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Studies of application possibilities of the products of microcrystalline chitosan biodegradation

RAPID COMMUNICATION

Summary — Some of the chitosan properties depend largely upon its molecular structure and in several applications of this polymer its lowered molecular weight is an asset. By degrading of microcrystalline chitosan in the presence of cellulolytic enzymes, the products having a reduced molecular weight were prepared, together with a water soluble blend of oligomers. The outcome of an initial assessment, made to evaluate the chance of a practical use of the obtained products of chitosan degradation, indicates that oligomers and partly degraded microcrystalline chitosan accelerate the germination of seeds, inhibit the growth of plant viruses, bacteria and milder.
Key words: chitosan, enzymatic degradation, oligomers, plant pathogens.

BADANIA MOŻLIWOŚCI ZASTOSOWAŃ PRODUKTÓW BIODEGRADACJI MIKROKRYSTALICZNEGO CHITOZANU

Streszczenie — Niektóre właściwości użytkowe chitozanu w istotnym stopniu zależą od struktury cząsteczkowej i ze względu na możliwości ich zastosowań korzystne jest obniżenie średniego ciężaru cząsteczkowego. W wyniku heterofazowej degradacji mikrokrystalicznego chitozanu, w obecności enzymów celulolitycznych otrzymano produkty o częściowo obniżonym ciężarze cząsteczkowym oraz mieszaninę oligomerów rozpuszczalnych w wodzie. Wstępna ocena możliwości wykorzystania otrzymanych produktów biodegradacji chitozanu do ochrony roślin wykazała, że oligomery i częściowo zdegradowany mikrokrystaliczny chitozan przyśpieszają kiełkowanie nasion, hamują rozwój wirusów (tabela 1), bakterii roślinnych oraz pleśni (tabela 2).
Słowa kluczowe: chitozan, degradacja enzymatyczna, oligomery, patogeny roślin.

The average polymerization degree of chitosan and its *N*-acetylation degree are the factors which considerably determine the possible use of polyaminosaccharide. A decrease in the molecular weight appears to be an asset in some applications of the polymer. Hence, new effective degradation methods of chitosan are sought in order to prepare oligomeric products which reveal in some applications a higher bioactivity than chitosan itself. The products characterized with polymerization degree below 20 and molecular weight up to 3900 are classified as oligoaminosaccharides.

In earlier research projects the susceptibility of chitosan towards hydrolytic, radiation and enzymatic degradation was investigated [1—5]. As the result of works carried out in Institute of Biopolymers and Chemical Fibres (IBWCh), it was possible to qualify the impact of the molecular and supermolecular structures of chitosan and microcrystalline chitosan (MCCh) upon bioactivity. Recently, research work has been conducted by us focused on the preparation of water soluble oligomer blends designed for agriculture uses.

The biochemical degradation of microcrystalline chitosan in the form of an aqueous gel-like suspension was carried out in a heterogenic system with the use of enzymes of the hydrolases group: cellulases and xylanases. Low-molecular fractions were obtained, both as degraded polymer and water soluble oligoaminosaccharides [6]. The amount of the low-molecular fractions depends upon the degradation conditions and the enzyme applied.

It has been found that if chitosan is degraded with the use of hydrolytic enzymes of the cellulases group, the process can be aimed at obtaining of polymer fractions with substantially lowered molecular weights or oligomeric fractions in the form of dissolving aminosaccharides, and the process output exceeds 90 %.

The aim of this study was an initial assessment of a practical use of products of microcrystalline chitosan biodegradation in plant protection.

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EXPERIMENTAL

Materials

In this study the following substances were used:

— chitosan in the commercial form from Vanson HaloSource Inc. (Redmond, WA, USA), characterized by viscosity-average molecular weight $\overline{M}_v \approx 400\ 000$, and degree of deacetylation $DD = 75.8$ %.

— neutral cellulase in the commercial form produced by AB Enzyme Oy, Finland; enzymatic preparation with an endo-1,4-β-glucanase activity of 186 Unit/cm^3.

— lactic acid and sodium hydroxide of analytical purity.

Degradation process

The suspension of microcrystalline chitosan (MCCh), manufactured according to a method developed at the Institute of Biopolymers and Chemical Fibres [7, 8], was exposed to degradation. The degradation was conducted in a heterogenic system containing 1 wt. % of the polymer, under dynamic conditions, at temp. of 50 °C, over 7 hours, at a ratio of enzyme and substrate of (E/S) = 2066 Unit CMC/g (the amount of enzyme units per 1 g of chitosan), and pH equal 7.0. The aqueous solution of chitosan oligomers, obtained as the result of degradation, was purified by ultrafiltration using Vivaflow 200 with membrane of polyetherosulphone of MWCO 5000. The selected chitosan oligomers' preparations were freeze dried. Three type of samples were investigated: not degradated (MCCh 1), partially degradated (MCCh 2 — insoluble in water fraction) and oligomers.

Methods of testing

The antivirus tests were carried out with the use of the two following plant (virus model systems): the bean/Lucerne mosaic virus (AIMV) and the tobacco plant/tobacco mosaic virus (TMV). The plants were sprinkled (by 0.025 wt. % water solution of chitosan or its degradation products), and next mechanically infected by the selected viruses. A phenomenon of oversensitivity, characterized by occurring of the local, necrotic stains was the effect of the infection. The activity of chitosan, *i.e.* the fact of hindering or lack of the virus infection (checked at the Institute of Plant Protection, Poznań) was estimated by comparison of the number of necrotic stains occurring on plants treated and not treated by chitosan.

Tests of the anti-mycotic activity of chitosan fraction were performed in the presence of the following plant soil pathogens: *rhizoctonia solam* and *myrothecium roridum*. The diameter of stains on the leaves of decorative pot plants, after 4 and 7 days of the test duration, was the measure of the efficiency of the formulations tested (carried out at the Research Institute of Pomology and Floriculture, Skierniewice).

Analytical methods

The physicochemical and structural properties of the chitosan formulations and the enzyme activity were estimated in IBWCh laboratories by research teams distinguished with G-016 certificate of Good Laboratory Practice within the area of physical, chemical, and biochemical research of biopolymers and enzymes.

The viscosity-average molecular weight was calculated on the basis of the limiting viscosity number (η), and the viscosity measurements were carried out with the use of a dilution viscometer equipped with a capillary of No. 1 and K ≈ 0.01 [9].

The degree of deacetylation (*DD*) was assessed by the spectrophotometric method consisting on the determination of the maximum of the curve obtained as the first derivative of UV spectrum, and mathematical calculation of the preparation's *DD* [10].

The cellulolytic enzymes' activity was estimated by the colorimetric method [11].

The protein content was determined by the Lowry's method [12].

Investigations of the structures of chitosan oligomers were carried out by the following methods:

— high-performance liquid chromatography (HPLC),

— high-performance anion-exchange chromatography (HPAEC) with the use of amperometric detection,

— mass spectroscopy with ion trap (ESI-MS),

— gel permeation chromatography/size exclusion chromatography (GPC/SEC),

— Fourier transform infra-red spectroscopy (FT-IR).

RESULTS AND DISCUSSION

Molecular structure

The molecular structure characterization of the chitosan degradation products, including oligomers, is important taking into account the recognition of the degradation process including its mechanism of proceeding. The ability to control the degradation process and to obtain the chitosan oligomers of a preset structure is essential considering their applications.

The analysis of water soluble degradation products by HPLC, HPAEC, GPC/SEC, and ESI-MS methods did not enable us to identify the particular oligomers precisely.

The investigations indicated that the degradation products where the blends of monomeric and oligomeric fractions. In turn, the oligomeric fraction was a blend of oligomers differentiated by their chemical structure and degree of polymerization (Figure 1). Considering that the degraded chitosan was characterized by a relatively

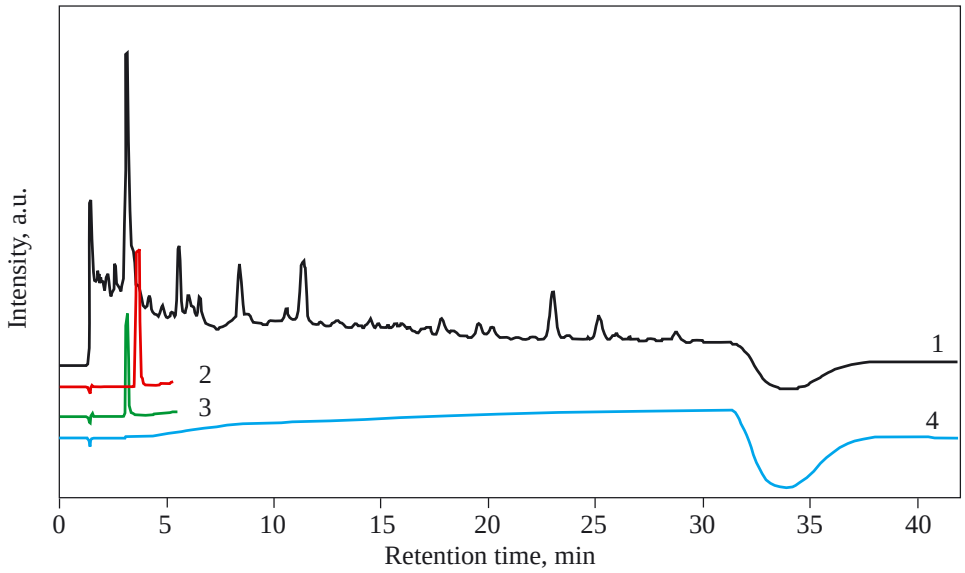

Fig. 1. HPAEC chromatograms of chitosan oligomers (1), glucosamine (2), and acetylglucosamine (3) in comparison with basic line (4)

low *DD* ≈ 75 %, D-glucose amines and its oligomers, *N*-acetylo-D-glucose amines and its oligomers, as well as mixed oligomers formed from the remains of D-glucose amines and *N*-acetylo-D-glucose amines were formed as a results of degradation. The precise identification of all elements of such complex blend is extraordinarily difficult and requires an initial separation into more homogeneous fractions.

Investigations concerning biological activity and possible application

Some introductory attempts were made to asses a possible application of the degraded chitosan products obtained in plant protection and medicine areas. Emphasis was put on the investigations aimed at protection against plant pathogens like bacteria and viruses.

The ability of chitosan biodegradation products to stimulate plant growth was assessed by germination force of test plant seeds. It has been found that the oligomers and partially degradated chitosan (MCCh 2) considerably stimulate the seeds germination at much lower concentrations than those required for the same effect by the use of chitosan before degradation (MCCh 1).

The activity of selected chitosan preparations against plant bacteria and viruses was tested at the Institute of Plant Protection in Poznań. The outcome of the tests is that chitosan oligomers effectively inhibit (100 %) the growth of lucerne mosaic virus (AIMV) (Table 1) and are less potent (32 %) in the case of the tobacco mosaic virus (TMV) which is resistant to plant protection agents. It could also be found that the water soluble products of chitosan degradation in the concentration range up to 0.5 % showed antibacterial activity against plant pathogens like *clavibacter michiganensis* subsp. *michiganensis*, *ervinia carotovora* subsp. *carotovora* and *escherichia coli*.

T a b l e 1. Influence of chitosan preparations on the number of stains on bean caused by lucerne mosaic wirus (AIMV)

Chitosan sample	Average number of stains/leaf	Inhibition of virus infection, %
Control[a)]	83.5	0
0.025 wt. % of oligomers	0.0	100
0.025 wt. % of MCCh 1	10.3	88
0.025 wt. % of MCCh 2		

[a)] Control sample was a pure water.

T a b l e 2. Effectiveness of chitosan and its oligomers in the protection of kalanchoe against *rhizoctonia solani*

Chitosan sample	Diameter of stains after days of application, mm	
	4	7
Control	5.8	7.0
MCCh 1	2.7	4.6
MCCh 2	2.4	3.0
Oligomers	3.0	4.8

Tests carried out in the Research Institute of Pomology and Floriculture in Skierniewice confirmed that all chitosan preparations investigated showed activity against the pathogenic plant fungi: *rhizoctonia solani* (Table 2) and *murothecium roridum*. Considering the commonly applied spray protection of plants, it is expected that the agents used in agriculture and gardening will show, apart from their high bioactivity, a good water solubility.

CONCLUSIONS

Low-molecular products of chitosan degradation seem to be a convenient material for the preparation of a human- and environmentally friendly plant protection agents. It is advisable to confirm, in further investigations, the activity against other than the above mentioned bacteria, fungi and viruses. The Plant Protection Institute and the Research Institute of Pomology and Floriculture express their readiness to carry on research in this direction including a wide groups of plants and pathogens.

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