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Review

ANTIFUNGAL PROPERTIES OF CHITOSANS

Adina MATICA, Gheorghița MENGHIU, Vasile OSTAFE ♣
West University of Timisoara, Department of Biology – Chemistry (Pestalozzi 16, Timisoara, 300315) & Advanced Environmental Research Laboratories (Oituz 4, Timisoara 300086, Romania)

ABSTRACT

Chitosan, a natural polymer derived from chitin by deacetylation, has proved antifungal activity against numerous type of fungi. The most accepted mechanisms of action of chitosan and chitosan based materials against fungal cell are briefly presented. The influence on antifungal activity of molecular weight of the polymer, the degree of deacetylation, pH, the influence of various type of derivatization and forms of presentation were also considered. Due to the lack of consistency of the published paper in this field, but especially because the antifungal activity of a product depend greatly on the tested specie, a prediction of the efficacy of chitosan based material cannot be made.

Keywords: chitosan, antifungal activity, chitosan derivatives, nanochitosan

♣ Correspondent author: +40-755240153, vasile.ostafe@e-uvt.ro

INTRODUCTION

Literature is abundant in examples of antifungal effects of chitosan and chitosan derivatives ([1]) The mode of action of these chitosan based-materials depend of various types of factors, among them the most important being the type of microorganisms. In this respect, there are two type of fungi - chitosan-sensitive fungi and chitosan-resistant fungi[2].

As in the case of antibacterial activity of chitosan [3] antifungal activity of chitosan can be explained by several mechanisms. The positive charges from chitosan chains affects the cell membranes due to electrostatic interactions with negatively charge phospholipids [2]. Depending on the molecular weight (MW) of polymer, chitosan can enter in the cell after disturbance of the membrane [4]. Once in the cell, chitosan can interact with DNA and / or RNA synthesis [5] and with protein synthesis [6]. In the case of fungi that are resistant to chitosan, it seems that the polymer is incapable to disturb enough the cell membrane and cannot penetrate the cell [2]. In the case of this type of fungi, it was not observed the leakage of the intracellular material due to destruction of the membrane, as it was proved in the case of sensitive fungi [7]. Due to the variances in fluidity of the cell membranes of sensitive and resistant fungi, the action of chitosan on these two types of fungi is different. It was revealed that the higher the amount of unsaturated fatty acids in the fungi cell membrane, the more sensitive are these types of cells. Using mutants with a reduced amount of unsaturated fatty acids, it was observed a decreased antifungal activity of chitosan, compared with the wild types of the same species. These experiments leaded to the conclusions that the action of chitosan on fungi depend on membrane composition and fluidity [8].

The fungi manifesting different sensibility to chitosan belong to different fungal family, taxonomical classification being realized based on the fatty acid composition [9]. It was hypothesized that one can predict or explain the antifungal activity of chitosan using the taxonomy based on fatty acid composition. Similarly, it was assumed that the uptake of chitosan by the cell is ATP dependent, at least for those fungal species that are sensitive to chitosan. By monitoring the chitosan uptake in the presence of azide or at low temperature, when the production of ATP is inhibited, it was shown that chitosan was unable to pass the plasma membranes. Comparing these results with the standard situation, i.e. at room temperature and in the absence of the azide inhibitor, it was possible to conclude that chitosan uptake by fungal sensitive cells is diffusion and ATP dependent [2].

ANTIFUNGAL MECHANISM OF CHITOSAN

Likewise to antibacterial activity of chitosan, the activity of this polymer and of chitosan based-derivatives against fungi is supposed to be rather fungistatic than fungicidal [10]. It seems that chitosan can communicate regulatory changes in both fungus and host [11,12]. In the presence of chitosan, there were started some biological processes, like induction of synthesis of chitinases together with activation of action on vesicular arbuscular mycorrhizal fungi and on entomopathogenic fungi [13].

Interaction of chitosan with various types of fungi depend on the cell wall composition,

and when the interaction can take place, the polymer interfere with fungal growth. There are microscopic evidences that show that chitosan oligomers may diffuse inside hyphae, where may interfere with the enzymes involved in fungus growth. The extent of degradation of fungal cells depend on several factors, among them most important being the concentration of the chitosan, its molecular weight (MW), its degree of deacetylation (DD) and the local pH [14].

Regarding the influence of chitosan concentration, besides the obvious observation that the antifungal activity increase with polymer concentration, it was observed the concentration of chitosan modify the length of the lab phase. As the pH of the medium increase the effectiveness of chitosan decrease[14].

Although the antifungal activity of chitosan was studied from more than 20 years now and many articles on this subject were published, it is rather hard to compere these results due to unclearly characterization of chitosans and to differences in experimental factors. Even so, some conclusions can be drown. The minimum inhibitory concentration of chitosans, against various type of fungi, vary in the range of 0.01 till 8 mg/mL. The antifungal activity of chitosan depend on MW, DD and pH. Unfortunately, the data is cumbersome and no clear relation cu MW and DD can be considered in all cases. It is surer to consider that the antifungal activity of chitosan is dependent on the type of fungi [1].

Theoretically, the source for chitosan production should not be a factor that influence the antifungal activity. Perhaps the manufacture procedure can have a small influence, due to the presence of some by-products or residual compounds, but none of the published articles has treated this subject. Nevertheless, there are published results that, at least indirectly, can conduct to the conclusion that the source of chitosan may affect the antifungal activity. For example, chitosan from shrimps inhibits fungus *Aspergillums niger* [15], whole chitosan produce from *Rhizomucor miehei* and *Mucor racemosus* present antifungal properties against *Candida albicans* and *Candida glabrata* [16]. Species like *Rhizoctonia solani, Thanatephorus cucumeris, Sclerotinia sclerotiorum* and *Sclerotium rolfsii*, among other species, are inhibited by chitosan extracted from larvae of the housefly [17]. However, the source of chitosan production cannot modify the mechanism of action of the polymer, as long as, the chitosans with the same purities, same MW and DD and in similar conditions (pH, temperature, etc.) are used.

When chitosans with different MW were tested against several species, there were noticed differences in susceptibility and influence of MW. For example, *Puccinia asparagi* and *Fusarium oxysporum* are sensitive to chitosan with low molecular weight (LMW), while against *Stemphylium solani* chitosan with high molecular weight (HMW) was more effective [18]. In another study is was reported that LMW chitosan was more active against mycelial growth Rhizopus stolonifera, but inhibition of spore development of the same mold was better realized by HMW chitosan [19].

It seems that LMW chitosan is more successful against mycelial growth, as LMW chitosan is more efficient in interacting and further disturbing the function of cellular membranes [1]. LMW chitosan is also more effective in inhibition major metabolic processes once penetrating the fungal cell. Probably both these mechanisms of action take place simultaneously, but the extent of each depend on the fungal strain. It appear also to be a synergy in the case when both these mechanisms take place, which might explain the maximum antifungal activity of LMW chitosan, compared to oligo-chitosans and HMW chitosan. In same articles there is not a clear difference between oligo-chitosans and LMW

chitosans, and the efficiency of oligo-chitosans is quite common mentioned [20].

Degree of deacetylation (DD) is also an essential factor for the antifungal activity. Generally, it is accepted that as the DD is increased the antifungal activity is also increased [21]. Combining the two factors, DD and MW, one may say that the antifungal activity is higher at higher DD and lower MW. This conclusion was proved on various species: *Candida albicans* [21], *Fusarium oxysporum, Aspergillus parasiticus* [22], *Aspergillus fumigatus,* [23] and *Aspergillus flavus* [24]. These results sustain the mechanism of action of chitosan based on electrostatic interaction of positive charges of the polymer with negatively charged phospholipids from cell membranes. Increasing DD means to increase the number of free amino groups that can interact with phospholipids heads.

N-substituted chitosan derivatives

Even though raw chitosan have an antifungal activity by itself, there are researches to improve this activity by chemical modification of chitosan. The addition to the polymer chain of substituents like heterocycles of aromatic moieties can lead to an increase of the antifungal activity. An increased activity against *Pythium debaryanum* and *Fusarium oxysporum* was obtained using different N-benzyl chitosan derivatives prepared by reductive amination [25]. Against Botrytis cinerea and Pyricularia grisea an improved antifungal activity was obtained for aliphatic N-substituted and N-benzyl chitosan derivatives [25]. Improved antifungal activity against *Aspergillus niger* was obtained with thymine based chitosan derivatives [26]. Imino-chitosan derivatives presented improved antifungal activity against *Aspergillus fumigatus* [27]. Other chitosan derivatives with improved antifungal activities are thiosemicarbazone-chitosan, 2-(α-arylamino phosphonate)-chitosan, hydroxylbenzenesulfonanilide chitosan [1].

Beside the new functional groups, the antifungal performances of these chitosan derivatives, still depend on MW and DD. Unfortunately, rarely the authors of new chitosan based products take into consideration the degree of substitution (DS), i.e. the number of the new functions introduced on the polymeric chain, although most of them determine the DS. But seldom they consider this as an important factor that influence the antimicrobial properties of the new product. The antifungal activity do not depend linearly on DS. For example, N-octyl-chitosan with DS 12% was the best antifungal product, when compared with similar products, but with DS of 8%, 21% and 22% [25]. From this study, it was concluded that it is necessary to find a good balance between the hydrophilicity and hydrophobicity of the chitosan derivatives. The new functions introduced on the polymeric chain may increase the hydrophobicity which may change the type of interaction with the fungal cell wall. That means that for N-substituted chitosan derivatives, it will be necessarily to find an optimal ratio between free amino groups and substituted amino groups, as these functions have an important role in the mechanism of action of chitosan derivatives in interaction with fungal cells. It has to be taken into consideration the fact that by quaternization the chitosan become more soluble in aqueous solutions, and for this reason a better interaction with all kind of molecules are expected to be more facilitated [28]. Amphiphilic derivatives, containing dodecyl and propyltrimethylammonium substituents, were soluble at neutral pH and presented a 4 times increased antifungal activity against *A. flavus* and *A. parasiticus* [29].

O-substituted chitosan derivatives

The derivatization of chitosan can be realized either at amino groups, by quaternization, when the positive charges remain charged at neutral pH, or by derivation the hydroxyl groups presented on the polymeric chain. Most of the articles presenting new O-substituted chitosan derivatives do not make a clear comparison of the properties of the new product and samples of raw chitosan, with the same MW and DD. In these cases is hard to decide if the new product is really a better antifungal product and if the derivatization was worth. For example, some different type of O-acyl chitosan derivatives were analyzed regarding the antifungal properties against *Botrytis cinerea* and *Pyricularia grisea* [30] and the antimicrobial activity was better compared with raw chitosan. If one take into consideration the fact that the reaction to synthesize O-acyl chitosan was realized in an acidic environment that can lead to a depolymerization, resulted that the new product have, in fact, a lower MW that the initial polymer. At least in this example, it cannot be considered that the improved antifungal activity is only due to derivatization. Another example when the depolymerization should be taken into consideration is the case when O-substituted chitosan derivatives were synthesized via protection of the amino group with methanesulfonic acid [31]. For all derivatives synthetized in this work, an increased activity was noticed, in top being O-benzyl chitosan derivative.

N,O-substituted chitosan derivatives

Derivative of chitosan containing N-benzyl carboxymethyl groups prepared by reductive amination of O-carboxymethyl chitosan manifested increased antifungal activity against *Alternaria solani* and *Fusarium oxysporum* [32]. Hydroxylbenzenesulfonanilide chitosan derivatives that were tested against *Fusarium oxysporum* and *Colletotrichum gloeosporioides* have also presented improved antimicrobial activity compared with the starting chitosan material [33]. Similarly, a quaternary carboxymethyl chitosan presented a higher activity against *Geotrichum candidum* and *Candida albicans* than the original chitosan [34].

Nanoparticles, films and application of chitosan coatings

In the last decade chitosan nanoparticles, nano-rods and films have attracted the interest of researchers and industries as there were developed many applications of these type of materials. One of the major interest is the used of chitosan nanoparticles as antifungal agent due to the fact that chitosan based materials in nanoforms and films presented an increased antifungal activity. For example, chitosan nanoparticles at concentration of 1 mg/mL inhibited mycelial growth with 82.2% for *Alternaria alternata*, with 87,6% for *Macrophomina phaseolina* and with 34.4% for *Rhizoctonia solani* [35]. The raw chitosan inhibited the mycelial growth with only 20% for all three fungi. The inhibition of spore germination for A. alternate was 87.1% compared with only 21% when raw chitosan was tested.

Films of chitosan and of chitosan derivatives also possess an improved antifungal activity when compared with soluble form of chitosans [36]. For example, films of chitosan and soluble chitosans with different molecular weight (LMW and HMW) presented and average radial inibhition of 88% against *Aspergillus niger*, 57% for *Alternaria alternate* and 0% for *Rhizopus oryzae*. In these cases it was not observed a influence of the molecular weight on the antifungal activity against the tested fungi. Beside the molecular weight, there are also other factors that can be considered when the nano-forms of chitosan based materials are tested, like viscosity, surfactants, procedures of assay and others. Most important factor seems to be the type of fungus. At least from the results published until now, it is impossible to predict if a certain type of film from chitosan or a chitosan derivative will have antifungal properties and the extent of these [1].

An application that is considered more often in the last years is to coat with chitosan films directly the vegetables and fruits. At least in the case of tomatoes, grapes and citrus fruits, this application proved to be beneficial for the extend of preservation of the merchandise. When table grapes were coated with films of chitosan the growth of *Botrytis cinerea* [37] and of Penicillium expansum [38] was reduced. Chitosan films were used on postharvest blueberries, when increased freshness, increased firmness and reduced weight loss were obtained compared with control samples [39]. At least in the case when the films of chitosans were used on citrus, a distinction was made between oligo-chitosan and HMW chitosan. While oligo-chitosan films retarded the ripening process more than HMW films, the high molecular weight form of chitosan was more effective against tested fungi (*B. cinerea, P. digitatum, P. italicum and B. lecanidion*) [40].

To control gray mold caused by *Botrytis cinerea*, the tomatoes were coated with different molecular weight films of chitosan (5 kDa; 37 kDa; 57 kDa; 290 kDa). The most efficient was chitosan coatings of 57 kDa chitosan, that means that, at least for tomatoes, when the molecular weight of chitosan used to coat the vegetable increased or decreased, the antifungal activity decreased as well. In experiments realized in vitro, the most effective against *B. cinerea* was chitosan with the lowest molecular weight [31]. Nevertheless, it is proved that chitosan coating and films have a certain capacity in retarding the ripening process and the potential to reduce the infection of fruits or vegetables during the period between harvest and consumption [1].

CONCLUSIONS

Chitosan, a natural polymer, with many applications in various fields of research and industries, have proved to be a good agent against various types of fungi. This antifungal activity was tested for various types of chitosans, with different molecular weight, different degree of deacetylation, with different functional groups added to the polymeric backbone, in different forms (soluble, nanoforms, films) and against different types of fungi. Most of published results tend to conclude that the activity against fungi can be increased by increasing the degree of deacetylation. This conclusion is in accordance with the mechanism of action of chitosan, which is believed to be the electrostatic interaction between the positive charges from the chitosan chains and the negative charges from the components of the cell wall and membrane.

Regarding the molecular weight, the most effective chitosan against fungi was the

polymer with LMW, although the differences in antifungal efficacy was rather low, when chitosans with LMW, MMW and HMW were tested. In the case of LMW chitosan, a dual mode of actions can be assumed – after electrostatic interaction with cell membrane and disruption of it, the LMW chitosan can enter in the cell and can interact with other molecules negatively charged like RNA, DNA, proteins. The published results seem to agree that the antifungal activity is highly dependent on the type of fungi.

Chitosan derivatives and nano-forms of chitosan based materials have better antifungal properties than neat chitosan, and this activity can be influence by the molecular weight, degree of deacetylation and nature of functional groups added to the polymeric chain. Most of the chitosan derivative and nano-chitosans are active at neutral pH.

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