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# Comparison of the potential activities of viral and bacterial chitinases

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## Abstract

**Background:** Chitin, a long-chain polymer of *N*-acetylglucosamine, is a major structural component of the insect exoskeleton and the peritrophic membrane (PM). Chitinases are able to effectively break down glycosidic bonds of chitin polymer thus can be used in agriculture to control plant pathogen insects. These enzymes can be synthesized by higher plants, animals, protista, bacteria, and viruses.

**Results:** In this study, viral and bacterial chitinases were compared for their potential activity on a laboratory test insect. The genes encoding chitinases of *Autographa californica nucleopolyhedrovirus* (AcNPV) and *Cydia pomonella granulovirus* (CpGV) were amplified from genomic DNAs by PCR and cloned into the pET-28a (+) expression vector. The chitinase proteins of these 2 viruses (AcNPV-Chi, CpGV-Chi) and *Serratia marcescens* chitinase C (ChiC) protein which was previously cloned were overexpressed in *Escherichia coli*. Expressed proteins were purified and confirmed by western blot analysis as 50, 63, and 68 kDa for AcNPV, CpGV, and *S. marcescens* chitinases, respectively. Enzyme activities of the chitinases were confirmed. Chitinases were also compared to each other in silico. The insecticidal effects of these proteins were evaluated on *Galleria mellonella* L. larvae. Bioassays were performed on the 3rd instar larvae for each chitinase protein in triplicate. The results showed that although there were differences in enzymatic activities and domain organizations, all 3 microbial chitinases produced almost the same level of insecticidal activity on the test insect. $LC_{50}$ and $LT_{50}$ values were compatible with the mortality results. These results were a preanalysis for comparing the effects of microbial chitinases.

**Conclusion:** Potential activity experiments should be carried out on more insects to provide detailed information on the insecticidal effects of bacterial and viral chitinases.

**Keywords:** Bacteria, Virus, Chitinase, Potential activity

## Background

Chitin is a linear polysaccharide of β 1–4-linked *N*-acetylglucosamine, a derivative of glucose, and is the second most abundant biopolymer on earth after cellulose. It is the major polysaccharide present mainly in insects, crustaceans, and fungi. Chitinase, an extracellular enzyme, is able to effectively break down glycosidic bonds of chitin polymer. Chitinase production is widely distributed in various organisms including those that do not contain chitin, like plants, vertebrates, bacteria, and viruses, and also those that contain chitin such as insects, crustaceans, and fungi. These organisms synthesize chitinases for different purposes such as morphogenesis, pathogenesis, parasitism, and defense (Berini et al. 2018).

Insects are organisms which include both chitin and chitinases together. Chitin is the structural component of the insect exoskeleton, gut lining (perithrophic membrane/PM), salivary gland, trachea, eggshells, and muscle attachment points (Muthukrishnan et al. 2012). At the same time, chitinases are essential for insect survival, molting, or development (Arakane and Muthukrishnan 2010). However, chitinases can also be used exogenously for the biological control of insect pests. Any defect in the cuticle or peritrophic membrane caused by

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chitinases will reduce nutrition and protection against microbial attacks.

Bacteria and viruses are other chitinase producers. Microbial chitinases weaken and disrupt the chitin structures in insects, thus increasing the risk of microbial infection of the host. Bacteria produce chitinases to obtain nitrogen and carbon as a source of nutrients or precursors and parasitism (Rathore and Gupta 2015). Several genera of bacteria, including *Serratia* (Bahar et al. 2012), *Bacillus* (Sasi et al. 2020), *Streptomyces* (Tran et al. 2019), *Enterobacter* (Sivaramakrishna et al. 2020), and *Vibrio* (Jahromi and Barzkar 2018), produce chitinase. A few viral chitinases, mainly species from Baculoviridae, are currently known (Ishimwe et al. 2015). Viral chitinases weaken the host barrier structure to facilitate virus infection and release progeny viruses from infected cells. The most widely studied chitinase from baculoviruses belongs to *Autographa californica nucleopolyhedrovirus* (AcNPV) species (Hawtin et al. 1997). Viral chitinases and most bacterial chitinases belong to family 18 glycohydrolases that belong to a family of multimodular proteins (Berini et al. 2018). AcNPV chitinase is closely related to *Serratia marcescens* ChiA thus suggesting that baculovirus chitinase may have been acquired from a bacterium found in insect bodies.

The negative effects of chemicals on the ecosystem and on human health force scientists to find alternative solutions against insect pests. Insect pathogenic bacteria and viruses or their bioactive agents are good options to be used as biological pest control materials.

This study aimed to investigate whether there is a difference between viral and bacterial chitinase enzymes in terms of their potential activity.

## Methods
### Viral and bacterial strains and insect culture
In the study, 2 viral and a bacterial chitinase proteins were investigated in terms of their potential activity. *Autographa californica nucleopolyhedrovirus* (AcNPV) and *Cydia pomonella granulovirus* (CpGV) species were used for viral chitinase sources. Bacterial chitinase protein (chitinase C) belongs to *Serratia marcescens* that was previously isolated from dead *Helicoverpa armigera* (Hubner, 1808) larvae. The *S. marcescens* chitinase gene (accession number: KF823632) was provided cloned into the pET-28a (+) expression vector (Danişmazoğlu et al. 2015). Lab-reared culture of *Galleria mellonella* (Linnaeus, 1758). was used in the activity assay. *G. mellonella* larvae were maintained on an artificial diet (22% glycerol, 24% wheat bran, 22% honey, 24% honeycomb, and 4% water) and reared at 26 ± 2°C, and 60–70% relative humidity, with a 14:10-h light:dark photoperiod.

### In silico analysis of chitinases
AcNPV (NC_001623.1), CpGV (NC_002816.1), and S. marcescens (KF823632) chitinase proteins were analyzed in terms of their domain content at the NCBI conserved domain search database. Amino acid sequences of chitinases were compared to each other using NCBI protein blast and multiple alignment tools.

### Construction of chitinase expressing bacterial vectors
Viral genomic DNAs were isolated from virus suspensions, using DNeasy Blood & Tissue Kit (Qiagen, 69506). Specific primers were designed for both AcNPV chitinase (Fw: 5′-GG**GGATCC**ATTCCCGGCACGC-3′; Rv: 5′-GG**CTCGAG**TTACAGTTCATCTTTAGGT-3′) and CpGV chitinase (Fw: 5′-GG**GGATCC**AAACCCGGCA CACC-3′; Rv: 5′-GG**CTCGAG**TCATACTGAATTGC ACAC-3′). *Bam*HI and *Xho*I restriction endonucleases were added to the 5′ ends of forward and reverse primers, respectively. Both chitinase genes (*AcNPV-chi*, *CpGV-chi*) were amplified by PCR from genomic DNAs. The reactions contain 10 ng of genomic DNA, 2.5 μl of both primers of 10 μM, 1 μl of 10 mM dNTPs, 10 μl 5X Phusion HF reaction buffer, and 1 U Phusion HF DNA polymerase. Lastly, the volume was adjusted to 50 μl with sterile dH₂O. The reaction program was as follows: an initial denaturation step at 98°C for 3 min followed by 30 cycles of 98°C for 50 s, 55°C for 50 s, 72°C for 1 min, and final extension step at 72°C for 10 min. The PCR products were ligated into the pJET1.2/blunt vector according to the manufacturer's instructions. Ligation reactions were transformed into *E. coli* DH10β cells, and positive clones were confirmed. The generated recombinant plasmids carrying *AcNPV-chi* and *CpGV-chi* genes were named as pAcNPV and pCpGV, respectively. The chitinase genes were sequenced by Macrogen Inc. (Amsterdam, the Netherlands). Following the sequence analysis, both chitinase genes containing BamHI and XhoI restriction sites at their 5′ and 3′ ends were introduced into the pET-28a(+) expression vector using the same sites. These plasmids were transformed into *E. coli* BL21 (DE3) competent cells, and recombinant plasmids were selected in the presence of Kanamycin (50 μg/ml).

### Over-expression, purification, and western blot analysis of the recombinant proteins
The AcNPV-Chi, CpGV-Chi, and *S. marcescens*-ChiC proteins were overexpressed as fusion proteins with the 6×His-tag at their N-terminal in *E. coli* BL21 (DE3) cells. Chitinase proteins were purified by using the MagneHis™ Protein Purification System Kit (Promega) and dialyzed for 24 h through 1 l of 1X PBS buffer, pH 7.5. The identification and purity of the samples were confirmed by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subsequently Coomassie
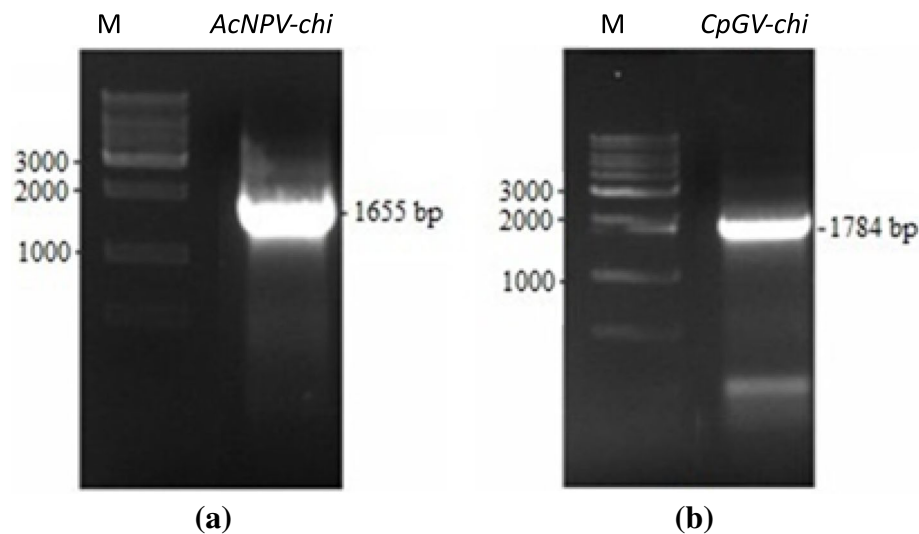
**Fig. 1** PCR amplified *AcNPV-chi* (**a**) and *CpGV-chi* (**b**) genes. M, 1 kb DNA ladder (Promega)

staining. Western blot analysis was also performed to demonstrate heterologous gene expression at the immunological level. Electrophoresed proteins were transferred to the nitrocellulose membrane. Immune-detection was performed using 1:1000 diluted polyclonal rabbit anti-His-taq antibodies (Abcam) and subsequently 1:1000 diluted polyclonal alkaline phosphatase-conjugated goat-anti-rabbit IgG (Millipore). The binding of the antibodies was then visualized with the NBT-BCIP substrate system (Roche).

### Chitinase activity assay

Chitinase activities were assayed by using the colorimetric 3,5-dinitrosalicylic acid (DNS) method (Monreal and Reese 1969) with some modifications. The purified bacterial and viral chitinases (0.5 μg) were reacted with 150



**Fig. 2** SDS-PAGE analysis of His-taq-purified viral and bacterial chitinase proteins. Lane 1, *S. marcescens*-ChiC (50 kDa); lane 2, AcNPV-Chi (63 kDa); and lane 3, CpGV-Chi (68 kDa). M, PageRuler Prestained (Thermo Scientific)

μl of colloidal chitin (12.5 mg/ml chitin) as a substrate and incubated at 30°C for 3 h. The reactions were terminated by boiling the mixture in the presence of 300 μl DNS acid reagent for 5 min. In the control reaction, only substrate and DNS were used. The hydrolysis of chitin was measured at 540 nm and run with a glucose standard. One unit of the chitinase activity was defined as the amount of enzyme that liberates 1 μM of reducing sugar per 1 min at 30°C.

### Potential activity test

Proteins were tested on *G. mellonella* at 5 different concentrations (1.5, 1.8, 2, 2.5, and 3 μg protein) per larvae. Bioassays were performed by 30 larvae (3rd instar) for each concentration in triplicate. The larvae were starved for 12 h and then fed with an artificial diet inoculated with desired concentrations of protein. After consumption of the inoculated diets, non-inoculated ones were added and incubated at 26 ± 2°C. Control group larvae were fed on only 1X PBS treated diets. Mortalities were recorded daily for 14 days. An experimental design, showing the insecticidal activity tests, is given in Supplementary Fig. 1.

### Statistical analysis

Mortality data were corrected using Abbott's formula (Abbott 1925); $LC_{50}$ and $LT_{50}$ values were calculated by Probit analysis using MS Excel (Finney 1952).

## Results

### In silico analysis of chitinases

Chitinase proteins were analyzed for their domain contents. The results showed that viral chitinases had similar domain structures to each other but different than
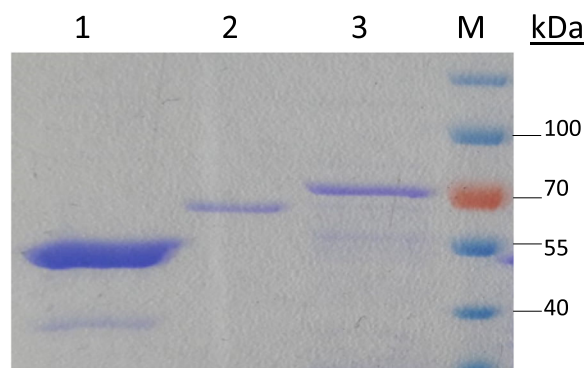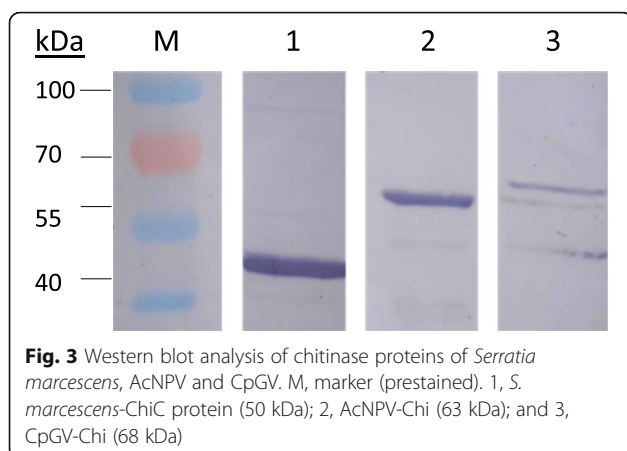
**Fig. 3** Western blot analysis of chitinase proteins of *Serratia marcescens*, AcNPV and CpGV. M, marker (prestained). 1, *S. marcescens*-ChiC protein (50 kDa); 2, AcNPV-Chi (63 kDa); and 3, CpGV-Chi (68 kDa)

that of bacterial chitinase (Supplementary Fig. 2). Viral chitinases contain a ChitinaseA_N domain, whose function is binding to chitin substrat, followed by a glyco_18 domain that catalyses the hydrolysis of 1,4-beta-linkages of *N*-acetylglucosamine in chitin and chitodextrins. *S.marcescens*-ChiC protein contains a Chi1 domain, whose function is hydrolyzing the chitin, followed by a COG3979 domain that cleaves soluble oligomers to the di- and trisaccharides. Multiple alignments of amino acid sequences of 3 chitinases together did not produce a significant similarity. Likewise, pairwise alignments of amino acid sequences of bacterial chitinase with either AcNPV or CpGV chitinases did not produce similarity. Although viral chitinases had similar domain organizations, their pairwise identity at amino acid level was only 60%.

### Expression, purification, and enzyme activities of chitinases

The sizes of the DNA fragments amplified by PCR for the *AcNPV-chi* and *CpGV-chi* genes corresponded with the expected sizes of 1655 and 1784 bp, respectively (Fig. 1a, b). These products were first cloned into the pJET1.2/blunt vectors and subsequently to the pET-28a (+) vectors (Novagen) individually. AcNPV-Chi, CpGV-Chi, and *S. marcescens*-ChiC proteins were expressed in *E. coli* with a N-terminal 6xHis-tag. Recombinant His-tagged proteins were purified, using the MagneHis™ Protein Purification kit. Purified proteins were analyzed by 10% SDS-PAGE. The electrophoresis process was performed using 2 gels on which samples were loaded identically. In one of the 2 gels stained with Coomassie Brillant Blue, *S. marcescens*-ChiC, AcNPV-Chi, and CpGV-Chi proteins were observed at sizes of 50, 63, and 68 kDa, respectively, as expected (Fig. 2). The other gel was used for western blot analysis using histidine antibody, which facilitates seeing these proteins by binding to the histidine tag attached to the N-terminal of the proteins. As a result of this analysis, expected chitinase protein sizes were observed on the membrane (Fig. 3). Chitinase activities of these purified proteins, tested by the DNS method, were determined as colorimetric in a spectrometer (Fig. 4).

### Potential activity of chitinase proteins

To analyze the potential activity of AcNPV-Chi and CpGV-Chi and *S. marcescens* ChiC proteins, *G. mellonella* larvae were fed on purified and dialyzed proteins. The results showed that the effects on *G. mellonella* larvae were not much different from each other. However, CpGV-Chi, at 2 and 2.5 μg/larvae concentrations, produced a slight high mortality on insects (Fig. 5). The daily number of dead insects was recorded. Mortality effects of chitinase proteins over time were also examined (Fig. 6). $LC_{50}$ and $LT_{50}$ values were correlated with the mortality results (Tables 1 and 2).
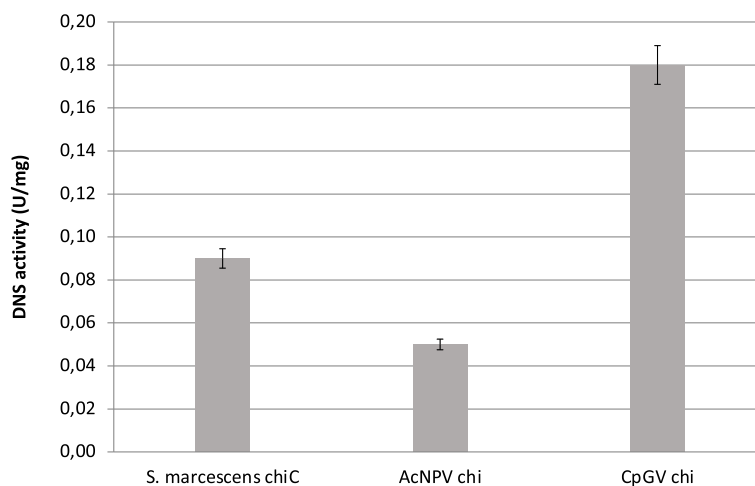


**Fig. 4** Hydrolyzing activities of *Serratia marcescens*-ChiC, AcNPV-Chi, and CpGV-Chi proteins against colloidal chitin
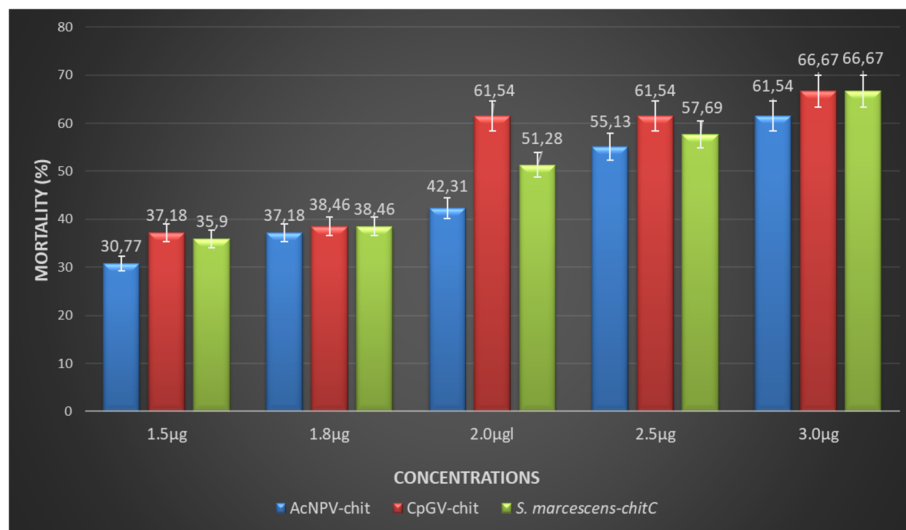
**Fig. 5** Insecticidal activity of purified AcNPV-Chi, CpGV-Chi, and *Serratia marcescens*-ChiC proteins at different concentrations on the 3rd instar *Galleria mellonella* larvae

## Discussion

Potential effects of chitinase proteins which belong to *Autographa californica nuleopolyhedrovirus* (AcNPV), *Cydia pomonella granulovirus* (CpGV), and *Serratia marcescens* were tested and compared to each other. The results showed that the potential effects of all 3 chitinases were comparable, with only a slightly high mortality rate caused by CpGV chitinase. Increased chitinase concentration produced high mortality rates.

Amino acid sequence alignments of chitinases showed that *S. marcescens*-ChiC and viral chitinases did not show significant similarity to each other. Also, domain positions were opposite in *S. marcescens*-ChiC and viral chitinases, while the glyco_18 and GH_18 domains located at the C terminal of viral chitinases showed similar domains located at the N terminal of *S. marcescens*-ChiC. *S.marcescens*-ChiA, structurally analyzed previously by Danişmazoğlu et al. (2015), had a similar domain organization to those of AcNPV-Chi and CpGV-Chi.

Enzyme activities of chitinases were confirmed using the DNS method before activity analysis. The results showed that CpGV-Chi activity was higher than *S. marcescens*-ChiC activity and much higher than AcNPV-Chi
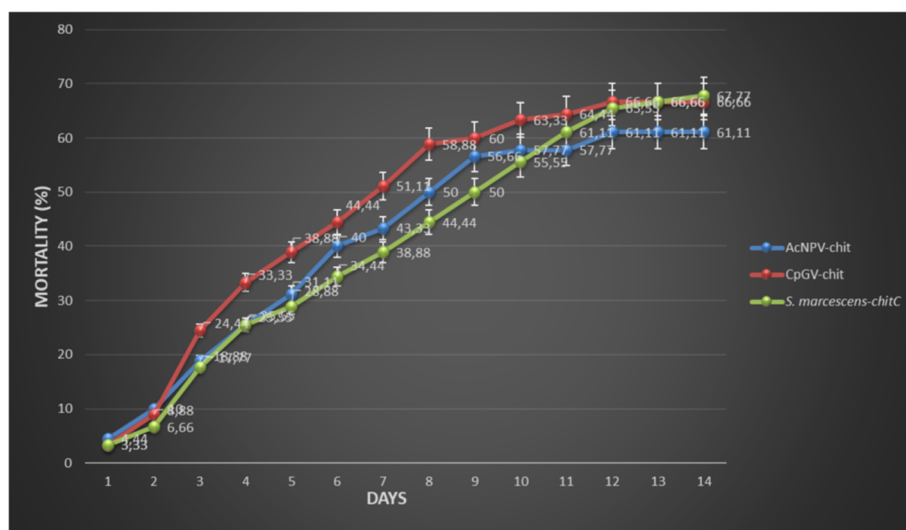


**Fig. 6** Mortality rates of purified AcNPV-Chi, CpGV-Chi, and *Serratia marcescens*-ChiC proteins on *Galleria mellonella* larvae depending on time. Mortality rates were carried out using the 2.5 μg per larvae protein concentration

**Table 1** LC$_{50}$ values of AcNPV-Chi, CpGV-Chi, and *Serratia marcescens*-ChiC proteins

| Chitinase | LC$_{50}$ (ng) | Slope±SE | df | X$^2$ |
|---|---|---|---|---|
| AcNPV-Chi | 2318.318 | 2.753±0.066 | 3 | 0.997 |
| CpGV-Chi | 1958.961 | 2.686±0.067 | 3 | 0.658 |
| S.marcescens-ChiC | 2095.064 | 2.734±0.066 | 3 | 0.944 |

activity. Although there was a small difference between bacterial and viral chitinases in terms of potential activity results, this difference was also detected in the in vitro enzyme activity assay.

Chitinase protein of AcNPV in conjunction with virus-encoded cathepsin caused liquefaction of the *Trichoplusia ni* (Hubner, 1803) larvae in the latter stages of infection (Hawtin et al. 1997). They showed that infections of *T. ni* larvae with mutant viruses lacking either chitinase or cathepsin did not cause liquefaction of dead insect bodies. In the present study, dead *G. mellonella* larvae showed softening, but the body remained intact for several days after death. Failure to observe body liquefaction in dead *G. mellonella* larvae may be due to not using cathepsin protein with chitinase in infections. In another study, the AcNPV chitinase A (*ChiA*) gene was expressed in *E. coli* cells, subsequently purified and tested on *Bombyx mori* (Linnaeus, 1758) larvae at a dose of 1 μg/g of larval body weight. They obtained 100% mortality and concluded that AcNPV-ChiA may offer interesting new opportunities for pest control (Raw et al. 2004). Daimon et al. (2007) constructed a recombinant *Bombyx mori* NPV (BmNPV) in which BmNPV ChiA was replaced by CpGV ChiA. This recombinant BmNPV was tested on *B. mori* larvae. Their results showed that CpGV ChiA were able to complement the absence of BmNPV ChiA in the terminal liquefaction process of infected larvae.

The potential activity of *S. marcescens* ChiC protein, used in this study, was previously studied by Danişmazoğlu et al. (2015), who tested the mortality not only on ChiC protein but also chiA and chiB proteins of *S. marcescens* on *Malacosoma neustria* (Linnaeus, 1758) and *Helicoverpa armigera* larvae. Among these 3 chitinase proteins, ChiC produced the highest potential activity against used insect larvae. At another study, the same *S. marcescens* chitinase B and C proteins were transformed

to *Bacillus thuringiensis* strains containing crystal proteins, and the potential activity of the recombinant bacteria was tested on *G. mellonella* larvae (Ozgen et al. 2013). Mortality rates recorded were between 45 and 55%. In the present study, the chitinase C protein of *S. marcescens* was tested on *G. mellonella* larvae and displayed around 50% potential activity at 2 μg protein amount per larvae. Insecticidal proteins, which provide important tools to control insect pests, can be used in conjunction with microorganisms. Virion-free proteins of a GV (GVPs) were used with an NPV on *H. armigera* larvae (Shigeyuki and Chie 2007). Their results showed that the addition of GVPs successfully enhanced NPV infectivity in *H. armigera* without any negative influence on NPV pathogenicity.

## Conclusion

Two viral and a bacterial isolates were compared to each other in terms of their potential activity. The results showed that all the 3 chitinases produced close insecticidal effects on *G. mellonella* larvae. Testing these proteins on more insects would be more informative to conclude which microbial chitinases are more effective. Chitinases and other insecticidal proteins alone or in conjunction with other control agents may contribute to the development of new biopesticides.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-021-00435-0.

**Additional file 1: Supplementary Fig. 1.** Experimental design of insecticidal activity test. Five different virus concentration (indicated on tubes) was used. Every virus concentration was tested on 30 larvae for one trial. Experiment was repeated three times.

**Additional file 2: Supplementary Fig. 2.** Conserved domain structures of *Serratia marcescens*-ChiC (A), AcNPV-Chi (B), and CpGV-Chi (C) proteins.

**Table 2** LT$_{50}$ values of AcNPV-Chi, CpGV-Chi, and *Serratia marcescens*-ChiC proteins[a]

| Chitinase | LT$_{50}$ (days) | Slope±SE |
|---|---|---|
| AcNPV-Chi | 8.600 (6.55–11.28) | 1.882 ± 0.060 |
| CpGV-Chi | 7.199 (5.62–9.20) | 2.086 ± 0.055 |
| S.marcescens-ChiC | 8.639 (6.76–11.04) | 2.110 ± 0.054 |

[a]LT$_{50}$ values were calculated using the 2.5 μg per larvae protein concentration.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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