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Comparison of the Ability of Partially *N*-Acetylated Chitosans and Chitooligosaccharides to Elicit Resistance Reactions in Wheat Leaves[1]

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Chitin, a linear polysaccharide composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) residues, and chitosan, the fully or partially *N*-acetylated, water-soluble derivative of chitin composed of (1→4)-linked GlcNAc and 2-amino-2-deoxy-β-D-glucopyranose (GlcN), have been proposed as elicitors of defense reactions in higher plants. We tested and compared the ability of purified (1→4)-linked oligomers of GlcNAc (tetramer to decamer) and of GlcN (pentamer and heptamer) and partially *N*-acetylated chitosans with degrees of acetylation (DA) of 1%, 15%, 35%, 49%, and 60% and average degrees of polymerization between 540 and 1100 to elicit phenylalanine ammonia-lyase (PAL) and peroxidase (POD) activities, lignin deposition, and microscopically and macroscopically visible necroses when injected into the intercellular spaces of healthy, nonwounded wheat (*Triticum aestivum* L.) leaves. Purified oligomers of (1→4)-linked GlcN were not active as elicitors, whereas purified oligomers of (1→4)-linked GlcNAc with a degree of polymerization ≥ 7 strongly elicited POD activities but not PAL activities. Partially *N*-acetylated, polymeric chitosans elicited both PAL and POD activities, and maximum elicitation was observed with chitosans of intermediate DAs. All chitosans but not the chitin oligomers induced the deposition of lignin, the appearance of necrotic cells exhibiting yellow autofluorescence under ultraviolet light, and macroscopically visible necroses; those with intermediate DAs were most active. These results suggest that different mechanisms are involved in the elicitation of POD activities by GlcNAc oligomers, and of PAL and POD activities by partially *N*-acetylated chitosan polymers and that both enzymes have to be activated for lignin biosynthesis and ensuing necrosis to occur.

Plant cells can respond to fungal infection by the activation of induced resistance reactions upon recognition of fungal cell wall components (Albersheim and Anderson-Prouty, 1975; Ride, 1992; Boller, 1995). In plant tissues extracellular chitinases (Collinge et al., 1993) and possibly chitosanases (Grenier and Asselin, 1990), in concert with β-(1→3)-glucanases (Mauch et al., 1988), are likely to partially degrade fungal cell wall polysaccharides. This not only leads to a weakening of the fungal cell wall (Arlorio et al., 1992) but also produces soluble and diffusable fragments that may indicate the presence of a potential pathogen to the plant tissues (Ham et al., 1991; Ryan and Farmer, 1991).

Chitin, a linear polysaccharide composed of GlcNAc residues, forms the fibrillar component of typical fungal cell walls. The fungal cell wall may in addition to chitin also contain chitosan (Peberdy, 1990), the deacetylated counterpart of chitin. Fungal chitosan is most likely biosynthesized from chitin by the action of chitin deacetylase, converting in chain GlcNAc residues to GlcN residues (Araki and Ito, 1975; Davis and Bartnicki-Garcia, 1984; Kafetzopoulos et al., 1993). Thus, fungal cell walls can in principle contain chitin and chitosans with DAs from 0% to 100%.

It has been shown previously that chitin (Pearce and Ride, 1982), chitosan (Pearce and Ride, 1982; Moerschbacher et al., 1986; Gotthardt and Grambow, 1992), and oligomers of β-(1→4)-linked GlcNAc (Barber et al., 1989; Gotthardt and Grambow, 1992), but not GlcN (Barber et al., 1989), are active elicitors of defense-related lignification in wounded (Pearce and Ride, 1982; Barber et al., 1989) and intact (Moerschbacher et al., 1986) wheat (*Triticum aestivum* L.) leaves and in suspension-cultured wheat cells (Gotthardt and Grambow, 1992). The activity of chitosan was thought to reside in the acetylated regions of the molecule, since fully deacetylated chitosan was inactive (Barber and Ride, 1988). Recently, enzymatic hydrolysates of highly

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Abbreviations: DA, degree of acetylation; DP, degree of polymerization; $\overline{DP}_n$, number of average degree of polymerization; GlcN, 2-amino-2-deoxy-β-D-glucopyranose; GlcNAc, 2-acetamido-2-deoxy-β-D-glucopyranose; PAL, Phe ammonia-lyase; POD, peroxidase.

acetylated chitosan (DA of 80%) were shown to be active elicitors in wounded wheat leaves (Mitchell et al., 1994). Problems in the interpretation of these studies arose because of the use of relatively poorly characterized chitins and chitosans as elicitors, as well as because of the relatively unspecific and qualitative rather than quantitative assay of elicitor action. Assays involving wounding of the wheat leaves further complicate the interpretation, since wounding alters the reactivity of the tissues (Barber et al., 1989).

In the current study we used, in addition to purified β-(1→4)-linked oligomers of GlcN and GlcNAc, chemically well-characterized and fully water-soluble chitosans. The chitosans had known (average) M_rs ($\overline{DP}_n$ = 540–1100) and a wide range of known (average) DAs (1%, 15%, 35%, 49%, and 60%). Furthermore, the distribution of GlcNAc residues along the polymer chain has been characterized and found to be random (Vårum et al., 1991a, 1991b). Samples were applied to the leaves by injection into the intercellular spaces using a hypodermic syringe, minimizing wounding of the leaves (Moerschbacher et al., 1989). The elicitation of symptoms known to be involved in the defense mechanism of wheat to fungal pathogens, such as an increase in enzyme activities involved in lignin biosynthesis, the deposition of lignin or a lignin-like polymer, and the formation of macroscopically and microscopically visible foliar necroses (Beardmore et al., 1983; Moerschbacher et al., 1988; Moerschbacher, 1989; Ride et al., 1989; Tiburzy et al., 1990; Moerschbacher and Reisener, 1997), could thus be quantified under near-natural conditions.

MATERIALS AND METHODS

Preparation and Characterization of GlcNAc and GlcN Oligomers

Purified GlcNAc oligomers with DPs from 4 to 10 were prepared as described by Bosso et al. (1986), and oligomers of glucosamine with DPs of 5 and 7 were prepared according to the method of Domard and Cartier (1989).

Characterization of the purified GlcNAc oligomers and glucosamine were obtained by ^{13}C-NMR and steric-exclusion chromatography (Bosso et al., 1986; Domard and Cartier, 1989), as well as by MS (Bosso and Domard, 1992).

Preparation and Characterization of Partially *N*-Acetylated Chitosan Polymers

Water-soluble polymeric chitosans ($\overline{DP}_n$ 540–1100) with DAs from 1% to 60% (Table I), in which the GlcNAc and GlcN residues are randomly distributed along the polymer chain, were prepared and the chemical composition (DA) and the intrinsic viscosities ([η]), which are converted to the number of average M_r or DP_n (Anthonsen et al., 1993) were determined. It should be emphasized that the M_r is an average and that the samples are polydisperse both with respect to M_r and chemical composition (Anthonsen et al., 1993; Vårum et al., 1994).

Table I. *Characterization of chitosans used in this study (Anthonsen et al. [1993]; Vårum et al. [1994])*

DA[a]	Intrinsic Viscosity	No. of Average M_r[b]	$\overline{DP}_n$[c]
%	mL g^{-1}	kD	
1	610	170	1100
15	740	190	1100
35	410	96	550
49	450	98	540
60	820	180	970

[a] Determined by NMR. [b] Determined from intrinsic viscosity measurements. [c] Calculated from the average M_r.

Bioassays for Elicitor Activity

Primary leaves of 7-d-old wheat (*Triticum aestivum* L. cv Prelude) plants grown in automatically regulated growth chambers were injected with the test solutions using a hypodermic syringe (Moerschbacher et al., 1989). Leaf segments were harvested into liquid nitrogen 24 h after injection.

A crude enzyme extract (0.1 M borate buffer, pH 8.8, 1 mM EDTA, 1 mM DTT, 25 mg mL^{-1} insoluble PVP, and 25 mg mL^{-1} Dowex 1 × 2 [Cl$^-$] quartz sand) was prepared, and the activities of PAL (EC 4.3.1.5.) and POD (EC 1.11.1.7.) were determined (PAL: 0.1 M borate buffer, pH 8.8, 6 mM Phe, 40°C, 290 nm; POD: 0.1 M phosphate buffer, pH 5.8, 18 mM guaiacol, 8 mM H_2O_2, 30°C, 470 nm) as described previously by Moerschbacher et al. (1988). The protein concentration of the extracts was estimated with Biuret reagent (Gornall et al., 1949).

Determination of Lignin Deposition

Lignin content of elicitor-treated leaves was estimated using the acetyl bromide procedure (Iiyama and Wallis, 1988). Cell walls were prepared from frozen wheat leaves by grinding in liquid nitrogen and then in methanol:water (1:1), followed by sequential extraction with methanol:water (1:1), methanol:chloroform (2:1), acetone, ethanol, and distilled water. Lyophilized and vacuum oven-dried cell walls (5–15 mg) were incubated for 30 min in 2.5 mL of acetyl bromide reagent (25% [v/v] acetyl bromide in acetic acid) and 0.1 mL of 70% perchloric acid at 70°C. Ice-cooled samples were then transferred to a mixture of 10 mL of 2 M NaOH and 12 mL of acetic acid, and the volume was adjusted with acetic acid to 50 mL. After 60 to 120 min, the extinction of the solution was read at 280 nm, and the lignin content was calculated using the molar extinction coefficient for graminaceous lignin of 20 g^{-1} L cm^{-1} (Iiyama and Wallis, 1988).

RESULTS

PAL and POD Elicitation by Purified GlcN and GlcNAc Oligomers

Fully acetylated GlcNAc oligomers with DPs from 4 to 10 were injected into the intercellular spaces of wheat leaves at a concentration of 1 mg mL^{-1}, and PAL and POD

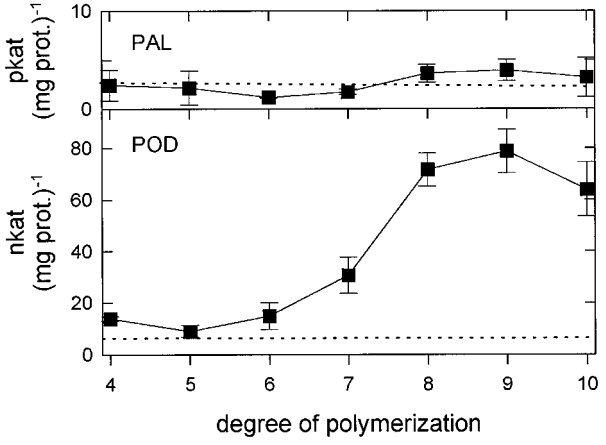

Figure 1. Influence of the DP on the activity of purified GlcNAc oligomers (1 mg mL^{-1}; 24 h after injection) as elicitors of PAL and POD in wheat leaves. Data are means ± SD of triplicate samples from one representative of two independent experiments. The dotted lines indicate enzyme activities of water-treated control plants. prot., Protein.

activities were determined 24 h after injection (Fig. 1). Only oligomers with a DP ≥ 7 induced POD activities, whereas PAL activity was not activated by any of the oligomers. Fully deacetylated GlcN oligomers of DPs 5 and 7 induced neither POD nor PAL activities in the leaves (data not shown).

Dose-response curves for PAL and POD activation by the GlcNAc oligomer with a DP of 8 and by the GlcN oligomer with a DP of 7 were established, with concentrations ranging from 0.01 to 1000 μg mL^{-1} (Fig. 2). Only the GlcNAc oligomer induced POD activities but not PAL activities at concentrations ≥ 500 μg mL^{-1}, whereas neither PAL nor POD activities were activated by the GlcN oligomer.

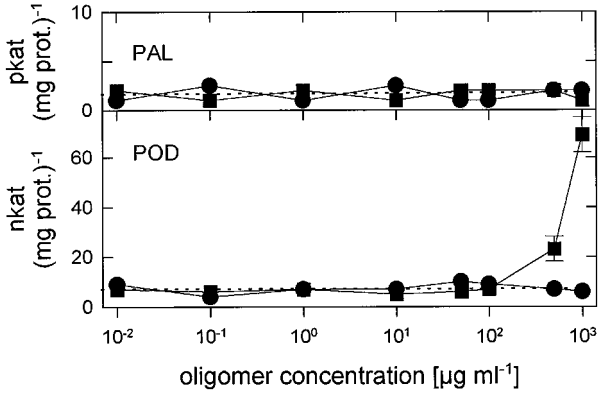

Figure 2. Dose-response curves of PAL and POD elicitation by the GlcNAc octamer (■) and the GlcN heptamer (●) 24 h after injection into the intercellular spaces of primary wheat leaves. Data are means ± SD of triplicate samples from one representative of two independent experiments. The dotted lines indicate enzyme activities of water-treated control plants. prot., Protein.

PAL and POD Elicitation by Polymeric Chitosans with Different DAs

Polymeric chitosans with $\overline{DP}_n$s from 540 to 1100 and DAs ranging from 1% to 60% were injected into intact wheat leaves at a concentration of 100 μg mL^{-1}, and PAL and POD activities were determined 24 h after injection (Fig. 3). Chitosans with a DA of less than 20% strongly induced PAL activity, but POD activities were only weakly activated. With increasing DA up to 35%, both PAL and POD induction increased. No further increase in PAL induction was seen with DAs of 50% to 60%, whereas POD induction increased dramatically.

Dose-response curves for PAL and POD activation by the chitosans with DAs of 1%, 35%, and 60% were established with concentrations ranging from 0.01 to 1000 μg mL^{-1} (Fig. 4). Almost fully deacetylated chitosan polymers (DA of 1%) elicited both enzymes, with half-maximum enzyme elicitation at a concentration of about 10 μg mL^{-1}. With increasing DA the chitosans became more potent elicitors of both enzymes, with about 1 μg mL^{-1} of the DA 35% chitosan achieving half-maximum enzyme elicitation. When the DA of the chitosans was increased even further, their elicitor activity decreased; about 100 μg mL^{-1} DA 60% chitosan was required for half-maximum PAL elicitation and about 20 μg mL^{-1} achieved half-maximum POD elicitation. PAL activation remained about constant at higher chitosan concentrations, whereas POD activation drastically declined: at 1 mg mL^{-1} DA 1% and DA 35% chitosans, POD activities were not higher than in water-treated control leaves.

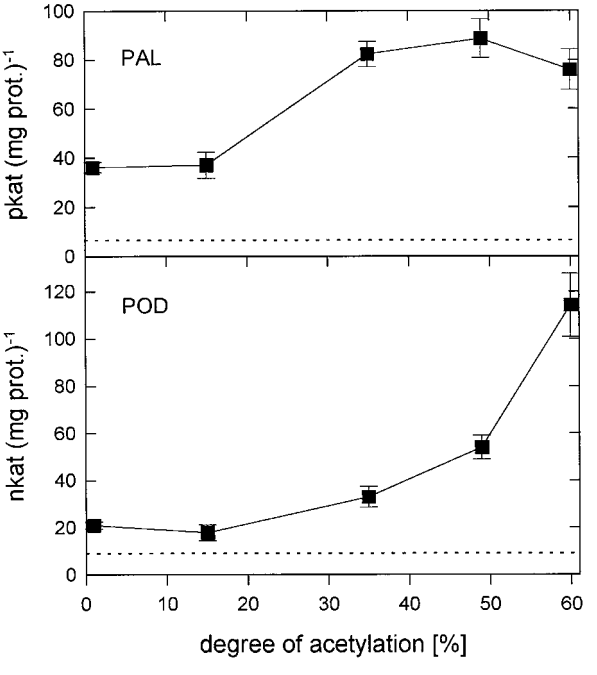

Figure 3. Influence of the DA on the activity of chitosan polymers ($\overline{DP}_n$ = 540–1100; 100 μg mL^{-1}; 24 h after injection) as elicitors of PAL and POD activities in wheat leaves. Data are means ± SD of triplicate samples from one representative of two independent experiments. The dotted lines indicate enzyme activities of water-treated control plants. prot., Protein.

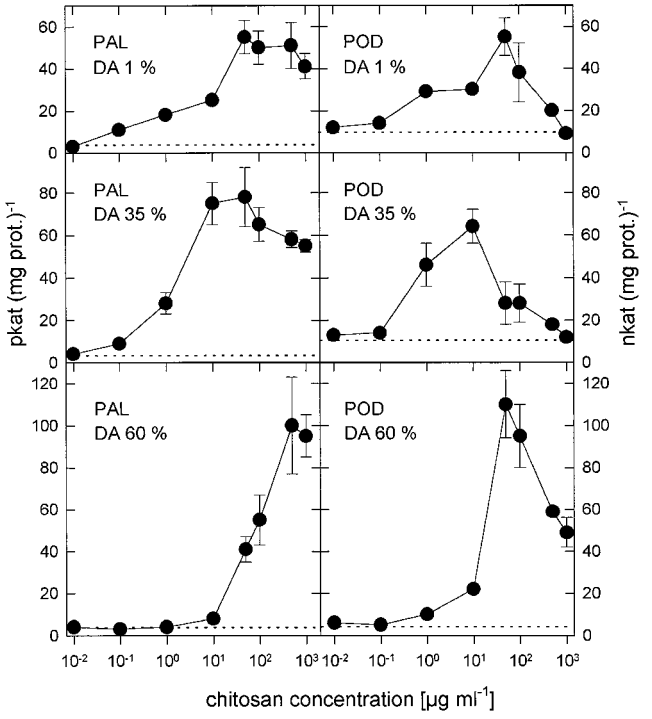

Figure 4. Dose-response curves of PAL and POD elicitation by polymeric chitosans with DAs of 1%, 35%, and 60% 24 h after injection into the intercellular spaces of primary wheat leaves. Data are means ± SD of triplicate samples from one representative of two independent experiments. The dotted lines indicate enzyme activities of water-treated control plants. prot., Protein.

Time-response curves for PAL and POD activation by the chitosans with DAs of 1%, 35%, and 60% at 100 μg mL^{-1} were established (Fig. 5). PAL activity was already increased 6 h after injection of either of the chitosans and reached maximum activities at 24 h after injection. In contrast, POD activities started to increase only from 12 to 24 h after chitosan injection but were still increasing 48 h past injection. A strong increase in POD activities was especially striking with the chitosan DA of 60%.

Macroscopically Visible Necrosis Elicited by GlcNAc Oligomers, GlcN Oligomers, and Partially Deacetylated Chitosan Polymers

When fully acetylated GlcNAc oligomers with a DP varying from 4 to 10 or fully deacetylated GlcN oligomers with a DP of 5 or 7 were injected into the intercellular spaces of wheat leaves at a concentration of 1 mg mL^{-1}, no symptoms were visible macroscopically up to several days after injection. In contrast, necroses were visible by 24 h after injection of 1 to 100 μg mL^{-1} partially deacetylated polymeric chitosans (Fig. 6). The chitosan with a DA of 35% clearly was the most potent elicitor, because the whole injected area exhibited severe necrosis. The almost fully deacetylated chitosan with a DA of 1% elicited strong necrosis in the zone surrounding the injection site, but the more distal portions of the injected area appeared more or less healthy. Symptoms were least marked with the most highly acetylated chitosan used (DA of 60%), which induced necrotic spots in a slightly chlorotic area around the injection site smaller than the chitosan-injected area of the leaves.

Fluorescence-Microscopically Visible Symptoms Elicited by GlcNAc Oligomers, GlcN Oligomers, and Partially Deacetylated Chitosan Polymers

No symptoms other than a narrow ring of necrotic cells surrounding the injection site were visible after injection of water, fully acetylated GlcNAc oligomers (DPs from 4–10), or fully deacetylated GlcN oligomers (DP of 5 or 7). In contrast, necrotic cells exhibiting alkali-stable (0.5 N NaOH, 48 h, 20°C), yellow autofluorescence indicative of lignin deposition appeared in the injected area after injection of partially deacetylated polymeric chitosans. In all cases, the extent of microscopically visible autofluorescence closely correlated with the severity of macroscopically visible necroses. The extent of necroses correlated more closely with the induced PAL activities than with POD activities (Fig. 7).

Lignin Deposition Elicited by Partially Deacetylated Polymeric Chitosans

Polymeric chitosans with $\overline{DP}_n$s from 540 to 1100 and DAs of 1%, 35%, and 60% were injected into intact wheat leaves at a concentration of 100 μg mL^{-1}, and the lignin content of the treated leaf areas was determined 24 h after injection

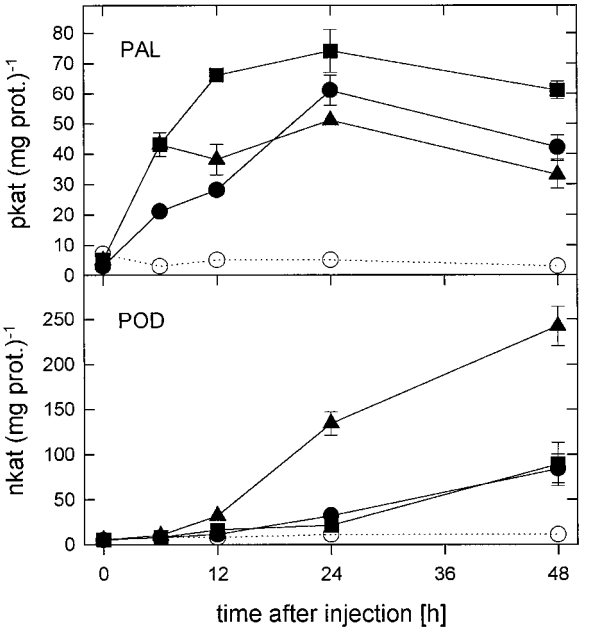

Figure 5. Time-response curves of PAL and POD elicitation by polymeric chitosans with DAs of 1% (circles), 35% (squares), and 60% (triangles) at 100 μg mL^{-1} upon injection into the intercellular spaces of primary wheat leaves. Data are means ± SD of triplicate samples from one representative of two independent experiments. The dotted lines indicate enzyme activities of water-treated control plants. prot., Protein.

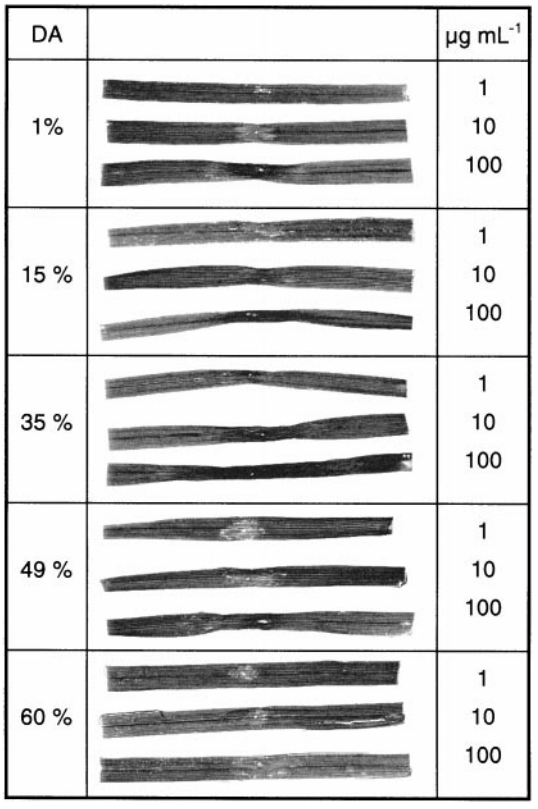

Figure 6. Macroscopically visible symptoms induced in wheat leaves by polymeric chitosans with DAs of 1%, 15%, 35%, 49%, and 60%, at 1, 10, and 100 μg mL^{-1}, 24 h after injection into the intercellular spaces of primary wheat leaves.

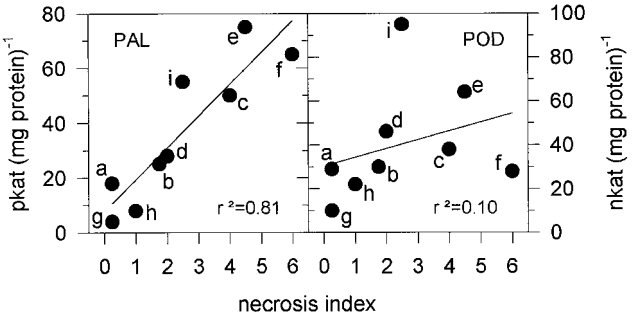

Figure 7. Correlation between the extent of foliar necroses and the induction of PAL and POD activities by chitosans with DAs of 1% (a–c), 35% (d–f), and 60% (g–i), at 1 (a, d, and g), 10 (b, e, and h), and 100 (c, f, and i) μg mL^{-1}, 24 h after injection into the intercellular spaces of primary wheat leaves. Necroses were arbitrarily quantified by macroscopic and UV-fluorescence microscopic examination on a necrosis index scale from 0 to 6. Macroscopic effects: 0, no symptoms visible; 2, central part of the injected area chlorotic; 4, central part of injected area necrotic, single chlorotic spots toward the periphery; 6, all of the injected area necrotic. Microscopic effects: 0, only cells directly at the injection site showed yellow autofluorescence under UV light; 2, many collapsed and yellow autofluorescing cells in the central part of the injected area, sparse clusters of such cells toward the periphery; 4, all of the cells in the central part of the injected area and many cells in the periphery collapsed and exhibited yellow autofluorescence; 6, all of the cells in the injected area collapsed and exhibited yellow autofluorescence.

(Table II). All chitosans induced a significant accumulation of lignin, with the most active being the chitosan with a DA of 35%.

DISCUSSION

Our studies corroborated some of the earlier findings, namely, elicitor activity of partially *N*-acetylated chitosan polymers and β-(1→4)-linked GlcNAc oligomers, but not GlcN oligomers. Surprisingly, however, differential effects of the elicitors toward different enzymes used as markers for active resistance responses were observed, and the macroscopic symptoms exhibited by the elicitor-treated leaves differed significantly. Fully deacetylated GlcN oligomers elicited neither PAL nor POD activities, fully acetylated GlcNAc oligomers strongly elicited POD activities at rather high concentrations, but PAL activity was not induced at all. Neither of the oligomers induced any macroscopically or microscopically visible symptoms. In contrast, partially acetylated chitosan polymers elicited both enzymes and led to lignin deposition and development of foliar necroses. These differences between the effects of GlcNAc oligomers and chitosan polymers in wheat leaves suggest different modes of action of these elicitors.

GlcNAc oligomers are most likely recognized by specific lectin-like receptors. Lectins specific for GlcN or GlcNAc have been implicated in the recognition of chitosan (Liénart et al., 1991) and chitin (Etzler, 1985) oligomers, respectively, and high-affinity binding sites for GlcNAc oligomers that are not inhibited by GlcN oligomers have been described in suspension-cultured rice and tomato cells that can be elicited by chitin but not by chitosan oligomers (Shibuya et al., 1993; Baureithel et al., 1994). In wheat leaves the GlcNAc-specific lectin wheat germ agglutinin might be involved in the recognition of chitin oligomers (Ride et al., 1989), triggering a specific induction of POD, which alone does not lead to lignin deposition. GlcNAc oligomers did induce lignification in prewounded wheat leaves (Barber et al., 1989), possibly because of the activation of PAL and the general phenylpropanoid pathway by mechanical wounding (Menden, 1995). The rather high concentrations of GlcNAc oligomers needed for elicitation in intact wheat leaves (1 mg mL^{-1} = 30 μg per leaf) are in the same range as reported for wounded wheat leaves (40 μg per wound, Barber et al. [1989]) and melon plants (50 μg per plant, Roby et al. [1987]). Suspension-cultured wheat cells, on the other hand, were much more sensitive, react-

Table II. *Lignin content of cell walls isolated from wheat leaves 24 h after injection of polymeric chitosans with different DAs*

Data are means ± SD of triplicate samples. The lignin contents determined in chitosan-treated leaves were significantly ($P < 0.05$) different from those of water-treated control plants.

Sample	Lignin Content	Control
	% (w/w) of cell wall	*%*
Water	10.92 ± 0.79	100
DA-1%	12.84 ± 0.82	118
DA-35%	14.51 ± 0.33	133
DA-60%	12.82 ± 0.28	117

ing in the nanomolar range (Vander, 1995), as reported for suspension-cultured cells of tomato and rice (Felix et al., 1993; Yamada et al., 1993).

In contrast, the observed elicitor activity of highly deacetylated chitosan polymers and the inactivity of GlcN oligomers may be explained by a mechanism independent of specific receptors (Kauss et al., 1989). The polycationic polymers may interact with negatively charged phospholipids, thus disturbing the integrity of the plant plasma membrane. Consequently, their elicitor activity should decrease with increasing DAs (Kauss et al., 1989). However, this was not the case in our study. The maximum PAL and POD activities induced increased with increasing DA, whereas lignin deposition and symptom development were strongest with chitosans of intermediate DA. The extent of foliar necrotization correlated more closely with the induced PAL activities than with POD activities, possibly because POD became trapped in the developing lignin polymer during heavy necrotization (McDougall, 1993). The chitosan with the highest DA (60%) induced only weak necroses despite substantial elicitation of both PAL and POD activities. It is interesting that this resembles the recently reported results of the toxicity of partially acetylated chitosans toward human epithelial cells, which decreased with increasing DA (Schipper et al., 1996). The discrepancy seen with the chitosan with a DA of 60% may hint at a different step, such as the reduction of the phenylpropanoic acids to the corresponding alcohols or the production of hydrogen peroxide, being the limiting step in the biosynthesis of lignin in these elicited leaves.

We suggest that the interpretation of the observed elicitor activities of partially acetylated chitosan polymers is complex because of their expected degradation by apoplastic wheat leaf endochitinases (Ride and Barber, 1990). Whereas the fully water-soluble chitosans used in this study were well characterized with respect to DA and the random distribution of GlcNAc residues along the polymer chain (Vårum et al., 1991a, 1991b, 1994), the substrate specificity of the wheat leaf chitinases and/or chitosanases toward partially acetylated chitosans is unknown. Consequently, the detailed chemical composition and DP of the oligomers resulting from a possible degradation of an injected chitosan by apoplastic wheat leaf chitinases are unknown. Our results suggest that some of the fragments resulting from enzymic degradation of partially acetylated chitosans may be highly elicitor active and that studies of the biological activity of purified partially acetylated oligomers and small polymers of β-(1→4)-linked GlcNAc and GlcN are needed to obtain a more detailed understanding of the resistance reactions in wheat leaves elicited by partially acetylated chitosans and, hence, fungal cell walls.

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