BIOLOGICAL PROPERTIES OF CHITOSAN DEGRADATION PRODUCTS

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1. Introduction

Chitosan, considering its unique properties, such as biodegradability, bioactivity, biocompatibility, the fibre-grade properties, coating ability, and good miscibility with other polymers, finds broad applications in different fields, such as medicine, agriculture, environmental protection, and food industry [1 – 3]. Also the chito-oligomers, i.e. the low-molecular chitosan fractions, which are degradation products of this polymer, have practical significance considering their high bioactivity [4].

Products with a polymerisation degree smaller than 20, and of an average molecular weight up to 3900 Da are included into oligoaminosacharides [5]. Chitosan oligomers are characterised by antibacterial and antifungial activity, among others, and the fractions of the lowest polymerisation degrees are soluble in water which facilitates their practical use [6, 7]. The radiation, oxidation, hydrolytic, and thermal degradation methods belong to the group of well known chemical, physical and biochemical methods of chitosan degradation, which results in changes of the structural and use properties of this polymer.

The enzymatic degradation of chitosan proceeds most effectively in the presence of specific but at the same time expensive enzymes from the chitinase and chitosanase groups [8], but can also be carried out with the use of enzymes from the hydrolase glycosidic (celulase) group which are broad available and cheap. Celulolitic enzymes of high hydrolytic activity are created by mildew fungi from the genera *Trichoderma*, *Penicillium*, *Aspergillum* and

Fusarium [9 – 10]. The majority of these micro-organisms produce celulolitic enzymes in the form of a complex, which has the activity of many enzymes affecting cellulose in different ways, for example such as endo-1,4-β-glucanases, egzo-1,4-β-glucosidases, egzocelobiohydrolases and β- glucosidases. The following action is typical: endoglucanases randomly cut the 1,4-β-glycosidic bonds of the inner part of the cellulose chain, especially in the amorphous regions which led to its statistic fragmentarisation, celobiohydrolases detach celobiose molecules from the not reducing chain-end in amorphous, as well as in crystalline regions, egzoglucosidases also detach glucose molecules from the not reducing chain-end, whereas β- glucosidase hydrolyse celobiose and short oligosacharides to glucose [11].

A similar mechanism should be expected during chitosan degradation with the use of the enzymes above-mentioned. A suitable selection of the celulolitic complex enables obtaining chitosan oligomers of assumed molecular structure by a controlled procedure.

Recently, in the Institute of Biopolymers and Chemical Fibres (IBWCh), Łódź, Poland) have been carried out investigations into the degradation of chitosan and recognition of application possibilities of the chitosan degradation products, especially of oligomeres which are water-soluble. A fragment of these research work was realised as part of the commissioned project 'Polymer materials from removable raw materials – the physicochemical basis of new technologies' co-ordinated by the Centre of Molecular and Macromolecular Research of the Polish Academy of Sciences (CBMiM PAN), Łódź, Poland. The aim of the research carried out by IBWCh were investigations into the enzymatic degradation of chitosan, in order to obtain low-molecular fractions characterised by appropriate use properties, suitable for agriculture and medical applications The biochemical degradation was conducted in a heterogenic system using a gel-like suspension of microcrystalline chitosan (MCCh) and enzymes from the hydrolase group (celulase and xylanase).

As part of this research work, investigation into the mechanism of enzymatic chitosan degradation were conducted together with the estimation of the physicochemical and structural properties of degradation products which was the subject of our earlier presentations [12 – 14]. In the work presented herein the results of biological investigation into the biodegradation products obtained are described, as well as the possibilities evaluated of their practical application as ecological stimulators of plant growth and preparations for protection plants against plant-pathogens, viruses, and bacteria.

2. Materials

Chitosan produced by Vanson HaloSource Inc., Redmont, WA, USA, characterised by an average molecular weight of M_v = 401 kDa and deacetylation degree of 75.8%.

This chitosan was used as raw material for manufacturing the microcrystalline chitosan form which was subjected to degradation.

Neutral celulase produced by AB Enzymes OY, Finland with endo-1,4-β-glucanase activity of 186 U/cm^3, a commercial enzymatic product used for biochemical chitosan biodegradation.

3. Research methods

3.1. Obtaining chitosan oligomers by biochemical biodegradation

Microcrystalline chitosan, which is used to obtain oligoaminosacharides, was obtained as the result of polymer agglomeration from the solvent solution, according to a method developed in the Institute of Biopolymers and Chemical Fibres [15, 16].

The enzymatic degradation of the MCCh suspension containing 1.0% wt. of the polymer was conducted under dynamic conditions at a temperature of 50 °C, for 7 hours, and at an enzyme to substrate module of (E/S) = 2066 U (CMC)/g. After the pre-set degradation time of 7 hours, the reaction mixture was filtered, in order to separate the oligomers soluble in water from the non-soluble fractions of partially degraded chitosan. Next, the aqueous solution of chitosan oligomers was purified from proteins originated in the enzyme, using a set equipped with a membrane of polyethersulphone with MWCO 5000, from Vivaflow.

3.2. Determining the physicochemical properties of chitosan and their degradation products

The estimation of physicochemical and structural properties of the chitosan preparations and evaluation of the enzymes activity were carried out in IBWCh research groups and laboratories granted by the certificate GLP G-016 of Good Laboratory Praxis into physical, chemical, and biochemical research of biopolymers and enzymes.

- The viscometric average molecular weight was calculated on the basis of the limiting viscosity number [η]. The viscosity measurements were carried out with the use of a dilution viscometer Nr 1, of K ≈ 0.01 (in accordance with the procedure GLP SPR/BPB/5).
- The deacetylation degree was assessed by the spectrophotometric method, basing on determining the curve maximum of the first derivative of the UV spectrum and mathematical calculating the degree (in accordance with the procedure GLP SPR/BLF/21).
- The celulolitic enzyme activity was evaluated by the colorimetric method (in accordance with the procedure GLP SPR/BBP/8, 9, 10, 11).).

3.3. Investigating the biological properties of chitosan and its oligomers

The biological properties of microcrystalline chitosan and the products of its degradation were estimated on the basis of the influence of these preparations on the germination ability of radish seeds, and the retardation ability to bacteria and mycotic diseases of plants, as well as of plant viruses.

The estimation of the germination ability of radish seeds was performed by the weight-pan method, with the use of filter paper discs saturated with a suspension of chitosan and oligomers of pre-determined concentration of 0.1%, 0.01%, and 0.001%. The influence of the preparations on the germination ability was estimated after 48 hours, on the basis of the number of germinated seeds, the green mass and the length of the germs in relation to the control test with use of water.

The anti-virus tests were carried out with the use of the two following plant-virus model systems: bean / AIMV (virus of the *Lucerne mosaic* – sensitive to chitosan derivatives), and tobacco / TMV (virus of the *Tobacco mosaic* – of small sensitivity to chitosan derivatives).

The plants mentioned above were sprayed, and next infected mechanically by the viruses. The infection effect was an oversensitivity phenomenon manifested by appearing of local necrotic stains. The chitosan activity was determined by comparison of the number of necrotic stains on the plants treated and non-treated by the chitosan preparations.

The antibacterial tests were carried out with the use of two plant bacteria, the *Clavibacter michganensis* subsp. *michganensis*, and the *Erwinia carotovora* subsp. *carotovora*. The bacteria suspension was inserted into the nutrient medium by the subside-inculation method and poured on a Petri dish. After the solidification of the nutrient, filter paper disks with a diameter of 5 mm, saturated with the solution or suspension of the appropriate preparation, were placed on the bacteria field prepared, three disks on each Petri dish. The dishes were incubated for 48 hours in an incubator at a temperature of about 30 °C. After incubation, the bacteria growth retardation zones were estimated. The tests were carried out in the Institute for Plant Protection in Poznań, Poland.

The tests of anti-mycotic activity of chitosan were carried out with the use of two plant soil pathogens, the *Rhizoctonia solani* and the *Myrothecium roridum*. The measure of the efficiency of the formulations tested was the diameter of stains on leaves of ornamental pot plants after 4 and 7 days of the test duration. The tests were carried out in the Institute for Fruit Farming and Floriculture in Skierniewice, Poland.

4. Test results and dicussion

The estimation of the biological activity was carried out of the microcrystalline chitosan before degradation (MCCh 1), of partially degraded microcrystalline chitosan (MCCh 2), and of degradation products in the form of oligomers soluble in water. Table 1 presents a characterisation of chitosan and the products of its degradation dedicated to application tests.

Table 1. *Basic physicochemical parameters of microcrystalline chitosan and products of its degradation.*

Preparation symbol	$\bar{M}_v$, kD	Deacetylation degree, %	Polymer content, %	Form
MCCh 1	320	75.8	2.52	suspension
MCCh 2	12	77.4	5.48	suspension
Oligomers	< 1.2	76.8	0.54	solution

4.1. Estimating the biological properties of chitosan preparations

As part of our work, the estimation of the influence of chitosan biodegradation products on the ability to stimulate the germination force of test pants (radish) was carried out. The influence of the initial MCCh was compared with those of its degradation products at concentrations of 0.1%, 0.01%, and 0.001%. The biological activity of the preparations tested was determined on the basis of the number of germinated seeds, the green mass of germs, and its length in relation to results obtained by control tests carried out with water (Table 2).

We indicated that all tested chitosan forms stimulate germination of radish seeds, but the chitosan oligomers with concentration of 0.01% were characterised by the most advanta-

Table 2. *Influence of the concentration of chitosan forms used while saturated the nutrient on the germination ability of radish seeds after 48 hours.*

Preparation symbol	Concentration, %	Number of germs in relation to control test, %	Length in relation to control test, %	Mass in relation to control test, %
Control test	0	100	100	100
MCCh 1	0.10 0.01	114.3 92.3	129.9 111.1	116.2 107.6
MCCh 2	0.010 0.001	97.4 105.7	133.3 145.0	107.7 120.8
Oligomers	0.010 0.001	108.6 111.4	154.7 139.0	126.2 121.5

geous action; the length increase of the germs was by about 55% and that of the mass by about 26% in relation to control tests. The partially degraded chitosan (MCCh 2) and the initial chitosan (MCCh 1) slightly less effective stimulated the seeds' germination. The chitosan oligomers stimulated the germination of the radish test seeds at a lower preparation dose in comparison with microcrystalline chitosan.

4.2 Evaluating the antivirus and antibacterial activity of MKCh and chitosan oligomers

The initial MCCh and the products of its enzymatic degradation, characterised by the parameters presented in Table 1, were used for evaluation of the antivirus and antibacterial activity towards plant pathogens.

The antivirus and antibacterial activity tests were carried out in the Institute for Plant Protection in Poznań, Poland. The antivirus test results are presented in Tables 3 and 4, whereas the antibacterial test results in Table 5.

Table 3. *Influence of chitosan preparations on the number of stains on bean leaves caused by the virus of lucerne mosaic (AIMV).*

Chitosan form	Average number of stains	Inhibition of the virus infection, %
Control test	83,5	0
0,025 % oligomers	0,0	100
0,025% MCCh 1	10,3	88

Table 4. *Influence of chitosan preparations on the number of stains on tobacco leaves caused by the virus of tobacco mosaic (TMV).*

Chitosan form	Average number of stains	Inhibition of the virus infection. %
Control test	70.1	0
0,025 % oligomers	47.7	32
0,025% MCCh 2	63.8	8

In the case of both plant-virus systems (Table 3 and 4), the chitosan oligomers were characterised by better effectiveness of protection against viruses than the preparations of micro-

Table 5. *Activity of chitosan preparations toward plant bacteria, in vitro; Cms - Clavibacter michganensis subsp. michganensis, Ecc - Erwinia carotovora subsp.carotovora, E. coli – Escherichia coli, (0.5) – at this concentration, the preparation still did not demonstrated bacteriostatic activity.*

Type of plant bacteria	Type of preparation - MIC		
	Chitosan oligomers	Glucosamine hydrochloride	Microcrystalline chitosan MCCh 2
Cms	0.1	(0.5)	(0.5)
Ecc	0.5	(0.5)	(0.5)
E. coli (control test)	0.5	(0.5)	(0.5)

crystalline chitosan (MCCh 1 and MCCh 2). In the case of the *Lucerne mosaic* virus, the chitosan oligomers soluble in water totally retarded the infection of this virus, whereas the initial MCCh 1 and the partially degraded MCCh 2 preparations were characterised by the ability to inhibition at the level of 88% in relation to control test. A lower antivirus activity was observed in the case of the resistant tobacco mosaic virus; the oligomers were characterised by an ability to inhibition at the level of 32%, whereas the partially degraded MCCh 2 only at the level of about 8% in relation to control tests.

While estimating the antibacterial activity of preparations, the minimum inhibitory concentration (MIC) was determined. The investigations carried out in a green house under adverse temperature conditions of a to high environmental temperature in the presence of the following plant bacteria; the *Clavibacter michganensis* subsp. *michganensis*, the *Erwinia carotovora* subsp. *carotovora*, and the control bacteria *Eschirichia coli* indicated that only the chitosan oligomeres are characterised by bacteriostatic activity, especially towards the Cms bacteria (*Clavibacter michganensis* subsp. *michganensis*). The remaining preparations (glucosamine hydrochloride and partially degraded microcrystalline chitosan) did not indicate any activity against plant bacteria within the range of concentrations up to 0.5% (Table 5). Considering the high temperature while carrying out the tests (environmental temperature above 30 °C) it was not possible to realise more complex investigations into antibacterial and antivirus tests under greenhouse conditions.

4.3. Estimating the MCCh' and chitosan oligomers' activity towards pathogens of ornamental plants

Activity tests of selected chitosan and oligomer preparations towards ornamental plants (kalanchoe and balsam – buzy Lizzie) pathogens of the type *Rhizoctonia solani* and *Myrothecium roridum* were carried out in the Institute for Fruit Farming and Floriculture in Skierniewice, Poland. The preparations listed in Table 1 were evaluated. The measure of efficiency of the preparations tested was the diameter of stains on the kalanchoe and balsam leaves. The test results are presented in Tables 6 and 7.

All chitosan preparations tested retarded in a similar way the growth of rhisoctonosis on the kalanchoe leaves already after 4 days of using. Contrary, the efficiency of these preparations in protection of the New-Guinea balsam was visible already after 7 days of application. During the tests carried out, we did not indicate a more advantageous action of chitosan degradation products towards soil pathogens compared with the initial chitosan.

Table 6. *Chitosan's and oligomers' efficiency in protection of Kalanchoe against Rhizoctonia solani (rhisoctonosis).*

Preparation form	Stain diameter in mm after, days	
	4	7
Control test	5.8 c	7.0 c
MCCh 1	2.7 a	4.6 b
MCCh 2	2.4 a	3.0 a
Oligomers	3.0 ab	4.8 b

Table 7. *Chitosan's and oligomers' efficiency in protection of New-Guinea balsam against Myrothecium roridum (ring discolouration).*

Preparation form	Stain diameter in mm after, days	
	4	7
Control test	9.0 a	14.8 c
MCCh 1	8.0 a	10.1 a
MCCh 2	9.2 a	12.0 b
Oligomers	9.0 a	10.3 a

5. Summary

As the result of investigations carried out we stated that the oligomeres and the partially degraded chitosan:

- effectively stimulate the germination of radish seeds at concentrations lower than those of non-degraded MCCh,
- totally retard (100%) the growth of *Lucerne mosaic* virus (AIMV), and in a smaller degree (32%) inhibit the growth of the resistant *Tobacco mosaic* virus (TMV),
- retard the growth of plant bacteria (*Clavibacter michganensis* subsp. *michganensis*, and *Erwinia carotovora* subsp. *carotovora*) within the concentration range of 0.5%,
- indicate an activity against pathogens of the plant fungi *Rhizoctonia solani* and *Myrothecium roridum*.

In the case that future investigations would confirm the activity of low molecular chitosan degradation products towards other bacteria, viruses and fungi, as well as considering their solubility in water, these products may be used for developing plant protection preparates, which would be easy in use and safe for humans and the environment. The Research Institute of Pomology and Floriculture and the Institute for Plant Protection declared their readiness to continue investigations into elaboration of new plant protection preparates dedicated for different plant and their pathogens.

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