Partially N-Deacetylated Chitin Fragments are Strong Elicitors for (+)-Pisatin Induction in Epicotyls of Pea

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Chitosan derivatives classed into three groups according to their molecular size were examined for the relationships between degree of N-acetylation and phytoalexin (+)-pisatin-inducing activity in pea epicotyl assay. Partially N-deacetylated chitin with degree of N-acetylation 56% (DAC 56%) was the most potent inducer of (+)-pisatin among sparingly water-soluble polysaccharides including chitosan (degree of N-acetylation 0%), DAC 32%, and chitin. Among the intermediate-sized molecules obtained from degradation of the chitosan and the DACs by $NaNO_2$, the chitosan fragment was the most powerful elicitor for (+)-pisatin induction. The $NaNO_2$-degraded DAC 56% exhibited a comparable activity to the $NaNO_2$-degraded chitosan at low concentrations. The $NaNO_2$-degraded fragments as well as the native polymer induced a relatively high amount of (+)-pisatin, while the acetone precipitate of chitosan oligomers from the HCl-catalyzed hydrolysate and their partially N-acetylated derivatives exhibited a moderate (+)-pisatin-inducing activity. These findings suggest the possibility that partially N-deacetylated chitin fragments could be one of the most promising elicitors in the pea system.

Introduction

Elicitors are signal molecules which induce physiological or biochemical responses associated with expression of resistance (Ryan, 1988; Darvill and Albersheim, 1984; Kobayashi *et al.*, 1993). Oligosaccharides derived from cell walls of fungi act as elicitors. The elicitor-active oligosaccharides are thought to be liberated from the fungal cell wall by the action of plant hydrolytic enzymes at the early stage of plant-microbe interaction and to activate a broad spectrum of defensive genes in plant tissues. Plants treated with elicitors elicit a wide variety of defensive responses including the induction of phytoalexins, hydrolytic enzymes such as chitinase, β-glucanase, and oxidation enzymes responsible for lignin accumulation and generation of active oxygen species.

Chitin, β-1,4-linked D-N-acetylglucosaminoglycan, is one of a major component of the fungal cell wall. Chitosan, β-1,4-linked-D-glucosaminoglycan, is generated by the enzymatic N-deacetylation of chitin by chitin deacetylase in fungi. The polycationic polymer is also produced industrially by thermochemical N-deacetylation of chitin (Martinou *et al.*, 1993). These two polysaccharides and their fragments have been reported to exhibit elicitor activities towards several plants (Pearce and Ride, 1982; Hirano *et al.*, 1990; Inui *et al.*, 1991; Yamada *et al.*, 1993; Ren and West, 1992; Hadwiger and Beckman, 1980; Kendra and Hadwiger, 1984; Walker-Simmons and Ryan, 1984).

As demonstrated in the studies on the antimicrobial and antitumor activities of chitin, chitosan, and their oligomers, their physiological potentialities were shown to have close correlation to their molecular size and degree of N-acetylation (Uchida, 1988; Nishimura *et al.*, 1984). These factors might also provide significant effects on the plant metabolism, prompting us to investigate the relationship between structural features of chitin-derived oligosaccharides and their elicitor activities in pea plant.

Chitosan, nitrous acid-cleaved chitosan with DP ~ 4 through DP > 6, and HCl-cleaved chitosan were reported to be strong elicitors of phytoalexin (+)-pisatin in immature pea pods, while chitin and low-molecular-weight chitin oligomers showed weak or no activities (Hadwiger and Beckman, 1980; Kendra and Hadwiger, 1984). However, the relationships of molecular size, degree of N-acetylation of the saccharides and their (+)-pisatin-inducing activities have not been examined.

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In this study, we investigated the (+)-pisatin-inducing potentiality of several chitin and chitosan derivatives with different molecular size and different degree of N-acetylation in our pea epicotyl assay, and found that partially N-deacetylated chitin derivatives are potent elicitors for the induction of (+)-pisatin and other unidentified phenolic compounds (Akiyama *et al.*, 1993).

Materials and Methods

Chemicals

Chitin was purchased from Wako Pure Chemical Industries, Ltd. Chitosan (degree of N-acetylation 0%) was obtained from Funakoshi Co., Ltd. For the large-scale preparation of chitosan oligomers by HCl hydrolysis, chitosan was provided by Tokyo Chemical Industry Co., Ltd.

(+)-Pisatin was purified from methanol extract of pea seedlings treated with 4 mM $CuCl_2$ aqueous solution as reported previously (Kobayashi *et al.*, 1993).

Conditions for plant growth

Pea seeds (*Pisum sativum cv.* USUI) obtained from Takayama Seed Co., Ltd. were surface sterilized by immersing in 70% EtOH for 5 min, followed by 5% H_2O_2 for 30 min and subsequently the seeds were rinsed three times with sterile distilled water. Surface-sterilized seeds were transferred onto a medium containing 0.1% $MgCl_2$ and 0.2% GELRITE (SAN-EI, Osaka) in test tubes (∅ 25 × 200 mm) and incubated in the dark at 24 °C for 10 days.

Preparation of partially N-deacetylated chitin (DAC)

Chitin (1.0 g) was dissolved in 100 ml of aqueous 50% (w/v) NaOH at room temperature for 1 h under reduced pressure with stirring. The solution was stirred at 75 °C under nitrogen gas for 30 and 120 min, respectively. The resulting solution was poured into 400 ml of ice-cooled MeOH and filtered. The residue was washed with MeOH−H_2O (1:1) until the aqueous suspension of the residue became neutral. The DAC powder was dried at 50 °C over P_2O_5 under reduced pressure.

Nitrous acid degradation of chitosan and DACs

Nitrous acid attacks GlcN units but not GlcNAc units in chitosan and DAC molecules, the following glycosidic linkage is cleaved, and a 2,5-anhydro-D-mannose residue is generated at the new reducing end. Limited degradation of chitosan and DAC with a trace of nitrous acid provides intermediate-sized molecules with much the same degree of N-acetylation as in the native polymers. The $NaNO_2$-degradation method has been employed to determine the degree of N-acetylation in partially N-deacetylated chitins by ^1H NMR spectroscopy (Vårum *et al.*, 1991). Judging from the fractionation precipitation with organic solvents, the mean value of their molecular weight was assumed to be much larger than that of the HCl-hydrolysis product of chitosan (data not shown).

One-hundred-milligram of chitosan or DAC was dissolved in 10 ml of 0.07 M HCl. Three-milligram of solid $NaNO_2$ was added, and the solution was stirred at room temperature for 4 h, then lyophilized.

Chitosan oligomers prepared by HCl hydrolysis and partial N-acetylation of the oligomers

Chitosan (25 g, Tokyo Chemical Industry Co., Ltd.) was hydrolyzed in 250 ml of 4 N-HCl at 65 °C for 12 h. After hydrolysis, the insoluble-residue was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The dried sample was dissolved in H_2O and decolourized once with activated charcoal. The solution was again diluted with H_2O to 200 ml in the total volume. To the solution, 800 ml of acetone was added, the resulting precipitate was collected by centrifugation, then freeze-dried after dissolved in a HCl-acidic solution. Precipitation of the chitosan hydrolysate with 80% acetone-H_2O promised to enhance the ratio of chitosan oligomers with DP 5 ~ 7. The oligomers, pentamer through heptamer, have been reported to be elicitor-active components from HCl-cleaved chitosan (Kendra and Hadwiger, 1984; Walker-Simmons and Ryan, 1984).

The chitosan oligomers were partially N-acetylated according to the method for selective N-acetylation (Barker *et al.*, 1958). One-hundred milligram chitosan oligomers were dissolved in 3 ml H_2O and mixed with 2.5 ml MeOH, 1 ml Bio-Rad AG 1−X8 (HCO_3^- form), and 15, 30, 100 µl

acetic anhydride. The mixture was stirred for 90 min at 0–5 °C.

Determination of degree of N-acetylation of DACs and N-acetylated chitosan oligomers by ^1H NMR spectroscopy

For determination of degree of N-acetylation of DACs with 500 MHz ^1H NMR spectrometry (Vårum *et al.*, 1991), 10 mg of $NaNO_2$-degraded DAC was dissolved in D_2O and lyophilized in order to minimize the HOD signal, and dissolved in D_2O at pD 3. The chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-d_4. The 500 MHz ^1H NMR spectra were recorded with a Varian VXR-500 Instrument at 90 °C. The degree of N-acetylation was determined by the comparison of the area of the signals due to the H-1 protons of the GlcNAc units (δ 4.55–4.65) with that of the GlcN units (δ 4.85) (Fig. 1). The degree of N-acetylation of DACs obtained from 30 min and 120 min-alkali treatment was 56% and 32%, respectively.

The degree of N-acetylation of the native chitosan oligomers and partially N-acetylated derivatives were determined by 500 MHz ^1H NMR spectrometry according to the method reported previously (Ishiguro *et al.*, 1992). Each lyophilized sample was dissolved in D_2O and the pD of samples was adjusted to pD 4 by addition of DCl. The same instrumental condition was adopted. The degree of N-acetylation was also determined by the comparison of the area of the signals due to the H-1 protons of the GlcNAs units (δ 4.55–4.65, 4.70 for β-anomeric protons, δ 5.19 for α-anomeric protons) with that of the GlcN units (δ 4.85–4.90 for β-anomeric protons, δ 5.43 for α-anomeric protons). The percentage N-acetylation as measured by ^1H NMR was 5, 37, 66, and 91%, respectively.

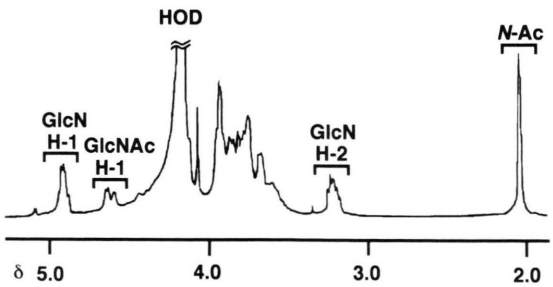

Fig. 1. ^1H NMR spectrum of $NaNO_2$-degraded partially N-deacetylated chitin with d.a. 32% at pD 3, 90 °C. pD, deuterium ion exponent.

Chitosan oligomer composition in 80% acetone-H_2O precipitation product of HCl-hydrolyzed chitosan

Chitosan oligomers in the 80% acetone-H_2O precipitation product of HCl-hydrolyzed chitosan were separated by a column of TSKgel Amide-80 (∅ 4.6 × 250 mm, TOSOH Co., Ltd.). Acetic acid (0.6%) and diethylamine (0.2%) in acetonitrile-water (35:65) were used as eluents. The flow rate was 0.5 ml/min and detected with a RI detector. The pseudo-molecular mass ion of each peak was measured by LC-MS (Finnigan MAT TSQ mass spectrometer equipped with API interface). Chitosan dimer through nonamer were detected in the sample (dimer; Rt = 8.5 min, $[M + H]^+$ 341, trimer; Rt = 10.3 min, $[M + H]^+$ 502, tetramer; Rt = 12.5 min, $[M + H]^+$ 663, pentamer; Rt = 14.8 min, $[M + H]^+$ 824, hexamer; Rt = 18.0 min, $[M + H]^+$ 985, heptamer; Rt = 22.0 min, $[M + H]^+$ 1146, octamer; Rt = 28.4 min, $[M + H]^+$ 1308, nonamer; Rt = 39.0 min, $[M + H]^+$ 1469). Glucosamine (= chitosan monomer) was also detected in TLC analysis.

Elicitor bioassay

Test materials in glass tubes (∅ 18 × 150 mm) were dissolved or suspended in 1 ml of distilled water and sterilized by autoclaving for 5 min. Five-mm sections of epicotyls of 10-day-old pea seedlings grown under an aseptic condition were prepared and transferred into the test tubes. The test tubes were incubated in the dark at 24 °C on a rotating cultivator (2 rpm). After 72 h incubation, the sections were weighed and returned into the original tubes. Each tube was filled with 5 ml of MeOH and then subjected to sonication for 10 min. After filtration the filtrate was evaporated to dryness and the residue was dissolved in 500 µl of MeOH, and the aliquot (50 µl) subjected to HPLC. HPLC analysis was carried out on a Inertsil ODS column (∅ 4.6 × 250 mm, 5 µm, GL Sciences) at a flow rate of 0.8 ml/min. The elution was performed in a gradient system with two solvents (solvent A: 3% acetic acid in 30% MeOH/H_2O, solvent B: 3% acetic acid in 90% MeOH/H_2O). The gradient was

achieved within 35 min. The column was washed for 10 min with solvent B and followed by a 15 min reequilibration with solvent A. Absorbance at 285 nm was monitored. Retention time for (+)-pisatin under this conditions was 36.1 min. For quantification of (+)-pisatin, the (+)-pisatin content was determined from the peak area of the sample with reference to the calibration of authentic (+)-pisatin.

Results

Characteristics of the elicitor bioassay

We found it essential for the reproducibility of the epicotyl elicitor assay that the growth of pea seedlings and the incubation of elicitor-treated epicotyl sections were carried out under an aseptic condition in order to avoid the contamination of environmental microbes. The time course for (+)-pisatin induction after treatment of the pea epicotyl sections with elicitor was determined (Fig. 2). The experimental conditions were the same as those described for the elicitor bioassay in "Materials and Methods" except that various elicitor-exposure times were employed. A $NaNO_2$-degraded chitosan (100 μg/ml) was used as the elicitor. (+)-Pisatin was induced at significantly elevated levels at 24 h after the elicitor-treatment, and reached a maximum at 48 h. Browning at the surface of the epicotyl cross-sections treated with the elicitor was first observed at 24 h and gradually intensified with the passage of incubation time. Fig. 3 shows a HPLC chromatogram of MeOH–H_2O extract of the epicotyl sections 72 h after

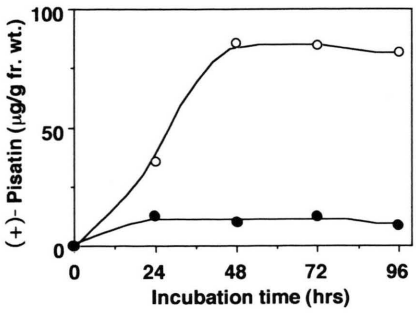

Fig. 2. Time course of the accumulation of (+)-pisatin. $NaNO_2$-degraded chitosan 100 μg/ml (○), control (●).

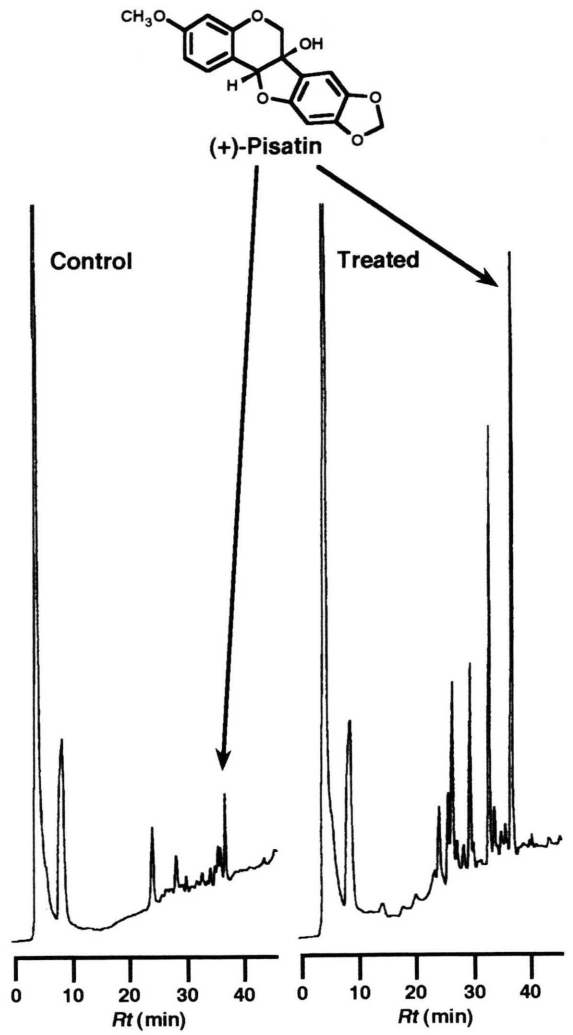

Fig. 3. HPLC profiles of (+)-pisatin and other unidentified compounds accumulated in pea epicotyl sections treated with $NaNO_2$-degraded chitosan (100 μg/ml) for 72 h. *Rt*, retention time.

treatment with the elicitor. In addition to (+)-pisatin, several unidentified phenolic compounds were also induced by the elicitor-treatment. A small amount of (+)-pisatin was induced in the control, probably owing to the wound stress afforded by preparing the epicotyl sections. Therefore, we evaluated the net (+)-pisatin content induced upon elicitor treatment as the value afforded by subtracting the amount of (+)-pisatin in the control from that in the elicitor-treated sections.

(+)-Pisatin-inducing activities of chitosan, DACs, and chitin

Chitosan (degree of N-acetylation 0%) and their partially N-deacetylated preparation (DAC 32%: degree of N-acetylation 32%, DAC 56%: degree of N-acetylation 56%), or fully N-acetylated preparation (chitin) were tested for their (+)-pisatin-inducing activities in 2 and 4 mg/ml suspensions (Fig. 4).

Among these samples, both DACs showed strong activities for (+)-pisatin induction. The DAC 56% with a higher degree of N-acetylation was more active than DAC 32%, especially at the lower concentration. Suspensions of chitosan showed weak induction in this assay. Their activities were nearly as much as those of chitin. The DACs caused severe browning at the cross-sections of epicotyl fragments at both concentrations, while neither chitosan nor chitin caused browning.

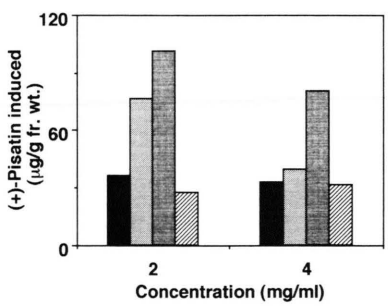

Fig. 4. (+)-Pisatin-inducing activity of chitosan (degree of N-acetylation 0%, ■), DAC 32% partially N-deacetylated chitin with degree of N-acetylation 32%, ▨), DAC 56% (partially N-deacetylated chitin with degree of N-acetylation 56%, ▩), and chitin (▨).

(+)-Pisatin-inducing activities of $NaNO_2$-degraded chitosan and DACs

The chitosan (degree of N-acetylation 0%), DAC 32%, and DAC 56% were degraded by a trace of $NaNO_2$ to intermediate-sized saccharides (Vårum *et al.*, 1991), and the $NaNO_2$-degraded samples were served for the evaluation of their (+)-pisatin-inducing activities at concentrations ranging from 7.8 to 250 μg/ml (Fig. 5-A).

The water-soluble $NaNO_2$-degraded chitosan exhibited a pronounced activity in (+)-pisatin induction, though the native water-insoluble chitosan polymer was a weak elicitor. The (+)-pisatin

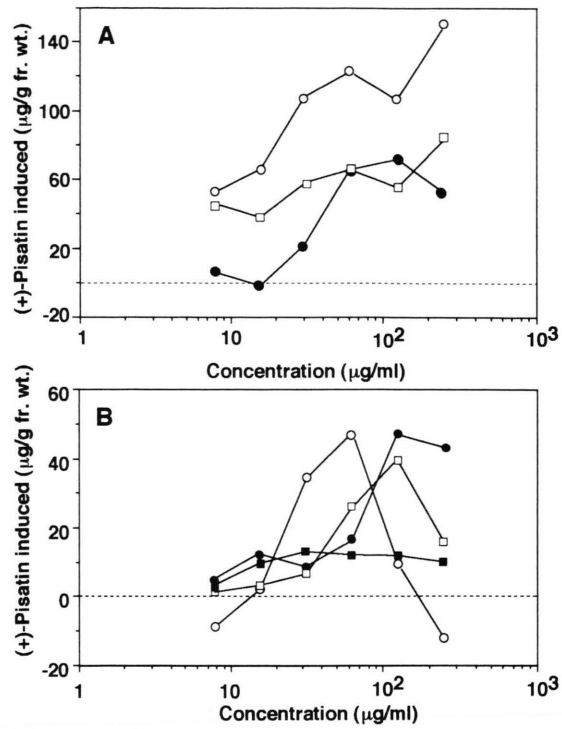

Fig. 5. A: (+)-Pisatin-inducing activity of $NaNO_2$-degraded fragments derived from chitosan (degree of N-acetylation 0%, ○), DAC 32% (partially N-deacetylated chitin with degree of N-acetylation 32%, ●), and DAC 56% (partially N-deacetylated chitin with degree of N-acetylation 56%, □). B: (+)-Pisatin-inducing activity of chitosan oligomers with degree of N-acetylation 5% (○), 37% (●), 66% (■), and 91% (□).

content in the elicitor-treated epicotyls significantly increased with elevating concentration. Severe browning at the cross-sections was observed at the concentrations of 62.5, 125, and 250 μg/ml.

The $NaNO_2$-degraded DAC 56% showed a moderate activity. A gradual increase in the (+)-pisatin content was observed as the concentration was elevated, and the carbohydrate was less active than the $NaNO_2$-degraded chitosan at high concentrations. The $NaNO_2$-degraded DAC also caused browning in epicotyl segments at 250 μg/ml.

At low concentrations the $NaNO_2$-degraded DAC 32% induced only a small amount of (+)-pisatin and was nearly as active as the $NaNO_2$-degraded DAC 56% at high concentrations where severe browning was seen at the cross-section.

(+)-Pisatin-inducing activities of chitosan oligomers with different degrees of N-acetylation

Chitosan oligomers containing monomer through nonamer and their 37, 66 and 91% N-acetylated derivatives were tested at concentrations ranging from 7.8 to 250 μg/ml (Fig. 5-B).

The chitosan oligomers induced a moderate amount of (+)-pisatin (50 μg/g fr. wt.) at the concentrations of 31.3 and 62.5 μg/ml. The 33% N-acetylated derivative showed a moderate activity even at higher concentrations (125, 250 μg/ml) than the native oligomers. The 66% N-acetylated derivative was totally inactive. The 91% N-acetylated derivative showed much the same activity as the 37% N-acetylated derivative at 125 μg/ml. At the concentrations where a moderate amount of (+)-pisatin was induced, browning was observed at the cross-sections.

Discussions

Oligosaccharide fragments derived from the fungal cell wall polymers such as chitin and β-glucan have been shown to be potent inducers of defensive responses in plants (Ryan, 1988; Darvill and Albersheim, 1984; Kobayashi *et al.*, 1993). Accumulation of phytoalexin (+)-pisatin is thought to be one of the important chemical defenses in *Pisum sativum* (Cruickshank, 1962). The results described in this paper demonstrated that partially N-deacetylated chitin fragments, together with chitosan fragments, act as elicitors for (+)-pisatin induction and also emphasized that their activities are closely related to both the molecular size and the degree of N-acetylation. The DACs were the most powerful inducers among the sparingly water-soluble polymers tested. Both chitosan and chitin showed a weak activity. Intermediate-sized chitosan fragments derived from degradation of the chitosan with a trace of sodium nitrite were more active than that of DACs, especially at the high concentrations. Chitosan oligomers obtained from acetone precipitation of HCl-cleaved chitosan hydrolysate induced a relatively small amount of (+)-pisatin compared with the native polymers and the $NaNO_2$-degraded fragments. These facts suggested that the presence of cationic amino groups was probably essential for (+)-pisatin-inducing activity of partially N-deacetylated chitin fragments as well as chitosan fragments. The treatment of *Glycine max* 63 cultured cells with water-soluble chitosan increased membrane permeability as shown by leakage of electrolytes, proteins, and UV absorbing materials, indicating a possible interaction of the carbohydrate with the anionic phospholipids on the plasma membrane (Young *et al.*, 1982). In pea pods, chitosan-induced defense responses were not correlated with membrane leakage (Kendra and Hadwiger, 1987). As seen in both sparingly water-soluble polymers and $NaNO_2$-degraded intermediate-sized fragments, there were no linear correlation between the degree of N-acetylation, *i.e.* the number of amino groups and the (+)-pisatin-inducing activity. This suggests that a certain length of sugar chain with specific distributions of the cationic residues is required for expression of (+)-pisatin-inducing activity.

In the pea epicotyl assay, treatment of the sections with the potent inducers of (+)-pisatin caused severe browning at the cut-surface of the epicotyls as well as induction of several unidentified phenolic compounds besides (+)-pisatin. The modified chitosan could activate oxidation or polymerization enzymes which lignify the cut-surfaces and induce antimicrobial phenolics (Kobayashi *et al.*, 1994). Such plant responses may play a significant role in chemical defense systems of *Pisum sativum*. Attempts to identify the newly induced compounds by the elicitors and to examine their biological activities are in progress.

Elicitor-active fragments of chitosan and partially N-deacetylated chitin are probably generated by the combined action of the hydrolytic enzymes such as chitinase, chitin deacetylase, and chitosanase (Ryan, 1988). The presence of chitinase and chitosanase in the pea pod tissue has been reported, while there is so far no report on chitin deacetylase in this plant (Nichols *et al.*, 1980). However, the glucosamine content of *Fusarium solani* f. sp. *phaseoli* dramatically increased within 2 h after the fungus contacted plant tissues, suggesting that the induction of chitin deacetylase could be closely related to the occurrence of the modified chitosan, and consequently antimicrobial phenolics accumulate (Hadwiger and Beckman, 1980).

In our preliminary investigation about the (+)-pisatin-inducing effect of N-acetylated sugar (monomer through hexamer) newly prepared, it was found that the partially N-acetylated chitosan

hexamer with low degree of N-acetylation induced a moderate amount of (+)-pisatin (40 μg/g fr. wt.) at much lower concentrations (9.4 μg/ml) than the corresponding native oligomers. These facts suggest that partially N-acetylated chitosan hexamer plays an important role in the defense by (+)-pisatin induction at the early stage of plant-microbe interaction. Further investigation about the structural features of the most potential elicitor-entity among the modified oligochitosans is currently under way.

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