed that there was a deletion of 638 nucleotides between positions 106,630 and 107,268, which spanned the *chiA* and *cath* promoters and the amino terminal coding region of each gene, and hence this plasmid contained incomplete copies of both *chiA* and *cath;* the same deletion was found in pAge. To produce a recombinant virus, *S. frugiperda* cells were cotransfected with pAge-*lac* and infectious AcMNPV DNA (King and Possee, 1992) using the lipofectin method. Subsequently, A*cchiA*$^-$*/cath*$^-$*.lacZ* was identified by incubation of plaque titrations of the progeny from the cotransfection with X-gal. Blue plaques were isolated and retitrated six times to homogeneity.

AcchiA$^-$. A plasmid with a deletion only in the AcMNPV *chiA* was produced by repeating the site-directed mutagenesis as described above to insert an *Age*I site 300 bp downstream of the translation start codon, while retaining intact *cath* to derive pAge-2. This was digested with *Age*I and *Sac*II to remove 1005 bp from the chitinase coding region. Synthetic linkers (*Age*I–*Sac*II; 5′ CCGGGGATCCTAGC 3′, *Bam*HI site underlined; complementary strand, 5′ TAGGATCC 3′) were ligated with the digested plasmid to provide a unique *Bam*HI restriction site to aid correct identification of the modified vector, which was designated pAge-*Bam.* This plasmid was mixed with linear (*Bsu*36 I-digested) A*cchiA*$^-$*/cath*$^-$*.lacZ* DNA and used to cotransfect insect cells. The progeny virus from the cotransfection was titrated in a plaque assay in the presence of X-gal to identify colorless plaques (A*cchiA*$^-$) consistent with the loss of the *lac*Z coding region (Kitts and Possee, 1993).

Virus infection of *T. ni* larvae and purification of polyhedra

Cultures of *S. frugiperda* cells inoculated with recombinant viruses (0.1 PFU/cell) were harvested 4 days postinfection and fed to fourth instar *T. ni* larvae, which were incubated at 24° until just prior to death. Polyhedra were purified as described by King and Possee (1992) to provide a primary *in vivo* derived virus stock. This was used to produce secondary virus stocks for further experiments with insects.

Chitinase and cathepsin assays

Samples from virus-infected cells or polyhedra (5×10^8) were assayed for chitinase activity using the microtiter plate method of McCreath and Gooday (1992) as de-

scribed previously (Hawtin *et al.,* 1995). The method is based on the detection of fluorescence released by one of four fluorogenic substrates which are 4-methylumbelliferyl glycosides of *N*-acetylglucosamine oligosaccharides (4MU-$(GlcNAc)_{1-4}$), referred to as substrates 1–4. The fluorescent aglycone is released in the presence of the following enzyme activities: *N*-acetylglucosaminidase (substrate 1, 4MU-GlcNAc), exochitinase (substrate 2, 4MU-$(GlcNAc)_2$), and endochitinase (substrates 3, 4MU-$(GlcNAc)_3$; and 4, 4MU-$(GlcNAc)_4$) (cf. Robbins *et al.,* 1988). A positive control for chitinase activity comprised a lyophilized powder obtained from Sigma (Product No. C 7809) which was reconstituted according to the manufacturer's instructions. For the cathepsin assays, approximately 3×10^7 *S. frugiperda* cells were mock infected or inoculated with AcMNPV, *cath*$^-$, Ac*chiA*$^-$, Ac*chiA*$^-$/*cath*$^-$.*lacZ* (10 PFU/cell) or both Ac*chiA*$^-$ and *cath*$^-$ (5 PFU/cell for each virus). After 1 h at ambient temperature to permit virus adsorption, the residual inoculum was removed and replaced with TC100/10% FCS and the cells were incubated until 40 h p.i. The cells were harvested and processed to monitor cathepsin activity precisely as described by Ohkawa *et al.* (1994).

SDS–PAGE and Western blot analysis

Chitinase synthesis in virus- and mock-infected cells using SDS–PAGE and Western blot analysis was assessed as described previously (Hawtin *et al.,* 1995). Samples from virus- and mock-infected insect larvae were prepared by maceration of whole insects in 500 μl of dissociation mix and boiling prior to analysis of 40 μl using SDS–PAGE.

Bioassays and insect liquefaction studies

Second instar *T. ni* larvae derived from eggs hatched on the same day were fed individually with a diet plug containing one of five concentrations of virus or PBS. Doubling doses of 7.5 to 240 polyhedra/larva were used, with at least 40 larvae for each dose. After 24 h, those insects which had consumed the diet plugs were transferred to fresh diet and incubated at 24°. Insects were monitored twice daily thereafter. Dead larvae were harvested and examined using Giemsa staining to identify polyhedra. For AcMNPV-infected larvae, death was associated with the onset of liquefaction. Insects infected with viruses lacking *chiA* or *cath* did not produce this phenotype, so death was judged to have occurred when the larvae failed to respond to mechanical stimulation with a blunt glass rod. The number of deaths caused by each concentration of virus were recorded and lethal dose $(LD)_{50}$ values, with associated confidence limits, were determined by probit analysis (Finney, 1971). The survival $time_{50}$ (ST_{50}) values for neonate *T. ni* infected with AcMNPV or Ac*chiA*$^-$ were determined as described by Stewart *et al.* (1991). To assess liquefaction, fourth instar larvae were fed 10^5 polyhedra and monitored twice daily.

RESULTS

Deletion of the AcMNPV *chiA*

We examined the role of chitinase in virus infection *in vitro* and *in vivo,* by producing a recombinant virus with an insertion in *chiA* of a complete *lac*Z coding region under the control of the polyhedrin gene promoter (Fig. 1). However, in the subsequent analysis of the recombinant virus, we identified a deletion of 638 nucleotides within a region of the virus genome upstream of the insertion site of the *lac*Z gene; 30 nucleotides of unknown origin replaced the authentic sequence. This deletion encompassed the transcription and translation start sites of both *chiA* and *cath* (Rawlings *et al.,* 1992) located 48 bp upstream and in the opposite orientation to *chiA* (Ayres *et al.,* 1994). Our recombinant virus was designated Ac*chiA*$^-$/*cath*$^-$.*lacZ* to denote the double mutation (Fig. 1). We derived a second virus recombinant from Ac*chiA*$^-$/*cath*$^-$.*lacZ* which retained *cath,* but had part of the *chiA* coding region removed to provide a single mutant. This virus was designated Ac*chiA*$^-$ (Fig. 1). Both Ac*chiA*$^-$/*cath*$^-$.*lacZ* and Ac*chiA*$^-$ replicated normally in *S. frugiperda* cells. A third virus, with an insertion of the β-galactosidase coding region, under the control of the AcMNPV *p10* promoter, in *cath* (AcDVC1 or *cath*$^-$; Slack *et al.,* 1995) was used to analyze the effect of disrupting cathepsin production.

Analysis of each recombinant virus DNA using Southern hybridization analysis confirmed the expected modification in the virus genome (data not shown). Assays of virus-induced chitinase in insect cells at 40 h p.i. showed that neither Ac*chiA*$^-$ nor Ac*chiA*$^-$/*cath*$^-$.*lacZ* produced functional chitinase (Fig. 2a). Exo- and endochitinase activities were detected in *cath*$^-$-infected cells and in cells infected with both Ac*chiA*$^-$ and *cath*$^-$. Analysis of virus-infected cells using immunoblotting or immunofluorescence with a chitinase-specific antibody failed to detect chitinase in cells infected with either Ac*chiA*$^-$ or Ac*chiA*$^-$/*cath*$^-$.*lacZ* (data not shown). To confirm that cathepsin was still produced by viruses lacking the chitinase gene, we monitored protease activity in cells 48 h after infection with AcMNPV, Ac*chiA*$^-$, *cath*$^-$, Ac*chiA*$^-$/*cath*$^-$.*lacZ*, or both Ac*chiA*$^-$ and *cath*$^-$. The results in Fig. 2b demonstrated that, as expected, *cath*$^-$- and Ac*chiA*$^-$/*cath*$^-$.*lacZ*-infected cell extracts contained minimal amounts of cathepsin, whereas cells infected with Ac*chiA*$^-$ or Ac*chiA*$^-$ and *cath*$^-$ produced near normal levels of the enzyme. Further enzyme assays (data not shown) demonstrated that the cathepsin activity in each sample was susceptible to E-64, a specific inhibitor of cysteine proteases.

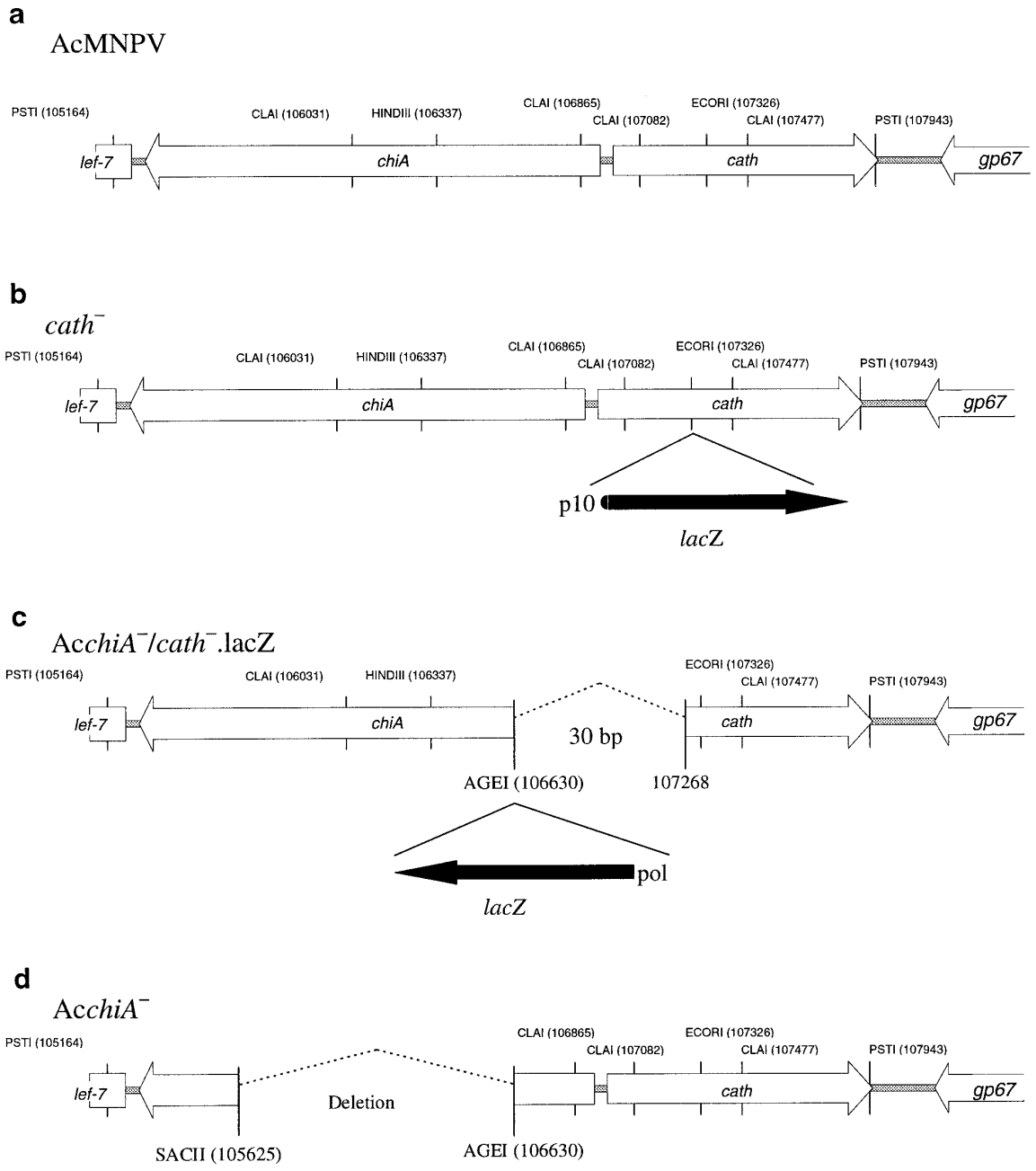

FIG. 1. Genomic organization of parental and recombinant baculoviruses with modified *chiA* and *cath.* (a) AcMNPV. Relative positions of the *chiA* and *cath,* flanked by part of *lef-7* and *gp67.* (b) *cath*⁻. The thick black arrow indicates the insertion of the *E. coli lac*Z coding region, under the control of the *p10* promoter, at the *Eco*RI site within *cath* (Slack *et al.,* 1995). (c) Ac*chiA*⁻*/cath*⁻*.lacZ.* The extent of the deletion in this virus is indicated by broken lines as well as the extra 30 bp of unknown origin. (d) Ac*chiA*⁻. This was derived by cotransfection of insect cells with linearized Ac*chiA*⁻*/cath*⁻*.lacZ* DNA and pAge-*Bam;* progeny virus was screened for plaques lacking β-galactosidase activity. Selected restriction enzyme sites are shown with appropriate genomic coordinates (Ayres *et al.,* 1994).

Chitinase activity in AcMNPV polyhedra

Polyhedra were purified from AcMNPV-infected *T. ni* larvae and assayed for chitinase activity (Fig. 3). Exochitinase activity, detected with substrate 2, was higher than endochitinase activity detected with substrates 3 and 4. In contrast with earlier results using virus-infected cell extracts (Fig. 1; Hawtin *et al.,* 1995) we also detected some *N*-actetylglucosaminidase activity in polyhedra (Fig. 3; substrate 1). We also showed that polyhedra purified from Ac*chiA*⁻-infected insects contained very low levels of chitinase activity (Fig. 3). Some *N*-acetylglucosaminidase activity remained associated with the occlusion bodies, suggesting that this enzyme derives from the host insect.

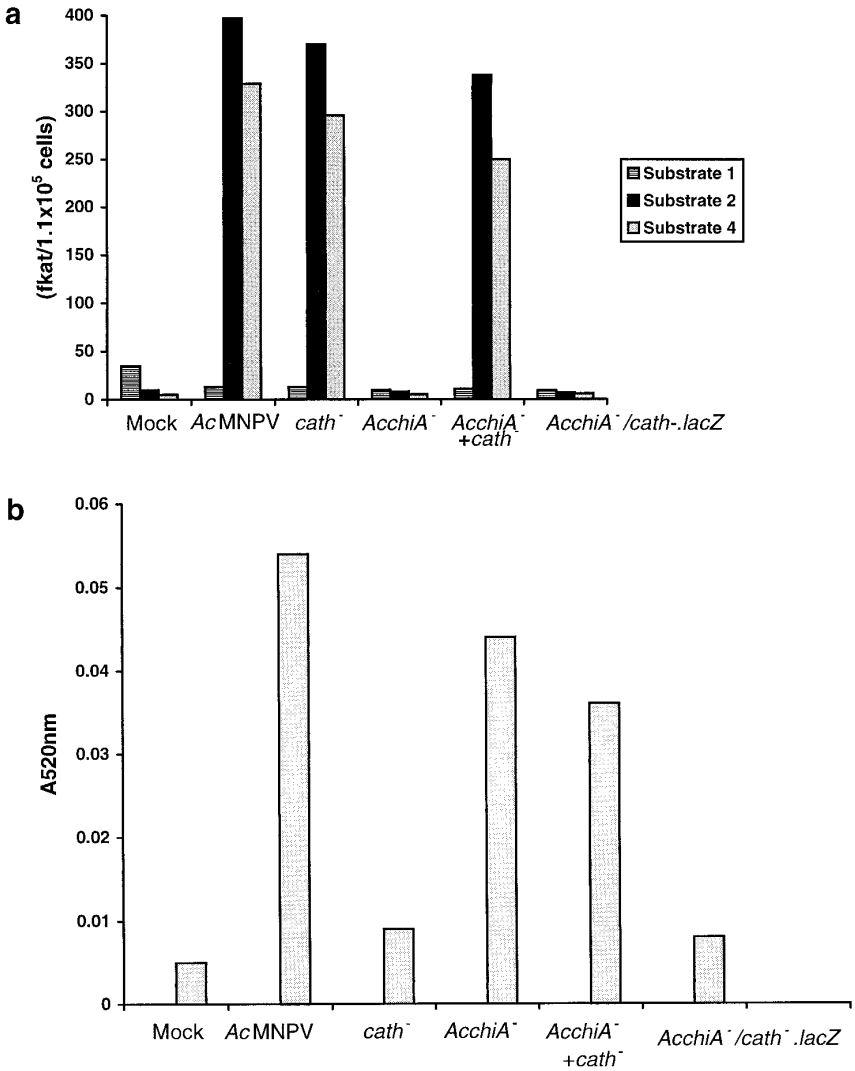

FIG. 2. Assay for chitinase and cathepsin activity in virus-infected cells. (a) Extracts were prepared from mock-infected *S. frugiperda* cells or cultures inoculated with AcMNPV, *cath*$^-$, Ac*chiA*$^-$, Ac*chiA*$^-$*/cath*$^-$*.lacZ* (10 PFU/cell), or Ac*chiA*$^-$ and *cath*$^-$ (5 PFU/cell for each virus) at 48 h p.i. and assayed for *N*-acetylglucosaminidase with the substrate 4MU-GlcNAc (substrate 1), exochitinase with the substrate 4MU-$(GlcNAc)_2$ (substrate 2), and endochitinase with the substrate 4MU-$(GlcNAc)_3$ (substrate 3) using the microtiter plate assay. Activities are expressed in femtokatals (fkat) per 1.1×10^5 cells. (b) Lysates were prepared at 40 h p.i. from cells infected with the same viruses and assayed for cathepsin activity. A katal represents the hydrolysis of a mole of substrate per second.

pH sensitivity of the AcMNPV chitinase

The chitinase induced in AcMNPV-infected cells was active over a much wider pH range than the mixture of *S. marcescens* chitinases (Figs. 4a and 4b). Exochitinase (Fig. 4b; substrate 2) and endochitinase activity (Fig. 4b; substrates 3 and 4) in *S. marcescens* samples decreased below pH 5.0. In contrast, virus exo- and endochitinase activities only decreased below pH 4.0 (Fig. 4a; substrates 2, 3, and 4). The virus chitinase was also more active under alkaline conditions. At pH 9.0, the level of virus chitinase activity was near maximal (Fig. 4a), whereas all chitinase activities of *S. marcescens* were progressively inhibited above pH 6.5, and at pH 9.0 were almost abolished (Fig. 4b). Exochitinase activity (Fig. 4a; substrate 2) in AcMNPV-infected cell samples was markedly diminished under conditions exceeding pH 9.5. The AcMNPV endochitinase activities decreased slightly above pH 9.5, but retained approximately 60% of maximum activity at pH 11.5. Uninfected cell extracts lacked significant chitinase activity at all of the pH values for each of the three substrates; only the results for substrate 3 are shown in Fig. 4a.

In vivo infectivity studies

We used the cell culture-derived polyhedra to obtain virus stocks of AcMNPV, Ac*chiA*$^-$, *cath*$^-$, and Ac*chiA*$^-$*/cath*$^-$*.lacZ* propagated in *T. ni* larvae. Similar yields of polyhedra were obtained from each group of virus-infected insects, suggesting that virus replication was not compromised. The infectivities of the viruses obtained *in vivo* were

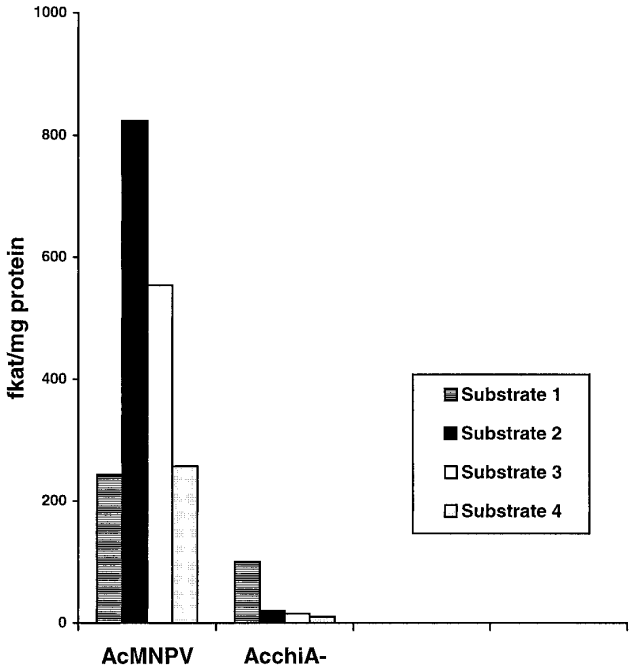

FIG. 3. Chitinase activity associated with polyhedra. Polyhedra freshly derived from AcMNPV- or Ac*chiA*$^-$-infected fourth instar *T. ni* larvae were assessed for *N*-acetylglucosaminidase, with substrate 1, exochitinase with substrate 2, and endochitinase with the substrate 4MU-$(GlcNAc)_3$ (substrate 3) and substrate 4 using the microtiter plate assay. Activities are expressed in femtokatals (fkat) per mg protein; 5 $\times$ 10^8 polyhedra were used for each assay.

compared by performing bioassays using second instar *T. ni* larvae (Table 1). The LD_{50} for Ac*chiA*$^-$ (40 polyhedra per larva) was not significantly higher than those for AcMNPV (29 polyhedra per larvae) or *cath*$^-$ (31 polyhedra per larvae). Further, the 95% confidence limits for each result overlap. We noted, however, that none of the insects infected with Ac*chiA*$^-$ or *cath*$^-$ liquefied after death. There was no significant increase in the time required for the virus to kill the host. The ST_{50} value was 114 h for AcMNPV and 116 h for Ac*chiA*$^-$ in neonate *T. ni.*

Liquefaction of virus-infected insect larvae

The process of liquefaction in virus-infected insects was investigated more thoroughly. Groups of fourth instar *T. ni* larvae were infected with AcMNPV, Ac*chiA*$^-$, *cath*$^-$, or Ac*chiA*$^-$/*cath*$^-$.*lacZ* (10^5 polyhedra/larva). Insect larvae infected with AcMNPV became pale and creamy in color prior to death, whereafter they blackened and rapidly liquefied. Insect larvae infected with Ac*chiA*$^-$, *cath*$^-$, or Ac*chiA*$^-$/*cath*$^-$.*lacZ* also became pale and creamy before death but blackened very slowly and did not liquefy after death. Representative insects are shown in Fig. 5a. Establishing the precise time of death of these virus-infected larvae was difficult because they remained intact. Routinely, we concluded that larvae were dead if they did not respond to mechanical stimulation. The integrity of the larval integument was maintained until at least 5 days after death as evidenced by the ability to pick up the larvae with a pair of forceps. In contrast, insects infected with AcMNPV could not be lifted with these instruments 1 day after death because of the extensive liquefaction.

Chitinase production in virus-infected insect larvae

We then determined if chitinase production in virus-infected insects could be monitored using the chitinase-specific antibody described previously (Hawtin *et al.,* 1995). Insects fed unmodified or recombinant viruses were harvested at daily intervals after infection and equal volumes from whole larval extracts fractionated in polyacrylamide gels. The Coomassie blue-stained gel in Fig. 6a shows some variation in the recovery of protein from individual insects, but illustrates very clearly the preponderance of polyhedrin protein in virus-infected larvae at 7 days p.i. It was also noted that AcMNPV- and *cath*$^-$-infected insects contained a protein of about 58 kDa, consistent with the production of chitinase (Fig. 6a; lanes 2 and 4). This protein was absent in insects infected with Ac*chiA*$^-$ (Fig. 6a, lane 3). Insects infected with both Ac*chiA*$^-$ and *cath*$^-$ also produced the putative chitinase protein (Fig. 6a, lane 5). The identity of the chitinase was confirmed by an immunoblot analysis (Fig. 6b), which detected a protein of about 58 kDa in AcMNPV- (Fig. 6b, lane 2), *cath*$^-$- (Fig. 6b, lane 4) infected insects and larvae infected with both *cath*$^-$ and Ac*chiA*$^-$ (Fig. 6b, lane 5). Chitinase was first detected in extracts from virus-infected insects at 5 days postinfection (data not shown). It was also noted that insects infected with both of the latter viruses liquefied after death (Fig. 5b). This served to confirm that the mutations in each of the virus recombinants could be complemented by the authentic gene in the other virus.

DISCUSSION

We have discovered that the AcMNPV-encoded chitinase plays an essential role in the liquefaction of virus-infected insect larvae. The association of chitinase with polyhedra and the fact that it remained active under very alkaline conditions suggested that it would also be important in the early stages of virus infection in the insect midgut. After ingestion, polyhedra dissolve in the alkaline midgut and the virus particles which are released must traverse the chitinous peritophic membrane before infecting the midgut epithelium. Deleting *chiA* from AcMNPV, however, had no significant effect on the LD_{50} or the ST_{50} of the virus recombinant. It would be interesting to isolate insect peritrophic membranes and observe how they interact with purified chitinase. Derksen and Granados (1988) reported that the chitinous peritrophic membrane of *T. ni* larvae was severely disrupted after feeding AcMNPV polyhedra (10^6 per larva). It is tempting to speculate that the chitinase retained within the occlu-

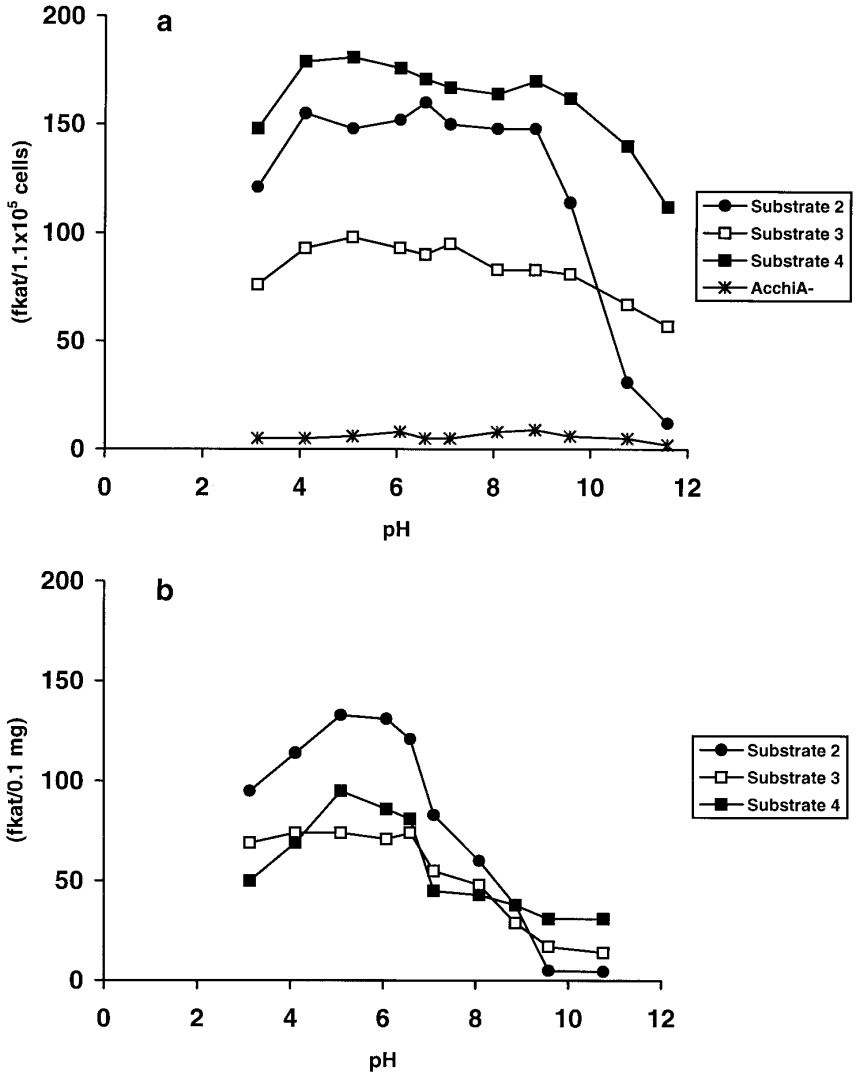

FIG. 4. Effect of pH on chitinases of AcMNPV and *S. marcescens.* Monolayers of *S. frugiperda* cells were inoculated with AcMNPV (10 PFU/cell) or mock infected, harvested at 36 h p.i., and prepared for chitinase assays. A *S. marcescens* mixed chitinase was formulated from a commercial preparation (see Methods). Virus-infected cell (a) and bacterial samples (b) were assessed for exochitinase (●) and endochitinase (□, ■). Mock-infected cells extracts were assayed for endochitinase activity with substrate 3 (*). Activities are expressed in (a) fkat/1.1 $\times$ 10^5 cells or (b) fkat/0.1mg.

sion bodies is reponsible for this effect. This could be readily tested by feeding insects with one of the chitinase-deficient mutants constructed in this study.

The AcMNPV chitinase is active under very alkaline conditions. We compared the activities of the AcMNPV-encoded chitinase with a mixture of chitinases from *S. marcescens* under a range of pH conditions. The pH optimum for the majority of microbial chitinases is between pH 4.0 and 5.0 (Jeuniaux, 1966). The chitinase activities produced by AcMNPV, although being at or near maximal over this pH range, did not alter dramatically between pH 4.0 and 9.0, contrasting with the *S. marcescens* chitinase sample which was analyzed in parallel. The bacterial preparation used comprised a mixture of enzymes, but chitinase A activity predominated (Fuchs *et al.,* 1986) and so direct comparison with the viral chitinase is reasonable. It was of particular note that the AcMNPV chitinase retained high activity at pH 10.0 and above. The discrepancy between the pH tolerances of the bacterial and virus chitinase activities is surprising given the sequence identity between the two enzymes (57%). Presumably, the regions of amino acid sequence which differ between the two proteins influ-

TABLE 1

LD_{50} (Polyhedra per Larvae) for AcMNPV, Ac*chiA*$^-$, and *cath*$^-$ in Second Instar *T. ni* larvae

Virus	LD_{50}	95% confidence limits	Slope
AcMNPV	29	25–36	2.1
Ac*chiA*$^-$	40	33–48	2.2
cath$^-$	31	26–37	2.5

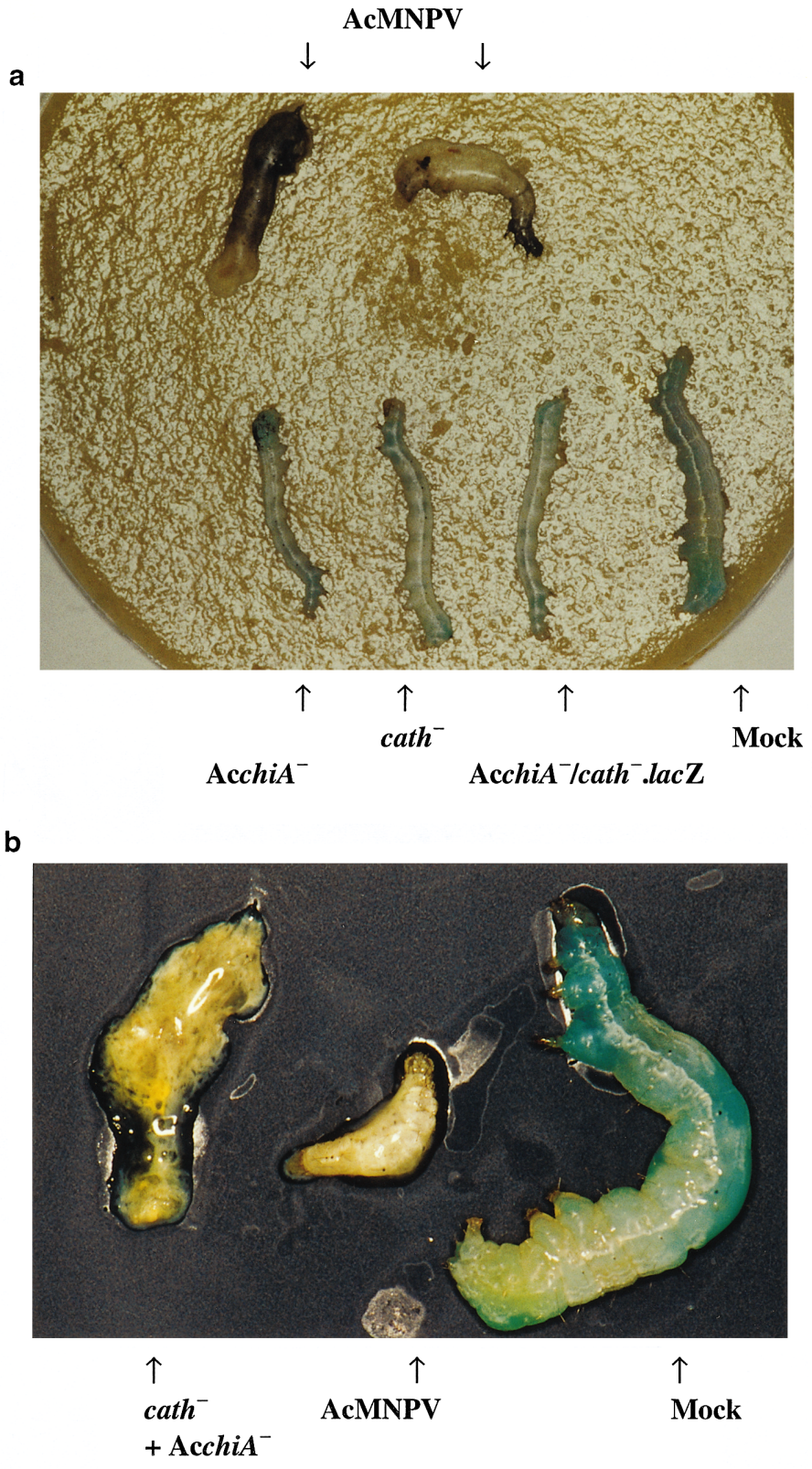

FIG. 5. Liquefaction of virus-infected larvae. (a) Individual fourth instar *T. ni* larvae were fed AcMNPV, Ac*chiA*$^{-}$, *cath*$^{-}$, or Ac*chiA*$^{-}$/*cath*$^{-}$.*lac*Z (10^5 polyhedra per larva) or mock infected (mock). Larvae were reared at 24° and monitored daily. A representative larva from each treatment was photographed at 8 days postinfection. (b) Insect larvae infected with *cath*$^{-}$ and Ac*chiA*$^{-}$, AcMNPV, or mock infected and photographed at 7 days p.i.

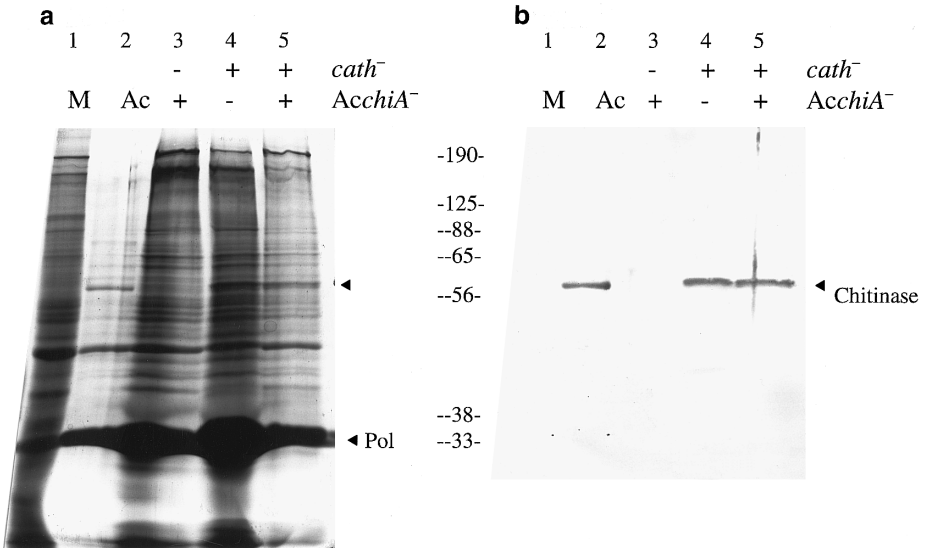

FIG. 6. Immunoblot analysis of chitinase in extracts from virus-infected larvae. Fourth instar *T. ni* larvae were fed AcMNPV (Ac), Ac*chiA*$^-$, or *cath*$^-$ as indicated (total virus dose 10^5 polyhedra per larva) or mock-infected (M). After 7 days at 24°, extracts from individual larva were fractionated in duplicate 12% polyacrylamide gels. (a) Coomassie blue-stained gel. (b) Immunoblot analysis using anti-chitinase antiserum (1/10,000) for primary detection and alkaline phosphatase-conjugated goat anti-guinea pig IgG polyclonal antiserum (1/1000) for the secondary reaction. The positions of the chitinase and polyhedrin (Pol) proteins are indicated. Molecular size markers (M; kDa) are shown.

ence their ability to function at high pH. If the AcMNPV *chiA* did originate from *S. marcescens,* natural selection may have favored an enzyme which was more active in the insect midgut since there is a pH gradient of 7.8 to 9.5 from the midgut epithelium to the midgut lumen in some lepidopterous larvae (Santos and Terra, 1986).

The LD_{50} values obtained with the *cath*$^-$ virus also suggested that cathepsin has no role in the ability of the virus to infect insect larvae. We do not know if cathepsin is packaged by virus polyhedra, but cathepsins are lysosomal enzymes which are inhibited at pH values approaching or exceeding 7 (Barrett and Kirschke, 1981; Bond and Butler, 1987). Assuming that the AcMNPV cathepsin retains these characteristics, it would not be expected to function in the extracellular, alkaline environment of the midgut lumen.

The lack of liquefaction seen in insect larvae infected with Ac*chiA*$^-$, *cath*$^-$, or Ac*chiA*$^-$/*cath*$^-$.*lacZ* was the most significant observation in this study. We also noted that liquefaction was restored when insects were coinfected with Ac*chiA*$^-$ and *cath*$^-$. This latter experiment answered the criticism that alteration of either *chiA* or *cath,* which are located only 45 bp apart in the virus genome, might affect expression of the unmodified gene. Other experiments conducted *in vitro* confirmed production of chitinase and cathepsin in cells infected with *cath*$^-$ and Ac*chiA*$^-$, respectively. These data suggested that chitinase and cathepsin were associated with the liquefaction of virus-infected larvae. The insect cuticle must be degraded in this process.

The insect cuticle is formed primarily of chitin cross-linked with sclerotized proteins. The overall structure varies considerably between different species and throughout larval development. The cuticle serves to protect the insect from infection by pathogens, physical damage, and desiccation. It also provides an anchor for the skeletal muscles. Based upon histological, structural, and mechanical data, the lepidopteran larval cuticle is frequently ascribed to a group of cuticular structures termed "arthroidal membranes and caterpillar-like cuticles." Such cuticles are soft and flexible with little crystalline order. The chitin loosely interacts with the protein component to produce a structure with little stiffness and great distensability, but which is reasonably strong (Kramer *et al.,* 1985).

Chitinases are directly implicated in the degradation of the insect cuticle at molting. During this process the epidermis detaches and retracts from the cuticle, molting gel being secreted into the intervening space. The epidermis secretes a new cuticle, which thickens and sclerotizes while the molting gel decreases in viscosity and becomes molting fluid. The molting fluid partially digests the old cuticle which is then resorbed into the epidermal cells (Kramer *et al.,* 1985). Chitinases have been detected in the molting fluid of the silkworm, *Bombyx mori* (Kimura, 1976), and the tobacco hormworm, *Manduca sexta* (Bade, 1974, 1975; Bade and Stinson, 1978a,b; Koga *et al.,* 1983).

The requirement for the "unmasking" by proteases of cuticular chitin, before chitinase may act, is an area of unresolved dispute (Lipke and Goeghan, 1971; Bade and Stinson, 1978a,b; St Leger *et al.,* 1986a,b,c,d). Proteases are present in the molting fluid produced by insects, acting synergistically with chitinases in cuticle degradation

(Passonneau and Williams, 1953; Jeuniaux and Amanieu, 1955; Katzenellen-Bogen and Kafatos, 1971; Bade and Shoumikas, 1974). Bade and Stinson (1978a,b) found that digestion of *M. sexta* cuticle by molting fluid was inhibited by antitrypsin agents. The appearance of protease in molting fluid prior to the appearance of chitinase was also reported. These observations led the authors to propose that, following penetration of the endocuticle by molting fluid, proteases unmask the cuticular chitin, allowing chitinase access to its substrate. Inhibition of the protease prevents the chitinase from reaching the chitin, which is masked by protein.

The absence of liquefaction in insects infected with *cath*$^-$ (Slack *et al.,* 1995) and Ac*chiA*$^-$/*cath*$^-$.*lacZ* suggests that cathepsin is also involved in insect "melting," possibly in synergy with chitinase. The cathepsin may strip the protein from chitin in the insect cuticle, enabling the chitinase to break down the naked chitin. An absence of liquefaction in *cath*$^-$-infected larvae may represent the inability of chitinase to reach the cuticular chitin in the absence of proteinase. The integrity of Ac*chiA*$^-$-infected larvae, despite the likely action of cathepsin at the cuticle, may reflect the role of chitin in retention of the insect cuticle, as described by Samsinakova *et al.* (1971).

There is a clear advantage to the virus in being able to escape efficiently from the cadaver. Virus polyhedra which remain trapped within the host are less likely to encounter a susceptible individual than virus released via liquefaction. While the discovery of two gene products with a role in the liquefaction of virus-infected insects is very interesting, we do not discount the possibility that other virus-encoded proteins might also be involved in this process. It has already been reported in the literature that AcMNPV mutants lacking *p35* do not liquefy their host (Clem and Miller, 1993), although this could be a consequence of retarded virus growth, rather than a direct result of the mutation. Clearly much remains to be done to understand this interesting aspect of the baculovirus replication process.

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