Minireview

An overview of viral chitinases: General properties and biotechnological potential

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Impact statement

Chitinases form an extensive group of glycoside hydrolases (GHs) and are widespread in nature. These enzymes are essential in many biological processes and can be found in several microorganisms, including viruses. Chitinases have many biotechnological applications, and viruses can be important sources of new enzymes. In this minireview, we cover the most recent information about chitinases, including their functions and applications, highlighting their impact on agriculture, industry, and medicine. We focus on viral chitinases and provide new *in silico* structural analysis showing the differences of chitinases found in the virosphere. Moreover, based on robust phylogenetic analysis, we discuss the possible origins of these viral enzymes, proposing new scenarios for the emergence of chitinases among viruses. Finally, we hope that the body of information in this study, allied with a better understanding and characterization of viral chitinases, will generate many possibilities around industrial and biotechnological processes involving those enzymes.

Abstract

Chitin is a biopolymer profusely present in nature and of pivotal importance as a structural component in cells. It is degraded by chitinases, enzymes naturally produced by different organisms. Chitinases are proteins enrolled in many cellular mechanisms, including the remodeling process of the fungal cell wall, the cell growth process, the autolysis of filamentous fungi, and cell separation of yeasts, among others. These enzymes also have properties with different biotechnological applications. They are used to produce polymers, for biological control, biofilm formation, and as antitumor and anti-inflammatory target molecules. Chitinases are classified into different glycoside hydrolase (GH) families and are widespread in microorganisms, including viruses. Among them, the GH18 family is highly predominant in the viral genomes, being present and active enzymes in baculoviruses and nucleocytoplasmic large DNA viruses (NCLDV), especially chloroviruses from *the Phycodnaviridae* family. These viral enzymes contain one or more GH domains and seem to be involved during the viral replication cycle. Curiously, only a few DNA viruses have these enzymes, and studying their properties could be a key feature for biological and biotechnological novelties. Here, we provide an overview of viral chitinases and their probable function in viral infection, showing evidence of at least two distinct origins for these enzymes. Finally, we discuss how these enzymes can be applied as biotechnological tools and what one can expect for the coming years on these GHs.

Keywords: Chitinases, virus, glycoside hydrolase, biotechnology, enzymes

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Introduction

Chitin is one of the most abundant biopolymers in nature and an essential structural component of arthropods and fungi. This polysaccharide forms tight arrangements that give high resistance against biotic and abiotic pressures. Furthermore, it is widely used in biomedicine, most often for chitosan production, a pseudo-natural cationic polymer with several applications.[1] They are degraded by chitinases, naturally produced by different organisms in nature. They are GH enzymes (EC 3.2.1.14) capable of breaking the chitin molecule into oligomers of lower molecular weight by the cleavage of β-1,4-O-glycosidic bonds. These enzymes are present in many bacteria, fungi, plants, and animals and are widely used as biotechnological tools.[2]

Chitinases are essential enzymes enrolled in different cellular mechanisms, including the remodeling process of the fungal cell wall, growth process, autolysis of filamentous fungi, and cell separation of yeasts.[3,4] Those of bacterial origin have also demonstrated substantial protective activity against fungal growth.[5] In plants, they are essentially produced as a defense mechanism to stress situations, whether related to pathogenic organisms or environmental and abiotic factors.[6] These enzymes also have interesting properties with different biotechnological applications. They are used to produce polymers, for biological control, biofilm formation, and as antitumor and anti-inflammatory target molecules.[7–10] In agriculture, the inhibitory properties of chitinases have an impact on the biological control of pests and fungal plant diseases.[11,12]

Chitinases are classified as endo or exochitinase, by amino acid sequence as GH18, GH19, GH23, and GH48 families, and by class I to VI based on their gene sequences. Among them, the GH18 family is highly predominant in microorganisms like bacteria from the *Salmonella* genus, the slime mold myxomycete *Physarum polycephalum*, and *Mycogone perniciosa* fungi.[13–16] Interestingly, these enzymes are rarely found in viruses. Chitinases have been described in members of the *Baculoviridae* family, including granuloviruses and nucleopolyedroviruses that infect arthropods such as butterflies and moths (Lepidoptera). Furthermore, these enzymes have been found in nucleocytoplasmic large DNA viruses (NCLDV), mainly chloroviruses, which infect green algae.[17–19] In baculoviruses, chitinases are related to the release of virion particles from the cells, and some of these enzymes have antifungal activity, evidencing a promising biotechnological potential for these viral enzymes.[20] The role of chitinases in chloroviruses is still unclear, and as for the baculoviruses enzymes, they remain poorly explored and characterized. With the recent discovery of new giant viruses (e.g. *Mimiviridae*) and the advance of genome sequencing technology, it is highly plausible that chitinases are more widespread among viruses than initially thought.

Here we provide a general overview of chitinases, focusing on the viral enzymes, and discuss how they act on substrates and their general properties. We show that the viral enzymes are structurally diverse as evidenced by *de novo* protein modeling, and we provide evidence of at least two distinct origins for viral chitinases. Finally, we discuss how these genes/proteins can be applied as biotechnological tools and what one can expect for the coming years on these GHs.

Microbial GH18 chitinases, mechanism of action and protein domains

Chitin is an abundant polymer found in different organisms and is essential for protection and structure. It is considered to be insoluble in water. In crustaceans and fungi, chitin is packed in fibers containing 18–25 chains arranged in an antiparallel fold, also known as α-chitin.[21,22] Chains organized in a parallel fold are called β-chitin, and they are less abundant in nature.[22,23]

Chitinases are hydrolytic enzymes capable of breaking chitin chains, resulting in oligomers of low molecular weight. They are hydrolase enzymes that can break (1→4) β-glycoside bond of *N*-acetyl d-glucosamine and are categorized into families GH18, GH19, GH23, and GH48, which exhibit distinct structures and have different hydrolysis mechanisms.[22,24,25] The GH18 family acts as a catalysis-assisted substrate.[25] They have a catalytic domain in triosephosphate isomerase (TIM), with eight α-helices and eight β-strands (α/β).[8] The catalytic amino acids, aspartate and glutamate, are highly conserved. Glutamate acts as a proton donor, and the carbonyl oxygen of the C-2 acetamido group of the substrate acts as a nucleophile.[2] Besides that, substrate binding residue contains serine and glycine residues (SxGG). This family is classified as subgroups A, B, and C, according to the similarity of amino acid sequences and ligands inserted into the TIM barrel.[26] The subgroup of chitinase A has a single GH18 hydrolytic domain architecture. Chitinase B has a hydrolytic domain and carbohydrate binding-type (CBM) domain at the C-terminal region, and subgroup C has the GH18 catalytic domain and several CBM at the C-terminal end.

The CBM domain is commonly found in amylases that degrade cellulose and some GH and can vary in organisms. They are distributed in one or more modules along the GH proteins and promote the proximity of the protein to its substrate.[27,28] The presence of the CBM domain in the *Trichoderma harzanum* chitinase could increase its hydrolytic activity in insoluble substrates. In the replacement of the CBM domain of endo-1,4-β-glucanase from *Bacillus subtilis* with CBM from *T. harzanum* fungi, the enzyme activity and binding affinity increased, evidencing how this domain can affect enzyme activity.[27,29,30] CBM domain is also present in viral chitinases, although its function is still unknown.

Viral chitinases have one (e.g. ChiA AcMNPV and chitinase ATCV-1) or two GH18 domains (e.g. Chitinase PBCV-1 and Pbi FR483), carbohydrate-binding type-2 domain (CBM2) and the total protein size can vary for each virus, reaching more than 800 amino acids (Figure 1). Repeat GH18 domains are also found in other organisms, and the role of this event is not well understood. These domains can have different amino acid sequences showing different evolutionary origins, and it is suggested that they can act synergistically to degrade chitin polymers.[31,32] The CBM2 domain contains six cysteine residues, and it is associated with GH18 hydrolases to degrade insoluble chitin via binding with highly conserved aromatic residues.[27,32] They are widely present in chloroviruses, suggesting that these domains can be important structural keys of chitinase enzymes to degrade the host cell wall.

Chitinases in the virosphere

Chitinases are not commonly present in viruses, and the enzymes identified so far are from the GH18 family. They were described in the *Baculoviridae* family, such as granuloviruses of *Cydia pomonela, Epinotia aporema*, and *Pieris rapae*, and in nucleopolyhedrovirus of *Dendrolimus kikuchii.* They are also observed in chloroviruses, like Paramecium bursaria chlorella virus 1 (PBCV-1), which infects algae from the *Chlorella* genus.[17,20,33–36] The first and widely described viral chitinase is ChiA from Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV).[37] Proteomic analysis of the AcMNPV budded virus revealed the presence of chitinase.[38] ChiA interacts biochemically with viral cathepsin inside the host since it is required for activating pro-cathepsin into its proteolytic form to modulate the liquefaction mechanism in host cadaver.[39] It is related to the liquefaction of infected host larvae after death to liberate occlusion bodies (OB) and viral particles, an essential step for maintaining the viral replication cycle.[20] This viral chitinase showed important insecticidal activity, as observed for chitinase from *Serratia Marcescens* bacteria, although both enzymes have different domain structures.[40]

Some chitinases of *Cydia pomonela* and *Epinotia aporema* granuloviruses lack a C-terminal KDEL motif, indicating that they can be secretory proteins, and the interaction with cathepsins seems to be conserved in all granuloviruses and

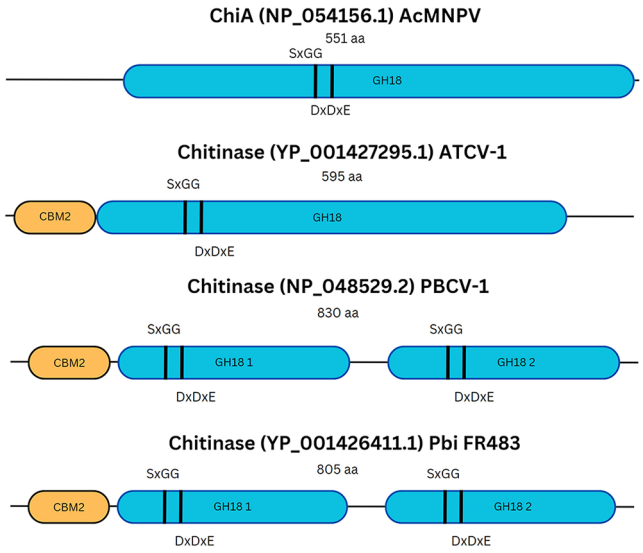

Figure 1. Representative structures of viral GH18 chitinases.
Amino acid sequences from nucleopolyhedrovirus and chloroviruses were taken from NCBI databases. Domain prediction was made by Prosite Expasy and InterProScan platforms. Schemes are meant for structural comparisons only and are out of scale. Only one representative chlorovirus from each clade was considered as a comparative model: PBCV-1 as NC64 viruses, ATCV-1 as SAG viruses, and FR483 as Pbi viruses.

nucleopolyhedroviruses.[20,41] In some granuloviruses, chitinases can also be found as intracellular proteins along the host cytoplasm.[34] Chitinase of Dendrolimus kikuchii Matsumura nucleopolyhedrovirus (DkNPV) has both endo and exochitinase properties and antifungal activity, thus exhibiting potential for biotechnological application as microbiological control.[40]

Horizontal gene transfer can occur between viruses and their hosts, and it's not surprising that baculoviruses have chitinase genes to go through the physical barrier and degrade the strong chitin polymer present in the insect cells.[42] On the contrary, chloroviruses infect algae from the *Chlorella* genus, whose cell wall is mainly composed of cellulose and secondary sugars, with the exception of *Micractinium conductrix*, the only algae that has a chitin-like glycan constituting the cell wall.[43] Thus, the presence of functional chitinase genes in these viruses remains uncertain. Nevertheless, in some circumstances, chitinases can also interact and degrade cellulose polymers, and this might be the case for the chloroviruses GHs.[44–46] Chloroviruses are nucleocytoplasmic large DNA viruses (NCLDV, *Nucleocytoviricota* phylum) from the *Phycodnaviridae* family that infect unicellular zoochlorella.[47] Giant viruses have large genomes, with a wide range of genes capable of coding diverse proteins, including those related to DNA repair, transcription factors, and even metabolic enzymes.[48,49] Interestingly, chloroviruses differ from other giant viruses since they are rich in enzymes involved in carbohydrate degradation, including GH18 chitinases and chitosanases.[50,51]

The presence of these enzymes can vary in each chlorovirus clade since they are host-specific, infecting *Chlorella variabilis*, *Chlorella heliozoae*, and *Micractinium conductrix*, commonly referred to as NC64A, SAG, and Pbi clades, respectively.[47] Chitinases from NC64A viruses CVK2 and PBCV-1 were described as being expressed in the late phase of infection and showed to be active enzymes.[19,35,52] These hydrolases were not found in proteome analysis of PBCV-1, suggesting their absence in virion particles and thus not involved in the initial degradation of the chlorella cell wall.[51] Considering that the transcripts encoding these enzymes and respective proteins occur in the late phase of infection, it is hypothesized that they are important for the degradation of the strong host cell wall to release the virion particles from the cells at the final stage of the replication cycle, a similar mechanism found in granuloviruses.[19] However, there is a lack of information about the molecular characterization of these enzymes, how they interact with the substrates, and their role in the viral cycle.[19,53]

For the viral chitinases of nucleopolyhedroviruses, granuloviruses, and chloroviruses representatives, we modeled the proteins by the *ab initio* method using AlphaFold to have insights into each viral chitinase 3D structure and to compare their similarities, overlapping their domains.[54] The baculoviruses enzymes have high structural similarities with each other but have divergent predicted structures compared with the PBCV-1 protein (Figure 2(A) to (F)). Nucleopolyhedroviruses and granuloviruses proteins showed great overplotting of the domains, α-helices, and β-chains (Figure 2(G)). The alignment between their structures with PBCV-1 chitinase evidenced a major difference among the proteins. The presence of two GH18 domains confers a larger volume to the 3D predicted structure of chlorovirus enzyme compared with baculoviruses chitinases, evidencing the high divergence among viral chitinases (Figure 2(F) and (H)).

Curiously, the gain of genes from the hosts is not the only mechanism that could explain how viruses have chitinases. Searching for viral chitinase homologs in public databases, we found that a few giant viruses also have putative GH18 hydrolases, such as Marseillevirus LCMAC201, *Tupanvirus salinum*, *Tupanvirus altamarinense*, *Homavirus sp.*, *Satyrvirus sp.* and *Fadolivirus*, even though the known host of some of these viruses (amoeba from *Acanthamoeba* genus) does not have GH18 hydrolases.[55] In that sense, an intriguing mystery resolves around the origin of the viral chitinases. Different phylogenetic studies using gene sequences of these proteins in baculovirus and chloroviruses provided distinct proposals for the emergence of these sequences, which are still poorly represented within databases. Previous phylogenetic reconstructions based on distance matrix and maximum parsimony methods suggested that baculoviruses acquired the chitinase genes from γ-proteobacteria through horizontal gene transfer.[41] The relationship between chitinases from baculovirus and proteobacteria was pointed out, especially from the species *Serratia marcescens*.[35,56] Although chitinase of this bacterium had 61% identity with ChiA of AcMNPV, it seems that they have different origins, representing different lineages.[33,57] These viruses have insects of the Lepidoptera order as hosts, and gene sequences for GH18 chitinases are also found in these animals. There is evidence indicating that the chitinase genes in these insects may have been acquired from a bacterium or a baculovirus.[20,57] However, new analyses adding more viral and bacterial sequences, and focusing on the GH18 domains, raised doubts regarding the

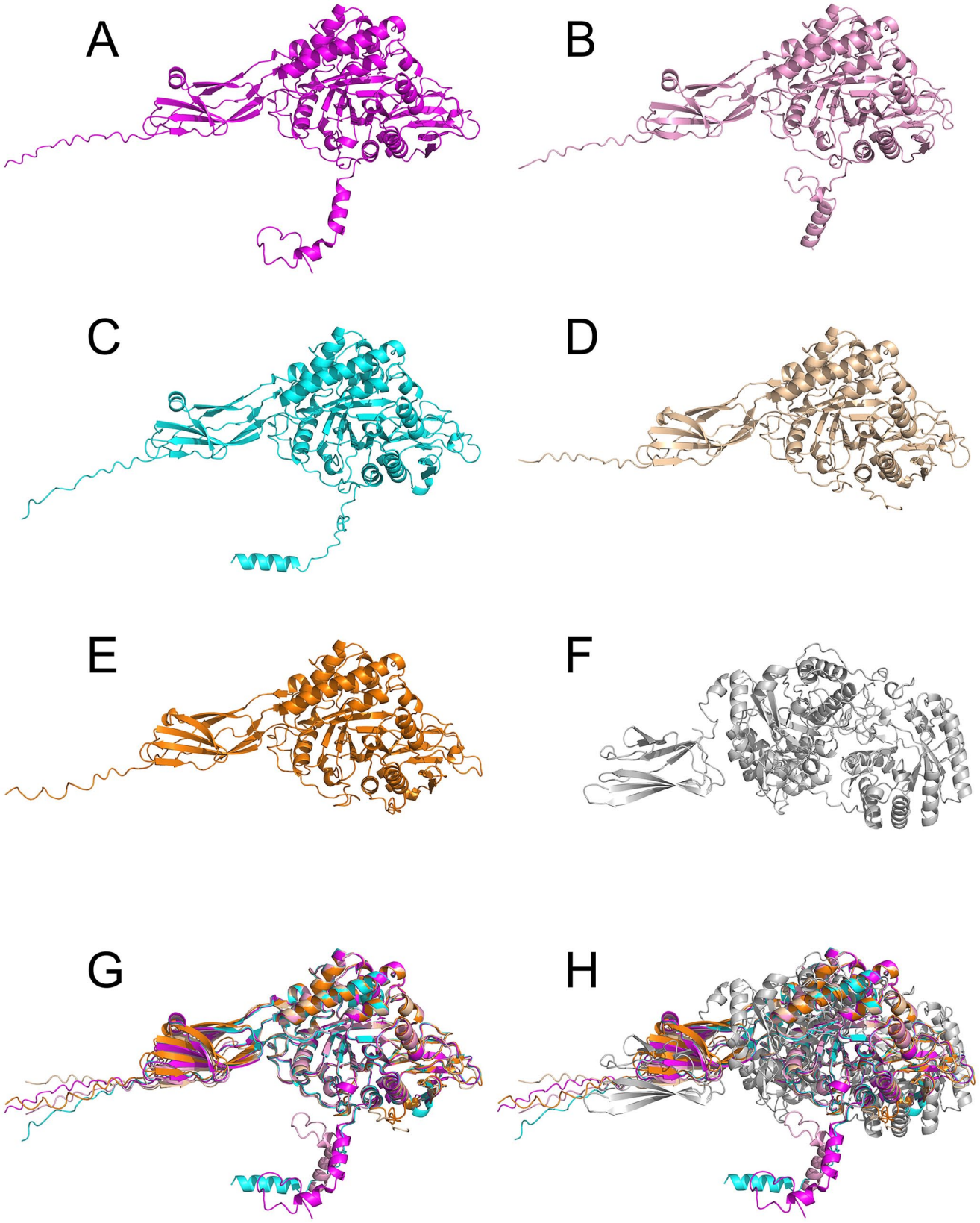

Figure 2. Models of viral chitinase proteins. Modeled structures obtained with AlphaFold are represented by cartoons. (A) *Cydia pomonella* granulovirus (magenta), (B) *Epinotia aporema* granulovirus (pink), and (C) *Pieris rapae* granulovirus (cyan), which has a longer C-terminal chain in comparison to (D) *Autographa californica* nucleopolyhedrovirus (wheat) and (E) *Dendrolimus kikuchii* nucleopolyhedrovirus (orange), (F) PBCV-1 (gray) has two GH18 domains, which highlights its structural differences with other viral chitinases, corroborated by the structures 3D alignment (G) with and (H) without PBCV-1. Images were generated with PyMOL (v0.99c).

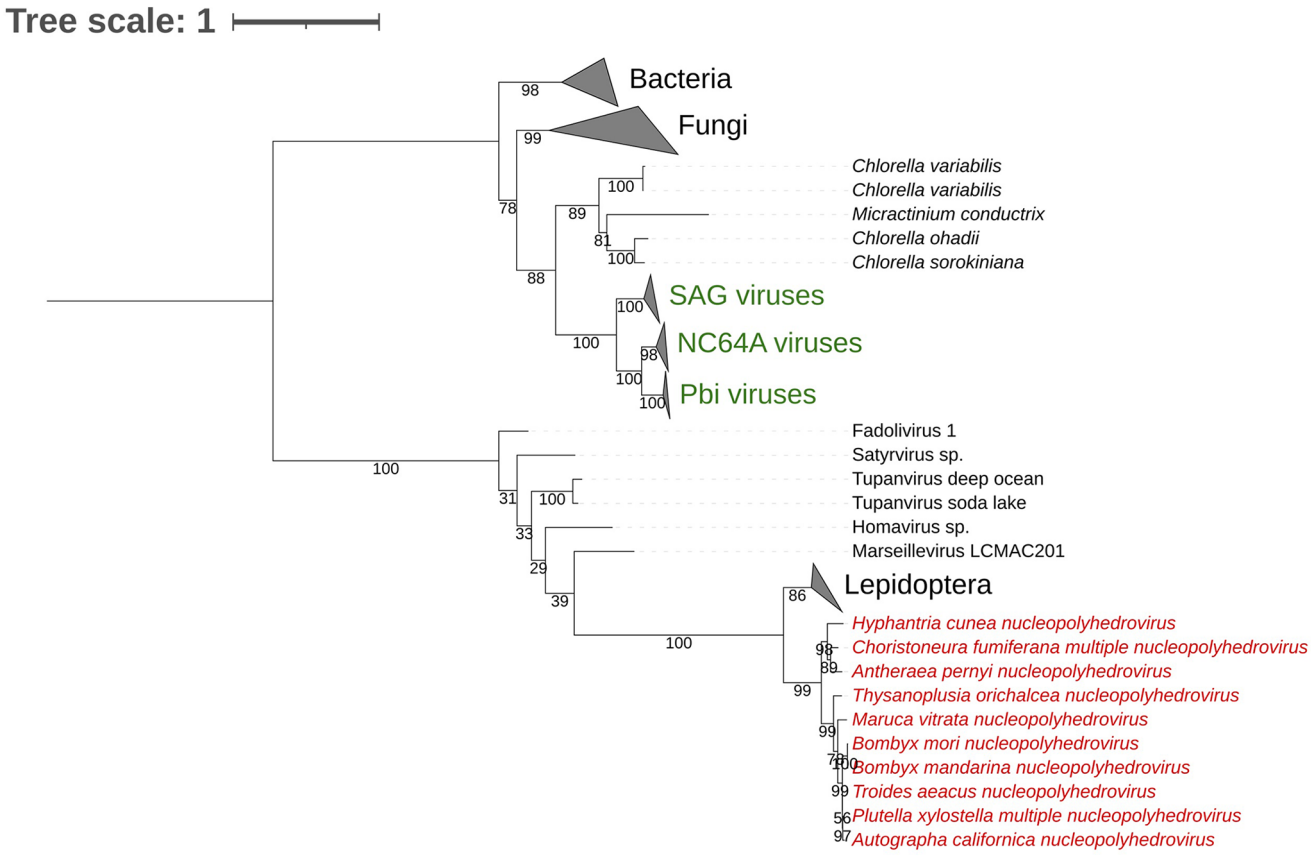

Figure 3. Phylogenetic reconstruction of viral chitinases.
Maximum likelihood tree constructed with 1000 bootstrap replicates based on the alignment of amino acid sequences of chitinases GH18 found in different viruses and cellular organisms. The tree was built using IQ-tree 2.2.0 and visualized using iToL. The scale bar represents the rate of evolution. Green: chloroviruses from distinct lineages; Red: baculoviruses.

origin of these genes in viruses.[33] Phylogenetic characterization of chloroviruses chitinases and chitosanase fueled the discussion on the origins of these viral genes, suggesting that the PBCV-1 *a181/182r* chitinase gene possibly shared an ancestor with a thermophilic bacterium and an insect of the Hymenoptera order, despite the common ancestor of a gene found in tobacco.[35] The discovery of chitinase in the *Chlorella* strain NC64A indicated a new scenario, given the high similarity between the algae hChti1 gene and the chlorovirus vChti1 gene, wherein the viral gene might have been horizontally transmitted between a specific strain of chlorella and their viruses.[58] Two other scenarios appear in previous studies indicating the possible origin of the chitinase genes in Chlorella and chlorovirus. The chitinase genes may have been acquired from an algal ancestor, and lineages other than Chlorella have lost genes through evolution. Another scenario implies that a horizontal gene transfer event has occurred, passing genes from fungi or prokaryotes to Chlorella. The viruses then acquired the host's genes.[58]

Considering that the origin of the viral chitinases is still under debate, we performed a new phylogenetic analysis and propose an alternative scenario. Differently from the previous study and taking advantage of the genomic advances over the past years that provided a lot of new sequences in the databases, we included in the analysis different viral genes found in distinct groups of viruses, that is, baculoviruses, chloroviruses, and the recently described giant amoebae viruses. Considering sequences from other microorganisms, like bacteria and fungi, and genes found in the viral hosts whenever available, we observed two distinct origins of the viral chitinases (Figure 3). For the chloroviruses, the most likely scenario is that an ancestor virus obtained the gene by HGT from an ancestor chlorella host only once in the evolutionary history, and this gene was maintained in the different lineages of chloroviruses, resulting in homologs in all relatives described so far. This is interesting since chloroviruses are highly host-specific, and it is most likely that the chitinase gene was present before the irradiation of the chloroviruses and the chlorella algae. For the baculoviruses, the scenario is a bit more intriguing. The chitinase genes of baculoviruses share a common ancestor with genes of insects of Lepidoptera order, indicating an HGT event where the viruses acquired the genes from their hosts and not from bacteria as previously suggested[27,39,41] (Figure 3). However, both baculoviruses and Lepidoptera share ancestors with giant amoebae viruses in this analysis, and one could suggest that the insects acquired the gene from the viruses. It is important to note that baculoviruses and nucleocytoviruses have different evolutionary histories, and there is no evidence that they share any common ancestor. Taking it altogether, it is clear that chitinases found in chloroviruses and baculoviruses have different origins. Structural analysis

of these proteins corroborates our phylogenetic data (Figure 2). Based on this analysis, we cannot confidently indicate the baculoviruses genes origin. An in-depth investigation including a more comprehensive dataset could provide a clear evolutionary picture, which is out of the scope of this work. Also, the identification of new giant viruses bearing a chitinase gene will be pivotal to elucidating the origin of this second group of GH18 viral chitinases.

Biotechnological application of chitinases

In the cellular context, chitinases participate in remodeling the fungal cell wall, in the microbial growth process, autolysis of filamentous fungi, and cell separation of yeasts.[3,4] Those of bacterial origin have also demonstrated high protective activity against fungal growth.[5] In plants, they are essentially produced as a defense mechanism to stress situations, whether related to pathogenic organisms or environmental and abiotic factors.[6] These enzymes have properties that allow them a diverse biotechnological application.

Chitinase can be useful in biofuel production as oligomers resulting from *Bacillus haynesii* chitinase hydrolysis demonstrate increased ethanol efficiency production from colloidal chitin.[59] Chitinases slowly degrade natural chitin from animals, insects, and plants. Some industrial processes have this polymer as a waste product, demanding an efficient and faster method. These are limiting factors to natural hydrolases. The identification and production of new active enzymes is a major demand.[60] As one of the most impactful activities for economy and culture, fishery produces tons of chitinous residues that are released in oceans or soil, causing extensive environmental damage. Microbial chitinases constitute a promising alternative for chitin recycling and degradation, and this process can lead to the synthesis of many products.[61] From chitin hydrolyzation by chitinase, N-acetyl-D-glucosamine and N-acetyl chitooligosaccharides, as well as GlcNAc can be produced and used in many areas and processes.[62,63] They are also potential anti-biofilm enzymes, demonstrating inhibition of *Francisella* bacteria biofilm, an organism associated with environmental persistence in aquatic habitats and water transmission.[64,65]

Monocultures and plants are highly susceptible to invasion by pests and infectious agents.[66,67] Pesticides of chemical origin have been used for over 80 years in agronomy.[17] In 2017, more than 2.3 million tons of pesticides were used worldwide, and despite their effectiveness, the excessive use of these products is harmful to the environment, plants, and animals. Contamination of the water and earth also plays a role in selecting resistant pests.[68,69] As a result, in the past few years, there has been significant industrial effort regarding more sustainable biological control methods.[70] Biopesticides have higher selectivity, mainly related to the interaction of the microorganism with its host. They are used in smaller amounts, presenting lower risks to the environment and human health.[71] This method is on the rise, and forecasts indicate that they will match the synthetic market in about 20–30 years.[72]

In this context, microbial chitinases can be applied as promising biological control. Their potential activity against fungi and insects has been described.[73] Studies using the ChiA from the baculovirus AcMNPV showed fungicidal activity on *Botrytis cinerea*, related to "gray rot" of fruits, and *Alternaria alternata*, which causes spots and death on plant leaves. In *in vitro* analyses, the purified protein inhibited fungal spore germination, and tobacco leaves expressing the protein showed a certain degree of resistance to infection by *B. cinerea*.[73] Tests with recombinant protein in the tissue of *Bombyx mori* larvae demonstrated alterations in the structure and permeability of the insect, and the protein consumption by the larvae showed a high mortality rate.[74] In addition, many studies involving the administration of a transgenic plant diet with ChiA and DkChi proteins (the latter present in *D. kikuchii*) showed increased mortality rates and decreased metabolic rates, such as growth and trypsin synthesis in different insects.[18,75,76]

As an alternative to chemical fungicides, methods using bacteria are used for biological control, and chitinases are one of the weapons for suppressing these organisms.[36] One of the techniques applied is the production of transgenic plants expressing chitinase genes from bacteria to create vegetables with resistance against pathogens.[77] Two bacteria from the *Enterobacteriaceae* family, *Serratia marcescens* and *Enterobacter agglomerans*, are models for eliminating many pathogenic fungi.[78] Another use of microbial chitinases is related to defense against insects and diseases. For example, *Bacillus* sp. has chitinases used in the control of *Aphis gossypii*,[79] and a chitinase from one of these species, *Bacillus thurigiensis*, is a component of a biopesticide against the diamondback moth larvae.[80] With the capacity of hydrolyzing chitin, chitinases can be used for creating pores in the peritrophic membrane, making the insect more susceptible to the effects of toxins and pesticides. The chitinase SBK1 in *Aeromonas hydrophila* showed positive results in insecticidal tests against *Culex quinquefasciatus*.[81] These enzymes also showed insecticidal effects against nematodes and can be used in the biological control of *M. arenaria* and *H. avenae*.[82,83]

In the past few years, these enzymes have been explored as microbiological control and biomedical molecules. *Trichosanthes dioica* seeds chitinase demonstrate antitumor activity in human breast and colorectal cancer cells ***in vitro*** and ascites carcinoma cells ***in vivo***.[84,85] Purified chitinase from bacteria and fungi also demonstrates antitumor potential.[86,87] They also exhibit anti-inflammatory properties, tissue remodeling, and injury.[88] Finally, human chitinase and *chitinase-like* proteins (CLP) have been studied as a therapeutic target as they showed to be enrolled in the modulation of inflammatory bowel diseases (IBD).[89]

Conclusions and perspectives

Microbial chitinases are essential enzymes for different cell processes. These proteins are enrolled in composition, protection, pathogenesis, and microbial survival against harsh conditions. Chitinases are important in terms of cellular biology and also in diverse applications in industry and biotechnology fields. These enzymes demonstrated potential application in the industry to produce biofuels, modified plants, and biomedicine as antitumoral and anti-inflammatory targets. Notably, viral enzymes have been used as

biotechnology tools for decades, especially in molecular biology, such as DNA ligases and replicases (e.g. reverse transcriptase). We can expect viruses to be impressive sources of new enzymes, and chitinases must be in sight. As evidenced here, only a few viral chitinases are known. Despite that, there is good evidence of their potential application in different fields, the most promising being the biological control of fungi and crop pests. These viral GHs have distinct origins, and the discovery and characterization of new viruses shall improve our comprehension of their biology and evolution. Most importantly, this will allow new products applicable in many fields of biological sciences, medicine, and the biotechnology industry. We have only scratched the surface in the field of viral chitinases, and the development of this field will open doors for advances in many different ways.

AUTHORS' CONTRIBUTIONS

EGO and CACF conducted the literature review, bioinformatics analysis, and wrote the first draft of the manuscript; RALR participated in the design of the study, interpretation of the data, and review of the manuscript. All the authors read and approved the final version of the manuscript. This project is registered at SISGEN, accession A8952E4.

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