Partially N-Deacetylated Chitin Oligomers (Pentamer to Heptamer) are Potential Elicitors for (+)-Pisatin Induction in Pea Epicotyls

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Partially N-deacetylated chitin oligomers (pentamer to heptamer) are potential elicitors for (+)-pisatin induction in pea epicotyls. Oligomers (dimer to heptamer) of chitosan and their partially/completely N-acetylated derivatives were examined for (+)-pisatin-inducing activities in pea epicotyl elicitor assay at concentrations ranging from 6.25 to 100 μg/ml. The structures of the oligomers were analyzed by 1H NMR and MALDI TOF MS spectrometers. The chitosan oligomers from dimer to tetramer were inactive, while the pentamer exhibited a moderate activity at 100 μg/ml. The hexamer showed a pronounced activity at 100 μg/ml, whereas the oligomer had no activity at the higher concentrations. Although the chitosan heptamer induced a moderate amount of (+)-pisatin (ca. 60 μg/g fr. wt.) at 50 μg/ml, the oligomer did not elicit pisatin formation at 100 μg/ml. The dimer to tetramer of both partially N-deacetylated chitin (DAC) and chitin were totally inactive. The DAC pentamers (d.a. 31%, 52%), DAC hexamers (d.a. 26%, 41%), and DAC heptamers (d.a. 26%, 36%) exhibited significant elicitor activities at lower concentrations (6.25–25 μg/ml) than the native chitosan oligomers. The chitin pentamer to heptamer had weak or no activity. Both glucosamine and N-acetylglucosamine were inactive in the assay. These facts suggest that the DAC pentamer to heptamer may act as elicitors for pisatin induction in pea-fungus interactions.

Introduction

Plants mobilize a wide variety of inducible defensive responses including the production of antimicrobial compounds, enhanced reinforcement of cell walls, and the production of antifungal proteins against microbial attack. Elicitors are signal molecules which trigger these events via intracellular signal transduction processes. Biotic elicitors are thought to be released from the cell components of pathogens and plants such as carbohydrates, lipids, and proteins during early stages of their interaction (Lamb et al., 1989).

Oligosaccharide fragments derived from chitin (β-1,4-linked N-acetyl-D-glucosaminoglycan) and chitosan (β-1,4-linked D-glucosaminoglycan) have been shown to act as elicitors in several plant-microbe interactions (Ryan, 1988; Darvill et al., 1992). The elicitor-active fragments are thought to be generated from the polysaccharides by the combined action of the host enzymes such as chitinase, chitin deacetylase, and chitosanase. The relationships between the degree of polymerization (d.p.) of the homo-oligomers, i.e. chitin oligomers and chitosan oligomers and their elicitor activities have been investigated in detail (Kendra and Hadwiger, 1984; Walker-Simmons and Ryan, 1984; Barber et al., 1989; Yamada et al., 1993). However, there have been a few reports on the structural requirements of partially N-deacetylated chitin oligomers for induction of defensive responses (Kauss et al., 1989; Kobayashi et al., 1994; Akiyama et al., 1994a).

We have found that DAC fragments as well as chitosan fragments strongly induced phytoalexin (+)-pisatin and antimicrobial flavonoids in pea epicotyl elicitor assay (Kobayashi et al., 1994; Akiyama et al., 1994a). Recently, we also found that the chemo-enzymatically synthesized DAC oligomers with degree of N-acetylation (d.a.) 13% and 36% exhibited strong activity for (+)-pisatin induction in the assay system and for (±)-kievitone induction in bean cotyledon elicitor assay (Aki-
yama et al., 1994b). These facts suggest that DAC oligomers act as elicitors for the induction of plant defensive responses. In this report, we examined oligomers (monomer to heptamer) of chitosan, DAC, and chitin for their elicitor activity in pea epicotyl assay, and found that DAC pentamer through heptamer possessed strong elicitor activities for pisatin induction in *Pisum sativum*.

**Materials and Methods**

**Chemicals**

Chitosan (d.a. 0%) was purchased from Funakoshi Co., Ltd. Meicelase P-1 (cellulase derived from *Trichoderma viride*) was provided from Sanko Junyaku Co., Ltd. All other chemicals were obtained from commercial sources. An activated charcoal for chromatography was supplied from Wako Pure Chemical Industries, Ltd.

**Measurements**

500 MHz ¹H NMR spectra were recorded on a Varian VXR-500 instrument at 90 °C. Values of δH(D₂O) are expressed in p.p.m. downfield from the reference to internal sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d₄ ([(CH₃)₂SiCD₂CD₂CO₂Na]. MALDI TOF MS spectra were measured on a Finnigan MAT Vision 2000 with 10 mg/ml 2,5-di-hydroxybenzoic acid in 30% CH₃CN–H₂O (0.1% TFA) as a matrix solution. Ions were accelerated to an energy of 6 keV before entering the TOF mass spectrometer. Under the conditions, chitosan oligomers were detected as a monosodium carbohydrate adduct ion. The data are as follows: chitosan dimer \( m/z 363.5 \ [M + Na]⁺ \), chitosan trimer \( m/z 524.4 \ [M + Na]⁺ \), chitosan tetramer \( m/z 685.4 \ [M + Na]⁺ \), chitosan pentamer \( m/z 846.2 \ [M + Na]⁺ \), chitosan hexamer \( m/z 1007.2 \ [M + Na]⁺ \), chitosan heptamer \( m/z 1168.7 \ [M + Na]⁺ \), chitin dimer \( m/z 447.4 \ [M + Na]⁺ \), chitin trimer \( m/z 650.4 \ [M + Na]⁺ \), chitin tetramer \( m/z 853.6 \ [M + Na]⁺ \), chitin pentamer \( m/z 1056.8 \ [M + Na]⁺ \), chitin hexamer \( m/z 1259.6 \ [M + Na]⁺ \), chitin heptamer \( m/z 1462.9 \ [M + Na]⁺ \). HPLC analysis was performed using a column of TSKgel Amide-80 (Ø 4.6 × 250 mm, TOSOH Co., Ltd.). The eluent was 35% CH₃CN–H₂O (0.2% phosphoric acid, 0.2% triethylamine). The flow rate was 0.5 ml/min at 40 °C and peaks were detected with a RI monitor. Retention times of chitosan dimer through heptamer were 7.8, 8.7, 10.8, 12.7, 14.8, and 17.4 min, respectively.

**Preparation and purification of chitosan monomer through heptamer**

Chitosan (d.a. 0%) 10 g was dissolved in 1 l of 0.1 M acetate buffer (pH 4.0). To the solution 1 g of Meicelase P-1 (cellulase from *Trichoderma viride*, a product of Meiji Seika) was added, and the mixture was stirred for 30 h at 50 °C. After heating at 100 °C for 10 min, insoluble materials were centrifuged off, and the supernatant was concentrated under diminished pressure. The solution was again diluted with H₂O to 200 ml in the total volume. To the solution 300 ml of acetone was added (60% acetone–H₂O), and precipitates were centrifuged off. To the supernatant 500 ml of acetone was added (80% acetone–H₂O), and the resulting precipitates were collected by centrifugation to afford a chitosan oligomer mixture mainly containing pentamer to octamer.

The chitosan oligomer mixture was first separated on an activated charcoal column chromatography employing gradient elution using an acetone–H₂O (50 mM diethylamine) system. In a typical run, the chitosan oligomer mixture 700 mg applied to a column (Ø 2.0 × 60 cm) as a solution in 10% acetone–H₂O (50 mM diethylamine) was separated by gradient elution from 10% acetone–H₂O (50 mM diethylamine) 700 ml to 45% acetone–H₂O (50 mM diethylamine) 700 ml. The flow rate was 0.5 ml/min, and the fraction size was 7 ml. The oligomer peaks were detected by a modification of Schales’ procedure (Imoto and Yagishita, 1971). Chitosan monomer to tetramer can be purified (>99%) by the chromatographic run.

The chitosan pentamer to heptamer-rich fractions obtained from the activated charcoal column were further purified by an ion-exchange column chromatography using TSKgel CM-Toyopearl 650S (TOSOH Co., Ltd.). A typical run is as follows; ca. 200 mg of chitosan oligomers was separated on a column (Ø 2.0 × 68 cm) by gradient elution from 50 mM acetate buffer (pH 5.0) 500 ml to 1 M NaCl in the buffer 500 ml. Fractions (5 ml) were collected at a flow rate of 0.5 ml/min. The fractions were analyzed by TLC [DC-Alufolien Kieselgel 60 F₂₅₄ plates (MERCK), n-BuOH–MeOH–28% NH₄OH (1:1:1) or (4:3:3), develop-
oped three times]. The oligomers were detected by spraying with diphenylamine-aniline-phosphoric acid. After desalted with gel filtration chromatography on TSKgel Toyopearl HW40F (TOSOH Co., Ltd.), the purified species were isolated by concentrating the solutions, followed by addition of acetone. The resulting precipitates were thoroughly washed with acetone, then freeze-dried after dissolution in an excess of a HCl-acidic solution.

Preparation of DAC oligomers and chitin oligomers

Dimers to heptamers of DAC and chitin were prepared by partial or complete N-acetylation of the corresponding oligomers according to the method for selective N-acetylation (Barker et al., 1958). The structures of oligomers were analyzed by 1H NMR and MALDI TOF MS spectrometers. The d.a. of the DAC oligomers were 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, 4.70 for β-anomeric protons, δ 5.19 for α-anomeric protons) with that of the D-glucosamine (GlcN) units (δ 4.85–4.90 for β-anomeric protons, δ 5.43 for α-anomeric protons) in the 1H NMR spectra (D2O, pH 4, 90°C).

Elicitor bioassay

Pea epicotyl elicitor assay and quantification of (+)-pisatin by HPLC were conducted as described previously (Kobayashi et al., 1994).

Results

Structural characteristics of DAC pentamer to heptamer

DAC dimer to heptamer were prepared by partial N-acetylation of the corresponding chitosan oligomers. Among them, DAC pentamers (d.a. 31%, 52%), DAC hexamers (d.a. 26%, 41%), and DAC heptamers (d.a. 26%, 36%) showed significant activities in pea epicotyl elicitor assay as described later [the degree of N-acetylation (d.a.) were determined by 1H NMR experiments]. The DAC oligomers prepared by the method for selective N-acetylation are heterogeneous in the degree and pattern of N-acetylation. MALDI TOF MS is a powerful tool for detecting the distribution pattern of the oligomer products with different d.a.. MALDI TOF MS spectra of the DAC pentamer with d.a. 31%, the DAC hexamer with d.a. 26%, and the DAC heptamer with d.a. 26%, together with the results of 1H NMR experiments, clearly showed that mono- and di-N-acetylated oligoglucosamine are major constituents in each DAC oligomer preparation, and that there are no detectable amount of the chitosan oligomers in the analytes (Fig. 1). In the same manner, the spectral analysis of the pentamer with d.a. 52%, the hexamer with d.a. 41%, and the heptamer with d.a. 36% revealed that di- and tri-N-acetylated chitosan oligomers are major constituents in each DAC oligomer. Information about a sequence of GlcN and GlcNAc residues in the DAC oligomers was obtained from 1H NMR spectral data. On the
assumption that the tautomeric equilibrium ratios α-anomer : β-anomer of the reducing GlcNAc and GlcN end units are equivalent, the relative rates of the reducing GlcNAc end units in each DAC oligomer are those listed in Table I. These data indicate that most of the oligomers in the DAC oligomer preparations have a GlcNAc units at the reducing end in the chain.

(+)-Pisatin-inducing activity of pentamers of chitosan, DAC, and chitin

Chitosan pentamer, DAC pentamers with d.a. 31% and 52%, and chitin pentamer were examined for their (+)-pisatin-inducing activities in pea epicotyl elicitor assay at concentrations ranging from 6.25 to 100 µg/ml [Fig. 2.(a)].

Chitosan pentamer showed a moderate activity only at 100 µg/ml. Both of the DAC pentamers induced a moderate amount of (+)-pisatin at lower concentrations than the native chitosan pentamer (25, 50, and 100 µg/ml). Chitin pentamer was totally inactive at all concentrations tested. No browning at cross-sections of epicotyl fragments was observed in all oligomers tested.

(+)-Pisatin-inducing activity of hexamers of chitosan, DAC, and chitin

Chitosan hexamer, DAC hexamers with d.a. 26% and 41%, and chitin hexamer were tested for (+)-pisatin-inducing activity in pea epicotyl elicitor assay at concentrations ranging from 6.25 to 100 µg/ml [Fig. 2.(b)].

Chitosan hexamer showed a strong activity only at 100 µg/ml where browning at the cross-sections of epicotyl fragments was observed. However, at higher concentrations (>150 µg/ml), no increase in pisatin formation was seen (data not shown). In a parallel experiment, a NaNO2-degraded DAC (degree of N-acetylation 32%), which has been showed to be a potent inducer of pisatin and antimicrobial flavonoids in *Pisum sativum* (Kobayashi et al., 1994; Akiyama et al., 1994a), induced 98 µg/g fr. wt. of pisatin together with the flavonoids. The DAC hexamer with d.a. 41% exhibited moderate activities at the concentrations ranging from 25–100 µg/ml. The DAC hexamer with d.a. 26% was less active than the DAC hexamer with higher d.a.. Both of the DAC oligomers caused browning at cross-sections at 100 µg/ml, and chitin hexamer was a weak elicitor for pisatin induction.

(+)-Pisatin-inducing activity of heptamers of chitosan, DAC, and chitin

Chitosan heptamer, DAC heptamers with d.a. 26% and 36%, and chitin heptamer were served for the evaluation of their (+)-pisatin-inducing activity at concentrations ranging from 6.25 to 100 µg/ml [Fig. 2.(c)].

Among the oligomers tested, the DAC heptamer with d.a. 26% was the most potent elicitor, which showed a comparable activity to a NaNO2-degraded DAC (d.a. 32%), which induced 120 µg/g fr. wt. of pisatin at 100 µg/ml in a parallel experiment. The DAC heptamer with d.a. 36% was less active than the DAC heptamer with d.a. 26%, but showed significant activity at concentrations from 6.25–100 µg/ml. The native chitosan heptamer exhibited elicitor activity only at 50 µg/ml, whereas no pisatin accumulation was observed at the higher concentration (100 µg/ml). The Chitin heptamer was a weak inducer of pisatin. Browning at cross-sections was seen at the concentrations where a moderate amount of pisatin was induced.

### Table I. Relative area of the anomeric proton signals of GlcN and GlcNAc units.

<table>
<thead>
<tr>
<th>Oligomers</th>
<th>GlcN H-1 α (δ 5.43)</th>
<th>GlcN H-1 β (δ 4.85–4.90)</th>
<th>GlcNAc H-1 α (δ 5.19)</th>
<th>GlcNAc H-1 β (δ 4.55–4.65)</th>
<th>d.a. (%)</th>
<th>GlcNAc H-1 α (GlcNAc H-1 α + GlcN H-1 α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAC pentamer-1</td>
<td>0</td>
<td>69.1</td>
<td>8.4</td>
<td>22.4</td>
<td>31</td>
<td>1.00</td>
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<tr>
<td>DAC pentamer-2</td>
<td>0</td>
<td>48.2</td>
<td>8.4</td>
<td>43.4</td>
<td>52</td>
<td>1.00</td>
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<tr>
<td>DAC hexamer-1</td>
<td>12.3</td>
<td>61.7</td>
<td>10.1</td>
<td>15.9</td>
<td>26</td>
<td>0.45</td>
</tr>
<tr>
<td>DAC hexamer-2</td>
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<td>54.7</td>
<td>12.5</td>
<td>28.4</td>
<td>41</td>
<td>0.74</td>
</tr>
<tr>
<td>DAC heptamer-1</td>
<td>6.7</td>
<td>66.8</td>
<td>12.4</td>
<td>14.0</td>
<td>26</td>
<td>0.65</td>
</tr>
<tr>
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<td>57.3</td>
<td>11.1</td>
<td>25.2</td>
<td>36</td>
<td>0.63</td>
</tr>
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</table>
Discussion

Chitin- and chitosan-derived oligosaccharides have been shown to be elicitors for defensive responses in several plants. Chitin is a structural polysaccharide of the cell walls of fungi and the exoskeletons of invertebrates. The polysaccharide is an idealized polymer formed by linear chain of poly-N-acetylglucosamine. However, particularly in arthropods, some of the constitutional sugars appears deacetylated, raising doubts about the fully acetylated states of the native polymer (Lezica and Allué, 1990). In addition, the use of drastic methods for chitin isolation is known to cause some degradation. Chitosan, the β-1,4-linked polymer of glucosamine, is produced by the enzymatic or chemical N-deacetylation of chitin. In general, chitosans obtained from living organisms and commercially available chitosans contain some extent of acetylated residues. These facts suggest that the heteropolymers could be employed in many studies on the chitin, chitosan elicitor. The biological active molecules may be, in all cases, oligosaccharide fragments of chitin and chitosan, as many plants constitutively express chitinase (Darvill et al., 1992). These observations suggest the possible participation of DAC oligomer fragments in plant defensive responses triggered by the polysaccharides. There have been a few reports on the elicitor activities of DAC oligomer fragments (Walker-Simmons et al., 1984; Kauss et al., 1989; Kobayashi et al., 1994; Akiyama et al., 1994a, b).

The results described in this paper demonstrated that DAC pentamer to heptamer are elicitors for pisatin induction in pea epicotyls. MALDI TOF MS analysis of the oligomers suggested that mono-, di-, and tri-N-acetylated chitooligosaccharides are the active principals for elicitor activity. Chitosan pentamer to heptamer also showed pisatin-inducing activity at higher concentrations than the corresponding DAC oligomers. However, relatively high concentrations (≥100 μg/ml) of the chitosan oligomers did not induce pisatin formation. It has been reported that relatively high concentrations of chitosan (>60 μg/ml) kill slash pine cells (Lesney, 1990). Chitin monomer to heptamer has little or no activity in pea epicotyl assay.

Pisatin-inducing activity of the DAC oligomers depended on their chain size. Pisatin accumulation was low with DAC oligomers smaller than pentamer, and the accumulation was increased up to DAC heptamers. The amount of pisatin (ca. 100 μg/g fr. wt.) induced by 100 μg/ml of the DAC heptamer with d.a. 26% was comparable to that induced by a NaNO₂-degraded DAC fragment (d.a. 32%). We reported that the fragment with much higher d.p. than heptamer was one of the strongest elicitor in the pea system (Kobayashi et al., 1994; Akiyama et al., 1994a). The elicitor activity of chi-
tosan oligomers towards pea epicotyls also depended on their d.p. as observed in pea pod assay. Chitosan heptamer was found to be the smallest sized unit for elicitation of pisatin induction in the pea pod assay, although dose-response for chitosan oligomer pisatin formation was not examined because of limitation in quantity of the oligomers (Kendra and Hadwiger, 1984). “Chitosan heptamers” with pisatin-inducing activity was isolated from the cell wall of Fusarium solani. However, Kendra et al. also mentioned that the presence of some acetylation and residual protein in the oligomer fractions could not be excluded, though the IR spectra differed significantly from those of chitin (Kendra et al., 1989). In pea epicotyl assay, significant activities were observed in chitosan pentamer to heptamer, among which the hexamer induced a comparable amount of pisatin to the NaN02-degraded DAC fragment.

In this experiments, the elicitor-active DAC oligomers as well as the chitosan oligomers were weak elicitors for the antimicrobial flavonoids induction [(+)-α,2′,4,4′-tetrahydroxydihydrochalcone, (-)-4′,7-dihydroxyflavanone, and 4,4′-dihydroxy-2′-methoxychalcone] in pea epicotyls at the concentration range tested. In our previous paper, we found that the NaN02-degraded DAC fragment showed pronounced activities at 62.5, 125, and 250 μg/ml, suggesting that longer chain length might be required for the flavonoid induction (Akiyama et al., 1994a).

The elicitor-active DAC oligomers showed pisatin-inducing activity at lower concentrations than the native chitosan oligomers. However, the concentrations are much higher than those compared to a hepta-β-glucoside and N-acetylchitoheptaose which showed elicitor activity at nanomole levels towards soybean cotyledons and rice cells, respectively (Darvill et al., 1992; Yamada et al., 1993). The DAC oligomer preparations obtained by chemical N-acetylation consist of stereoisomeric oligomers with different extent and pattern of N-acetylation. On the assumption that only one of the stereoisomers acts as an elicitor, the oligomer may exert a pisatin-inducing activity at nanomole levels.

The relationships between sequences of GlcNAc and GlcN in the DAC oligomer elicitors and pisatin-inducing activity are still unclear. The DAC heptamer was found to be the smallest sized unit which maximally elicits pisatin induction, while significant activity was also observed in the DAC pentamer. MALDI TOF MS analysis indicated that mono-, di-, and tri-N-acetylated pentamers are possible candidates for the elicitation of pisatin induction. The pentamers can serve as a model sample for clarifying the structural requirements for DAC oligomer elicitors. 1H NMR analysis of the elicitor-active DAC pentamers indicated the high frequency of the occurrence of a GlcNAc unit at the reducing end, suggesting that the presence of the GlcNAc unit might be required for their activities. The synthesis of the DAC oligomers with a GlcNAc unit at the reducing end is being undertaken by the enzymatic method utilizing a lysozyme-catalyzed transglycosylation reaction (Akiyama et al., 1994b).

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