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Review Article

Chitinases as Antibacterial Proteins: A Systematic Review

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ABSTRACT
Chitinases are hydrolases that catalyze the cleavage of the β-1,4-O-glycosidic linkages in chitin, a polysaccharide abundantly found in nature. Chitin is an important structural component of the cell wall of most fungi and the exoskeleton of arthropods, including insects and crustaceans. These enzymes are widespread in the living world, being found in organisms from all three domains of life. Due to their hydrolytic activity on chitin, chitinases have great biotechnological potential in different areas, such as human health, agriculture and food technology. The antifungal, insecticidal and nematicidal effects of many chitinases have been intensively investigated in the scientific literature, aiming to exploit these properties to protect crops against phytopathogenic fungi and insect pests and parasitic nematodes. On the other hand, the effects of chitinases on bacteria have been underexploited, possibly because chitin is not present in bacterial cell walls. The aim of this study was to search the scientific literature for works describing chitinases with antibacterial activity. Three bibliographic databases were searched using the keywords "chitinase" and "antibacterial" as descriptors and the chosen articles were selected according to specific inclusion and exclusion criteria. As a result, we identified only 5 reports wherein 6 purified chitinases have been shown experimentally to have antibacterial activity. Three out of these 6 antibacterial chitinases were shown to be bifunctional enzymes, which have chitinase and lysozyme activity. The possible mechanism of action of these antibacterial chitinases is discussed, highlighting their potential as antibacterial agents.

Key words: Antimicrobial, Chitin, Lysozyme, Peptidoglycan, Hydrolases.

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INTRODUCTION

Chitin is a linear and water-insoluble polysaccharide constituted by *N*-acetyl-β-D-glucosamine (GlcNAc) units linked through β-1,4 linkages. This polymer is the most abundant amino polysaccharide in nature and after cellulose, is the second most plentiful biopolymer on earth. It is an important structural component of the cell wall of many fungi and the exoskeleton of arthropods, such as insects and crustaceans, like crabs, shrimps and lobsters. Chitin is also found in the shell and radula of mollusks and the cuticle and egg shell of nematodes.[1] Recently, Tang and co-workers have obtained evidences that chitin is endogenously produced in non-mammalian vertebrates, including fishes and amphibians.[2]

Chitinases (EC 3.2.1.14) are glycoside hydrolases (GHs) that catalyze the cleavage of the β-1,4 glysosidic bonds between the GlcNAc residues that form the chitin chains. Based on the similarities of their amino acid sequences, most chitinases are grouped into the GH18 and GH19 families according to the current classification of the Carbohydrate-Active Enzymes (CAZy; http://www.cazy.org) database.[3,4] Chitinases are found in a wide range of organisms including humans, seed plants, insects, bacteria and fungi. These enzymes are involved in a variety of biological processes, such as the remodeling of chitin in the cell walls of fungi and the exoskeleton of arthropods during the periods of growth and development,[5] the utilization of chitin as a source of carbon and nitrogen by many bacteria [6] and participation in defense mechanisms against pathogens.[7] In plants, for example, besides being expressed in different tissues and organs during the regular growth and development, some chitinases act as pathogenesis-related proteins, whose expression is upregulated in response to chitin-containing pathogens. The hydrolytic action of these induced chitinases on the chitin fibers of the pathogen's cell wall impairs its growth and spread, whereas the chitin oligomers released are recognized by plant chitin receptors, which trigger other defense reactions.[8] In carnivorous plants, some chitinases also play a digestive role, being used along with other hydrolytic enzymes to digest caught prey in their pitchers.[9]

The great interest in the study of chitinases primarily relies on their enzymatic action on chitin. Endo-chitinases, for example, randomly cleave chitin chains at internal sites, producing low molecular mass chito-oligomers with 2 to 6 GlcNAc units, whereas exo-chitinases catalyze the progressive release of *N*,*N*'-diacetylchitobiose $[(GlcNAc)_2]$ or *N*,*N*',*N*''-triacetylchitotriose $[(GlcNAc)_3]$ from the chitin chains.[10] Some exo-type chitinases attack the chitin chains at the non-reducing end, but others cleave the polymer at the opposite end.[11] Due to their ability to degrade chitin, many chitinases can cause damages to the cell walls of fungi and cuticles of insects. The antifungal and insecticidal activities of these enzymes have attracted the attention of the biotechnologists, which has led to the development of transgenic crops with enhanced resistance to fungal pathogens and insect pests.[12] Chitinases with toxic effects towards plant-parasitic nematodes have also been investigated as an alternative strategy to protect crops from the serious damages these organisms cause in many parts of the world.[13] The nematicidal effect of chitinases is also due to their degradative activity on chitin chains, which causes destruction of the nematode's cuticle, intestine and egg shell.[14] Furthermore, it is also well documented that *N*-acetyl-chitooligosaccharides, which can be obtained from chitin by treatment with chitinases, have antibacterial, antifungal, metastasis suppression and other biological

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activities, thus showing great biotechnological potential in areas as diverse as food technology, human health and agriculture.[15]

The aim of this study was to search the scientific literature for works describing chitinases with antibacterial activity and summarize their main findings, discussing the probable mechanisms responsible for the antibacterial activity of these proteins and their potential applications.

MATERIALS AND METHODS

Searches were performed on the following bibliographic databases: PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (https://apps.webofknowledge.com/) and Scopus (http://www.scopus.com). Full-text articles, published until November 2017 and containing the words "chitinases" and "antibacterial" in their titles or abstracts were selected for further analysis. The appropriate papers were selected based on 2 criteria: (a) the reported antibacterial tests were performed using pure protein samples, as evidenced by denaturing gel electrophoresis, for example; and (b) the tested protein was a genuine chitinase, able to degrade chitin or chitin derivatives, as demonstrated by experimental data.

RESULTS

By searching the scientific literature, we have identified only 5 works that reported the purification and partial characterization of chitinases with antibacterial activity (Table 1). These chitinases included 3 proteins from bacterial species, ChiS from *Bacillus pumilus* SG2 [16] and FI and FII from *Pseudomonas aeruginosa* K-187,[17] 2 proteins from fungi species, *Aspergillus terreus* [18] and *Monascus purpureus* CCRC31499,[19] and one protein (CpCHI) from papaya (*Carica papaya*).[20] In most of these papers (4 out of 5), the antibacterial activity of the protein was determined using the agar disk-diffusion method, whereas in one of them,[20] the authors used a dilution method. The chitinases FI and FII from *P. aeruginosa* K-187 showed a large spectrum of antibacterial effect, being able to inhibit the growth of different Gram-positive and Gram-negative species.[17] In all these works, the chitinolytic nature of the purified proteins was demonstrated using enzymatic assays, in which their ability to degrade either colloidal chitin or glycol chitin was verified. For ChiS and CpCHI, the authors also reported the amino acid sequences of the studied proteins, as deduced from their DNA coding sequences, which confirmed their identities as chitinases, belonging to the GH18 (ChiS) and GH19 (CpCHI) families. Three (ChiS, FI and FII) out of these 6 antibacterial chitinases had lysozyme activity, which was measured by the ability of the tested proteins to cause bacterial cell lysis, as evidenced by the decrease in optical density of a cell suspension of the target microorganism exposed to the enzyme. The *M. purpureus* chitinase did not show lysozyme activity, when tested towards a cell suspension of *Micrococcus lysodeikticus*, whereas for CpCHI and the chitinase from *A. terreus*, this biochemical activity was not investigated.

Besides the reports listed above, Wang and co-workers [21] described the purification of Hypotin, a protein from peanut (*Arachis hypogaea*) seeds which showed antifungal activity towards a variety of fungi species as well as antibacterial activity towards *Staphylococcus aureus*. The classification of Hypotin as a chitinase was based on the similarity of its N-terminal amino acid sequence with already known plant chitinases. However, those authors did not show any experimental evidence on the ability of Hypotin to degrade chitin or chitin derivatives, and their work was not included in our analysis.

It is noteworthy to mention that we also identified one paper that described the purification of a 55-amino acid residues peptide from the marine mussel *Mytilus coruscus*, which was named mytichitin-CB, that showed antifungal as well as antibacterial activity towards Gram-positive species.[22] Cloning of the cDNA encoding mytichitin-CB revealed that mytichitin-CB is derived from the processing of a chitotriosidase/chitinase precursor with 446 amino acid residues. Nevertheless, no antibacterial activity of the chitotriosidase/chitinase itself was demonstrated, and this work was not included in the present analysis.

DISCUSSION

Very few works have reported the characterization of chitinases with antibacterial activity (Table 1), contrary to their antifungal activity, which has been documented since 1980s.[23] Unlike fungi, bacteria cell walls do not contain chitin. Although genes encoding chitin synthase (CHS; EC 2.4.1.16), which catalyzes the elongation of chitin, have been recently discovered in some bacterial genomes,[24] species containing CHS genes represent only 0.9% of the 1218 bacterial genomes analyzed and to date there is no experimental evidence that chitin occurs in these few species. This is probably the main reason why many researchers have neglected bacterial species as possible targets for the action of chitinases. However, it has been shown that some chitinases from diverse sources, besides being able to cleave the O-glycosidic bonds in chitin chains, also have lysozyme activity.[25,26,27] Hevamine, a GH18 chitinase firstly purified from the latex of *Hevea brasiliensis*, is probably the better known chitinase with lysozyme activity.[28,29]

Most bacterial cells are encased by a cell wall constituted mainly by a mesh-like layer of peptidoglycan (PG), also known as murein, which guarantees cell integrity and shape. PG macromolecule is constituted by glycan chains cross-linked by short peptides. These glycan chains are composed of alternating residues of GlcNAc and *N*-acetylmuramic acid (MurNAc) linked by β-1,4 bonds.[30,31] MurNAc is the ether of lactic acid and GlcNAc, in which a D-lactate residue is attached to the C-3 atom of the glucopyranoside ring. Indeed, the first step during PG biosynthesis is the conversion of UDP-GlcNAc to UDP-MurNAc.[32]

Lysozymes (muramidases) (EC 3.2.1.17), are glycoside hydrolases that cleave the β-1,4 linkages in murein, causing bacterial cell lysis, as first observed by Alexander Fleming.[33] Besides their direct bacteriolytic action, these proteins also have an immunomodulatory function in the host's response to infection.[34]

Three (ChiS, FI and FII) out of the 6 antibacterial chitinases listed in Table 1 showed lysozyme activity which could be related to their bactericidal properties. ChiS is a GH18 enzyme, like hevamine, but the amino acid sequences of FI and FII were not reported, and the GH families to which they belong are not known. GH18 members adopt a conserved $(\beta/\alpha)_8$-barrel fold that do not share similarity with the lyzosyme-type fold. Furthermore, the cleavage specificities of hevamine and lysozymes for PG are distinct: hevamine hydrolyzes the linkage between the C-1 of GlcNAc and the C-4 of MurNAc,[35] whereas lysozymes cleave the PG chains between the C-1 of MurNAc and the C-4 of GlcNAc.[36,37,38,39] Considering the conservation of the three-dimensional structure of GH18 members from diverse taxa,[40] one can speculate that GH18 chitinases which are able to hydrolyze PG molecules exhibit a cleavage specificity similar to that reported for hevamine. In summary, the denomination of these bifunctional GH18 enzymes as chitinases/peptidoglycan hydrolases seems to be more appropriate than the term chitinases/lysozymes.

CpCHI, a GH19 papaya chitinase, exhibited antibacterial activity towards *E. coli*, but the authors did not investigate if the enzyme had lysozyme activity.[20] Besides GH18 enzymes with hydrolytic activity on PG, bacterial and plant GH19 chitinases with the same activity have been also found.[26,41] GH19 chitinases, chitosanases (E.C. 3.2.1.132; family GH46) and lysozymes (families GH22, GH23 and GH24) share a similar fold, and are classified in the lysozyme superfamily.[42] Therefore, CpCHI and other GH19 chitinases probably hydrolyze PG chains, which causes bacterial cell lysis, through the same molecular mechanism as lysozymes.

Table 1: Scientific papers that have reported the characterization of chitinases with antibacterial activity, as reviewed in the present work.

Authors and year of publication	Origin of the studied chitinases	Species to which the chitinases showed antibacterial activity	Main findings
Wang and Chang 1997[17]	*Pseudomonas aeruginosa* K-187, a Gram-negative bacteria	*Bacillus cereus* CCRC 14689, *B. subtilis* CCRC 10029, *B. thuringiensis* subsp. *israelensis* CCRC 11501, *B. thuringiensis* subsp. *kurstaki* CCRC 11498, *B. bassiana* CCRC 31767, *Enterobacter faecalis* CCRC 10789, *Escherichia coli* CCRC 51445, *Lactobacillus bavaricus* CCRC 12933, *L. lactis* subsp. *lactis* CCRC 10791, *Micrococcus lysodeikticus* CCRC 11034, *Staphylococcus aureus* CCRC 10451 and *S. aureus* CCRC 10777	Two chitinases (FI and FII) were purified from *P. aeruginosa* K-187. Both proteins showed hydrolytic activity towards colloidal chitin, and the optimum pH and temperature for enzymatic activity were 8 and 50 °C (FI) and 7 and 40 °C (FII). Both chitinases also exhibited lysozyme activity, as measured by the ability of the proteins to cause bacterial cell lysis.
Wang *et al.* 2002[19]	*Monascus purpureus* CCRC31499, a fungus from the division Ascomycota	*Bacillus cereus* CCRC10603, *B. subtilis* CCRC10255, *E. coli* CCRC 10239, *P. aeruginosa* K-187, *S. aureus* CCRC10780 and *Streptomyces actuosus* A-151	A 81 kDa chitinase was purified from the fungus *M. purpureus*. The enzyme was able to degrade colloidal chitin, with optimum pH and temperature for hydrolytic activity of 7 and 40 °C. The enzyme exhibited antifungal and antibacterial activity, but had no lysozyme activity when tested towards *M. lisodeikticus*.
Chen *et al.* 2007[20]	Papaya (*Carica papaya*), a seed plant from the family Caricaceae	*Escherichia coli* ED2566 and *E. coli* AD494	A 26 kDa papaya chitinase (CpCHI) was produced in *E. coli* Tuner (DE3), purified and partially characterized. The recombinant enzyme was able to degrade glycol chitin, and the optimum pH and temperature for enzymatic activity were 6.0 and 30 °C. Besides showing antifungal activity, CpCHI was able to inhibit the growth of *E. coli* (IC_{50} = 2.5 μM)
Ghasemi *et al.* 2011[16]	*Bacillus pumilus* SG2, a Gram-positive bacteria	*Xanthomonas translucens* pv. *hordei*, *X. axonopodis* pv. *citri*	A 75 kDa chitinase (ChiS) from *B. pumilus* SG2 was expressed in *E. coli* M15. The purified recombinant chitinase had hydrolytic activity towards colloidal chitin (the optimum pH and temperature for activity were 6.0 and 50 °C), and it also showed lysozyme activity towards all tested bacteria (*X. translucens* pv. *hordei*, *X. axonopodis* pv. *citri*, *B. licheniformis*, *E. coli* C600, *P. aeruginosa* and *P. putida*). ChiS exhibited antifungal and antibacterial activity.
Farag *et al.* 2016[18]	*Aspergillus terreus*, a fungus from the division Ascomycota	*P. aeruginosa*, *S. aureus* and *Salmonella typhi*	A 60 kDa chitinase was purified from the fungus *A. terreus*. The purified protein had hydrolytic activity towards colloidal chitin and the optimum pH and temperature for enzymatic activity were 5.6 and 50 °C. The enzyme showed antifungal and antibacterial activity.

One antibacterial chitinase, purified from the fungus *M. purpureus*, did not show lysozyme activity, when tested towards cell suspensions of *M. lysodeikticus.*[19] Fleming was the first scientist to isolate *M. lysodeikticus*, and due to its high susceptibility to lysozyme, he used this Gram-positive species in his pioneer work,[33] and the same microorganism has been used until today in lysozyme activity assays. Wang *et al.* used *M. lysodeikticus* to determine if the *M. purpureus* chitinase had lysozyme activity, but the antibacterial tests were performed using different Gram-positive and Gram-negative bacteria. Bacteria can evolve efficient mechanisms which protect them from the lytic activity of lysozyme,[43] including *M. lysodeikticus*.[44,45] Therefore, the absence of lysozyme activity of *M. purpureus* chitinase should be carefully interpreted and re-examined, using the same species to which the protein exhibited antibacterial effects. Moreover, large variations in the PG fine structure exist between species, which may occur even within the same species, as a function of medium composition and culture age.[31] This may explain previous results in which ChiS chitinase showed lysozyme activity towards 6 bacterial species, but only 2 of these species were killed by the same enzyme.[16]

Concerning the potential exploitation of antibacterial chitinases as antimicrobial agents, the GH18 enzymes are particularly interesting, because they are likely to degrade bacterial cell walls using a mechanism that is distinct from that used by lysozymes. This possibility seems very attractive when we consider that some human pathogenic bacteria, such *S. aureus* and other species, are resistant to lysozyme.[46]

CONCLUSION

Only a few chitinases have been investigated as potential antibacterial proteins. However, a growing number of publications have reported that many of these chitinolytic enzymes, besides being able to degrade chitin, also have the ability do cleave peptidoglycan chains, thus promoting the lysis of bacterial cells. The peptidoglycan hydrolase activity of chitinases should be investigated in more detail, as their antibacterial activities could be exploited to control pathogenic bacteria.

CONFLICT OF INTEREST

There is not conflicting interest.

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ABBREVIATIONS

GlcNAc: *N*-acetyl-β-D-glucosamine; **GHs:** glycoside hydrolases; **CAZy:** Carbohydrate-Active Enzymes; **(GlcNAc)2:** *N,N′*-diacetylchitobiose; **(GlcNAc)3:** *N,N′,N″*-triacetylchitotriose; **CHS:** chitin synthase; **PG:** peptidoglycan; **MurNAc:** GlcNAc and N-acetylmuramic acid residues.

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