Virus Genes
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Analysis of a chitinase from EpapGV, a fast killing betabaculovirus

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Abstract The main function of baculoviral chitinase protein (V-CHIA) is to promote the final liquefaction of infected host larvae, facilitating the dispersion of occlusion bodies (OBs) in the environment. In this study, a *v-chiA* from Epinotia aporema Granulovirus (EpapGV) was identified and characterized. The 1,713 base pairs long open reading frame encodes a protein of 570 amino acids with a predicted molecular weight of 63 kDa. EpapGV V-CHIA sequence alignment resulted 62 % identical to Pieris rapae GV and Blastp search revealed a high conservation among all baculovirus chitinases. Amino acid sequence analysis indicated that the C-terminal KDEL present in most alphabaculovirus chitinases is absent in EpapGV V-CHIA, as well as in the rest of the betabaculoviruses. Phylogenetic analysis was performed with bacterial, lepidopteran, and baculoviral chitinase sequences available in databases. Using an AcMNPV bacmid (bAp-GOZA) a recombinant Ac-chiAEpapGV was obtained in order to overexpress EpapGV V-CHIA in cell culture. The presence of chitinase was detected in purified AcMNPV-chiAEpapGV OBs. Peritrophic membranes of *Anticarsia gemmatalis* larvae fed with recombinant OBs showed an altered structure. The results presented in this study show that EpapGV chitinase overexpression in recombinant baculovirus can cause association of this protein with OBs, and suggest that this could be used to evaluate the protein role in early stages of baculoviral infections.

Keywords V-CHIA · Baculovirus · EpapGV · Chitinase · Peritrophic membrane

Ricardo Salvador and M. Leticia Ferrelli contributed equally to this study.

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Chitin is an abundant polysaccharide in nature restricted to arthropods, nematodes, yeasts, and some other fungi [1]. In insects, chitin and covalently attached polypeptides are the primary components of their exoskeleton and the peritrophic membrane (PM) lining the midgut [2, 3]. Some organisms have developed the ability to degrade chitin by proteins known as chitinases. In insects, these enzymes play an important role in their growth and morphogenesis [4]. Most baculoviruses express chitinase, which participate in the liquefaction of infected larvae, late in infection. The degradation of the dead insect's cuticle facilitates the dispersal of the occlusion bodies in the environment [5, 6]. The first baculovirus chitinase (V-CHIA) was described in Autographa californica MNPV and classified as member of the family 18 of the glycohydrolase superfamily [7, 8]. Thus far, at least five chitinases from nucleopolyhedroviruses (NPVs, from Alphabaculovirus) were studied, but the information regarding betabaculovirus chitinases is scarce. A chitinase gene was identified in Cydia pomonella granulovirus (CpGV) by Kang et al. [9]. Later, in 2007, Daimon et al. characterized the V-CHIA of CpGV by constructing a recombinant Bombyx mori NPV (BmNPV) in which BmNPV *v-chiA* was replaced by CpGV *v-chiA*. They

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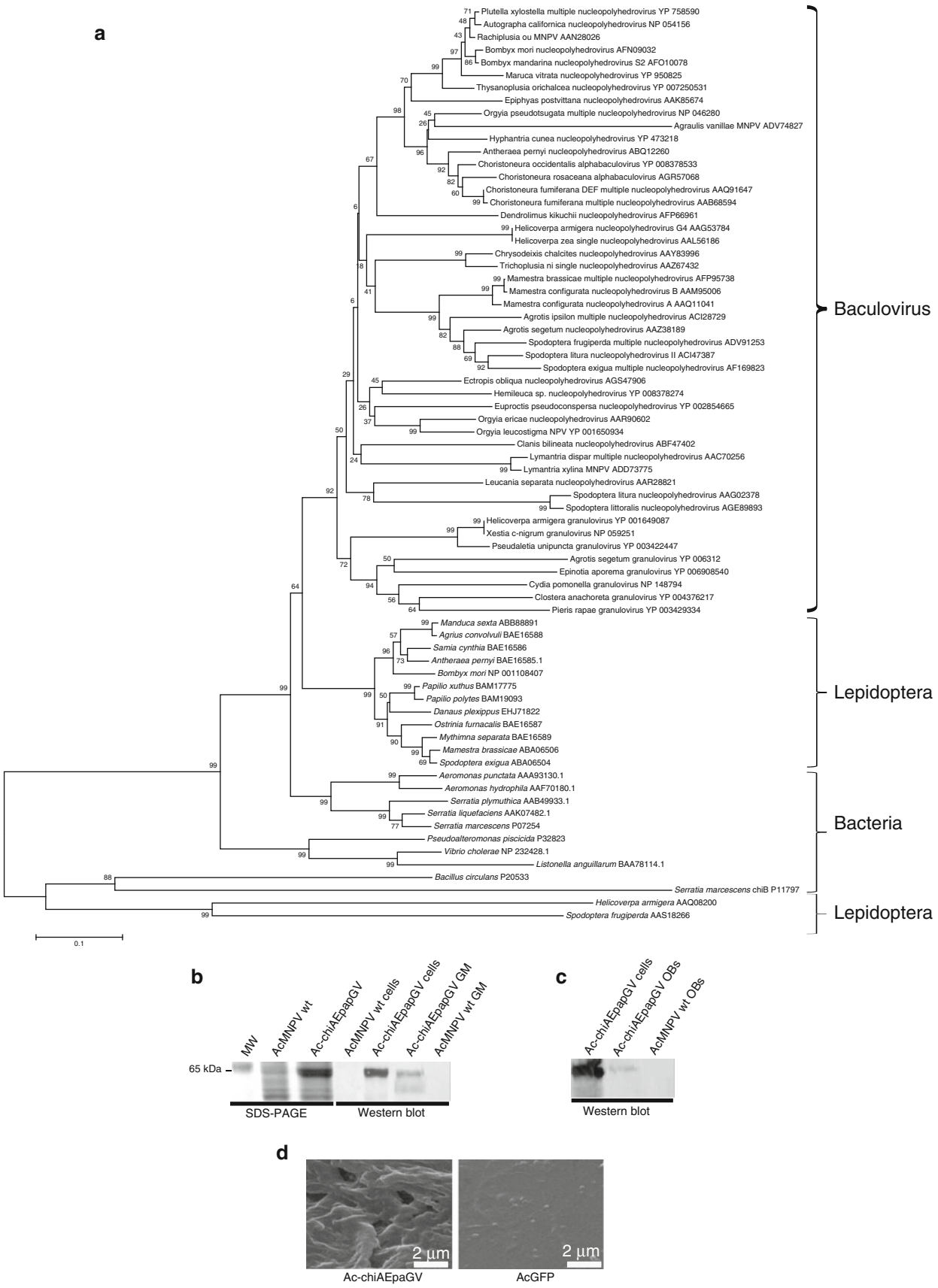
demonstrated the CpGV V-CHIA endo- and exochitinase activities, its role in the liquefaction process and the early secretion of this protein, associated with the lack of KDEL endoplasmic reticulum retention motif [10]. Recently, another betabaculovirus chitinase, encoded in Pieris rapae GV, was also characterized [11].

Epinotia aporema granulovirus (EpapGV) is highly pathogenic for the bean shoot borer (*Epinotia aporema* Wals), a serious pest of soybean and other legume crops in South America. EpapGV causes a polyorganotropic infection and the liquefaction of the host after death [12]. Recently, the complete sequence analysis of EpapGV genome [13] revealed the presence of two genes known to cause the liquefaction effect in baculovirus: *v-chiA* (EpapGV ORF 32) and *v-cath* (EpapGV ORF 31), which encode a putative chitinase and a cysteine protease, respectively. Both genes are adjacent to each other in opposite orientations, as it is found in many baculoviruses, with an intergenic distance of 136 bp. Sequence analysis of EpapGV chitinase ORF revealed that it is 1,713 base pairs long and a hypothetic late promoter motif (TTAAG) was detected 6 nt upstream of the start codon. This gene encodes a predicted 570 amino acid long protein with a molecular weight estimated in 63 kDa. EpapGV chitinase had the highest identity (62 %) with PiraGV chitinase, followed by CpGV and ClanGV with 61 % identity. The amino acid sequence analysis showed that the C-terminal KDEL or related ER retention motif is absent. This characteristic is shared with all chitinases found in betabaculovirus genomes [11]. The catalytic domain was identified between positions 153 and 522 and the active site (FDGVDLDWE), between residues 301 and 309. A typical N-terminal domain found in bacterial and viral chitinases was located in the region spanning amino acid residues 15–148, and the putative signal peptide sequence, between amino acid positions 1 and 15 (online resource 1).

Phylogeny of baculoviral chitinase sequences was analyzed in previous reports: Wang et al. [14] suggested the monophyletic relationship between baculoviral and bacterial chitinases, and Daimon et al. [15] proposed that the bacterial-type chitinase genes of lepidoptera might have been acquired from a bacterium or a baculovirus. Those phylogenetic studies contained little information on betabaculovirus chitinases since very few sequences of this genus were available. In order to further support the suggested relationship among baculoviral, lepidopteran, and bacterial chitinases, we report a comprehensive new phylogenetic analysis based on more sequence data by means of MEGA 5 program with default parameters [16] (Fig. 1a). A neighbor joining (NJ) tree was inferred, and the topology was analyzed using bootstrapping (1,000 replicates). The analysis showed that chitinase sequences were clustered in well-supported groups. Baculoviral (alphabaculovirus and betabaculovirus) chitinases formed a monophyletic group that clustered with bacterial-type lepidopteran V-CHIAs, indicating that these chitinases had a common origin. This baculoviral and lepidopteran group clustered with one clade of bacterial chitinases but within a larger clade that include other bacterial sequences. These results are in agreement with previous studies [7, 9, 14, 15] reinforcing the hypothesis that baculovirus and lepidopteran chitinases have a bacterial origin, and were acquired by horizontal gene transfer.

Fig. 1 **a** Phylogenetic analysis of chitinase sequences. Method: Neighbor-Joining, Bootstrap (1,000 replicates). Bootstrap numbers are shown**.** **b** SDS-PAGE and western blot analysis of cell lysates (cells) and growth media (GM) of High Five cells infected with recombinant and wild type (wt) viruses. **c** Western blot analysis of recombinant and wild type OBs. **d** Electron microscopy of PMs extracted from *A. gemmatalis* larvae fed with recombinant Ac-chiAEpapGV OBs and AcGFP OBs (used as a negative control)

In order to characterize the EpapGV V-CHIA protein a recombinant AcMNPV was constructed (Ac-chiAEpapGV) containing the complete EpapGV *v-chiA* ORF under the control of the *polyhedrin* promoter. In brief, *v-chiA* ORF was amplified with specific primers (5′-TGGTACCGTAT GAAACTAGCAATTGTGTC-3′ and 5′-GGGAGCTCGC TATCCTTTACAGAACGCG-3′) and cloned in pBacPak9 (Invitrogen). The resulting plasmid was cotransfected in High Five cells [17] with bApGOZA DNA [18]. bApGOZA is an AcMNPV bacmid that is nonviable if transfected alone because it is defective for the 1629 gene. It gains viability upon homologous recombination with the transfer plasmid which restores the 1629 deletion. It is worth noting that this bacmid retains the native chitinase gene of AcMNPV. As the parental genome is nonviable, only recombinant virions were recovered from the growth medium (GM). Recombinant V-CHIA expression was verified in SDS-PAGE, yielding a 63 kDa product, as expected from the predicted amino acid sequence and verified by western blot with anti-HaSNPV V-CHIA antibodies [14]. On the contrary, the native AcMNPV V-CHIA was not detected (Fig. 1b). The cross reaction of these antibodies with EpapGV V-CHIA evidences a close relationship between alpha- and betabaculovirus chitinases. Subsequently, the chitinase activity present in the growth medium of Ac-chiAEpapGV infected cells was evaluated under different conditions of temperature and pH (Online resource 2). The highest enzyme activity was detected at 27 °C. On the other hand, the data showed that the chitinolytic activity is maintained at pH values ranging from 5 to 10. The highest activity was observed at pH 7. The recombinant virus, which expresses both endogenous AcMNPV and EpapGV chitinases, showed higher activity than the wild type (wt) AcMNPV used as a control. Therefore, the difference observed can be attributed to an overexpressed active EpapGV V-CHIA. Similar western


a
Plutella xylostella multiple nucleopolyhedrovirus YP 758590
Autographa californica nucleopolyhedrovirus NP 054156
Rachiplusia ou MNPV AAN28026
Bombyx mori nucleopolyhedrovirus AFN09032
Bombyx mandarina nucleopolyhedrovirus S2 AFO10078
Maruca vitrata nucleopolyhedrovirus YP 950825
Thysanoplusia orichalcea nucleopolyhedrovirus YP 007250531
Epiphyas postvittana nucleopolyhedrovirus AAK85674
Orgyia pseudotsugata multiple nucleopolyhedrovirus NP 046280
Agraulis vanillae MNPV ADV74827
Hyphantria cunea nucleopolyhedrovirus YP 473218
Antheraea pernyi nucleopolyhedrovirus ABQ12260
Choristoneura occidentalis alphabaculovirus YP 008378533
Choristoneura rosaceana alphabaculovirus AGR57068
Choristoneura fumiferana DEF multiple nucleopolyhedrovirus AAQ91647
Choristoneura fumiferana multiple nucleopolyhedrovirus AAB68594
Dendrolimus kikuchii nucleopolyhedrovirus AFP66961
Helicoverpa armigera nucleopolyhedrovirus G4 AAG53784
Helicoverpa zea single nucleopolyhedrovirus AAL56186
Chrysodeixis chalcites nucleopolyhedrovirus AAY83996
Trichoplusia ni single nucleopolyhedrovirus AAZ67432
Mamestra brassicae multiple nucleopolyhedrovirus AFP95738
Mamestra configurata nucleopolyhedrovirus B AAM95006
Mamestra configurata nucleopolyhedrovirus A AAQ11041
Agrotis ipsilon multiple nucleopolyhedrovirus ACI28729
Agrotis segetum nucleopolyhedrovirus AAZ38189
Spodoptera frugiperda multiple nucleopolyhedrovirus ADV91253
Spodoptera litura nucleopolyhedrovirus II ACI47387
Spodoptera exigua multiple nucleopolyhedrovirus AF169823
Ectropis obliqua nucleopolyhedrovirus AGS47906
Hemileuca sp. nucleopolyhedrovirus YP 008378274
Euproctis pseudoconspersa nucleopolyhedrovirus YP 002854665
Orgyia ericae nucleopolyhedrovirus AAR90602
Orgyia leucostigma NPV YP 001650934
Clanis bilineata nucleopolyhedrovirus ABF47402
Lymantria dispar multiple nucleopolyhedrovirus AAC70256
Lymantria xylina MNPV ADD73775
Leucania separata nucleopolyhedrovirus AAR28821
Spodoptera litura nucleopolyhedrovirus AAG02378
Spodoptera littoralis nucleopolyhedrovirus AGE89893
Helicoverpa armigera granulovirus YP 001649087
Xestia c-nigrum granulovirus NP 059251
Pseudaletia unipuncta granulovirus YP 003422447
Agrotis segetum granulovirus YP 006312
Epinotia aporema granulovirus YP 006908540
Cydia pomonella granulovirus NP 148794
Clostera anachoreta granulovirus YP 004376217
Pieris rapae granulovirus YP 003429334
Manduca sexta ABB88891
Agrius convolvuli BAE16588
Samia cynthia BAE16586
Antheraea pernyi BAE16585.1
Bombyx mori NP 001108407
Papilio xuthus BAM17775
Papilio polytes BAM19093
Danaus plexippus EHJ71822
Ostrinia furnacalis BAE16587
Mythimna separata BAE16589
Mamestra brassicae ABA06506
Spodoptera exigua ABA06504
Aeromonas punctata AAA93130.1
Aeromonas hydrophila AAF70180.1
Serratia plymuthica AAB49933.1
Serratia liquefaciens AAK07482.1
Serratia marcescens P07254
Pseudoalteromonas piscicida P32823
Vibrio cholerae NP 232428.1
Listonella anguillarum BAA78114.1
Bacillus circulans P20533
Serratia marcescens chiB P11797
Helicoverpa armigera AAQ08200
Spodoptera frugiperda AAS18266
Baculovirus
Lepidoptera
Bacteria
Lepidoptera
0.1
b
MW
AcMNPV wt
Ac-chiAEpapGV
AcMNPV wt cells
Ac-chiAEpapGV cells
Ac-chiAEpapGV GM
AcMNPV wt GM
65 kDa
SDS-PAGE
Western blot
c
Ac-chiAEpapGV cells
Ac-chiAEpapGV OBs
AcMNPV wt OBs
Western blot
d
2 µm
2 µm
Ac-chiAEpaGV
AcGFP

blot and activity results were obtained in a Spodoptera litura NPV chitinase study [19].

Baculoviral chitinase from AcMNPV can cause damages in the peritrophic membrane: In a study by Rao et al. [20], the baculovirus chitinase expressed in bacteria was purified and used to evaluate the action on the insect chitinous PM, the first barrier that virions need to cross to infect midgut epithelial cells. The effects observed showed that the baculoviral chitinase altered the PM permeability in vitro in a dose-dependent manner using the *E. coli* expressed protein. In other study where the *v-chiA* gene is under the control of its native promoter, the AcMNPV chitinase activity was detected associated to polyhedra purified from infected larvae [21]. However, no significant effect was observed in bioassays performed with recombinant *v-chiA* defective AcMNPV and *wt* AcMNPV OBs. These observations might be due to a very low concentration of V-CHIA in AcMNPV polyhedra [21]. In our hands, EpapGV V-CHIA was detected in Ac-chiAEpapGV polyhedra probably due to the overexpression of the *v-chiA* gene under the control of the strong polyhedrin promoter (Fig. 1c). Taking into account these observations, we tested the EpapGV V-CHIA containing polyhedra for the ability to damage the PM of *Anticarsia gemmatalis* larvae. Scanning electron microscopy showed PM alterations on larvae treated with Ac-chiAEpapGV OBs. By contrast, larvae treated with OBs from a recombinant Ac-GFP (constructed using the same vector and bacmid as described for Ac-chiAEpapGV virus) showed no damage (Fig. 1d). This observation suggests that changes on the PM can be attributed to the overexpression of V-CHIA. Previous studies showed that the larvicidal activity of a nuclear polyhedrosis virus toward the gypsy moth larvae (*Lymantria dispar*) was increased fivefold when it was coadministered with bacterial chitinase [22]. In that case, chitinases were supposed to cause perforations in the gut PM [3]. Recently, Wang et al. [19] obtained a recombinant AcMNPV that overexpresses SpliNPV V-CHIA. Biossays performed using these recombinant OBs improved the insecticidal activity of AcMNPV against *Spodoptera exigua* larvae. Taken together, these results suggest that Ac-chiAEpapGV could have insecticidal activity associated to damages on the PM. More experimental data are necessary to elucidate this.

In our study, we demonstrated that EpapGV contains a functional chitinase in agreement with the observed liquefaction of infected *E. aporema* larvae [12]. The EpapGV V-CHIA was active under alkaline conditions similar to those found in lepidopteran midgut. This feature, together with the effect on the PM shown here, indicates that this protein could be included in viral formulations in order to increase the oral infectivity of baculoviral agents. Studies on alphabaculovirus chitinases focused on the potential use of these proteins to improve the viral activity applied to biological control [20, 22]. As an alternative to formulation of biopesticidal mixtures (OBs + chitinase), our results suggest that the overexpression of a protein allows its association with OBs. Therefore, genetic modifications could be designed to enhance the oral infectivity of baculoviruses including viral genes under the control of strong promoters.

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Virus Genes

Analysis of a chitinase from EpapGV, a fast killing betabaculovirus

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Online resource 1

Gene sequence and structure. Sites and main domains presenti n predicted protein are depicted. Late promoter element is indicated in red.

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                                M  K  L  A  I  V  S  I  L  C  C  V  V  T  A  K  P  G  V  P  S  I  E  W  A  E  N  K  Y  A  L  V  S  V  R  P  D  A  A  T·
1    AAAATAATA ATTAAGTAT GAAACTAGC AATTGTGTC TATT TTA TG TTGTGTAGT TACCGCAAA ACCAGGAGT GCCCAGTAT AGAATGGGC GGAGAACAA GTATGCTCT GGTTAGTGT CAGACCGGA TGCCGCCAC
     ·T  Y  E  T  L  I  E  R  V  S  D  S  V  S  V  S  L  K  W  N  V  W  S  G  G  S  G  S  S  A  E  L  L  F  D  N  K  A  V  K  L  A  T  E  Q  E·
136  GTATGAGAC ATTGATTGA GCGCGTCAG CGACAGTGT TAGCGTGTC GCTTAAATG GAACGTGTG GTCTGGCGG CAGCGGTAG CTCTGCTGA ACTGTTGTT CGATAACAA AGCGGTCAA GCTGGCCAC CGAGCAAGA
     ·E  L  Q  Q  K  Q  I  N  H  L  V  N  R  G  G  R  Y  K  A  A  V  R  L  C  D  D  D  G  E  C  S  V  S  S  A  I  N  V  V  V  A  D  T  D  G  S·
271  GCTGCAGCA GAAACAAAT AAACCACTT AGTCAATCG CGGCGGGCG ATACAAGGC AGCGGTGCG GTTGTGCGA CGACGACGG CGAATGTTC GGTCAGCAG CGCGATCAA CGTGGTAGT GGCGGACAC CGACGGTTC
     ·S  H  L  A  P  L  P  I  E  W  N  E  N  N  V  P  Y  Q  R  G  G  G  T  V  V  A  A  Y  F  V  E  W  G  V  Y  G  R  Q  Y  P  A  N  K  L  P  I·
406  CCACCTGGC TCCGCTGCC GATCGAGTG GAACGAGAA CAACGTTCC GTACCAGCG CGGCGGCGG AACCGTGGT GGCCGCTTA CTTTGTCGA GTGGGGTGT GTATGGTAG ACAGTATCC GGCCAACAA ACTACCCAT
     ·I  P  N  L  T  H  L  L  Y  G  F  V  P  I  C  G  G  D  G  I  N  D  S  L  K  Q  I  P  N  S  F  E  V  L  Q  K  S  C  S  N  R  A  D  F  S  V·
541  ACCAAATCT GACACATTT GCTGTACGG CTTTGTGCC CATTTGCGG CGGAGACGG TATCAACGA TAGCCTAAA ACAGATCCC CAATTCTTT TGAGGTCCT CCAAAAGTC ATGCTCAAA CCGAGCCGA CTTTAGTGT
     ·V  S  I  H  D  I  W  G  A  L  Q  K  P  Q  K  G  V  E  A  W  N  E  P  Y  K  G  N  F  G  Q  L  M  A  I  K  R  H  H  P  H  L  S  I  L  P  S·
676  GAGCATACA CGATATATG GGGCGCGCT GCAAAAACC GCAAAAGGG TGTAGAGGC GTGGAACGA GCCGTACAA GGGTAACTT TGGCCAGTT GATGGCCAT CAAACGACA CCATCCCCA CCTATCCAT ACTGCCCTC
     ·S  I  G  G  W  T  L  S  D  P  F  F  F  M  H  D  P  A  K  R  A  V  F  V  E  S  V  R  E  F  L  Q  T  W  K  F  F  D  G  V  D  L  D  W  E  F·
811  GATTGGTGG GTGGACTCT GAGCGATCC ATTCTTTTT TATGCACGA CCCGGCCAA GCGTGCCGT GTTTGTAGA GTCTGTGCG CGAATTTCT GCAAACGTG GAAGTTCTT TGACGGAGT CGATTTGGA CTGGGAGTT
     ·F  P  G  G  K  G  A  N  P  A  I  G  N  P  D  I  D  A  K  T  Y  T  A  L  L  R  E  L  R  F  M  L  D  E  L  A  E  K  N  D  R  V  Y  Q  L  T·
946  TCCGGGTGG AAAGGGAGC CAACCCCGC GATCGGTAA CCCCGACAT TGACGCAAA AACGTACAC CGCGCTGTT GCGCGAACT AAGATTTAT GCTGGACGA GTTGGCGGA AAAAAACGA TCGCGTCTA CCAACTAAC
     ·T  T  A  I  S  A  G  H  D  K  I  Q  V  V  N  Y  A  S  A  Q  K  H  L  D  H  I  F  V  M  S  Y  D  F  R  G  A  W  S  N  T  D  V  G  H  Q  T·
1081 CACCGCTAT TAGCGCCGG GCATGATAA AATACAAGT GGTCAACTA CGCTTCGGC CCAAAAACA TCTGGACCA CATTTTCGT TATGAGTTA CGATTTTAG GGGCGCCTG GTCCAACAC CGACGTGGG CCATCAGAC
     ·T  P  L  Y  G  D  D  Q  L  N  A  H  H  A  I  T  Q  L  L  N  Q  N  V  D  S  K  K  L  V  L  G  V  A  M  Y  G  R  G  W  T  G  M  Q  Q  P  S·
1216 ACCGCTTTA CGGCGACGA CCAACTCAA CGCACATCA CGCCATCAC ACAACTGCT CAACCAAAA TGTCGATAG CAAAAAACT CGTGTTAGG CGTCGCCAT GTACGGGCG AGGGTGGAC CGGTATGCA GCAACCGTC
     ·S  G  P  A  T  G  P  V  T  G  T  W  E  D  G  V  V  D  Y  R  D  I  L  R  R  Y  N  T  T  A  Y  D  D  A  A  E  A  A  Y  V  F  S  N  G  D  Y·
1351 GGGACCCGC CACTGGACC TGTAACCGG CACCTGGGA GGACGGCGT AGTCGATTA TAGGGACAT TTTACGCCG GTACAACAC CACCGCATA TGACGACGC CGCCGAGGC AGCCTACGT TTTCAGCAA CGGCGACTA
     ·Y  I  S  Y  D  S  V  R  S  V  L  A  K  G  A  Y  V  L  E  H  K  L  G  G  L  F  A  W  E  I  D  A  D  N  G  D  L  L  N  A  M  N  Q  G  L  G·
1486 TATTTCATA CGATAGTGT ACGATCGGT GCTAGCTAA GGGCGCCTA TGTTCTAGA ACATAAGCT AGGTGGACT GTTTGCGTG GGAGATTGA CGCCGACAA CGGGGATTT GCTCAACGC CATGAATCA GGGGTTGGG
     ·G  N  K  K  V  S  L  C  K  L  E  T  N  K  Y  A  K  Y  L  C  P  L  K  P  A  I  L  Y  G  L  D  A  F  C  K  G  *
1621 CAACAAAAA AGTATCGCT GTGTAAGCT AGAAACTAA TAAATATGC CAAATATCT GTGCCCGCT CAAACCCGC AATCCTTTA TGGACTTGA CGCGTTCTG TAAAGGATA G
```

Signal peptide | N-terminal domain | Catalytic domain | Active site

Virus Genes
Analysis of a chitinase from EpapGV, a fast killing betabaculovirus
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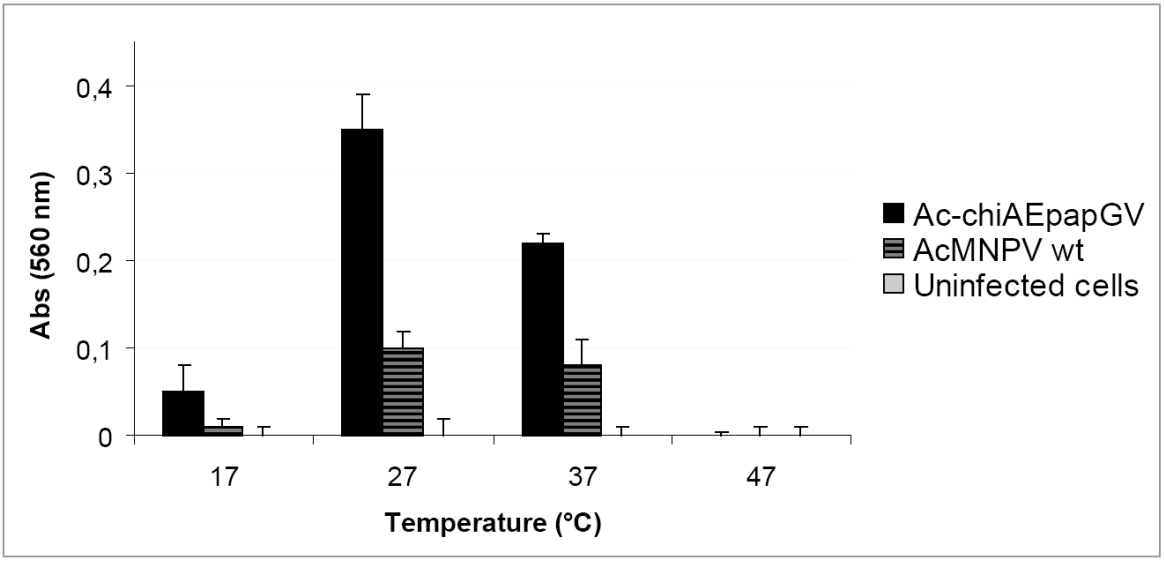
Online resource 2

ChiA activity at different temperatures and pH

In order to evaluate the activity of EpapGV CHIA at different temperatures High Five cells were infected with recombinant Ac-chiAEpapGV (MOI of 5), using the wt AcMNPV virus as a negative control. At 72 hours p.i. the growth medium was collected and 1 ml of sample containing similar protein quantities (5 mg/ml) were mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.5) and 5 mg of chitin-azure (Sigma). Mixture obtained was incubated at different temperatures for 5 days. The tubes were centrifuged and the supernatants were used for measurement of absorbance at 560 nm. Abs (560) of cell free medium was used for normalization.



Virus Genes

Analysis of a chitinase from EpapGV, a fast killing betabaculovirus

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In order to evaluate the activity of EpapGV CHIA at different pH values High Five cells were infected with recombinant Ac-chiAEpapGV (MOI of 5), using the wt AcMNPV virus as a negative control. At 72 hours p.i. the growth medium was collected and 1 ml of sample containing similar protein quantities (5mg/ml) were mixed with 1 ml of cell culture medium at different pH values and 5 mg of chitin-azure (Sigma). Mixture obtained was incubated at 27° C for 5 days. The tubes were centrifuged and the supernatants were used for measurement of absorbance at 560 nm. Abs (560) of cell free medium was used for normalization.