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MACROMOLECULAR CHEMISTRY AND POLYMERIC MATERIALS

Synthesis and Biological Activity of Metal Chitosan Complexes

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Abstract—A series of complexes of chitosans of various molecular weights (3000–150000) with copper, iron(II), and zinc sulfates were examined. Participation of amino groups of the polymer in coordination bonding with the metal was proved by IR spectroscopy. The affinity of chitosan for iron ions was enhanced by introducing phenolic fragments into the polymer.

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Chitosan attracts attention of a wide range of researchers owing to a set of valuable chemical and biological properties [1, 2]. Availability of raw materials for its preparation and polysaccharide nature determining its affinity for living bodies make chitosan an accessible and promising polymer for agriculture, in particular, in plant growing for treatment of seeds and vegetating plants [3].

Chitosan derivatives are major components of cell walls of many parasitic microorganisms and phytopathogens. Plant treatment with chitosan-based preparations simulates contact of plants with a phytopathogen and activates their natural protective resources [4]. Advantages of this approach consist in doubtless environmental safety, prolonged action of preparations taken in low concentrations, and stimulation of the resistance to any stress situations such as night frosts, drought, and water logging.

One of possible ways to enhance valuable biological properties of chitosan is its chemical modification via reactive functional groups present in the chitosan macrochain. Particularly attractive is preparation of soluble metal chitosan complexes whose application will provide plants with microelements and thus enhance the capability of plants for adaptation to unfavorable factors of the environment [5].

EXPERIMENTAL

Commercial chemicals [chitosan (MW 150000, degree of deacetylation 85%, Aldrich), sodium nitrite, *p*-hydroxybenzaldehyde, sodium borohydride, copper sulfate pentahydrate, zinc sulfate, iron(II) sulfate heptahydrate, sodium hydroxide, potassium hydroxide, glacial acetic acid] were used without additional purification. The solvents (acetone, ethanol) were purified by standard procedures [6].

The UV spectra were recorded on an SF-56 device (Leningrad Optical and Mechanical Association, Russia) in 1-cm quartz cells. The content of incurporated *p*-hydroxybenzaldehyde (BA) fragments in chitosan was calculated using the extinction of a model compound, 4-(dimethylaminomethyl)phenol, at a wavelength of 274 nm in a mixed solvent CH_3COOH : H_2O : C_2H_5OH = 0.2 : 1 : 1. The IR spectra of chitosan and its complexes (KBr pellets) were recorded in the range 5000–400 cm^{-1} with a Specord IR-75 spectrophotometer. The ^1H NMR spectra were taken on a Bruker DPX-300 spectrometer (300.13 MHz), solvent 1 M DCl/D_2O. The signal positions were determined using as reference the signals of methyl protons of the nondeacetylated units of the polymer (2.06 ppm) [7]. The viscosity of polymer solutions was determined with an Ubbelohde viscometer at 25°C in a mixed solvent 4% CH_3COOH : 0.6 M NaCl = 1 : 1. The molecular weight was calculated by the procedure suggested in [8].

Oxidative degradation of chitosan. Chitosan (5 g) was dissolved in 50 ml of 4% acetic acid. To the resulting solution we added 5 ml of a solution containing appropriate amount of $NaNO_2$, after which

we added in succession 2.7 ml of glacial acetic acid and 25 ml of distilled water. The reaction mixture was stirred at 20°C for 2–2.5 h. The reaction was stopped by adding an alkali solution (4 g of NaOH in 6 ml of water) until a colorless precipitate of chitosan formed (pH ~7.0–7.5). The mixture was diluted with distilled water, and the chitosan precipitate was separated by centrifugation. The precipitate was washed with water to neutral reaction of wash waters and dried at 40°C. Yield 44–90%. ^{1}H NMR spectrum of sample Chit-1, δ, ppm: 2.06 s (0.42 H, CH_3CONH), 3.19 s (1.00 H, H^2 in GlcN), 3.35 s (0.14 H, H^2 in GlcNAc), 3.4–4.1 m (~5H, H^{3,4,5,6} in GlcN and GlcNAc).

Modification of chitosan with *p*-hydroxybenzaldehyde. Prior to modification, chitosan was activated by converting into a gelatinous form. For this purpose, 0.5 g of chitosan (3.1 mmol of units) was dissolved in 10 ml of 4% acetic acid, 10 ml of water was added, and the product was precipitated by dropwise addition of a KOH solution (0.5 g in 5 ml of water) to pH 10.0. The polymer precipitate was separated by centrifugation and washed with ethanol. To the activated chitosan we added 0.4 g of *p*-hydroxybenzaldehyde in 10 ml of ethanol, and the mixture was allowed to stand for 24 h at 20°C. Then 0.2 g of sodium borohydride was added to the reaction mixture with vigorous stirring. After 12 h, the product was separated by centrifugation, repeatedly washed with ethanol, and dried at 40°C. Yield (product Chit-BA) 85%. ^{1}H NMR spectrum, δ, ppm: 2.06 s (CH_3CONH), 3.20 s (H^2 in GlcN), 3.35 s (H^2 in GlcNAc), 3.4–4.1 m (H^{3,4,5,6} in GlcN and GlcNAc), 4.39 s (NCH_2Ar), 6.97 d (H^{3,5} in Ar, J = 8 Hz), 7.37 d (H^{2,4} in Ar, J = 8 Hz).

Preparation of chitosan (Chit, Chit-BA) complexes with metals. Chitosan (0.5 g) was dissolved in 100 ml of 4% acetic acid, appropriate metal sulfate ($CuSO_4 \cdot 5H_2O$, $ZnSO_4$, $FeSO_4 \cdot 7H_2O$) was added (reactant weight ratio chitosan : salt = 1 : 1), and the mixture was neutralized with an alkali (10% KOH) to pH 5.6. Then the mixture was stirred at 20°C for 3 h, after which the polymer was precipitated with acetone, separated by centrifugation, washed with ethanol, filtered off, and vacuum-dried. Yield 0.7–1.0 g. The content of metals in the chitosan complexes was determined by UV spectroscopy, following standard procedures, with preliminary mineralization of the samples [9].

Biological tests. Fungicidal activity of chitosan and its complexes was studied using the agar block technique [9] and was characterized by the degree of inhibition of the growth of Fusarium oxysporum fungus mycelium relative to the control on the 5th and 10th days of cultivation.

The phytotoxicity of the composite agent Chit-4-BA-Fe : Chit-4-Cu : Chit-4-Zn = 1 : 1 : 1 and the growth-stimulating activity of Chit-4 and Chit-4-BA samples was evaluated by the effect of their working solutions on the growth and development of wheat and cucumber plantules, respectively. To this end, seeds (20 seeds in each of four replicate tests) were placed in humid chambers with working solutions of the agents (sterile Petri dishes with a layer of wool and filter paper) and incubated at 20–25°C. Water was used in the control experiment.

The antistress activity (drought and salt resistance) was evaluated by the procedure described in [10]. Wheat seeds preliminarily kept for 16 h in solutions of the agents were placed on rolls of filter paper (20 seeds on each roll). Seeds soaked in water served as control. The rolls (three rolls in each run) were placed in a vessel with a 2% solution of sucrose or NaCl. The germination and the linear size of plantules and roots were determined. The parameters of seed germination in distilled water served as control.

The goal of this study was the development of metal chitosan complexes using samples of different molecular weights and evaluation of the fungicidal and antistress activity of formulations based on them. The choice of copper, iron, and zinc salts for preparation of metal chitosan complexes was governed by the fact that these microelements are required for normal development of plants, and their deficiency leads to various pathological processes [5, 11].

The molecular weight of chitosan, largely determining its biological properties, is the main criterion of the efficiency of using this polymer in plant growing as an agent stimulating the growth and development of plants and enhancing the immunity of plants to various diseases [12, 13].

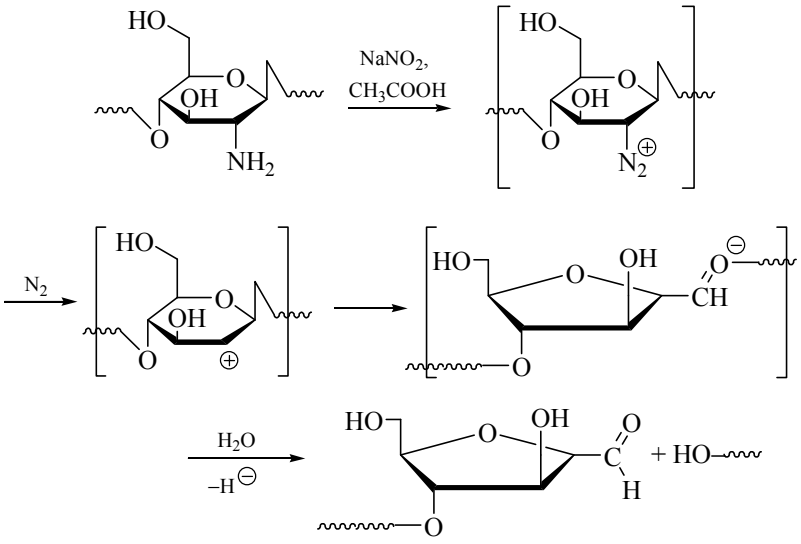
Chitosan exhibits biological activity in a wide range of molecular weights, from oligomers with MW 1200 to samples with a high degree of polymerizatoin (MW 350000) [7, 8]. The choice of the optimal MW of chitosan ensuring its highest performance is primarily determined by the type of the test culture used. Also, the biological properties of chitosan largely depend on the quality and origin of the raw material used for its production [2].

Based on the previous results [14] and taking into account the published data, we synthesized samples of chitosans with MW varying in a wide range and then used these samples for preparing metal complexes.

The most widely used procedure for preparing chitosan samples with various molecular weights is oxidative degradation occurring by the radical mechanism [2]. Chemical degradation of chitosan is performed using oxidants (hydrogen peroxide, ozone, potassium persulfate) at 50–70°C [15]. Under these conditions, the occurrence of side reactions such as oxidation of hydroxy and amino groups of chitosan is highly probable [2, 16].

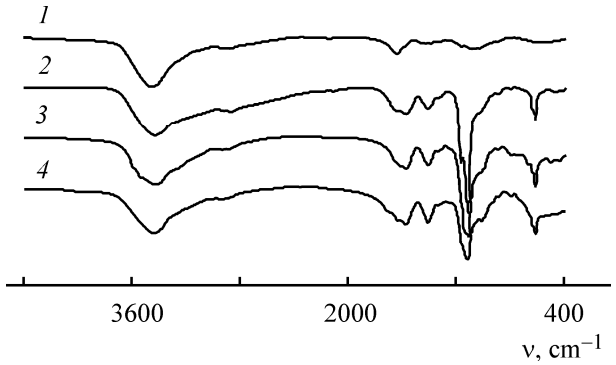
In our study we used the procedure of chitosan degradation under the action of sodium nitrite in acid solution, occurring via cationic rearrangements. In contrast to the above-mentioned chemical degradation procedures, in this procedure active centers at which the polymer chain is broken are formed selectively from amino groups by decomposition of diazonium salts. The advantages of this procedure are mild reaction conditions, absence of side oxidation processes, and availability of the reagents.

As seen from Table 1, the major factor determining the molecular weight of the product is the amount of sodium nitrite introduced into the system. In addition, with an increase in the amount of $NaNO_2$ in the reaction mixture the fraction of oligomers that are lost in the course of product isolation increases, which leads to a considerable decrease in the yield of the desired polymer:



Owing to the presence of functional groups, chitosan is highly capable of metal coordination [2]. The bond of the metal ion with nitrogen is enhanced by coordination of hydroxy groups. From the initial chitosan (MW 150000) and samples prepared by degradation (MW 3000, 5000, 10000, 54000), we synthesized complexes with copper, iron, and zinc. To prove the formation of metal chitosan complexes and confirm their structure, we used IR spectroscopy as the most informative method for this kind of objects. The figure shows the IR spectra of chitosan (MW 54000) and its metal complexes.

In formation of a complex by coordination of amino groups to the metal, the absorption maxima corresponding to stretching vibrations of N–H and O–H bonds (3500–3300 cm^{-1}) and to bending vibrations of



IR spectra of (*1*) chitosan of MW 54000 and of its complexes with (*2*) iron, (*3*) copper, and (*4*) zinc. (ν) Wavenumber.

Table 1. Conditions of oxidative degradation of chitosan and characteristics of the samples obtained

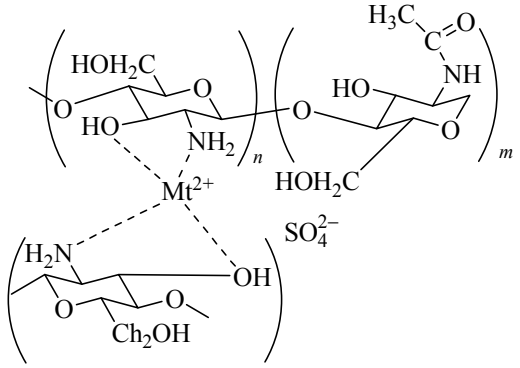
Sample	$NaNO_2$/Chit,[a] mol/mol	Reaction time, h	MW of sample	Yield, %
Chit-1	0.2500	2.5	3000	44
Chit-2	0.1250	2.5	5000	44
Chit-3	0.0625	2.0	10000	65
Chit-4	0.0140	2.0	54000	89

[a] Amount of sodium nitrite per chitosan unit.

Table 2. Characteristics of metal chitosan complexes prepared

Sample	MW	Content of indicated metal in complex, wt %		
Chit-0	150000	9.0	1.8	2.2
Chit-1	3000	11.0	2.1	4.2
Chit-2	5000	11.0	2.0	5.8
Chit-3	10000	9.0	1.0	6.4
Chit-4	54000	9.8	1.9	3.3

NH bonds (1635–1630 cm^{-1}) in amino and acetamido groups are shifted toward lower wavenumbers. The band at 1635–1630 cm^{-1} transforms into a shoulder at 1627–1612 cm^{-1} corresponding to bending vibrations of NH bonds in amino and acetamido groups of chitosan that are not involved in the complexation. A maximum appears at 1560 cm^{-1}, corresponding to NH bending vibrations in metal-coordinated amino groups of chitosan. The bands at 1116 and 617 cm^{-1} correspond to stretching and bending vibrations of sulfate ions. Based on the IR spectra, we suggest the following structure of metal chitosan complexes:



The content of metals in the complexes was determined by photometric methods with preliminary mineralization of samples by boiling with concentrated nitric acid. As seen from Table 2, copper is incorporated in the largest amount (9–11%), and iron, in the smallest amount (~2%). Low content of iron in chitosan complexes may be due to weak tendency of Fe^{2+} and Fe^{3+} ions to form complexes with amines [17]. For example, comparison of the instability constants of complexes of double-charged iron (5.25×10^{-5}), copper (1.6×10^{-11}), and zinc (1.2×10^{-6}) with ethylenediamine (M : L = 1 : 1) shows that the iron complexes are the least stable.

We evaluated the fungicidal activity of chitosan samples and their metal complexes by the degree of inhibition of the growth of Fusarium oxysporum fungus mycelium (Table 3). The biological tests showed that chitosan samples of different MWs exhibit high fungicidal activity, retarding the growth of fungus mycelium by 61.1–83.1%. It should be noted that the Chit-4 sample showed high antifungal activity even on the tenth day of cultivation, whereas the activity of the other samples tended to decrease. Incorporation of copper enhances the antifungal activity of the chitosan samples. On the contrary, the antifungal activity of the iron-containing chitosan samples is lower than that of the corresponding metal-free samples, irrespective of the molecular weight of the polymer.

It is known that iron deficiency in plants leads to a decrease in the activity of key enzymes participating in photosynthesis and to the development of chlorosis [5]. The iron chitosan complexes obtained contained a small amount of iron and under application conditions tended to undergo oxidation with the formation of insoluble iron(III) hydroxides. In this connection, we attempted to synthesize stable complexes of chitosan with a high iron content.

One of possible ways to increase the iron content and enhance the stability of an iron chitosan complex is introduction into the polymer chain of phenolic ligands with which iron, as a rule, forms stable complexes [18]. It was shown previously that introduction of phenolic fragments into the chitosan structure exerts a growth-stimulating effect on the plant [14]. Thus, appearance of phenolic fragments in the chitosan chain should lead not only to an increase in the iron content, but also to enhancement of growth-stimulating properties of chitosan samples.

To introduce phenolic ligands into chitosan, we performed modification of Chit-3 and Chit-4 samples

Table 3. Fungicidal activity of chitosan samples

Cultivation time, days	Sample	MW	Inhibition of mycelium growth, % of control			
			Chit	Chit–Cu	Chit–Zn	Chit–Fe
5	Chit-1	3000	61.1	81.0	78.9	51.8
	Chit-2	5000	79.6	85.7	87.8	55.5
	Chit-3	10000	83.1	87.5	83.5	54.7
	Chit-4	54000	82.3	87.5	82.7	55.0
	Chit-0	150000	79.1	85.0	81.8	64.4
10	Chit-1	3000	44.4	75.0	74.2	36.6
	Chit-2	5000	78.0	84.0	78.9	35.6
	Chit-3	10000	77.0	75.0	78.2	35.5
	Chit-4	54000	80.0	85.0	71.5	66.7
	Chit-0	150000	72.2	82.0	70.5	54.5

Table 4. Characteristics of chitosan modification products and their effect on the size of roots of cucumber plantules

Sample	Increase in root length, % relative to control, at indicated sample concentration, %					Metal content, wt %	
	0.1	0.05	0.02	0.01	0.001	Cu^{2+}	Fe^{2+}
Chit-4	6.2	15.4	0.4	0.0	–5.1	9.8	1.9
Chit-4-BA	18.5	29.6	9.7	7.6	2.4	10.0	11.0

Table 5. Effect of composite agent on the germination and length of wheat roots and plantules under the conditions of aqueous (2% sucrose solution) and salt (2% NaCl solution) stresses

c, %	Phytotoxicity		Aqueous stress			Salt stress		
	germination, %	plantule length, mm	germination, %	root lengthening, %	plantule lengthening, %	germination, %		root length, mm
						H_2O	2% NaCl	
0.1	25.0	24.0	N/d[a]	N/d[a]	N/d[a]	N/d[a]	N/d[a]	N/d[a]
0.01	70.0	52.5	60.0	2.0	4.0	70.0	35.5	5.0
0.001	80.0	60.3	80.0	53.1	22.5	80.0	55.5	10.0
0.0001	85.5	59.2	82.3	2.0	10.7	85.5	65.0	15.0
0	77.5	56.8	62.5	0.0	0.0	77.5	36.5	7.3

[a] (N/d) Not determined.

with p-hydroxybenzaldehyde, followed by reduction of the forming aldimine bonds with sodium borohydride. In the ^{1}H NMR spectra of the modification products, Chit-3-BA and Chit-4-BA, we observed a system of two doublets at 6.97 and 7.37 ppm with $^3J_{HH}$ = 8 Hz from aromatic ring protons. This allowed quantitative evaluation of the content of BA fragments by comparing the integral intensities of these signals with those of the signals of protons at C^2 atoms of chitosan units (2.86 ppm). The amount of BA fragments introduced into Chit-3-BA and Chit-4-BA samples, determined spectrophotometrically (23.7 and 19.5 mol %,

respectively), reasonably agrees with the results of ^{1}H NMR analysis (24 and 19 mol %, respectively).

As we expected, introduction of phenolic fragments into chitosan led to enhancement of the growth-stimulating properties (Table 4). A comparative test of Chit-4 and Chit-4-BA samples on cucumber plantules showed that the Chit-4-BA sample in the examined concentration range exerts an appreciably stronger root-growth-stimulating effect.

We prepared iron and copper complexes based on Chit-4-BA sample. The presence of BA fragments in chitosan does not affect the copper content in the complex, whereas the iron content in this case appears to be higher by a factor of 5.

Using Chit-4-BA sample containing 11% Fe and complexes of Chit-4 with copper and zinc, we prepared a composite agent with equal weight content of these components. The results of studying the effect of this composite agent taken in various concentrations on the growth of wheat plantules and the results of evaluating the antistress activity are given in Table 5.

As can be seen, soaking of wheat seeds in 0.01–0.0001% solutions of the composite agent does not exert a negative effect on the germination and growth of wheat plantules. The solution with a higher content of the composite agent (0.1%) exhibits phytotoxicity, inhibiting the growth of wheat plantules and negatively affecting the germination. For studying antistress properties, we chose three concentrations: 0.01, 0.001, and 0.0001%. Data on the effect of various concentrations of the composite agent on the germination and growth of wheat in early steps of the organogenesis under the conditions of aqueous and salt stress are given in Table 5.

CONCLUSIONS

(1) In the prepared complexes of chitosans of various molecular weights, the copper content is the highest among the examined metals, which suggests higher affinity of copper for amino groups of chitosan, compared to that of iron and zinc.

(2) Introduction of hydroxyphenyl groups into chitosan allows the iron content of the complex to be increased by a factor of 5.

(3) All the metal chitosan complexes prepared exhibit fungicidal activity. Presowing treatment of seeds with a composite agent based on metal chitosan complexes enhances the capability of wheat plantules in early steps of the development to resist stress factors (soil drought and salinization).

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