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EXPERIMENTAL ARTICLES

In vitro Antifungal Activity of Metal Complexes of a Quaternized Chitosan Derivative with Copper Ions

A. V. Il'ina[a], B. Ts. Shagdarova[a, *], A. P. Lun'kov[a, b], S. N. Kulikov[c, d], and V. P. Varlamov[a]

[a] *Institute of Bioengineering, Research Center of Biotechnology Russian Academy of Sciences, Moscow, Russia*
[b] *Dmitry Mendeleev University of Chemical Technology of Russia, Moscow, Russia*
[c] *Kazan Research Institute of Microbiology and Epidemiology, Kazan, Russia*
[d] *Kazan Federal University, Kazan, Russia*
**e-mail: shagdarova.bal@gmail.com*

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Abstract—Antifungal activity of synthetic metal complexes of quaternized N-(propyl) chitosan derivatives with Cu(II) against yeastlike (*Saccharomyces cereviseae, Rodothorula rubra,* and *Candida albicans*) and mycelial fungi (*Fusarium oxysporum, Alternaria alternata, Cladosporium herbarum*) was studied. In vitro application (at 250–500 μg/mL) of the metal complex of quaternized N-(propyl) derivative of low-molecular chitosan with 53% substitution and 1.3% copper ions proved efficient against *F. oxysporum*, one of ten most common fungal plant pathogens. Water-soluble quaternized N-(propyl) chitosan derivatives with 40–58% degree of substitution were synthesized using glycidyltrimethylammonium chloride under optimally adjusted conditions. Metal complexes of the chitosan derivative with 53% degree of substitution with Cu(II) ions were obtained by dialysis. The quantity of copper ions in the metal complexes was determined by atomic emission spectrometry. The structure of chitosan derivatives was confirmed by spectral analysis (IR, ^{1}H NMR).

Keywords: chitosan, antifungal activity of chitosan, alkylated quaternized chitosan derivatives, chitosan metal complexes

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The search for and the development of new efficacious antifungal compounds is a highly important task due to the potential hazard that various species of fungi may represent in the agricultural sphere and in the field of healthcare. Various species of fungi damage agricultural plants, decreasing their productivity, cause spoilage of harvested crops, and may contaminate plant products with mycotoxins (Kulikov et al., 2006). Moreover, micromycete fungi are among the most common sources of allergens in the environment. The incidence of mycogenic allergy may vary from 6 to 24% in the general population and up to 44% in patients with atopic pathology (Khaldeeva et al., 2011); about 25% of bronchial asthma patients are sensitized to mold micromycetes (Mari et al., 2003). The yeasts of the genus *Candida* are among the most common agents causing deep and superficial chronic forms of diseases (Lisovskaya et al., 2016).

To decrease the burden on the ecological state of the environment, which is caused by the application of various pesticides, including synthetic fungicides, at present a fresh impetus has been given to active development and production of new fungicides based on biological compounds. Development of the fungicides based on chitosan, a natural polyaminosaccharide, as well as on chitosan-based derivatives and composites, is considered one of the promising avenues (Seyfarth et al., 2008; Rahman et al., 2014). The antifungal properties of chitosan are determined by its polycationic characteristics. The molecular mass and the degree of deacetylation of chitosan also influence the antifungal effect (Kulikov et al., 2009 and Rahman et al., 2015).

The presence of numerous amino groups in the chitosan polymer chain makes it possible to obtain chitosan-based derivatives with improved physicochemical characteristics, e.g., with higher solubility and biological activity (Il'ina et al., 2008). Chitosan functionalization is usually carried out by the amino group at C2 atom, as well as by the hydroxyl groups at C6 and C3 atoms of the glucosamine residue.

Obtaining chitosan derivatives containing quaternary nitrogen atoms is one of the ways of increasing the biocide activity of chitosan. Quaternized N-alkylated and arylated derivatives of high-molecular chitosan (700 kDa) have a considerably higher inhibitory activity against such plant pathogens as *Botrytis cinerea* and *Colletotrichum lagenarium* (Guo et al., 2007). Quaternized N-(propyl)- and (pentyl)-trimethylammonium chitosan bromide (20–32 kDa)

revealed superior antifungal properties against *Aspergillus flavus* (de Oliveira Pedro et al., 2013). Badawy, using chitosan with a molecular mass (MM) of 360 kDa with 90% degree of deacetylation (DD), synthesized a number of quaternized N-alkyl derivatives with a C4–C8 chain length. According to the results of their studies, the peak of antifungal activity in the experiments with the plant pathogens *Botrytis cinerea* and *Fusarium oxysporum* at the stage of spore germination was shown for chitosan C5- and C8-derivatives (Badawy, 2010). Later, the authors reported the synthesis of a quaternized chitosan derivative using ethyl iodide (Badawy and Rabea, 2014). In order to obtain quaternized N-aryl chitosan derivatives, the relevant aromatic aldehydes were used. The antifungal activity studied on tomato (*Solanum lycopersicum* L.) leaves against *B. cinerea* was shown to increase compared to the original chitosan (MM 50–190 kDa, 91% DD) which was used to synthesize the derivatives (Badawy and Rabea, 2014). Thus, quaternized N-alkyl and aryl derivatives may contribute to increasing the fungicide properties of the chitosan polymer.

It is known that compounds containing certain d-metals, e.g., copper, may also possess fungicidal properties (Schmuhl et al., 2001; Mekahlia and Bouzid, 2009). The structure of the chitosan molecule contains several types of the functional groups: $-NH_2$, $-OH$, and $>NH$ (electron pair donors), which are capable of forming complexes with metal ions. In this connection, obtaining the polymer–metal complexes of d-metals with chitosan, including alkylated polymer derivatives, merits attention from the point of view of their potentially valuable fungicidal properties.

The goal of the present work was to obtain the metal complexes of a low-molecular quaternized N-(propyl) chitosan derivative with copper ions and to investigate their antifungal activity.

MATERIAL AND METHODS

Reagents. High-molecular weight crab chitosan with a molecular mass of 500 kDa and 87% degree of deacetylation (Bioprogress, Russia) was used in the work. Glycidyltrimethylammonium chloride (GTMAC) manufactured by Sigma (United States) was used for obtaining alkylated chitosan derivatives. Hydrochloric acid and copper sulfate were chemically pure (KhimMed, Russia).

Microorganisms. The yeast-like fungi *Saccharomyces cerevisiae* VKM Y-830, *Rodothorula rubra* VKM Y-341, *Candida albicans* ATCC 10231, as well as mycelial fungi *Fusarium oxysporum* VKM F-1182, *Alternaria alternata* VKM F-1120, and *Cladosporium herbarum* VKM F-23, were used in the experiments to determine antifungal activity. The fungal strains were maintained on Sabouraud agar.

Obtaining low-molecular chitosan. Using chemical hydrolysis with inorganic acid (HCl, 6 M, 90°C, 3 h), low-molecular chitosan with the following physicochemical characteristics: MM, 20 kDa; DD, 98%; and polydispersion index, 1.4 was obtained from chitosan with MM 500 kDa and 85% DD according to the technique described by Shagdarova et al. (2016).

The synthesis of N-[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride. Quaternized N-(propyl) chitosan derivatives were synthesized according to the technique proposed by Lun'kov et al. (2016) and Xiao et al. (2012). Chitosan (1 g) was dispersed in 10 mL of distilled water at 85°C; after that, GTMAC was added thrice at 2-h intervals. The reagent ratio used was proposed in the technique described by Cho et al. (2006). The total reaction time was 7 h. The end product was dialyzed against water and freeze-dried.

The degree of quaternization of the N-(propyl) chitosan derivatives obtained was determined using the technique of Qin et al. (2004). The sample was dissolved in 1% acetic acid and then titrated with $AgNO_3$ solution.

Water solubility of the samples was determined according to the method of Varma et al. (2004). A weighed portion of the sample was dissolved in water (pH 6.5) and mixed for 2 h. The solutions obtained were centrifuged; the pellet was dried and then weighed.

The sample solubility at different pH values was studied according to the technique of Varma et al. (2004). The transmission coefficient of test solutions was measured at 600 nm.

Obtaining metal complexes of quaternized N-(propyl) derivatives of low-molecular chitosan with copper ions. In order to obtain a metal complex, we used the solution of N-[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride (10 mg/mL in distilled water), which was introduced into a dialysis tube made of regenerated cellulose (25 × 16 mm, MM = 14 kDa); Cu(II) salt solutions at concentrations of 0.004 and 0.02 M served as dialyzing buffers. The dialysis conditions were as follows: pH 5.6–5.7, 23°C, 300 rpm, 24 h, 1 : 20 vol/vol. On completion of dialysis, the solutions of metal complexes were freeze-dried.

The quantity of copper ions in chitosan metal complexes was determined by the results of atomic emission spectrometry (AES). To the sample of chitosan derivative (100 mg) accurately weighed to 0.1 mg, 5 mL of concentrated nitric acid (69%, high-purity solvent for analytical research, ACS-ISO gradation; Panreac, Spain) was poured. After complete dissolution of the sample, the solution volume was adjusted to 50 mL with deionized water. The solution obtained was subjected to 5-fold dilution with deionized water and analyzed using an ICP-AES spectrometer (Agilent ICP-OES 5110).

The study of antifungal activity. The antifungal action of quaternized N-(propyl) derivatives of low-molecular chitosan (ChQ) with 53% DS, as well as of its complexes with copper at a concentration of 1.3 and

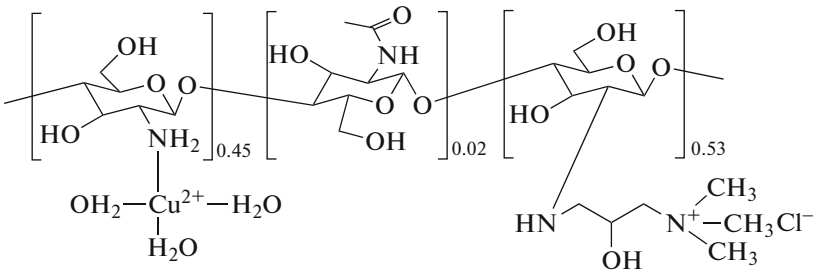

Fig. 1. Tentative structure of the metal complex of an alkylated chitosan derivative with copper ions.

6.5% against yeastlike fungi, was studied in liquid Sabouraud medium at pH 5.5–5.7, as described earlier (Kulikov et al., 2014). For this purpose, a solution of the chitosan derivative was added to the medium to the final concentration of 100, 250, or 500 μg/mL. The freshly prepared suspension of fungal cells in the same medium was added to the end concentration of 2.5–5 × 10^3 cells/mL. After 48 h of incubation at 30°C, growth of the culture was assessed by changes in its optical activity at 620 nm.

The antifungal action of the substances against mycelial fungi was investigated in liquid Sabouraud medium at pH 5.5–5.7, as described by Kulikov et al. (2010). For this purpose, a solution of the chitosan derivative was introduced into the medium to the end concentration of 100, 250, or 500 μg/mL. The freshly prepared suspension of fungal spores in the same medium was added to the end concentration of 1–2.5 × 10^5 spores/mL. After seven days of incubation at 30°C, growth of the culture was assessed by the amount of grown biomass (in mg of dry weight).

RESULTS AND DISCUSSION

Low-molecular weight chitosan was obtained in the form of a base (with free amino groups) using the method of chemical polymerization according to the technique described by Shagdarova et al. (2016). In order to preserve the total positive charge of the molecule at pH > 6.4 and to increase solubility, quaternized N-(propyl) chitosan derivatives were synthesized. In contrast to the schemes of synthesis proposed by other investigators (with at least three stages) (Badawy, 2010; Badawy and Rabea, 2014), we obtained chitosan derivatives at one stage, without side reactions. Commercially available glycidyltrimethylammonium chloride (GTMAC) was used as an alkylating reagent. The process was carried out in an aqueous medium (pH ≈ 6.8). The conditions for synthesis were selected so that the reaction could only proceed for the chitosan amino groups. By varying the molar ratio of the alkylating reagent to chitosan, quaternized N-(propyl) chitosan derivatives with 40–58% DS were obtained. The structure of chitosan derivatives was confirmed by spectral analysis (IR, ^{1}HNMR). The derivatives obtained were highly water-soluble (pH 6.5, 25°C): from 12 to 30 mg/mL, depending on the degree of substitution.

The chitosan metal complexes are known to be usually synthesized at pH 4–6 (Varma et al., 2004; Bratskaya and Pestov, 2016). At the pH values of 5.6–5.7, which were close to the pK_a of chitosan (6.4–6.5), the number of free amino groups in a chitosan molecule increased and the solubility sharply decreased. The introduction of positively charged ammonium groups into the biopolymer molecule resulted in its solubility increasing to 30 mg/mL. The quaternized N-(propyl) chitosan derivatives with 40–58% DS were highly water-soluble at pH 5.5–7.4 and contained a sufficient number of free amino groups required for complex formation with metal ions. A chitosan derivative with 53% DS and 45% of free amino groups was used for further studies.

The complexes containing a chitosan derivative and copper ions (Fig. 1) were obtained by dialysis, which provided for both the introduction and removal

Table 1. Copper ion content in alkylated chitosan derivatives

Sample	Estimated Cu(II)/ChQ* ratio		Found Cu (II)/ChQ, ratio, AES**	
	weight, g	molar, M	weight, %/g	mole, M × 10^{-4}
ChQ 0.004	0.3/1	1.2/1	1.3/0.1	0.05/1
ChQ 0.02	1.5/1	6/1	6.5/0.1	0.25/1

* ChQ, quaternized N-(propyl) chitosan.
** AES, atomic emission spectrometry.

Table 2. Growth of yeastlike fungal cultures in the presence of chitosan derivatives (OD_{620})

Strain	Concentration, μg/mL	Variants				
		Ch*	ChQ**	ChQ, 1.3% Cu(II)	ChQ, 6.5% Cu(II)	control
S. cereviseae	100	1.600	0.520	0.345	0.818	2.202
	250	0.900	0.400	0.297	0.495	2.004
	500	0.350	0.188	0.190	0.169	1.985
R. rubra	100	1.300	0.650	0.433	0.878	1.990
	250	0.880	0.326	0.255	0.655	2.123
	500	0.490	0.185	0.175	0.170	2.166
C. albicans	100	2.010	0.900	0.950	1.050	2.170
	250	1.115	0.300	0.309	0.325	2.168
	500	0.375	0.177	0.199	0.189	2.100

* Ch, original chitosan.
** ChQ, quaternized N-(propyl) chitosan.

Table 3. Growth of mycelial fungal cultures in the presence of the substances (in mg of dry biomass)

Strain	Concentration, μg/mL	Variants				
		Ch*	ChQ**	ChQ, 1.3% Cu(II)	ChQ, 6.5% Cu(II)	control
F. oxysporum	100	10.4	3	1.8	7.3	11.4
	250	11	0.9	0	2.5	10.1
	500	5	0	0	0	11.1
A. alternata	100	10.4	5	4.2	10.4	10.2
	250	7.1	2.1	1.8	6.1	10
	500	3.2	0	0	0	10.5
C. herbarum	100	9.5	2.5	1.5	7	9.1
	250	6.8	1.4	0	2.3	8.1
	500	2.3	0	0	0	8.8

* Ch, original chitosan.
** ChQ, quaternized N-(propyl) chitosan.

of small molecules from the sample solution in the dialysis tube due to their free transfer through the semipermeable membrane in both directions.

The UV spectra of metal complexes of alkylated chitosan derivatives with Cu(II) ions gave evidence of the formation of polymer–metal complexes; absorption bands of the ChQ–Cu(II) samples appeared in the 190–235-nm and 250–285-nm regions.

The quantity of copper ions in two samples of the metal complexes determined by atomic emission spectrometry was 1.3 and 6.5% of Cu(II) ions per 0.1 g of alkylated chitosan derivative (Table 1).

After freeze-drying, solutions of the complexes were investigated for antifungal activity. At 500 μg/mL, the quaternized N-(propyl) chitosan derivative (ChQ) was shown to completely inhibit the growth of all tested fungi, both yeastlike and mycelial ones (Tables 2, 3). At 100 and 250 μg/mL, a substantial difference was observed between the samples: the ChQ 0.02 sample was significantly less efficient against all tested cultures, whereas the ChQ 0.004 sample produced a more marked antifungal effect. No significant difference in relation to *C. albicans* was found between the samples, which may be

attributed to active formation of biofilms, which are highly resistant to antifungal substances.

The results obtained confirmed that both metal complexes of the quaternized N-(propyl) chitosan derivatives (53% DS) with 1.3 and 6.5% of copper ions, as well as quaternized N-(propyl) chitosan, exhibited pronounced antifungal activity against yeastlike and mycelial fungi compared to the original chitosan. The manifested activity was concentration-dependent and peaked when the substances were used at 250 and 500 μg/mL. When chitosan derivatives were used in the form of metal complexes containing 1.3 and 6.5% of copper, the fungicidal properties were significantly increased. The growth of yeastlike fungi decreased to a minimum, and biomass formation by micromycetes stopped completely. The chitosan polymer derivatives obtained, including their metal complexes with copper, may serve as a promising basis for the development of environmentally friendly biogenic drugs to fight pathogenic and plant pathogenic fungi.

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