Mutagenesis of the active site coding region of the Autographa californica nucleopolyhedrovirus chiA gene

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The chitinase of Autographa californica nucleopolyhedrovirus (AcMNPV) is required for the characteristic liquefaction of baculovirus-infected insect larvae. Alignments of the putative active sites of a range of chitinases revealed two highly conserved residues, glutamate and aspartate, which have been proposed to constitute the catalytic residues of the active site. These residues were mutated in the AcMNPV chitinase. Three recombinant viruses were generated, AcchiA D311G, AcchiA E315G and AcchiA D311G E315G, which contained mutations at either the glutamate, the aspartate or both. It was demonstrated that chitinase protein production was unaffected by the mutation of these residues. However, mutation of both residues resulted in the attenuation of chitinolytic activity and the cessation of liquefaction of Trichoplusia ni larvae infected with AcchiA D311G E315G. Mutagenesis of the glutamate residue led to a reduction in exochitinase activity and a delay in the appearance of endochitinase activity. In addition, larvae infected with this virus, AcchiA E315G, liquefied more slowly than those larvae infected with wild-type AcMNPV. Mutagenesis of the aspartate residue resulted in a reduction of exochitinase activity but an unexpected enhancement of endochitinolytic activity. Liquefaction of AcchiA D311G-infected larvae was observed at the same time as that of AcMNPV-infected larvae.

Introduction

The chitinase (chiA) and cathepsin (cath) genes of Autographa californica nucleopolyhedrovirus (AcMNPV) are necessary for the liquefaction of virus-infected larva (Slack et al., 1995; Hawtin et al., 1997). These enzymes probably serve to degrade the larval cuticle, which contains both chitin and protein, thereby promoting horizontal transmission of the virus. The AcMNPV-induced chitinase possesses both exo- and endochitinolytic activities (Hawtin et al., 1995). However, the disruption of AcMNPV chiA leads to the elimination of all chitinase activity (Hawtin et al., 1995), indicating that both exo- and endochitinase functions reside within one enzyme. This conclusion is supported by the apparent absence of other chitinase genes in the AcMNPV genome (Ayers et al., 1994). This is in contrast with many chitinase-producing organisms that synthesize a range of enzymes with separate exo- or endochitinase functions (Fuchs et al., 1986; Payne et al., 1990; Watanabe et al., 1990, 1992; Sticher et al., 1992; St Leger et al., 1993; Gooday, 1994).

Chitinase genes have been identified in many other baculoviruses (B. Arif & H. Peng, unpublished GenBank accession no. U72030; T. Wu & D. Tribe, unpublished GenBank accession no. U67265; Ahrens et al., 1997; Kang et al., 1998; Gomi et al., 1999; Kuzio et al., 1999). Comparisons between the available sequence data show a remarkable similarity between the baculovirus chiA genes. An alignment of the predicted amino acid sequences of the AcMNPV chitinase with chitinases from a range of bacteria, yeast and nematodes revealed high similarity (60-5%) with the chiA gene product from the bacterium Serratia marcescens (Hawtin et al., 1995). The most extensive similarity was observed in the region encompassing the putative active site. This region includes an invariant aspartate separated from an invariant glutamate by three residues. These amino acids are thought to contribute to the active site of family 18 chitinases (Henrikkat, 1990; Kuranda & Robbins, 1991; Watanabe et al., 1992). The crystal structure of the S. marcescens chiA gene product has been determined at 2·3 Å resolution (Perrakis et al., 1994). The structure of the chiA
gene product in a complex with the substrate \( N,N',N''\)-tetraacetylchitotetraose has suggested that Glu-315 might be the catalytic residue of the active site. Site-directed mutagenesis of the glutamate (Glu-204) and aspartate (Asp-200) residues in this product of the chiA gene of Bacillus circulans suggested that these residues are essential for chitinase activity (Watanabe et al., 1993). In addition, the natural substrates of both chitinase and lysozyme are structurally similar. Site-directed mutagenesis of the Asp-35 and Glu-52 codons of chick egg-white lysozyme revealed that mutation of Glu-52 resulted in almost total cessation of activity, while mutation of Asp-35 significantly decreased activity of the enzyme (Malcolm et al., 1989).

Given the above evidence, it was proposed that Asp-311 and Glu-315 of the AcMNPV chiA gene product might be critical for enzyme activity. However, conclusive proof that these residues constitute the active site of the AcMNPV chitinase cannot be derived from analysis of sequence data. Therefore, the effects of these residues on the activity of the enzyme were investigated by site-directed mutagenesis in a manner similar to that employed by Malcolm et al. (1989) and Watanabe et al. (1993). We also monitored the effect of making these changes on the ability of the chitinase to cause liquefaction in AcMNPV-infected larvae.

**Methods**

**Cells and viruses.** The AcMNPV C6 clone (Possee, 1986) was propagated in Spodoptera frugiperda (Sf) cells (IPLB-SF21; Vaughn et al., 1977). Recombinant viruses were amplified in a similar manner, except that X-Gal was added to the plaque assays for those viruses containing the lacZ coding region.

**Plasmid transfer vectors**

**pchiA-lacZ.** An internal portion of the chitinase coding sequence of 828 bp, spanning the putative active site, was obtained by digestion of the plasmid pUC119-PrlM (Hawtin et al., 1995) with BamHI and HindIII to release an 828 bp fragment. This was ligated into pUC18, previously digested with BamHI and HindIII, to yield the plasmid pUC18Chit. A ClaI restriction endonuclease site present in the central region of the putative active site was converted to a BgIII site by the insertion of a linker (CGAGATCT). This plasmid was subsequently linearized by digestion with BgIII and a lacZ cassette was inserted. The 3.4 kb lacZ cassette comprised the Escherichia coli lacZ coding sequence, under the control of the polyhedrin promoter, and the SV40 terminator sequence. It was obtained by restriction of pAcRP23lacZ (Possee & Howard, 1987) with BgIII and BamHI. Ligation of the lacZ cassette into BgIII-digested pUC118Chit produced pchiA-lacZ (Fig. 1).

**pchiA\(^{D311G, E315G}\).** PCR primers (5' TCCCAATCGATGCTCTACAC 3' and 5' CCGGAAGCTTGAATTCCGTG 3') amplified a product of 360 bp that contained a HindIII site at the 5' end and a ClaI site at the 3' end (underlined) to facilitate ligation of the fragment into pBSK. Purified virus DNA was used as template DNA. A mutation was introduced via PCR to change an aspartate residue to a glycine (GAC to GGA; shown in bold). Sequencing confirmed that the correct nucleotide had been altered and that no further nucleotides had been mutated. This 360 bp fragment was then ligated into ClaI, HindIII-restricted pUC18Chit to produce pchiA\(^{D311G, E315G}\).

**pchiA\(^{E315G}\).** Oligonucleotide primers (5' TGGAGACATCGATGCTCTACACGATTTCCG 3' and 5' GTCGACCGCGCGCGTACATG 3') amplified a fragment of 402 bp from pUC119-PslM, which included a mismatch of glutamate to glycine (GAG to GGA; shown in bold). The primers were designed to include Clal and SacII restriction sites (underlined) at the 5' and 3' ends of the fragment, respectively. This was ligated into pBSK\(^{+}\), previously digested with Clal and SacII. The PCR product was subsequently sequenced to confirm that only the correct mismatched nucleotide had been incorporated. This fragment was then cloned into pUC118Chit that had been double-digested with Clal and SacII, to produce pchiA\(^{E315G}\) (Fig. 1).

**pchiA\(^{D311G, E315G}\), pchiA\(^{E315G}\).** This plasmid was digested with Clal and HindIII and the 360 bp PCR product, containing the mutagenized site for aspartate (see pchiA\(^{D311G, E315G}\) above), was ligated into this plasmid.

**Generation of recombinant baculoviruses AcchiA\(^{D311G, E315G}\).** The plasmid pchiA-lacZ was mixed with AcMNPV DNA and used to co-transfect Sf21 cells (King & Possee, 1992). The progeny virus was titrated by using plaque assays and stained with X-Gal to identify blue plaques representing recombinants where the lacZ cassette should have been inserted within chiA. Working stocks of this virus, AcchiA-lacZ, were derived after further rounds of plaque purification to remove parental virus. Genomic DNA prepared from AcchiA-lacZ was linearized with Bsi361, mixed with pchiA\(^{D311G, E315G}\) or pchiA\(^{D311G, E315G}\) and used to co-transfect Sf21 cells. The progeny virus was titrated by plaque assay; stained with both X-Gal and neutral red and colourless, polyhedron-positive plaques were selected and subsequently purified to homogeneity. A seed stock of each virus was produced and used to derive working stocks of AcchiA\(^{D311G, E315G}\), AcchiA\(^{D311G}\) and AcchiA\(^{E315G}\). The structural integrity of the virus genomes was confirmed by restriction enzyme digestion (data not shown).

**Enzyme assays.** Sf21 cells were infected at an m.o.i. of 10 p.f.u. per cell or mock-infected, and harvested at the appropriate time post-infection (p.i.). Cell pellets were either sonicated or subjected to three rounds of freeze–thawing before assaying. Two separate assays were used to monitor chitinase activity. The first was based on measuring activity with \(^{2}H\)-labelled colloidal chitin as a substrate. The labelled chitin was prepared by the method of Molano et al. (1977) by treating chitosan (molecular mass approximately 750 kDa; Fluka) with \(^{2}H\)lactic anhydride (Amersham) to yield a product with a spec. act. of approximately 1 \(\mu\)Ci/mg. The labelled chitin was stored in 0.2% sodium azide at 4 °C and washed in 50 mM sodium phosphate buffer, pH 6.5, immediately before use. The labelled chitin was resuspended in 50 mM sodium phosphate buffer, pH 6.5, by vortexing prior to the addition of virus-infected cell lysate. After incubation with shaking at 30 °C for 30 min, the reaction was terminated by the addition of 5% trichloroacetic acid. The samples were centrifuged (15,000 g; 3 min) and the supernatants were transferred to vials containing scintillation fluid. Radioactivity released from the labelled substrate was quantified by liquid scintillation spectrometry in a Wallac 1217 Rackbeta counter. Samples were also assayed for chitinase activity using the microtitre plate method of McCreath & Gooday (1992) as described previously (Hawtin & Howard, 1987). This method enables exochitinase, endochitinase or N-acetylglucosaminidase activities to be determined. The substrates used in the assay were 4-methylumbelliferyl glycosides of N-acetylgalactosamine oligosaccharides (4MU-(GlcNAc)\(_n\)) (Sigma). Cell lysates were incubated with the appropriate substrate for 30 min at 37 °C and then the reaction was terminated by the addition of 0.5 M NaOH, and the fluorescence was read immediately. The cysteine protease assay was performed as described by Ohkawa et al. (1994). Approximately 3 × 10\(^7\) Sf21 cells (either mock-infected or virus-infected at an m.o.i. of 10) of each sample were used in this assay.
Mutagenesis of AcMNPV chiA

Fig. 1. Genomic organization of parental and recombinant baculoviruses with modified chiA. (a) Part of the AcMNPV genome containing the chiA and cath genes. (b) AcchiA−lacZ. A ClaI site within the central region of the putative active site of chiA was converted to a BglII site via the insertion of a linker. The thick black arrow indicates the insertion of the lacZ coding region, under the control of the polyhedrin gene promoter, at this BglII site. (c) Schematic drawing of the chiA active site region of AcMNPV and mutant viruses constructed with substitutions at either the aspartate (AcchiA D311G), glutamate (AcchiA E315G) or both residues (AcchiA D311G E315G). Positions of the PCR primers are indicated (open arrows, forward primers; filled arrows, reverse primers).

Results

Production of recombinant baculoviruses

The AcMNPV chiA gene was modified by inserting a copy of the lacZ coding region, under the control of a copy of the polyhedrin gene promoter, at a ClaI site that bisects the active site of the chitinase (AcchiA−lacZ, Fig. 1). Linear genomic DNA from AcchiA−lacZ was then mixed with plasmid transfer vectors containing chiA with modifications to the coding region of the active site (Fig. 1a) and used to co-transfect insect cells to obtain recombinant viruses. The removal of the lacZ coding region from the virus via homologous recombination provided a convenient selection system. Three viruses were constructed, AcchiA D311G (Asp-311 → Gly), AcchiA E315G (Glu-315 → Gly) and AcchiA D311G E315G (Glu-315 → Gly and Asp-311 → Gly), in order to determine whether or not these residues are required for chitinase activity.

Assessment of chitinase protein production by AcchiA−lacZ, AcchiA D311G, AcchiA E315G and AcchiA D311G E315G

Cells infected with AcchiA−lacZ or AcMNPV or mock infected were harvested at 48 h p.i. The samples were analysed by SDS–PAGE followed by Coomassie staining or Western blot analysis. Fig. 2(a) shows a Coomassie blue-stained gel. A 58 kDa chitinase was seen in the wild-type-infected cell sample but was absent in the AcchiA−lacZ- and mock-infected samples. The product of the lacZ gene, β-galactosidase, was visible as a 116 kDa protein in AcchiA−lacZ-infected cell samples. This protein was not detected in wild-type or mock-infected cell
samples. Western blot analysis was carried out by using anti-chitinase antiserum. The blot confirmed that the 58 kDa protein detected in the AcMNPV-infected cell sample was chitinase. This protein was not observed in the other samples. However, an approximately 25 kDa protein that cross-reacted with the anti-chitinase antiserum was identified in cells infected with AcchiA·lacZ (Fig. 2c). This was probably a truncated protein produced by the initial translation of the 5’ region of chitinase, and did not have chitinase activity (data not shown).

The effects of the active site mutations on chitinase protein production were assessed. Sf21 cells were inoculated with AcMNPV, AcchiA<sup>D311G</sup>, AcchiA<sup>E215G</sup> or AcchiA<sup>D311G,E215G</sup> (m.o.i. = 10) and harvested at 24 h p.i. The proteins were subsequently analysed by Western blotting with anti-chitinase antiserum (Fig. 2b). A protein of approximately 58 kDa, which cross-reacted with the anti-chitinase antiserum, was detected in cells infected with AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup> or AcchiA<sup>D311G,E315G</sup> and in wild-type-infected cells, but not in uninfected cell samples (Fig. 2b; compare lanes 1–4 with lane 5). The levels of chitinase protein produced by the mutant viruses were comparable to those resulting from a wild-type AcMNPV infection. Therefore, it appears that protein pro-

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**Fig. 2.** Analysis of chitinase expression in virus-infected cells. Sf21 cells were infected with virus at an m.o.i. of 10 or mock infected. After 48 h, cells were harvested and fractionated on polyacrylamide gels. (a) Coomassie blue-stained gel (lanes 1–3) and immunoblot analysis (lanes 4–6) with chitinase antiserum (1:10000) used for primary detection and alkaline phosphatase-conjugated goat anti-guinea pig IgG polyclonal antiserum (1:10000) used for secondary reaction. Lanes: 1 and 4, mock-infected cells; 2 and 5, AcMNPV-infected cells; 3 and 6, Ac<sup>chiA</sup><sup>−</sup>·lacZ-infected cells. Arrow denotes β-galactosidase in Ac<sup>chiA</sup><sup>−</sup>·lacZ-infected sample; arrowhead indicates protein that cross-reacted with chitinase antiserum, which is suggested to be a truncated protein produced by initial translation of the 5’ region of chitinase in Ac<sup>chiA</sup><sup>−</sup>·lacZ-infected cells. (b)–(c) Coomassie blue-stained gel (b) and immunoblot analysis (c). Lanes: 1, AcMNPV-infected cells; 2, AcchiA<sup>E315G</sup>-infected cells; 3, AcchiA<sup>D311G</sup>-infected cells; 4, AcchiA<sup>D311G,E315G</sup>-infected cells. The position of the chitinase protein is indicated. Positions of molecular mass markers (in kDa) are shown.

**Fig. 3.** Assay for cathepsin activity in virus-infected cells (n = 3). Lysates were prepared at 40 h p.i. from cells infected with viruses (m.o.i. = 10) or mock infected and were assayed for cathepsin activity as described by Okawa et al. (1994). Western blot analysis was carried out by using anti-chitinase antiserum. The blot confirmed that the 58 kDa protein detected in the AcMNPV-infected cell sample was chitinase. This protein was not observed in the other samples. However, an approximately 25 kDa protein that cross-reacted with the anti-chitinase antiserum was identified in cells infected with AcchiA·lacZ (Fig. 2c). This was probably a truncated protein produced by the initial translation of the 5’ region of chitinase, and did not have chitinase activity (data not shown).

**Fig. 4.** Chitinase activity assayed by measuring release of soluble radioactive material from 3H-labelled colloidal chitin over the linear range of the assay. Samples of either mock- or virus-infected Sf21 were assayed at 24 h p.i. Results are expressed as percentages of readings obtained from AcMNPV-infected cells (n = 3).
duction and stability were not affected by the mutations introduced into chiA.

**Cysteine protease activity in virus-infected cells**

The integrity of \( \nu \)-cath in \( \text{Acchi}^{\Delta D11G}, \text{Acchi}^{\Delta E15G} \) and \( \text{Acchi}^{\Delta D11G\Delta E15G} \) was assessed by monitoring cysteine protease activity in virus-infected cells. Cathepsin is required for the liquefaction of baculovirus-infected larvae. It was essential to establish that the gene was functional prior to assessing the effect of the mutations within the active site of chitinase on this process. SF21 cells were infected with the recombinant viruses at 10 p.f.u. per cell. At 40 h p.i., the cells were harvested and assayed for cysteine protease activity (Ohkawa et al., 1994). Fig. 3 shows the results from three independent assays and

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**Fig. 5. Assessment of chitinase activity by fluorogenic assay.** SF21 cells were either infected with AcMNPV, Acchi\( E315G \), Acchi\( D311G \), or Acchi\( D311G\ E315G \) or mock infected (n = 3). Cells were harvested at the times indicated and assayed for \( N \)-acetylglucosaminidase (a), exochitinase (b) or endochitinase (c and d) activities by using the microtitre plate assay of McCreath & Gooday (1992) over the linear range of the assay. Note the differences in scales for different substrates.
Table 1. Liquefaction of virus-infected larvae

*T. ni* larvae (50 per virus) were infected with $10^5$ polyhedral inclusion bodies of virus as designated and were monitored frequently for signs of liquefaction. The table indicates when liquefaction was first observed (+) in each cohort of larvae (three separate cohorts were tested). Liquefaction was not observed in any of the control, non-infected larvae.

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<th>Virus</th>
<th>Days p.i.</th>
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<td>AcMNPV</td>
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<tr>
<td>AcchiA&lt;sup&gt;E315G&lt;/sup&gt;</td>
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<td>AcchiA&lt;sup&gt;D311G&lt;/sup&gt;</td>
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demonstrates that the levels of cathepsin activity in cells infected with AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup> or AcchiA<sup>D311G,E315G</sup> were comparable to the levels detected in wild-type virus-infected cells. Statistical analysis of the data by one-way ANOVA revealed that the differences between the mock-infected and virus-infected cells were significant (P < 0·05). However, the variations observed between the different virus-infected cells were not significant (P > 0·05). This result indicates that cathepsin was produced to normal levels in cells infected with viruses containing point mutations in the chitinase gene.

Assessment of chitinase activity in AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup> and AcchiA<sup>D311G,E315G</sup>

Two assays were employed to assess the effect of mutations in the active site of chitinase on enzyme activity. The first assay used radiolabelled chitin as a substrate and measured the release of soluble radioactive material. Briefly, Sf21 cells were infected at an m.o.i. of 10, harvested at 24 h p.i. and sonicated. Chitinase activity was assayed by measuring the release of soluble radioactive material from $^3$H-labelled colloidal chitin. The data, from three independent assays, are presented as a percentage of the chitinase activity in AcMNPV-infected cells (Fig. 4). In cells infected with AcchiA<sup>E315G</sup>, there was a twofold reduction in enzyme activity when compared with the levels of activity from wild-type-infected cells. Cells infected with AcchiA<sup>D311G</sup> showed an increase in the level of chitinase activity (to approximately 165%). When both putative catalytic residues were mutated, in AcchiA<sup>D311G,E315G</sup>, there was a threefold reduction in the levels of enzyme activity when compared with wild-type.

This assay, however, did not discriminate between exo- and endochitinolytic activity. Therefore, samples were also tested in a fluorescence assay that, by utilization of different substrates, enables exochitinases, endochitinases and N-acetylglucosaminidases to be discriminated. In order to distinguish between exo- and endochitinolytic activity, Sf21 cells were infected with AcMNPV or the three chitinase mutants, harvested at various times p.i. and assayed according to the microtitre method (three independent infections were assayed). The method is based on the detection of fluorescence released by one of four fluorogenic substrates that are 4-methylumbelliferyl glycosides of N-acetylglucosamine oligosaccharides [4MU-(GlcNAc)<sub>1–4</sub>], 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)<sub>3</sub> and endochitinase (substrates 3, 4MU-(GlcNAc)<sub>3</sub> and 4, 4MU-(GlcNAc)<sub>4</sub>). Very low levels of N-acetylglucosaminidase activity that remained constant throughout infection were detected in both uninfected and virus-infected cells (Fig. 5a). The levels of N-acetylglucosaminidase activity detected from cells infected with AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup> or AcchiA<sup>D311G,E315G</sup> were lower than those observed from mock- or AcMNPV-infected cells. However, the overall range of activity detected from substrate 1 was weak (Fig. 5a). Chitinase activity was first detected in AcMNPV-infected cells at 9 h p.i. and reached a maximum at about 18 h p.i. High levels of both exo- and endochitinase activity were detected in AcMNPV-infected cells (Fig. 5b–d).

The mutation of the putative catalytic residues of the active site showed several effects on chitinolytic activities. The alteration of the glutamate residue in AcchiA<sup>E315G</sup> resulted in a decrease in exochitinase activity (Fig. 5b) and a reduction of the level of endochitinase activity detected at earlier time-points by using substrate 3 (Fig. 5c). The mutagenesis of aspartate in AcchiA<sup>D311G</sup> also resulted in a decrease in exochitinase activity (Fig. 5b) but seemed to cause an increase in the amount of endochitinase activity observed with both substrates (Fig. 5c, d). When both the glutamate and aspartate residues were mutated, only very low levels of chitinase activities were detected (Fig. 5b–d).

Effects of AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup> and AcchiA<sup>D311G,E315G</sup> on liquefaction of larvae

Analysis of the LD<sub>50</sub> values obtained for each virus indicated that there was no significant difference between the viruses with mutations in chiA and AcMNPV. This suggested that the alteration of the residues in the chitinase active site had not affected infectivity (data not shown). However, when a susceptible larva succumbs to AcMNPV infection, liquefaction of the host is observed. Chitinase is required for this process. Therefore, the effects of the mutation of residues of the active site on larval liquefaction were examined. Second instar *T. ni* larvae were fed diet plugs that had been soaked with either AcMNPV, AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup> or AcchiA<sup>D311G,E315G</sup> polyhedra ($10^5$) or PBS, and were monitored daily for signs of liquefaction and/or death. The experiment was repeated three times and the earliest time to liquefaction of a cohort of infected larvae was recorded for each virus. The results are
Fig. 6. Photographic record of virus infection of *T. ni* larvae that were being monitored for the onset of liquefaction (see Table 1). Larvae were infected *per os* with 10⁷ polyhedral inclusion bodies of AcMNPV, AcchiₐE₃₁₅G, AcchiₐD₃₁₁G or AcchiₐE₃₁₅G D₃₁₁G or mock-infected with PBS.

Discussion

This paper describes the effects on enzyme activity and biological function of altering the probable active site of the AcMNPV chitinase. Alignments of the putative active sites of a range of chitinases revealed two highly conserved residues, a glutamate and an aspartate (Kuranda & Robbins, 1991; Watanabe *et al.*, 1993; Hawtin *et al.*, 1995), that are proposed to be the catalytic residues of the active site. Mutagenesis of...
these residues in AcMNPV chitinase was carried out to assess the effects on protein production, enzyme activity and the liquefaction of virus-infected insects. Three recombinant viruses were constructed, AcchiA<sup>D311G</sup>, AcchiA<sup>E315G</sup>, and AcchiA<sup>D311G,E315G</sup>, that contained mutations at the glutamate, the aspartate or both. These viruses were employed to evaluate the effects of these mutations on chitinase function. Chitinase protein production was unaffected by the mutation of these residues. Chitinase enzyme activity was assessed by two assays. Both assays are highly sensitive to chitinase activity. However, the second assay allowed the differentiation of N-acetylglucosaminidase, exo- and endochitinase activities.

The results of the chitinase assays suggest that altering the glutamate at position 315 of the protein leads to decreased exo-chitinase activity and also a reduction in the amount of endochitinase activity detected at earlier times p.i. when using substrate 3. Watanabe et al. (1993) demonstrated that mutagenesis of the glutamate of <i>B. circulans</i> chitinase caused a significant reduction in the amount of chitinase activity detected. The alteration of Glu-315 also resulted in a delay in liquefaction of virus-infected larvae when compared with wild-type-infected insects. This suggests that Glu-315 is a major determinant of chitinase activity. Mutagenesis of the aspartate residue, position 311, resulted in a reduction of the exochitinase detected. However, this mutation unexpectedly appeared to enhance endochitinolytic activity. This may be a consequence of the fact that the AcMNVPV chiA gene product possesses both exo- and endochitinase activities that appear to be carried by a single polypeptide. Liquefaction of AcchiA<sup>D311G</sup>-infected larvae was observed at the same time as in the AcMNPV-infected larvae. The mutagenesis of both target residues, glutamate and aspartate, resulted in the attenuation of chitinolytic activity. In agreement with this, the larvae infected with AcchiA<sup>D311G,E315G</sup> failed to liquefy. The results indicate that the glutamate and aspartate residues are determinants of chitinase activity and are variably required for liquefaction of the virus-infected host insect.

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References


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