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Reviews

Chitosan as Antimicrobial Agent: Applications and Mode of Action

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Chitosan, a hydrophilic biopolymer industrially obtained by *N*-deacetylation of chitin, can be applied as an antimicrobial agent. The current review of 129 references describes the biological activity of several chitosan derivatives and the modes of action that have been postulated in the literature. It highlights the applications of chitosan as an antimicrobial agent against fungi, bacteria, and viruses and as an elicitor of plant defense mechanisms.

1. Introduction

Chitosan is a polycationic polymer with a specific structure and properties.[1] It contains more than 5000 glucosamine units and is obtained commercially from shrimp and crabshell chitin (a *N*-acetylglucosamine polymer) by alkaline deacetylation[2−4] (NaOH, 40−50%) (Figure 1). Recent advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan.[5−6]

Chitosan is insoluble in most solvents but is soluble in dilute organic acids such as acetic acid, formic acid, succinic acid, lactic acid, and malic acid. The use of chitosan is limited because of its insolubility in water, high viscosity, and tendency to coagulate with proteins at high pH. Many efforts to prepare functional derivatives by chemical modifications to increase the solubility in water have been reported.[7−15]

Chitosan is the *N*-deacetylated derivative of chitin, although the degree of the *N*-deacetylation is almost never complete. A sharp nomenclature border has not been defined between chitin and chitosan on the basis of the degree of *N*-deacetylation.

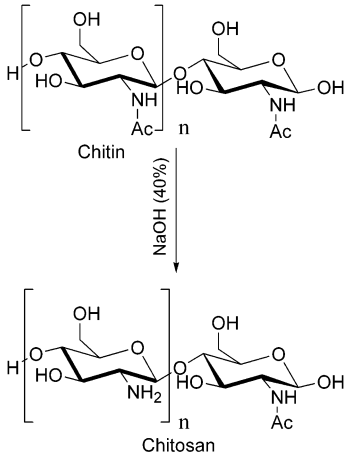

Figure 1. Preparation of chitosan from chitin.

Chitosan and chitin are commercially interesting compounds because of their high nitrogen content (6.89%) compared to synthetically substituted cellulose (1.25%). This makes chitosan a useful chelating agent. Because most of the polymers are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose, dextrose, chitin, chitosan, and their derivatives. However, these naturally abundant

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materials are also limited in their reactivity and processability.

The characteristics of chitosan that may be varied as required for a particular application are the degree of deacetylation (compared to chitin) and the molecular weight. The viscosity of solutions containing chitosan is affected by the degree of deacetylation, the molecular weight, the concentration, the ionic strength, the pH, and the temperature. Generally, an increase in temperature causes a decrease in the viscosity of the solution. The effect of the pH on the viscosity depends on the particular acid used. Native chitosan is soluble in organic acids when the pH is <6 and insoluble in water, in alkaline medium, or in organic solvents. However, water-soluble salts of chitosan may be formed by neutralization with acids such as hydrochloric acid, acetic acid, lactic acid, or formic acid.

In this review, the potential applications and modes of action of chitosan and its derivatives as antimicrobial compounds will be described.

2. Biological Activity

Chitosan is inexpensive and nontoxic and possesses reactive amino groups. It has been shown to be useful in many different areas—as an antimicrobial compound in agriculture, as a potential elicitor of plant defense responses, as a flocculating agent in wastewater treatment, as an additive in the food industry, as a hydrating agent in cosmetics, and more recently as a pharmaceutical agent in biomedicine.[16−25]

In this context, the antimicrobial activity of chitosan and its derivatives against different groups of microorganisms, such as bacteria and fungi, has received considerable attention in recent years.

2.1. Fungicidal Applications of Chitosan and Its Derivatives. The antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, algae, and some bacteria. However, the antimicrobial action is influenced by intrinsic factors such as the type of chitosan, the degree of chitosan polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions (e.g., substrate water activity or moisture or both). Although both native chitosan and its derivatives are effective as antimicrobial agents, there is a clear difference between them. Their different antimicrobial effect is mainly exhibited in live host plants. The fungicidal effect of *N*-carboxymethyl chitosan (NCMC) is also different in vegetable as compared to graminea hosts. In addition, oligomeric chitosans (pentamer and heptamer) have a better antifungal effect than larger units. The chitosan antimicrobial activity is more immediate on fungi and algae than on bacteria.[26]

Chitosan has been shown to be fungicidal against several fungi[4] (Table 1). The minimum inhibitory concentrations (MICs) reported for specific target organisms range from 0.0018% to 1.0% and are influenced by a multitude of factors such as the pH of the growth medium, the degree of polymerization of chitosan, and the presence or absence of interfering substances such as lipids and proteins.[27−35]

The inhibitory effect of chitosan was also demonstrated with soilborne phytopathogenic fungi.[36] The inhibitory activity of chitosan was higher at pH 6.0 (pK_a value of chitosan = 6.2) than at pH 7.5, when most amino groups are in the free base form.[36] The maximal antifungal and pisatin-inducing activities of chitosan were exhibited by chitosan oligomers of seven or more residues.[37] The soilborne phytopathogenic fungi *Fusarium solani* and *Colletotrichum lindemuthianum* were inhibited by chitosan and *N*-carboxymethyl chitosan.[36,38,39]

Table 1. MIC of Native Chitosan against Fungi[4]

fungi	MIC[a] (ppm)
Botrytis cinerea	10
Fusarium oxysporum	100
Drechstera sorokiana	10
Micronectriella nivalis	10
Piricularia oryzae	5000
Rhizoctonia solani	1000
Trichophyton equinum	2500

[a] MIC = minimum growth inhibitory concentration.

Chitosan has been utilized in soil amendment, in seed treatment, and as a foliar treatment to control the fungus *F. oxysporum*. Chitosan concentrations ranging from 0.1 to 1 mg/mL indicated that higher protection occurred when seed coating and soil amendment were performed with concentrations of 0.5 and 1 mg/mL. Although chitosan at a concentration of 0.1 mg/mL induced a delay in disease development (root lesions visible by 4 days after inoculation), emergence of wilting symptoms occurred between 7 and 10 days postinoculation, while death of about 80% of the plants was recorded 1 week later.[40]

F. acuminatum, *Cylindrocladium floridanum,* and other pathogens of interest in forest nurseries were inhibited by chitosan in vitro.[41] Similarly, *Aspergillus flavus* was completely inhibited in field-growing corn and peanut.[42,43]

Five chemically modified chitosans were tested for their antifungal activities against *Saprolegnia parasitica* by the fungal growth assay in chitosan-bearing broth. Results indicated that, as for the chitosan-bearing broth assay, *S. parasitica* did not grow normally; on the first day for methylpyrrolidinone chitosan and *N*-phosphonomethyl chitosan and on the second day for *N*-carboxymethyl chitosan, a tightly packed precipitate was present at the bottom of the test tubes instead of the fluffy fungal material as in the control. In contrast, *N*-dicarboxymethyl chitosan seemed to favor fungal growth, while dimethylaminopropyl chitosan did not significantly differ from the control data.[44]

The use of bioactive substances such as chitosan to control postharvest fungal disease has attracted much attention due to imminent problems associated with chemical agents, which include development of public resistance to fungicide-treated produce, an increasing number of fungicide-tolerant postharvest pathogens, and a number of fungicides that are still under observation.[45,46] Chitosan (1 mg/mL) reduces the in vitro growth of numerous fungi with the exception of Zygomycetes, that is, the fungi containing chitosan as a major component of their cell walls.[47] Hence, chitosan has potential as an edible antifungal coating material for postharvest produce. Recent investigations on chitosan coating of tomatoes have shown that it delayed ripening by modifying the internal atmosphere, which reduced decay.[45,46]

Also, the effect of chitosan coating on decay of strawberry fruits held at 13 °C was investigated.[45] Strawberry fruits were inoculated with spore suspensions of *Botrytis cinerea* or *Rhizopus stolanifer* and were subsequently coated with chitosan solutions (10 or 15 mg/mL). After 14 days of storage, decay caused by *B. cinerea* or *R. stolanifer* was markedly reduced by chitosan coating. Another important benefit of chitosan is the increased crop yield when used as a coating on wheat seeds. The authors further confirm the importance of a large number of alternating positively charged groups along the length of the polymer chain because low antifungal activity was observed with *N*,*O*-carboxymethyl chitosan compared to that of chitosan itself.[46]

Controlling the seed-borne infection *F. graminearum* by chitosan was able to increase the crop yield by 20%.[48] After seed treatment of wheat, peas, and lentils during a 5 year trial, plant yield increased 20−30%, and the potential use of chitosan in postharvest preservation of fruits and vegetables was proposed.[49,50]

Chitosan treatment (2−8 mg/mL) of wheat seeds significantly improved seed germination to recommended seed certification standards (>85%) and vigor at concentrations >4 mg/mL in two cultivars of spring wheat (Norseman and Max) by controlling seed-borne *F. graminearum* infection. The germination was <80% in the control and >85% in benomyl- and chitosan-treated seeds. The reduction of seed-borne *F. graminearum* was >50% at higher chitosan treatments compared to the control.[51]

An in vivo study[52] reported signs of infection in chitosan-coated fruits after 5 days of storage at 13 °C compared to 1 day for the control treatment. After 14 days of storage, the chitosan coating at 15 mg/mL reduced the decay of strawberries caused by the same fungi by more than 60%. It was also observed that coated fruits ripened normally and did not show any apparent sign of phytotoxicity. In another study,[53] the preservative effect of chitosan was shown on low-sugar candied kumquat (fruit). The growth of *A. niger* was inhibited by the addition of chitosan (0.1−5 mg/mL) to the medium (pH 5.4), whereas at less than 2 mg/mL, chitosan was not effective in inhibiting mold growth and aflatoxin production by *A. parasiticus*. In a similar study,[38] *N*-carboxymethyl chitosan reduced the aflatoxin production in *A. flavus* and *A. parasiticus* by more than 90%, while fungal growth was reduced to less than half. It was reported that apples coated with chitosan had a reduced incidence of molds occurring on the apples over a period of 12 weeks.[54] A study carried out on chitosan coating for the inhibition of Sclerotinia rot on carrot showed that the incidence of rotting was significantly reduced (from 88% to 28%) by coating carrot roots with 2% or 4% chitosan.[55]

The antifungal properties of chitosan are of interest in the food industry especially because chitosan is a safe biopolymer suitable for oral administration.[56] In apple juice, 15 yeasts and molds associated with food spoilage including *Mucor racemosus* and *Byssoclamys* spp. were inactivated by chitosan at various concentrations, pH values, and temperatures.[31] Similar results were obtained with *A. niger* and *A. parasiticus* in oriental food.[53] Some inhibitory effect on the fish pathogenic Oomycete *S. parasitica* has been reported.[57] The hyphae affected by chitosan at 500−600 mg/L shrunk markedly and contracted.

Chitosan has been successfully used as food wraps.[58] The use of *N*,*O*-carboxymethyl chitin films to preserve fruits over long periods has been approved in both Canada and the U.S.A.[59] Because of its ability to form a semipermeable film, chitosan coating is suggested to modify the internal atmosphere, as well as decrease the transpiration loss[60,61] and delay the ripening of fruits.[45,61] Rigid chitosan films can be formed using cross-linking agents such as glutaraldehyde,[62] divalent metal ions, polyelectrolytes,[63] or even anionic polysaccharides.[64] The preparation of chitosan and chitosan-laminated films with other polysaccharides has been reported by various authors; these include chitosan films,[65−67] chitosan/pectin laminated films,[64] and chitosan/methylcellulose films.[68] Several approaches have been used to form these edible films or coatings, including simple coacervation, in which a single hydrocolloid is transferred from aqueous suspension or caused to change its phase by evaporation of the solvent. In addition, complex coacervation, in which two solutions of oppositely ionized hydrocolloids are united, leads to interaction and precipitation of the polymer complex. Also simple cooling of a warm hydrocolloid suspension inducing a sol−gel transformation has been practiced.

Chitosan films are tough, long lasting, flexible, and very difficult to tear. Most of their mechanical properties are comparable to many medium-strength commercial polymers.[65] It was reported that chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values.[67] However, extremely good barriers were observed for the permeation of oxygen, while relatively low vapor barrier characteristics were exhibited.[65,69] Extension of the storage life and the better control of decay of peaches, Japanese pears, and kiwi fruits by application of chitosan film have been documented.[70] Similarly, cucumbers, bell peppers,[60] strawberries,[60] and tomatoes[52] could be stored for long periods after coating with chitosan. These results may be attributed to decreased respiration rates, inhibition of fungal development, and delay of the ripening due to the reduction of ethylene and carbon dioxide evolution.[45,60,70]

Chitosan and chitosan-laminated films containing antimicrobial agents provide a type of active package so that the preservatives released from the film deposit on the food surface and inhibit the microbial growth.[68,71] The sorbate-loaded edible barrier for mold inhibition on food surfaces was evaluated,[72] and the use of glucose oxidase/glucose was advocated as a dip for the extension of the shelf life of fish.[73] The presence of preservatives in chitosan films reduces the intermolecular electrostatic repulsion in the chitosan molecules and facilitates formation of intramolecular hydrogen bonds.[74] It was observed that the packaging film prepared from methylcellulose, chitosan, and preservatives possesses antimicrobial activity.[68]

2.2. Bactericidal Applications of Chitosan and Its Derivatives. Chitosan inhibits the growth of a wide variety of bacteria[4] (Table 2). Chitosan has been studied in terms of bacteriostatic/bactericidal activity to control growth of algae and to inhibit viral multiplication.[75−78] Moreover,

Table 2. MIC of Chitosan against Bacteria[4]

bacteria	MIC[a] (ppm)
Agrobacterium tumefaciens	100
Bacillus cereus	1000
Corinebacterium michiganence	10
Erwinia sp.	500
Erwinia carotovora subsp.	200
Escherichia coli	20
Klebsiella pneumoniae	700
Micrococcus luteus	20
Pseudomonas fluorescens	500
Staphylococcus aureus	20
Xanthomonas campestris	500

[a] MIC = minimum growth inhibitory concentration.

chitosan has several advantages over other type of disinfectants because it possesses a higher antibacterial activity, a broader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells.[79,80]

Chitosan derivatives containing quaternary ammonium salts, such as *N*,*N*,*N*-trimethyl chitosan, *N*-propyl-*N*,*N*-dimethyl chitosan and *N*-furfuryl-*N*,*N*-dimethyl chitosan were prepared and tested for their activity against *Escherichia coli*.[11] It was shown that the antibacterial activity of quaternary ammonium chitosan in acetic acid medium is stronger than that in water. Their antibacterial activity increased as the concentration of acetic acid is increased. It was also found that the antibacterial activity of quaternary chitosan against *E. coli* is stronger than that of chitosan itself.[11]

Certain antibacterial activities of diethylaminoethyl chitin, diethylaminoethyl chitosan, and triethylaminoethyl chitin were evaluated. The triethylaminoethyl chitin was the most active agent. It had a greater activity against *Staphylococcus aureus* than against *E. coli.* A concentration of 500 ppm was needed to kill all *S. aureus* within 120 min. Different molecular weight hydrolysates of diethylaminoethyl chitin showed a dependence of the antibacterial activity on molecular weight of the hydrolysate.[81]

In addition, the carbohydrate-branched derivatives 1-deoxyglucit-1-yl chitosan and 1-deoxylactat-1-yl chitosan had activity against *Bacillus circulans* but not against *E. coli*, while chito-oligosaccharides of varying degrees of polymerization (DPs) showed activity against *E. coli* but not against *B. circulans*.[82]

A much higher concentration of chitosan (1−1.5%) is required for complete inactivation of *S. aureus* after 2 days of incubation at pH 5.5 or 6.5.[29] Furthermore, chitosan concentrations of 0.005% were sufficient to elicit complete inactivation of *S. aureus*.[83] This was in accordance with the findings[84] on the effect of chitosan in meat preservation. The antimicrobial effect on different cultures of bacteria on raw shrimp with different concentrations of chitosan was studied, and variations in their degree of susceptibility to chitosan were observed.[25,85] According to these findings, chitosan concentrations of 0.02% were required to display a bactericidal effect against *B. cereus*, while *E. coli* and *Proteus vulgaris* showed minimal growth at 0.005% chitosan and complete inhibition at 0.0075%. It was also reported that *B. cereus* was inhibited by chitosan. However, much lower concentrations (0.005%) were required, perhaps due to the low molecular weight (35 kD) of chitosan used in this experiment.[83]

Numerous studies have also shown the effect of chitosan on *E. coli* inhibition. Complete inactivation was observed after a 2-day incubation period with chitosan concentrations of 0.5% or 1% at pH 5.5. Complete inactivation could be reached even after the first day if the chitosan concentration was more than 1% in the broth.[29] Meanwhile, a chitosan concentration of 0.1% was required to inhibit *E. coli* growth,[84] and only 0.0075% chitosan was needed to inhibit the growth of *E. coli*.[85] These variations were suggested to be due to the existing differences in the degree of acetylation of chitosan; chitosan with a degree of acetylation of 7.5% was more effective than chitosan with a degree of acetylation of 15%.

The antimicrobial effect of water-soluble chitosans[28] such as chitosan lactate, chitosan hydroglutamate, and chitosan derived from *Absidia coerulea* fungi was determined on different bacterial cultures. It was observed that chitosan glutamate and chitosan lactate were bactericidal against both gram-positive and gram-negative bacteria in the range of 1−5 log cycle reductions within 1 h. In that same study, the authors reported that chitosan was no longer bactericidal at pH 7 because of two major reasons, namely, the presence of a significant proportion of uncharged amino groups and the poor solubility of chitosan. These results are in agreement with findings of a similar study[86] in which a concentration of 0.2 mg/mL chitosan lactate appeared most effective against *E. coli* with a corresponding population drop of 2 and 4 log cycles within 2 min and 1 h exposure, respectively. These authors observed that chitosan glutamate was also effective against yeast cultures such as *Saccharomyces cerevisiae* and *Rhodotorula glutensis*, and inactivation was rapid and complete within 17 min when cultures were exposed to 1 mg/mL chitosan lactate. This was in contrast to the results[28,86] in which chitosan hydroglutamate was a more effective antagonist than chitosan lactate.

In another study,[30] the antibacterial effects of 69% deactivated shrimp chitosan, 0.63% sulfonated chitosan (SC1), 13.03% sulfonated chitosan (SC2), and sulfobenzoyl chitosan on oyster preservation were reported. Except in the case of *B. cereus*, bacterial growth was effectively inhibited by at least one of the above four compounds tested at 200 ppm. Although the sulfonation increased the solubility of chitosan, totally different antibacterial activities were observed for SC1 and SC2. For most of the bacterial cultures, SC1 had a very pronounced minimal inhibitory concentration (MIC) effect even at the 200 ppm level, while SC2 exhibited no antibacterial effect at concentrations below 2000 ppm. It was suggested that because SC2 has more sulfonyl groups, it carries a higher negative charge than SC1; thus, there would be a greater repulsive force between negatively charged SC2 molecules and bacterial cell walls.[30]

Chitosan derivatives were claimed as antimicrobials for fish and shellfish against infection from *Vibrio anguillarum*, *Edwardsiella tarda*, *Pasteurella piscicida*, and several bacteria, in agreement with data obtained on brook trout.[87,88]

On the other hand, chitin and chitosan are accepted diet supplements for cultured fish.[89]

3. Mode of Action of Chitosan

3.1. Antimicrobial Activity. Because of the positive charge on the C-2 of the glucosamine monomer below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin.[30] The exact mechanism of the antimicrobial action of chitin, chitosan, and their derivatives is still unknown, but different mechanisms have been proposed. Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents.[27,30,53,90,91] Chitosan acted mainly on the outer surface of the bacteria. At a lower concentration (<0.2 mg/mL), the polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations, the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension.[28,86]

Chitosan interacts with the membrane of the cell to alter cell permeability. For example, fermentation with baker's yeast is inhibited by certain cations, which act at the yeast cell surface to prevent the entry of glucose. UV-absorption studies indicated that chitosan caused considerable leakage of proteinaceous material from *Pythium oaroecandrum* at pH 5.8.[92]

Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth.[38] It also activates several defense processes in the host tissue,[45] acts as a water binding agent, and inhibits various enzymes. Binding of chitosan with DNA and inhibition of mRNA synthesis occurs through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of mRNA and proteins.[28]

It has been proposed that when chitosan is liberated from the cell wall of fungal pathogens by plant host hydrolytic enzymes, it then penetrates to the nuclei of fungi and interferes with RNA and protein synthesis.[93]

Chitosan, however, shows its antibacterial activity only in an acidic medium because of its poor solubility above pH 6.5. Thus, water-soluble chitosan derivatives (soluble in both acidic and basic physiological circumstances) may be good candidates as a polycationic biocide. Chitosan also inhibits toxin production by *A. alternata* and macerating enzyme production by *Erwinia* in addition to eliciting phytoalexin production.[94,95]

The effects of chitosan on growth inhibition of fungi such as *B. cinerea* in tomato and strawberries were correlated with the reduction of aflatoxin, elicitation of phytoalexin and phenolic precursors, enhanced production of chitinases, and other factors relevant to the plant defenses; direct contact of *A. flavus* with chitosan was reported to produce weakening and swelling of the hyphae. The fungistatic properties of chitosan against *R. stolonifer* were related to its ability to induce morphological changes in the cell wall.[45,46]

The effect of the molecular weight on some antibacterial and antifungal activities has been explored.[96] Chitosan with a molecular weight ranging from 10 000 to 100 000 would be helpful in restraining the growth of bacteria. In addition, chitosan with an average molecular weight of 9300 was effective in restraining *E. coli*, while that with a molecular weight of 2200 accelerated growth.[97] Moreover, the antibacterial activity of chitosan is influenced by its degree of deacetylation, its concentration in solution, and the pH of the medium. Antibacterial activities were also found to be increasing in the order *N*,*O*-carboxymethylated chitosan, chitosan, and *O*-carboxymethylated chitosan.[4]

Quaternary ammonium polymers have previously been considered bacteriostatic, not bactericidal, because they require long contact times to kill microorganisms, and generally they do not have a broad spectrum activity. Some of these polymers have been reported to have antimicrobial activity.[98] The antimicrobial action is believed to occur when the compounds are absorbed onto the bacterial cell surface, increasing the permeability of the lipid cell membrane and causing death through the loss of essential cell materials. In addition, these derivatives of chitosan are generally more active against gram-positive bacteria than their corresponding monomers particularly. This effect is believed to be due to adsorption of the polymers onto the bacterial cell surface and membrane with subsequent disruption of membrane integrity. Antimicrobial activity generally increases as the content of the quaternary ammonium moiety increases. It has been unexpectedly discovered that 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan and related chitosan derivatives exhibit antimicrobial activity at concentrations as low as 10−20 ppm. The other is 1 order of magnitude lower than the concentrations at which any previous chitosan derivative has been reported to exhibit antimicrobial activity. These chitosan derivatives may be included in formulations where it is desirable to minimize bacterial attack.[99,100]

The antibacterial activities of quaternary ammonium chitosan salts were evaluated against *S. aureus* and *E. coli*, gram-positive and gram-negative bacteria, respectively. It was found that the antibacterial activity increased with increasing chain length of the alkyl substituent, and this was attributed to the contribution of the increased hydrophobic properties of the derivatives. These results clearly demonstrated that hydrophobicity and cationic charge of the introduced substituent strongly affect the antibacterial activity of quaternary chitosan derivatives.[101]

In addition to the formation of gas-permeable films, chitosan has a dual function: (a) to direct the interference of fungal growth and (b) to activate several defense processes.[102] These defense mechanisms include accumulation of chitinases, synthesis of proteinase inhibitors, and lignification and induction of callous synthesis.[46] When applied on wounded wheat leaves, chitosan induced lignification and consequently restricted the growth of nonpathogenic fungi in wheat. Chitosan inhibited the growth of *A. flavus* and aflatoxin production in liquid culture, preharvest maize, and groundnut, and it also enhanced phytoalexin production in germinating peanut.[38,39] Chitosan also inhibited growth and toxin production by *A. alternata* f. sp. lycopersici in culture.[94,95]

3.2. Elicition of Plant Defense Mechanisms by Chitosan. Elicitors are substances that can induce defense responses

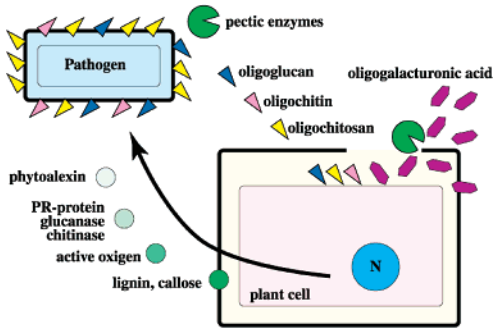

Figure 2. Production mechanism of oligosaccharide elicitors in a plant cell.[110]

when applied to plant tissues or cultured plant cells (oligosaccharides, glycoproteins, peptides, and lipids). The well-studied oligosaccharide elicitors include oligoglucan, oligochitin, oligochitosan, and oligogalacturonic acid. When a plant that has developed a resistance mechanism is challenged by a pathogen, rapid and highly localized cell death (hypersensitive cell death) occurs at the attempted infection sites and a variety of biochemical defense responses occur in the surrounding cells. These include the production of reactive oxygen species, structural changes in the cell wall, accumulation of defense-related proteins, and phytoalexin biosynthesis.

Chitosan has been extensively evaluated to determine its ability to elicit natural plant defense responses. Physiological and biochemical changes that occur within plants due to elicitation by chitosan have been reported.[103−109] One primary physiological change that has been observed when plants are treated with chitosan is the reduction of stomatal aperture, reducing fungal access to the inner leaf tissues. Guard cells in plant leaves, produce H_2O_2, which mediates the elicitor (chitosan)-induced decrease of stomatal apertures in response to chitosan treatment.[103,104]

The synthesis of phenolic acids is stimulated in primary leaves following chitosan treatment, and levels of these phenolic acids, especially ferulic acid, increased significantly with increasing chitosan concentration. The lignin content of primary leaves also showed a similar pattern. The synthesis of precursors of lignin such as *p*-coumaric, ferulic, sinapic acids, and phenolic acids having antimicrobial activity was also stimulated by chitosan treatment. The induction of phenolic acids and lignin was significantly lower in cultivar of spring wheat Max compared to Norseman. Chitosan also inhibited fungal transmission to the primary roots of germinating seedlings. Results suggest that chitosan controlled seed-borne *F. graminearum* infection and increased the resistance in seedlings by stimulating the accumulation of phenolics and lignin.[105]

In addition, chitosan oligosaccharides elicit the accumulation of lignin, callose, phytoalexin, "or" or "and" protease inhibitors in various plant tissues and induce early cellular responses. The mechanisms of action by which chitosan induces this lignification has been studied in a wide range of crops.[106−109]

The elicitors of the fungal cell wall are released from the wall by cell-wall-degrading enzymes (chitinases, glucanases) secreted by the plants upon infection (Figure 2). On the other hand, oligogalacturonic acids are degradation products of the plant cell wall, the result of microbial pectic enzymes secreted by pathogens.[110] In addition, oligogalacturonide (DP 10−12) induces phytoalexin formation in soybean tissues and stimulates lignin and protease inhibition in several other plants. Plants are capable of responding not only to fragments of the pathogen but also to their own cell wall components produced by the enzymes of the pathogen. It has been clearly proved that oligosaccharides elicit the plant−pathogen interactions.[110]

The culture filtrate of *Phytophthora megasperma sojae*, a fungal pathogen of soybean, has the ability to stimulate the accumulation of a soybean phytoalexin in soybean tissues. The elicitor turned out to be a component of the mycelia cell wall of the pathogen. The minimum structure required for the eliciting activity was a branched 1−3,1−6-hepta-β-glucoside. The maximum concentration necessary for the elicitation was 1 nM. This concentration suggests that the hepta-β-glucoside acts as an infection signal and soybean has a mechanism to perceive and transduce this specific signal. The elicitor is effective not only in soybean but also in several other leguminous plants.[110−112]

Oligosaccharides are effective in nanomolar to micromolar concentrations, giving them a true plant growth regulator status.[113,114] It has also been found that some oligosaccharide compounds affect only certain species in a predicted way.[111]

Oligosaccharins is the name given to a small group of oligosaccharides that cause hormonal effects on plants. It seems natural that a plant would differently respond to an attack from one organism than it would to another. The defense mechanism toward insects is probably not going to be effective against bacteria. When a plant is attacked by a fungus, an oligosaccharin, β-glucanase, is biosynthesized by the plant cell wall. This substance then proceeds to degrade the fungal cell wall, fragments from which amplify the effects. Some of these fragments are β-glucans, another oligosaccharin, which initiate the production of phytoalexins, leading to lignin synthesis and to the promotion of ethylene formation.[114,115]

It has been suggested that specific structural features are needed for the oligosaccharins to bind and to produce phytoalexin.[116] It has also been shown that the size of the oligosaccharin can play a role in the kind of effect it has on the plant. The effect of chitin with a degree of polymerization (DP) of less than 4 does not seem to be biologically important, while those chitins with a DP > 6 appear to be the most active. The induction of phytoalexins appears to require an oligosaccharin with a DP between 3 and 15, but this ranges depending on the species. The lignin production is started by those with a DP in the range of 8−15. This seems to be a fairly steady range for most species that are known to utilize lignin for defensive purposes. Proteinase inhibitors can be elicited by some of the smaller oligosaccharins, even by those with a DP as low as 2.[111,117]

Chitin and chitosan are cell wall structural components found in many fungi. When oligosaccharins are released from these compounds, they have been shown to elicit phytoalexin accumulation in pea pods, to lead to accumulation of

proteinase inhibitors in tomato and potato leaves, and to lead to the synthesis of a β-1,3-glucan, callose, in parsley.[111,114]

If, as suggested above, oligosaccharin receptors are located in the cell membranes of plants, there must be some mode of action for a signal to be sent out and to start the production of the various chemicals used for defense. Some of the events that take place occur very quickly and affect the cell membrane in such a way to make them less susceptible to attack. The speed with which this happens suggests that there may be a direct link between the oligosaccharins and the cell membrane. One of the actions that may cause this is the rapid depolarization of the membrane caused by the oligosaccharin. Fungal elicitors from the material of two plant pathogens began depolarization of root cells within seconds after application. About a minute later, a steady state was reached that lasted for 25 min before they returned to their original state. When the elicitors were removed before the 25 min were over, repolarization began immediately. Another response to the elicitor also involves depolarization but includes another step. Ten minutes after depolarization, the membrane became hyperpolarized, and this seems to have an influence in the production of phytoalexins.[113]

At present, the signaling pathway from the point at which the oligosaccharin contacts the cell surface to that at which the final response is realized is not well conducted. Moreover, when the oligosaccharin binds to a protein on the cell surface it is followed by an unknown step that is thought to activate more than one defense response.[118] This unknown step triggers a specific gene activity. This gene activity begins the production of proteins involved in the synthesis of phenylpropanoid enzymes, HO-cinnamoyl-CoA and HO-cinnamoyl-polymers. The phenyl propanoids are an initial step in the formation of soluble coumarin phytoalexins, which can be exported out of the cell. The HO-cinnamoyl-CoA leads to polymers similar to lignin. The HO-cinnamoyl-polymers are exported to the cell wall and are thought to be possible phytoalexins that are derived from the cell wall.[118]

Chitosan leads directly to 1,3-β-glucan synthase that leads to callose deposition in the cell wall. Some proposed that this callose deposition may include a change in protein phosphorylation/dephosphorylation, an increase of internal calcium concentration, a decrease in internal potassium concentration, a decrease in internal pH, and a change in the jasmonic acid production. It is unclear which combination of these events and how much of each is necessary to cause the desired response.[118,119]

Preliminary studies have been performed to examine the importance of calcium concentration in cells in connection with oligosaccharin mechanisms. When a calcium blocker was added to carrot cell cultures, the normal accumulation of phytoalexins was no longer observed following the infection of the cells. It was concluded that the calcium concentrations are important in phytoalexin elicitor responses. An increase in intercellular levels of cyclic adenosine monophosphate (cAMP) was noticed when the cells were exposed to an elicitor. It is proposed that calcium and cAMP may act as second messengers in phytoalexin synthesis.

Another study[120] investigated the effect of pH on the defense responses. Oligosaccharins with a DP from 6 to 15 were used to elicit responses from tobacco cells in culture. Following the application of the elicitors, the cell culture medium became alkaline, reaching maximum levels after 50 min. The pH slowly returned to its original state after another 150−200 min. In addition, after application of the elicitor, the external potassium levels increased. The intercellular cytosolic levels of potassium, however, dropped after exposure to elicitors but then rose again in the following hours. These data suggest a possible place for both potassium concentration and pH level changes as second messengers on the oligosaccharin pathway.

In addition, the plant's response can be manipulated genetically by the transfer of "R" genes (single dominant genes for race-specific disease resistance) or by treatment with elicitors such as chitosan. Both of these manipulations can result in the rapid activation of a subset of genes called PR (pathogenesis related) genes, generally regarded as the genes that functionally develop disease resistance. Multiple modes appear that can increase PR gene function by chitosan, including activating the surface or membrane receptors and internal effects on the plant's DNA conformation, which in turn influence gene transcription.[121] Most recently, it was confirmed that chitosan affects the plant DNA and reaches the plant nucleus. Chitosan can break DNA strands and complex with DNA (in competition with positively charged nuclear proteins). The result also showed that chitosan treatment inhibits the accumulation of RNA, homologous with a nuclear protein called high mobility group (HMG) protein, HMG I/Y, in pea tissue. This protein is an "architectural" transcription factor.[122,123]

3.3. Antiviral Activity of Chitosan. Antiviral activity of chitosan depends on the average degree of polymerization, the degree of *N*-deacetylation, the positive charge value, and the character of the chemical modifications of the molecule. Possible mechanisms of suppressing viral infections by chitosan are also discussed.[124−129]

Major factors of suppressing phage infections by chitosan are phage particle inactivation and inhibition of bacteriophage reproduction at the cellular level. Evidently chitosan may be used for induction of phagoresistance in industrial microorganism cultures to prevent undesirable phagolysis caused by inoculum contamination by virulent bacteriophages or by spontaneous prophage induction in lysogenic culture.

Chitosan possesses an antiviral activity by its ability to induce resistance toward viral diseases in plants, to inhibit viral infections in animal cells, and to prevent the multiplication of bacteriophages in infected cultures of microorganisms.[124,125]

The ability of chitosan to suppress viral plant infections does not depend on the virus type; chitosan affects the plant itself inducing resistance to the viral infection in plants. Imitating the contact of the plant with a phytopathogen, chitosan induces a wide spectrum of protective reactions in the plant, which limit a systemic spread of the viruses and viroids over the plant and lead to the development of the systemic acquired resistance.

Chitosan applied by spraying or inoculating leaves protected various plant species against local and systemic infection caused by alfalfa mosaic virus (ALMV), tobacco

necrosis virus (TNV), tobacco mosaic virus (TMV), peanut stunt virus (PSV), cucumber mosaic virus (CMC), and potato virus X (PVX). The efficiency of chitosan to inhibit viral infections depends on the host−virus combination, chitosan concentration, and application method.[126,127]

Using chitosan or its acetate and hydrochloride salts prevents phage infection of *E. coli*. It was shown that chitosan inhibited the productive infection caused by the bacteriophage, the efficiency of inhibition of bacteriophage depending directly on the final concentration of chitosan in the medium. Neither chitosan nor its salts significantly inhibited the growth of the bacterial culture. Chitosan added to nutrient media inhibits the multiplication of virulent bacteriophages in infected microbial cultures thus preventing lysis of microorganisms.[128]

In mammals, application of chitosan can stimulate the immune response to virus antigens. The ability to induce the interferon production could be supposed as another mechanism of antiviral activity of chitosan in animals. In addition, certain sulfated derivatives of chitosan were shown to inhibit the reproduction of some retroviruses in vitro.[129]

Chitosan's impact on viral infections in animals is determined by its ability to affect both the inductive phase of the immune response in animals and numerous effector mechanisms of the immune system. Chitosan's ability to induce interferon production may represent another important factor of antiviral resistance. Chitosan sulfated derivatives, specifically inhibiting retrovirus reproduction, were also synthesized.[129]

4. Conclusion

Even though chitosan and its derivatives have been considered as versatile biopolymers in agriculture applications, its potential uses as an antimicrobial compound and its mode of action need to be further studied in depth. In that sense, research and development should pay interest in finding novel derivatives of chitosan to increase the antimicrobial activity in accordance with low mammalian toxicity. Most physiological activities and functional properties of chitosan and its derivatives clearly depend on their molecular weight. Furthermore, it is a current matter of discussion as to whether these biopolymers may have the potential to influence physiological functions or metabolism in the microorganisms. Therefore, a significant increase in the number of scientific studies to obtain evidence to support this can be expected.

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