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Molecular-Weight-Dependent Fungicidal Activity of Chitosan

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Abstract—The synthesis of a series of low-molecular-weight chitosans and the results of their bioassay against several phytopathogenic fungi are described.

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Chitin, an aminopolysaccharide constituting the base of the exoskeleton in crustaceans and insects and the cell walls of fungi and other organisms, is the most abundant natural polysaccharide [1]. Partially or completely deacetylated chitin (poly-/β-(1 ⟶ 4)-2-amino-2-deoxy-D-glucan) is named chitosan (Fig. 1).

Low-molecular-weight chitosans (LMWCs), with their high solubility and the low viscosity of their aqueous solutions, at physiological pHs have a bioactivity that depends on the degree of chitosan acetylation, the distribution of glucosamine moieties along the chitosan backbone, and other factors. Synthesis parameters also influence the resulting activity of LMWCs, because the synthesis method (enzymatic or chemical hydrolysis) determines the chemical structure of the reducing end of the chitosan polysaccharide chain.

In this work, a set of LMWCs was prepared by controlled acid hydrolysis of high-molecular-weight chitosan derived from the following chitinous bioresourses: shrimp (*Pandalus borealis*), squid (*Loligo vulgaris*), cuttlefish (*Sepia oficinalis*), edible mollusk (*Cerastroderma edule*), and river crayfish (*Actacus leptodactylus*).

First, chitin was obtained from the aforementioned natural sources. Chitin preparation involves solid–liquid heterogeneous chemical reactions. Here, the particle size is the critical factor [2]. In this work, biowastes were reduced to particle sizes of 1 to 3 mm; then, they were subjected to alkaline deproteinization with 0.5 M sodium hydroxide at 80°C for 2 h. After alkaline deproteinization was over, the supernatant was separated and deproteinized chitin particles were washed until the wash-water pH was 7.0.

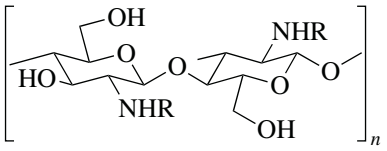

Fig. 1. Structure of chitin (R = Ac) and chitosan (R = H).

We used 0.5 M hydrochloric acid for chitin demineralization. Acid increments were added to a stirred water–chitin mixture until carbon dioxide evolution ceased; then, the mixture was allowed to stand for 1–2 h. Minor butanol was added to reduce foaming.

Chitin deacetylation was carried out with 50% NaOH at 90°C for 3 h. The resulting chitosan was washed with water and dried in air. The results of our experiments (table) showed that it is inappropriate to use mollusks for full-scale chitosan production: the chitosan yield from this source was low because of the high calcium carbonate content.

High-molecular-weight chitosan (HMWC) isolated from different sources was then used to prepare LMWC. Hydrolysis of HMWC and its derivatives was performed with hydrochloric acid of various concentrations.[1] Our prepared LMWC samples were analyzed by gas-permeation chromatography to determine their molecular weights M_w and polydispersion indices. The polydispersion indices of the samples were in the range 1.4–2.2.

Then, we studied the growth-inhibiting activity of the chitosans synthesized on fungi *Penicillium vermaesseni*. The comparison of the activities of 0.1% solutions of several LMWCs with M_w ranging from 4.1 to 90 kDa against *Penicillium vermaesseni* demonstrated that chitosans with molecular weights of 4 to 10 kDa had the highest activity (Fig. 2).

The growth-inhibition effect of 0.1% solutions of the chitosan sample with the molecular weight equal to 7.57 kDa was also observed against lower plant-pathogenic fungi such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Verticillium dalie*, and *Pythium ultimum* (Fig. 3).

[1] Spent hydrochloric acid from this operation can be recycled in the demineralization of chitin-containing wastes and the neutralization of alkali used for chitin deacetylation, thus decreasing the acid and alkali contamination of sewages.

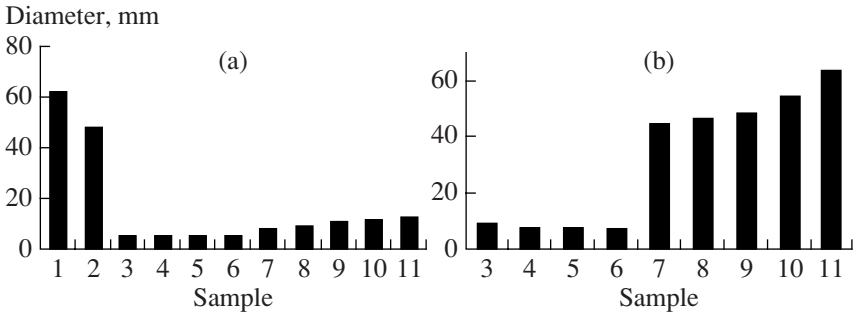

Fig. 2. Activity of a 0.1% LMWC solution against *Penicillium vermaesseni*: (*1*) blank, (*2*) $GlcNH_2$, and (*3–11*) chitosans with M_w (kDa) equal to (*3*) 4.10, (*4*) 5.15, (*5*) 7.57, (*6*) 9.92, (*7*) 15.03, (*8*) 24.23, (*9*) 29.19, (*10*) 40.45, and (*11*) 90.00. Measurement duration: (a) 5 and (b) 15 days.

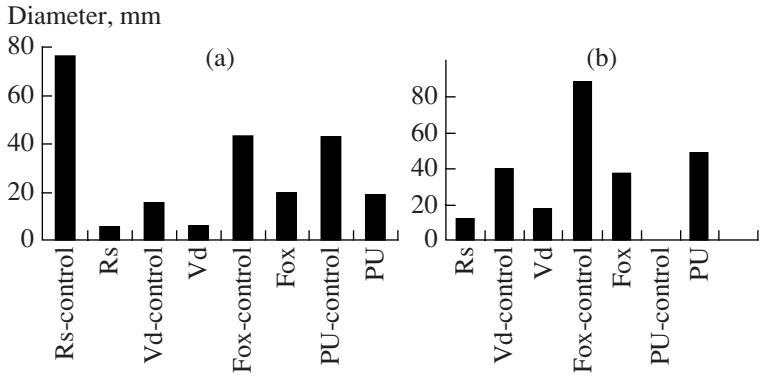

Fig. 3. Activity of a 0.1% LMWC (7.57 kDa) solution against *Rhizoctonia solani* (Rs), *Verticillium dalie* (Vd, *Fusarium oxysporum* (Fox), and *Pythium ultimum* (Pu). Measurement duration: (a) 5 and (b) 15 days.

In summary, we prepared a set of LMWCs; some of them demonstrated a high growth-inhibiting activity against some fungi, including phytopathogenic species.

EXPERIMENTAL

The chitosan molecular weight was determined by high-performance liquid chromatography. The weight-average molecular weight M_w, number-average molecular weight M_n, and polydispersion M_w/M_n for the LMWCs prepared were determined at 30°C using an Ultrahydrogel 500 column (Waters) in the 0.05 M acetic acid–0.15 M ammonium acetate system with pH 5.2 and the elution rate 0.5 mL/min. Chromatograms were monitored and analyzed using MultiChrom 1.6 software (Ampersand, Moscow). The column was calibrated against dextran molecular weight standards (1080, 4440, 9890, 43500, 66700, 123600, and 196300 kDa) purchased from Sigma.

The fungicidal activity was determined by measuring the radial growth of a fungus colony. Test chitosan samples were dissolved in 0.25 M hydrochloric acid; then, the solution pH was adjusted to 5.0–5.5 using 1 M NaOH. Antifungal Assay Agar (from Sigma), a 7.5% agar medium containing 1 mg/mL chitosan, was sterilized at 110°C for 20 min and then distributed to sterile Petri dishes 9 cm in diameter. The dished were inoculated by a fungus colony 6 mm in diameter. A set of three experiments was carried out for each sample. The blank was the same agar medium without chitosan. All dished were incubated in the dark at 20°C. The radial growth of colonies was measured daily during 15 days.

Precursor consumption required for preparing 1 kg chitosan

Sample no.	Source	Amount of the source (kg) required for preparing 1 kg chitosan
1	Shrimp: whole	90–110
2	Shrimp: debris	50–70
3	Squid: gladius	4–5
4	Cuttlefish: cuttlebone	80–85
6	Mollusk	1600
7	River crayfish	80–100

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