*Research Note*

# Chitosan Polymer Sizes Effective in Inducing Phytoalexin Accumulation and Fungal Suppression Are Verified with Synthesized Oligomers

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**Biologically derived chitosan has been reported to induce pisatin and disease resistance response proteins in pea tissue and also to inhibit the germination and growth of some fungal pathogens. Stereo-controlled synthesis of chitosan tetramer, hexamer, and octamer allowed the precise verification of oligomer size required for biological activity. The octameric oligomer optimally induced pisatin accumulation and inhibited fungal growth, verifying previous results obtained with column-purified oligomers derived from crab shells.**

Chitosan (β-1,4-linked glucosamine polymer), first implicated in host-parasite interactions when it was found to be involved in the interaction between pea and *Fusarium solani* (Hadwiger and Beckman 1980; Kendra *et al.* 1989), has subsequently been shown to influence other plant-pathogenic infections (Benhamou and Theriault 1992; Conrath *et al.* 1989; Cuero *et al.* 1991; El Ghaouth *et al.* 1992a,b; Hadwiger *et al.* 1989). Chitosan derived from *Fusarium* cell walls or from crab or shrimp shell chitin actively induces the accumulation of phytoalexins (Kendra *et al.* 1989), disease resistance response proteins (Loschke *et al.* 1983), and their corresponding mRNAs (Fristensky *et al.* 1985). Chitosan also has direct antifungal properties, which range from fungistatic to fungicidal, against *F. solani* and other plant pathogens (Allan and Hadwiger 1979; Kendra *et al.* 1989). Chitosan-like polymers are released from fungal walls when in contact with plant tissue and in the presence of β-glucanase and chitinase enzymes purified from pea tissue (Kendra *et al.* 1989). Chitosan is readily detectable in the pea-fungus interface by immunohistological methods (Hadwiger *et al.* 1981). Chitosan derived from the cell wall chitin of *F. s.* f. sp. *phaseoli,* a pathogen of beans, applied to pea tissue in advance of *F. s.* f. sp. *pisi* inoculum, induced resistance to this pea pathogen (Kendra *et al.* 1989).

The induction of defense-related responses by chitosan has been observed in other plants, such as soybean (Kohle *et al.* 1984) and parsley (Conrath *et al.* 1989). Chitosan-derived oligosaccharides induce proteinase inhibitors in tomato leaves (Walker-Simmons *et al.* 1983). Chitin (predominantly acetylated chitosan) increases lignification in wheat plants (Barber *et al.* 1989), and chitosan oligomers elicit lignification in pea tissue (Hadwiger *et al.* 1989). These observations, taken with chitosan's regulatory properties, suggest it might have a major signaling role in plant-fungus interactions.

Oligomer size is an important aspect of chitosan's action. Heptamer and larger oligomers maximally induce increases in pisatin in peas (Kendra and Hadwiger 1984). Chitosan oligomers of heptamer or greater length suppressed growth of *F. s.* f. sp. *phaseoli* at 4 μg/ml in nutrient medium. Previous determinations of the optimal chitosan oligomer size for biological activities have utilized sizing columns to separate the various sizes of chitosan oligomers generated from commercial crab shell chitosan cleaved with nitrous acid or hydrolyzed with HCl. Polycations such as chitosan have the potential to tenaciously complex with an array of negatively charged impurities. Thus, these impurities could potentially influence size separations. Hypothetically, the chitosan oligomer from a crab shell source could harbor a trace impurity reponsible for the observed biological activity. Unfortunately, sizing columns ineffectively separate larger oligomers, and thus the contributions of oligomers larger than the heptamer to the biological action have not been defined, because they are not readily separable from the heptamer fraction.

The chemical synthesis of different sizes of chitosan oligomers (Kuyama *et al.* 1993) makes it possible to assay polymers of precise sizes for biological activity. The specific recognition of the target receptor sites becomes more accurate when the biological activity is attributable to an elicitor preparation with a single polymer length.

In this note we examine the relative activities of synthesized chitosan oligomers in inducing pisatin accumulations in pea tissue and in inhibiting the growth of *F. s.* f. sp. *phaseoli* macroconidia. The stereo-controlled synthesis of chitosan oligomers was as described previously (Kuyama *et al.* 1993). The first series of oligomers were synthesized with a basic structure of $GlcNH_2\beta(\rightarrow 4GlcNH_2\beta)_n \rightarrow 4GlcNH_2\beta \rightarrow OMP$, where the terminal OMP = 4-$CH_3O$-$C_6H_4O$ and $n$ = 2, 4, 6, 8, and 10, corresponding to individual tetrameric, hexameric, octameric (Fig. 1A), decameric, and dodecameric oligomers.

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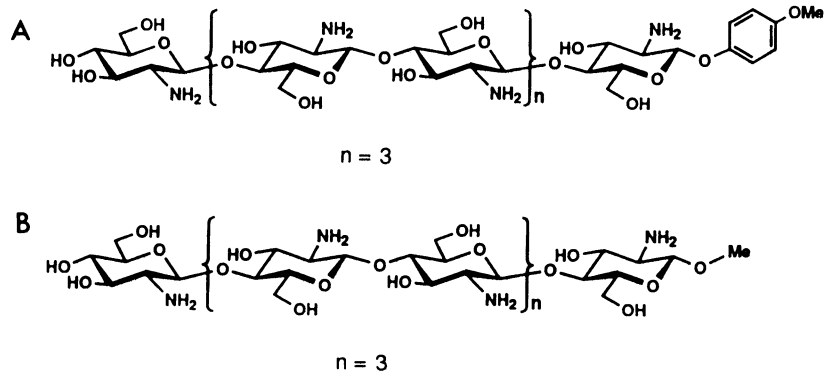


**Fig. 1.** Structures of the chitosan octameric oligomers derivatized with 4-$CH_3O$-$C_6H_4O$ (**A**) and $OCH_3$ (**B**) at carbon 1 of the terminal reducing sugar.

A second oligomer series, consisting of tetrameric, hexameric, and octameric (Fig. 1B) glucosamines, was synthesized with a small aglycon, $CH_3$, terminating the oligomer in place of OMP.

To determine the effect of chitosan oligomer treatment on induction of pisatin synthesis in peas, a chitosan solution (100 µl) was applied to the endocarp surface of uniform-sized immature pea pods (cultivar Alcan) in duplicate five-pod halflots totaling 0.25 g, fresh weight. The treatments consisted of a half-fold dilution series of oligomer concentrations in sterile $H_2O$ ranging from 2,000 to 62 µg/ml. The pods were incubated for 24 hr at 22° C, after which pisatin was extracted and quantitated as described previously (Hadwiger and Beckman 1980).

Pisatin accumulated in response to treatment with the chitosan octamer (at concentrations of 500–2,000 µg/ml) possessing the terminal methyl aglycon to levels that approached those obtained with a high concentration of spores ($3.8 \times 10^6$ per milliliter) of *F. s.* f. sp. *phaseoli* (Table 1). In contrast, none of the entire oligomer series with the terminal OMP detectably increased pisatin accumulations (data not shown), indicating that the methoxyphenol group negates or masks the eliciting properties of chitosan. The smaller (hexameric and tetrameric) oligomers with the terminal methyl aglycon were also unable to induce pisatin. Therefore, it appears that the synthetic oligomers require a length of at least eight sugar units to elicit pisatin production in peas. These results confirm the oligomer size optima for eliciting pisatin previously acquired with chitosan oligomers (heptamer or larger fraction) purified in a sizing column (Kendra and Hadwiger 1984). They further indicate that a major modification of the terminal reducing sugar with a large hydrophobic aglycon is capable of destroying elicitor activity.

The bean pathogen *F. s.* f. sp. *phaseoli* strain W-8 (American Type Culture Collection accession number 38135), an incompatible pathogen on peas, was utilized as the test organism for assays of antifungal activity of the chitosan oligomers. A half-fold dilution series of each oligomer was prepared in microtiter plates using a microdiluter capillary transfer pipette (Cooke laboratory products, Dyantech Laboratories, Alexandria, VA). The concentration of oligomers ranged from 500 to 0.24 µg/ml in quarter-strength Vogel's complete medium. Because of the limited quantities of synthesized oligomers available, the dilution series was only replicated once in two separate experiments. The first experiment employed approximately 100 macroconidia of *F. s.* f. sp. *phaseoli* per well, and the second approximately 30 macroconidia per well (Table 2). Germination and growth were initially observed with a dissecting microscope within 28 hr after the macroconidia were added to the treatment wells. Subsequently, growth was evaluated visually, by the degree of mycelial matting 64 hr after treatment began. Visually clear wells were recorded as having no growth. The concentration of chitosan that fell halfway between the minimum concentrations giving no growth and total matting of the well was used to estimate the 50% effective dose ($ED_{50}$).

**Table 1.** Effect of synthesized glucosamine oligomers on 24-hr accumulation of pisatin in pea endocarp tissues

| Treatment[a] | Concentration applied (mg/g) | Pisatin (µg/g fresh weight) |
|---|---|---|
| $H_2O$ | ... | 0 |
| *Fusarium solani* f. sp. *phaseoli* ($3.8 \times 10^6$ spores) | ... | 299 ± 7 |
| Shrimp chitosan | 1.0 | 148 ± 25 |
| Tetramer | 1.0 | 8 ± 1 |
| | 0.5 | 2 ± 0 |
| | 0.25 | 6 ± 1 |
| | 0.125 | 3 ± 2 |
| Hexamer | 1.0 | 5 ± 0 |
| | 0.5 | 3 ± 1 |
| | 0.25 | 0 |
| | 0.125 | 0 |
| Octamer | 2.0 | 207 ± 3 |
| | 1.0 | 206 ± 32 |
| | 0.5 | 48 ± 12 |
| | 0.25 | 4 ± 1 |
| | 0.125 | 14 ± 6 |
| | 0.062 | 0 |

[a] A 100-µl volume of each treatment was applied to 250 µg of pea endocarp tissue. Tetramer, hexamer, and octamer are glucosamine oligomers terminated with a $CH_3$ aglycon. Shrimp shell chitosan was solubilized as described previously (Kendra and Hadwiger 1984).

Inhibition of the growth of *F. s.* f. sp. *phaseoli* was optimal in treatments with the chitosan octamer terminated with the methyl aglycon (Table 2). In a separate test of growth suppression read at 42 hr, native chitosan, octamer-$CH_3$, octamer-OMP, and decamer-OMP completely inhibited fungal growth at 30 µg/ml, whereas tetramer-OMP, hexamer-OMP, and dodecamer-OMP inhibited growth at 60, 250, and 125 µg/ml, respectively. These results suggest that the additional modification of the oligomer with the OMP causes a major

**Table 2.** Concentration (μg/ml) of synthetic glucosamine oligomers for inhibition of growth of *Fusarium solani* f. sp. *phaseoli*

| Supplement to medium | Experiment 1[a] | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | 28 hr after treatment | | 64 hr after treatment | | 29 hr after treatment | | 65 hr after treatment | |
| | Total inhibition | $ED_{50}$[b] | Total inhibition | $ED_{50}$ | Total inhibition | $ED_{50}$ | Total inhibition | $ED_{50}$ |
| None | ...[c] | ... | ... | ... | ... | ... | ... | ... |
| Tetramer[d] | ... | ... | ... | ... | ... | ... | ... | ... |
| Hexamer[d] | ... | ... | ... | ... | ... | ... | ... | ... |
| Octamer[d] | 31 | 15 | 500 | 125 | 31 | 15 | 250 | 125 |
| Native chitosan | 31 | 15 | 62 | 31 | 31 | 15 | 125 | 62 |

[a] In experiments 1 and 2, 100 and 30 spores per microtiter well were tested, respectively.
[b] $ED_{50}$ = effective dosage providing approximately 50% inhibition of the growth that would occur in a medium without glucosamine oligomer.
[c] No growth inhibition was observed.
[d] The glucosamine oligomers were terminated with an *O*-methyl glycoside.

deviation from that characteristic of native chitosan oligomers. The deviant behavior may be more representative of the OMP than of its associated oligomer. Conversely, the simple *O*-methyl modification is likely to be trivial, and the biological activity of the oligomer may very closely resemble that of corresponding sizes of oligomers released in the plant-fungus interaction (Kendra *et al.* 1989). Both of the synthesized chitosan oligomer series have been derivatized on the reducing end of the terminal sugar. Interestingly, reducing-end modifications had no reported effect on the elicitation of soybean phytoalexin by a synthesized oligoglucoside (Hahn *et al.* 1992).

The availability of these specific chitosan oligomers will enable more precise determinations of chitosan's mode of action. The sharp size demarcations of biologically active sizes suggest that structural aspects of these molecules are more important than simply their cationic charge content. This oligomer specificity should also aid in the identification and purification of cellular receptors which initiate the biological response. More immediately, the confirmation of both the elicitor size and the absence of biological impurities in the elicitor obtained by chemical synthesis is added assurance that the induction is indeed related to the chitosan oligomer and not to some hypothetical impurity associated with biological preparations of these oligomers.

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