Effects of Molecular Weight and Concentration of Chitosan on Antifungal Activity Against Aspergillus Niger

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ABSTRACT

The antifungal activities of chitosan with different molecular weights and concentrations against Aspergillus niger were studied in vitro. The results showed that the antifungal activity of chitosan against Aspergillus niger is molecular weight and concentration dependent. The smaller the molecular weight, the stronger would be the antifungal activity. Chitosan with higher molecular weight and concentration has no antifungal but promotion activity towards Aspergillus niger. It is only the chitosan with proper molecular weight and concentration which possesses preferable antifungal activity towards Aspergillus niger. The effects of chitosan on Aspergillus niger and hyphal ultrastructure were examined to gain more information on its mode of action. The ultrastructure morphology investigated by transmission electron microscopy results indicated that chitosan acts on Aspergillus niger by inhibiting the growth of sporules. The fluorescein isothiocyanate labelled chitosan observation has elucidated the antifungal activity of chitosan to be caused mainly by inhibiting the DNA to RNA transcription. So the antifungal activity of chitosan towards Aspergillus niger was the combined effect of the above two observations. The study has provided sufficient scientific evidence for careful application of chitosan in food and medical industry.

INTRODUCTION

Chitosan, a deacetylated derivative of chitin, is found in the cell walls of some fungi (Aspergillus niger, Basidiomycetes spp, etc). Chitosan has been evaluated for various uses in food, medical, pharmaceutical, agricultural and chemical industries because of its non-toxic, biocompatible, mucoadhesive, and biodegradable properties [1-4]. Dissolved chitosan has antimicrobial and metal-binding properties and has been used as an antimicrobial additive to adsorb metals from food-processing wastewaters [5-7]. In addition, because of its free amino groups, chitosan can be dissolved in acidic aqueous solutions...
and form gels, films, sutures, beads, and fibres [1]. Chitosan can induce all kinds of botanic response of disease resistance [8-10], and inhibit the growth of pathogenic bacteria [11-13]. Numerous studies on antifungal activity of chitosan against plant pathogens have been carried out [14-17] and discussed [18,19]. Chitosan’s inhibition was observed in different development stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors. It has been commonly recognized that antifungal activity of chitosan depends on its molecular weight, deacetylation degree, pH of chitosan solution and, certainly the target organism. Mechanisms proposed for the antifungal activity of chitosan have focused mainly on its effect on fungal cell wall [20] and cell membrane [21,22]. Antifungal activity of chitosan has also been observed against several fungi [17]. Recently, penetration of fluorescent labelled chitosan oligomers with molecular weight under 8000 into living cells of Escherichia coli was observed and oligochitosan was suggested to inhibit bacteria from inside the cell [23,24]. This is clearly different from chitosan that is adsorbed onto bacterial walls, finally covering the wall, disrupting the membrane and leading to cell leakage [25-28].

Aspergillus niger is a filamentous fungus commonly found as a sporophyte growing on dead leaves, stored grain, compost piles and other decaying vegetation [29]. It is a common contaminant of food and a plant pathogen, causing a black mould on certain types of fruit and vegetables such as peanuts, onions, grapes, mangoes and tomatoes. Besides, it causes skin and pulmonary infections. This pathogen is worldwide identified to be difficult to control. In order to avoid fungal contamination, extensive search has been made [30-32]. Natural product such as chitosan may provide an alternative way to protect food from spoilage by fungi. The antifungal property of chitosan has been studied. Shao et al. investigated antifungal activity of chitosan made kumquat as object. The results have indicated that chitosan can stop the growth of Aspergillus niger, Aspergillus parasiticus and aflatoxin, with optimum antifungal concentration being 3-5 mg/mL, and absolute antifungal concentration being 4-5 mg/mL [33]. Chien et al. studied the antifungal activity of chitosan at various concentrations against fungi including Penicillium italicum, Botrydiplodia lecanidion and Botrytis cinerea. It was found that chitosan, depending on type and concentration, caused 25.0-90.5% growth inhibition on test organisms after 5 days of cultivation at 24ºC. Chitosan treatment significantly reduced (P < 0.05) the percentage decay of Tankan fruit during storage at 24ºC [34].

Up to the present, many researchers have targeted bacteria rather than fungi as target organisms for chitosan [35,36] and very few investigations have been performed on antifungal activity and its related mechanisms against Aspergillus niger. The objective of this study was to evaluate the effect of molecular weight and concentration on antifungal activity of chitosan against Aspergillus niger, which would be helpful for using chitosan as a natural preservative in foods prone to spoilage by yeasts and moulds and development of a high effective chitosan hydrolyte enzyme.

EXPERIMENTAL

Materials

Chitosan, from a shrimp shell with a molecular weight of 1000 kDa and 800 kDa were purchased from Yuhuan Ocean Biochemical (Zhejiang, China), Chitosan with molecular weight of 200 kDa, 140 kDa and 50 kDa were prepared in our laboratory [37], and the degree of deacetylation for all tested chitosans was 95%. Aspergillus niger was provided by the Chinese Medical Hospital of Tianshui, Gansu, China. Stock solution of chitosan (1.0%, w/v) was prepared in 1.0% (v/v) acetic acid. All other chemicals were of analytical grades and were used without further purification.

Evaluation of Antifungal Activity

The antifungal activity of chitosan solution against Aspergillus niger was evaluated as follows [38]: 12 mL of thawy wort culture medium was added in batches into 3 mL of chitosan solutions with concentrations of 5.0%, 4.0%, 3.0%, 2.0%, 1.0%, 0.5%, and 0.25% (w/v), and the final chitosan concentrations were made to 1.0%, 0.8%, 0.6%, 0.4%, 0.2%, 0.1% (w/v), and 0.05%, respectively. The mixed solution was then autoclaved at 121ºC for 15 min to prepare the culture medium. The control was a 12 mL culture
medium and 3 mL 1% acetic acid. In this study the effect of chitosan molecular weight and a concentration of 0.2% (w/v) were studied on antifungal activity.

The Aspergillus niger was inoculated in wort culture medium and activated at 30ºC for 72 h. A representative colony was picked off with a wire loop and placed in sterile physiological saline. The final cell suspension was adjusted to the number of 10^8 colony/mL. Then a 100 µL of suspension was added on the medium, cultivated at 30ºC for 96 h. In a control group, 3 mL of 1% acetic acid instead of the chitosan solution was added to the mixture. Each batch of experiments was carried out three times.

**Antifungal Rates**

Aspergillus niger was cultivated separately in 100 mL thawy wort of culture medium for preparing primary seed. In the next shaking experiment, 1 mL of suspension of primary seed was transferred into Erlenmeyer flasks containing liquid medium. The incubation condition was conducted with 120 rpm of shaking rate at 30ºC for 4 days. This cultivation method was used to investigate inhibitory activity of chitosan on growth of Aspergillus niger, and from that, the minimal inhibitory concentration (MIC) was determined. The chitosan samples after being dissolved in 1% acetic acid and sterilized by using Millipore Millex-GS filter (pore size 0.22 µm). The antifungal percentage of chitosan was calculated by weighing dried biomass of fungal strains by formula [39]:

\[
\frac{[\text{biomass of control (g/L)}] - [\text{biomass of treated sample (g/L)}]}{[\text{biomass of control (g/L)}]} \times 100
\]

**Effect of Chitosan on the Ultrastructure of Aspergillus Niger**

Aspergillus niger was prepared for electron microscopy study as previously described [7]. Aspergillus niger was grown in wort culture medium to obtain a culture with the concentration of 10^8 colony/mL. One milliliter of each culture was centrifuged at 1100 rpm × g. The resulting pellet was resuspended in 1 mL of 2% (w/v) chitosan solution, and the final concentration of chitosan was 1.0% (w/v). After shaking and incubation at 37ºC for 24 h, the suspension was centrifuged. The cells were washed twice with 5 mmol/L sodium phosphate buffer (pH 7.2, PBS) and then were fixed with 3.0% glutaraldehyde in 5 mmol/L PBS. Samples were post-fixed with 1% (w/v) OsO₄ in 5 mmol/L PBS for 1 h at room temperature, and washed three times with the same buffer, dehydrated separately at 4ºC for 10 min in a graded series of ethanol solutions (70, 80, 90, 100%, v/v), then embedded in Epon 812 low-viscosity embedding medium. Thin sections of the specimens were cut with a diamond knife on an Ultracut Ultramicrotome (SuperNova; Reichert-Jung Optische Werke, Wien, Austria) and the sections were double-stained with saturated uranyl acetate and lead citrate. The grids were examined with a JEM-1230 transmission electron microscope (Hitachi, Tokyo, Japan) at an operating voltage of 75 kV.

**Fluorescence Observation of Antifungal Activity of FITC-labelled Chitosan**

*Preparation of FITC-labelled Chitosan*

Chitosan, 0.2 g (molecular weights of 50 kDa and 1000 kDa) was dissolved in 20 mL of a H₂O:C₂H₅OH
=1:1 (v/v) mixed solution at pH 9.0, and 0.04% of FITC was reacted with the chitosan at an ice-cold temperature under stirring overnight. An FITC-chitosan was precipitated with ethanol and air-dried after extensive rinsing with ethanol. Schematic illustration of the chemical synthesis of FITC-labelled chitosan is shown in Figure 1.

Fluorescence Observation
Five percent of the culture medium adapting Aspergillus niger was inoculated in the medium containing 0.01% of the FITC-chitosan under a shake culture at 30°C for 24 h in the dark. Aspergillus niger cultured with FITC-chitosan was collected by centrifugation (3000 rpm for 15 min) and rinsed extensively with physiological saline. The aggregated organisms were dispersed by ultrasonication in the physiological saline and loaded on the fluorescence free slide glass followed by air-drying. Then, a cover glass was set after treatment with 50% aqueous glycerol containing sodium azide and localization of the fluorescence was observed by a confocal laser scanning microscope (Model: TSC-NT 165123, US) [40].

Statistical Analysis
In the present study, triplicate trials were performed for each experiment. The mean values and the standard deviation were calculated from the data obtained. These data were analyzed by the LSD method (DPS v7.55 edition).

RESULTS AND DISCUSSION

Effect of Molecular Weight of Chitosan on Antifungal Efficiency
Figure 2 shows the antifungal activity of chitosan with different molecular weights against Aspergillus niger. Five types of chitosan with molecular weights ranging from 50 kDa to 1000 kDa were studied for their antifungal activities. It is easy to see that the molecular weight of chitosan is the key factor in antifungal activity of chitosan against Aspergillus niger. The experimental results showed that the smaller the molecular weight is, the stronger would be the antifungal activity. The chitosan with molecular weight 50 kDa owned the best antifungal activity and could completely inhibit the growth of Aspergillus niger within 72 h, with only few single lawns appearing within 72–96 h. Also, the chitosan with molecular weight of 140 kDa and 200 kDa could completely inhibit the growth of Aspergillus niger within 72 h, but a lot of single lawns appeared within 72–96 h, the colour was white and no spores were formed. On the contrary, the chitosan with molecular weights of 800 kDa and 1000 kDa were not antifungal but promoted activity towards Aspergillus niger. The growth of Aspergillus niger was very extensive and fast at 72 h, with the culture medium being covered by plenty of spores, which far exceeded the control.

Summing up the result, it was seen that the antifungal activity of chitosan, which is a polycationic compound due to a large amount of -NH₃⁺ (lengthen-

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**Figure 2.** The antifungal activity of chitosan towards Aspergillus niger, with different molecular weights from a to f being 50 kDa, 140 kDa, 200 kDa, 800 kDa, 1000 kDa, and control, respectively.
ing of the polymer) in acidic solution, may depend on the concentration of the -NH\textsubscript{2} of the polymer. Also, when the molecular weight is under 50 kDa, the antifungal activity of chitosan increases with increasing the -NH\textsubscript{2} content. When the molecular weight exceeds 50 kDa, amino groups of chitosan may be too many, which promote a fictitious cross-linked structure through their strong intramolecular hydrogen bondings and then they are no longer available to attach to Aspergillus niger cell surface. Therefore, the antifungal activity of chitosan decreased with increasing its molecular weight when it was above 50 kDa.

**Effect of the Chitosan Concentration on Antifungal Efficiency**

The effect of chitosan concentration on the antifungal activity against Aspergillus niger is shown in Figure 3. With the increase of chitosan concentration, all had the stimulating effect on the growth of Aspergillus niger. When the concentration was 0.1% (w/v), the antifungal activity of chitosan sample with 50 kDa molecular weight was at its highest point. While the concentrations were up to 0.8% to 1.0% (w/v), all samples exceeded the control behaviour. The results showed that with the increase of chitosan concentration, the antifungal activity was decreased sharply. When the chitosan concentration was 0.1% (w/v), it could almost inhibit the Aspergillus niger completely.

The parallel experiment showed that chitosan at the concentration of 0.05% (w/v) within 96 h could not completely inhibit the growth of Aspergillus niger, and about ten lawns appeared and white spores grew, but 0.1% (w/v) chitosan could grow spores while no lawns appeared. For 0.2% chitosan, eight lawns appeared only in one of five culture plates, the complete antifungal probability was 80%. The 0.4% (w/v) chitosan also showed better fungistatic activity and about 60 little lawns appeared in each plate. In case of 0.6% (w/v) chitosan sample plenty of lawns were formed, which were big but no spores appeared. The 0.8% (w/v) chitosan sample was covered by Aspergillus niger, which was big and thick, and about 80% spores were formed. The 1.0% (w/v) chitosan sample was completely covered by thicker Aspergillus niger with 100% spores. Therefore, the concentration of 0.1% (w/v) was the best and at minimum level. In our experiment, we cannot check the best antifungal activity because of the poor solubility of chitosan at higher concentration. Chitosan concentrations ranging between 0.8% and 1.0% (w/v) could not show antifungal activity compared with the control sample. Therefore, we assume that the possible reason for the antifungal activity of chitosan was not related to its concentration, and may be it attributed to the increased viscosity of the chitosan solution at higher concentration, which restricted the number of effective contacts of chitosan with Aspergillus niger cell surface.

**Antifungal Rates of Chitosan with Different Molecular Weights and Concentrations**

As shown in Tables 1 and 2, with the increase of chitosan molecular weight and concentration, the antifungal activity is decreased. The molecular weights range from 50 kDa to 200 kDa have shown improved inhibition activity, but the molecular weight of...
Table 1. Antifungal rates of chitosan with different molecular weights at 0.2% (w/v) concentration against Aspergillus niger.

<table>
<thead>
<tr>
<th>Molecular weights (kDa)</th>
<th>Antifungal rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>98.24 ± 1.59a*</td>
</tr>
<tr>
<td>140</td>
<td>82.33 ± 2.52b</td>
</tr>
<tr>
<td>200</td>
<td>73.58 ± 4.16b</td>
</tr>
<tr>
<td>800</td>
<td>p**</td>
</tr>
<tr>
<td>1000</td>
<td>p**</td>
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(*) LSD test, P=0.05, a→b Mean standard deviation (n=3). Means in same column with different superscript letters are significantly different.

(**) Denotes accelerative activity, and the antifungal rates to Aspergillus niger were negative.

800 kDa and 1000 kDa have shown much enhanced, namely accelerative activity. With relation to concentration, the higher the chitosan concentration, the weaker was the antifungal activity. At higher concentrations of 0.8% and 1.0%, there was also an accelerated activity shown towards Aspergillus niger. All data indicated that there were significantly different (P < 0.05) antifungal rates for each chitosan and concentration treatment.

Effect of pH Value on Antifungal Efficiency

The pH value of culture medium was adjusted to 3.0, 4.5, 5.5, 6.5, and 7.5, respectively by 1.0 mol/L hydrochloric acid or sodium hydroxide. Correspondingly the antifungal rates were 100%, 92%, 73%, 45%, and 21% (Figure 4), which demonstrated that the antifungal activities of chitosan strengthened with the decrease in pH. While the pH was above 6.5, the antifungal rate was decreased sharply. Because the isoelectric point of chitosan was 6.3, the solubility of hydrated chitosan salts is significantly diminished beyond this pH [41] and it has been suggested by Popper et al. [42], that antifungal activity is directly proportional to the amount of chitosan present in solution. However, under pH 6.3, the solubility of hydrated chitosan salts is significantly enhanced; and the amount of amino group is increased as well. In addition, organic acids could restrain the growth of microorganism, especially for mildews [12]. In this study, the antifungal properties of chitosan against Aspergillus niger were the resultant force of chitosan and organic acid (1.0% acetic acid).

Effect of Chitosan on Morphology and Ultrastructure of Aspergillus Niger

In TEM photographs, the cell membrane and cell wall of Aspergillus niger are clearly noticeable in normal Aspergillus niger (Figure 5a). In contrast to control (Figure 5a), chitosan with 50 kDa strongly destroyed the cell membrane of Aspergillus niger (Figure 5b), there were cavums in Figure 5b, which indicated that a part of chitosan had entered the inner body of...
Aspergillus niger and influenced and even destroyed the cell nucleolus. Moreover, the outer cell membrane (Figure 5b) of Aspergillus niger became thicker, and the number of spores became fewer than the control (Figure 5a). The chitosan with molecular weight of 1000 kDa, however, was no antifungal activity towards Aspergillus niger, which greatly promoted the growth of Aspergillus niger and accelerated its cell division (Figure 5c). All the above observations demonstrated clearly that chitosan inhibits the growth of Aspergillus niger by controlling the conidiophore.

**Fluorescence Observation on FITC-labelled Chitosan**

The fluorescence micrographs of Aspergillus niger-accumulated FITC-labelled chitosan with molecular weights of 1000 kDa and 50 kDa are displayed in Figures 6a and 6b, respectively. In these micrographs, the fluorescence (bright) areas are FITC-labelled chitosan, and the bright elliptic area is Aspergillus niger cell. In Figure 6a, FITC-labelled chitosans are observed mainly outside Aspergillus niger, and few observed inside while being in division stage. However, the observations made on Aspergillus niger cell from the outside to inside as in Figure 6b, it is evident that the FITC-labelled chitosan (50 kDa) was observed inside the cell and while the cell was being destroyed. Thus, permeated chitosan (molecular weight of 50 kDa, i.e. Figure 6b) was suggested to block the DNA transcription and therefore to inhibit the growth of Aspergillus niger. As this fluorescence study corresponds closely to the results of antifungal activity of chitosan, it seems that it mainly acts by preventing DNA transcription to RNA.

**Mechanism Investigation of Chitosan Against Aspergillus Niger**

Several mechanisms for antifungal action of chitosan have been proposed. For example, it has been suggested that chitosan may inhibit microbial growth by acting as a chelating agent rendering metals, trace elements or essential nutrients unavailable for the organism to grow at normal rate [41]. The growth rates of fungal hyphae have been shown to be sensitive to all factors which influence intracellular calcium ion, including variations in extracellular calcium.

![Figure 5](image1.png)

**Figure 5.** Effect of chitosan on morphology and ultrastructure of Aspergillus niger with concentration of 0.1% at 96 h, control (a), chitosan with molecular weights of 50 kDa (b), and 1000 kDa (c), bar = 1 μm, mag ×30000.

![Figure 6](image2.png)

**Figure 6.** Fluorescence observation on FITC-labelled chitosan with molecular weights of 1000 kDa (a), and 50 kDa (b), bar = 20.00 μm.
concentrations and the presence of calcium transport inhibitors [43]. Therefore, it is conceivable that chitosan limits the growth of filamentous fungi indirectly by making calcium and other essential minerals and nutrients inaccessible. Such a mechanism would be consistent with the reduced growth rates of *M. racemosus* in the presence of chitosan as it was observed in this study. Also, several authors have proposed that the antimicrobial action of chitosan against filamentous fungi could be explained by a more direct disturbance of membrane performance [44,45].

Most of the studies conducted so far have concluded that the inhibition activity is linear with respect to the concentration of chitosan, because the chitinase in fungi would be produced at high concentration of chitosan, consequently leading to the degradation of chitin and chitosan of fungal cell wall [13]. However, our results suggest that chitosan exerts its inhibition activity by holding back the growth of spores of *Aspergillus niger*, which is the major mechanism for chitosan action against *Aspergillus niger*. Whilst, almost all reports conclude that the inhibition activity is linear with respect to concentration, in our study, however, it is indicated that there is no such relationship, and it is only at a definite molecular weight and concentration which best inhibition activity is observed.

*Aspergillus niger* is a fungus whose cell wall mainly consists of chitin and chitosan [29], and therefore it holds plenty of chitinase, by which chitosan excites much of its activity in fungus. Chitosan is a mixture composed of several chitosans with different molecular weights, also expressed as average molecular weight. Therefore, a chitosan with large molecular weight includes not only large molecules but also small ones as well. The low molecular weight chitosan, owing to its small size, was easy to enter into the interspaces of cell wall to give a target inhibition to *Aspergillus niger* by disturbing its metabolism. But the high molecular weight chitosan could not enter into the interspaces of cell wall just by forming a film on the cell membrane to jam the input and output exchanges of nutrition. The high molecular weight chitosan, therefore, cannot excite the activity of chitinase heavily and exhibit lower inhibition activity towards *Aspergillus niger*. In that respect, the excitation ability of chitosan to *Aspergillus niger* was limited, and only the chitosan with proper molecular weight and concentration can highly excite the expression of chitinase in *Aspergillus niger*, make the cell distortion and finally killing the cell. At high concentration, a small part of chitosan was concerned with the excitation of chitinase, but a majority of chitosan was utilized as nutrients and accelerated the growth of *Aspergillus niger*.

**CONCLUSION**

The antifungal activity of chitosan against *Aspergillus niger* was molecular weight and concentration dependent. By comparing chitosans together, with different molecular weights and concentrations, on bactericidal activity of *Aspergillus niger*, we could conclude that the antifungal activity of chitosan decreased with the increase of molecular weight and concentration. Chitosan with higher molecular weight and concentration have no inhibitory activity but promotion towards *Aspergillus niger*. It is only a chitosan with proper molecular weight at proper lower concentration that has preferable inhibition ability against *Aspergillus niger*. In our study, the best molecular weight and concentration were 50 kDa and 0.1%, respectively. TEM study showed that chitosan exerts its antifungal activity against *Aspergillus niger* by inhibiting the spores’ growth. The fluorescence observation showed that the antifungal activity of chitosan seems to be caused mainly by inhibition of DNA transcription to RNA.

So far, the study on antifungal activity and action mechanism of chitosan towards fungi has not been elucidated, and through some experiments we assume that chitosan activates the chitinase in epiphyte to inhibit its growth. However, the activity of chitinase in epiphyte is limited, as it is molecular weight and concentration dependent. The results of this study suggest new methods to investigate the mode of action of chitosan towards fungi.

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