Species-Specificity of an Elicitor-Active Oligosaccharide, LN-3, to Leguminous Plants

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Z. Naturforsch. **51c**, 371–378 (1996); received January 12/February 13, 1996

Elicitor, Species-Specificity, Phytoalexin Accumulation, Bean, Pea

LN-3, a linear pyridylaminated hepta-β-glucoside previously found to show elicitor activity in alfalfa cotyledons, was examined for phytoalexin-inducing activity in pea epicotyl and bean cotyledon assays. LN-3 did not show (+)-pisatin-inducing activity in pea epicotyls. In the bean cotyledon assay, however, the (±)-kievitone content gradually increased with increasing LN-3 concentration, and reached a maximum (*ca.* 17 μg/g fresh wt) at 100 μg/ml. Half-maximal elicitor activity was seen at *ca.* 16 μM. After three legumes, alfalfa, pea and bean, were treated with LN-3, the recovery of the remaining LN-3 or its fragments was examined. Almost 100% of LN-3 or its fragments was recovered from the pea test solution; in contrast, recoveries from alfalfa and bean were only 63.8 and 38.1%, respectively. HPLC and LC-MS analyses of the recovered samples indicated that LN-3 was hydrolyzed to give mono- and/or diglucoside(s) in the alfalfa and the bean solutions, while in the pea experiment a small portion of LN-3 was hydrolyzed to give sugar fragments with different degrees of polymerization <7.

Introduction

Oligosaccharide fragments from cell walls of fungi and plants, including chitin, chitosan, pectic acid, and β-glucan, elicit a wide variety of defence responses in plants. Homogenous oligosaccharides from the hydrolysates of chitin, chitosan, and pectic acid are easier to prepare and identify than the heterogeneous oligosaccharides originating from β-glucan, and the elicitor activities of such simple oligosaccharides have been investigated in some detail.

Chitin- and chitosan-derived oligosaccharides must generally have a degree of polymerization (DP) >4 to induce a biological response (Darvill *et al.*, 1992). Chitin oligomers with DP >6 induced phytoalexin formation in suspension-cultured rice cells (Yamada *et al.*, 1993), and chitosan oligosaccharides with DP >7 elicited pisatin accumulation in pea (Kendra and Hadwiger, 1984; Kendra *et al.*, 1989). We investigated the (+)-pisatin-inducing activity of several chitin and chitosan derivatives with different degrees of polymerization and N-acetylation, and newly found that partially N-deacetylated chitin derivatives are potent elicitors of (+)-pisatin induction (Kobayashi *et al.*, 1994; Akiyama *et al.*, 1994, 1995).

Oligogalacturonic acids elicit various defence responses, including the accumulation of phytoalexins, induction of wall-degrading enzymes, synthesis of proteinase inhibitor proteins, and induction of lignin biosynthesis (Ryan, 1988; Darvill *et al.*, 1992). The size range of oligogalacturonic acids that activate defence responses is usually quite narrow, their DPs generally being between 10 and 15 (Darvill *et al.*, 1992), although the proteinase inhibitor proteins were found to be induced by diuronides as well as larger oligomers in tomato leaves (Bishop *et al.*, 1984).

In contrast to the considerable amount of work that has been done on homogeneous elicitors, the molecular size-activity relationship of β-glucan oligomers has received relatively little attention. A branched hepta-β-D-glucoside from the mycelial walls of a soybean pathogen, *Phytophthora megasperma* f. sp. *glycinea*, is the only heterogeneous elicitor whose structure is fully understood (Sharp *et al.*, 1984a, b). Recently, the elicitor activities of a family of chemically synthesized oligo-β-glucosides were examined in a soybean cotyledon assay, and the structural elements for the elicitor activity of the oligoglucosides were determined (Cheong

* This study represents a section of dissertation submitted by A. T. to Osaka University in partial fulfilment of requirement for the Ph.D. degree.

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0939–5075/96/0500–0371 $ 06.00

et al., 1991). In most β-glucan elicitor studies, crude elicitor fractions have been used because of the difficulty of preparing pure elicitors. However, to be able to adequately investigate plant species-specificity of elicitors, the purity and identity of the elicitors must be the prime considerations. We have thus been attempting to obtain elicitor-active oligosaccharides from new sources other than the cell walls of fungi or plants.

We previously developed a convenient method for the preparation of a pure elicitor-active oligosaccharide, LN-3, from laminaran, a naturally occurring seaweed polysaccharide (Kobayashi *et al.*, 1993). The enzymatic hydrolysate of laminaran showed significant elicitor activity in alfalfa cotyledons. Introduction of the pyridylamino (PA) group into the elicitor-active oligosaccharides enhanced the original activity several-fold. LN-3 was shown to be a linear pyridylaminated hepta-β-glucoside consisting of a PA-laminaribiose unit at the reducing end, and a glucose and two laminaribiose units with three β-1,6-linkages (Kobayashi *et al.*, 1995). The minimum effective concentration of LN-3 for alfalfa cotyledons was 650 nM. Recently, we also succeeded the preparation of a great deal of an elicitor-active oligosaccharide (1.47 g) from laminaran (40 g) with the guidance of elicitor activity in the bean cotyledon assay (Tai *et al.*, 1996). Physicochemical means suggested that the oligosaccharide was a β-1,3- and β-1,6-linked triglucoside, a partial structure of LN-3. The triglucoside was a minimum essential structure for elicitation of phytoalexin accumulation in bean cotyledons.

In this report, we demonstrate the phytoalexin-inducing activities of LN-3 in pea and bean, and examine the fate of LN-3 during elicitor assays.

Materials and Methods

Preparation of the elicitor-active oligosaccharide LN-3

Laminaran purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo was hydrolyzed with β-1,3-glucanase. Guided by our previous work on elicitor activity in alfalfa cotyledons, the hydrolysate was fractionated by means of charcoal and gel-filtration column chromatography. The pyridylamino group was introduced into the elicitor-active oligosaccharides in order to facilitate isolation. LN-3 was purified by HPLC as described previously (Kobayashi *et al.*, 1993).

Isolation and identification of phytoalexin (±)-kievitone

(±)-Kievitone was induced by treating bean cotyledons of 330 plants with partially N-deacetylated chitin (Kobayashi *et al.*, 1994). Elicitor-treated bean cotyledons were extracted with 4 l of MeOH. The extract was concentrated to give a thick aqueous solution, and then partitioned with EtOAc (300 ml x 3). The organic phase was concentrated to dryness. The sample (790.3 mg) was chromatographed on a silica gel [Wakogel C-100 (Wako Pure Chemical Industries, Ltd., Osaka)] column (ϕ 1.3 x 72 cm) eluted stepwise with 0, 5, 10, 20, 40, 60, 80 and 100% EtOAc in *n*-hexane. The fractions eluted with 60% EtOAc-*n*-hexane (69.64 mg) were rechromatographed on an ODS (Millipore Preparative C18, 125 Å, 55–105 μm) column (ϕ 0.4 x 100 cm) employing gradient elution from H_2O (500 ml) through 70% MeOH-H_2O (500 ml). From the fractions eluted with *ca.* 60% MeOH-H_2O, 10.48 mg of kievitone was obtained.

^{1}H NMR spectra were recorded with a Varian VXR-500 Instrument. Mass spectra were measured with a JEOL SX-102A. UV spectra were obtained on a Shimadzu UV-3000 spectrophotometer. Optical rotation was measured with a Jasco DIP-360.

(±)-Kievitone: EIMS (direct inlet) 70 eV *m/z* (rel. int.): 356 $[M]^+$ (100), 338 (16), 311 (19), 299 (24), 286 (20), 221 (69), 205 (38), 192 (37), 177 (39), 165 (98), 153 (25), 136 (32), 123 (14), 107 (11). $[\alpha]_D$ +1.71° (MeOH, *c* 0.105). UV λ_{max} (MeOH) nm (log ε): 227sh (4.37), 291 (4.27); λ_{max} (MeOH+NaOH) nm (log ε): 331 (4.45). ^{1}H NMR (500 MHz, CD_3OD) δ: 1.69 (3H, s, Me), 1.77 (3H, s, Me), 3.23 (2H, m, H-1″), 4.22 (1H, dd, *J* = 5.5, 10.8 Hz, H-3), 4.46 (1H, dd, *J* = 5.5, 10.8 Hz, H-2a), 4.57 (1H, t, *J* = 10.8 Hz, H-2b), 5.20 (1H, m, H-2″), 5.97 (1H, s, H-6), 6.30 (1H, dd, *J* = 2.3, 8.2 Hz, H-5′), 6.37 (1H, d, *J* = 2.3 Hz, H-3′), 6.88 (1H, d, *J* = 8.2 Hz, H-6′)

Elicitor bioassays

Alfalfa cotyledon and pea epicotyl elicitor assays were performed as described previously (Kobayashi *et al.*, 1993, 1994). The bean cotyledon

elicitor assay was done as follows. Bean seeds (*Phaseolus vulgaris* L.) obtained from Takii & Company, Ltd., Kyoto were surface-sterilized with 70% EtOH for 5 min and 5% H_2O_2 for 30 min, and then washed extensively with sterile distilled water. The seeds were transferred onto a germination medium containing 0.1% $MgCl_2$ and 0.2% GELRITE (San-Ei Gen F.F.I., Inc., Osaka) in test tubes (ϕ 25 x 130 mm) and incubated in the dark at 25 °C for 6 days. Six-day old cotyledons were collected and cut longitudinally in half. Half a cotyledon was placed in 1 ml of the test solution in a test tube (ϕ 18 x 130 mm) and then incubated in the dark at 25 °C on a rotating cultivator (2 rpm). After 48-h incubation, the cotyledon was weighed and returned to the original tube. Each tube was filled with 5 ml of MeOH and then subjected to sonication for 20 min. After filtration, the filtrate was concentrated to dryness and the residue was dissolved in 2 ml of MeOH. Twenty µl of the methanolic solution was subjected to HPLC analysis using an Inertsil ODS column (ϕ 4.6 x 250 mm, 5 µm, GL Sciences Inc., Tokyo) and a flow rate of 0.8 ml/min. The elution was performed in a linear gradient system with two solvents (Solvent A: 1% acetic acid in 30% MeOH/H_2O, Solvent B: 1% acetic acid in 90% MeOH/H_2O). The gradient was achieved within 35 min. The absorbance at 285 nm was monitored. The retention time for (±)-kievitone under this condition was 35.5 min. For quantification, the (±)-kievitone content was determined from the peak area of the sample with reference to the calibration of authentic (±)-kievitone.

Analysis of fragments from LN-3 hydrolyzed by plant hydrolytic enzymes

The concentration of LN-3 in elicitor assays was set at 100 µg/ml. After 48-h incubation, the plant segment was removed from the test tube and 20 µl of the remaining solution was subjected to HPLC analysis using an Inertsil ODS column (ϕ 4.6 x 250 mm, 5 µm) and a flow rate of 0.8 ml/min at 30 °C. The elution was performed in a linear gradient system with two solvents (Solvent A: 1% acetic acid in 5% MeOH/H_2O, Solvent B: 1% acetic acid in 10% MeOH/H_2O). The gradient was achieved within 40 min. For fluorescence detection, an excitation wavelength of 320 nm and an emission wavelength of 400 nm were used.

Analyses of the fragments from the nonreducing end were carried out as follows. After 48-h incubation, 300 µl of the test solution was placed in a 1.5-ml sample tube and concentrated to dryness. The residue was acetylated with pyridine and acetic anhydride (40 µl each) for 12 h at room temperature. MeOH and toluene were added to the reaction mixture and then the solution was concentrated to dryness. The dried residue was dissolved in 500 µl of water, saturated with sodium hydrogen carbonate. The solution was extracted with 1 ml of EtOAc (x 3). The extracts were combined and dried over sodium sulfate. The sample was placed in a 0.3-ml Mini-Vial (GL Sciences Inc.) and concentrated to dryness. The residue was dissolved in 50 µl of methanol, and 50 µl of 0.2% sodium methoxide in methanol was added. The mixture was allowed to stand for 1 h at room temperature. Acetic acid (1 µl) was then added and the solution was concentrated to dryness. The residue was pyridylaminated (Suzuki *et al.*, 1991). The reaction mixture was purified on a TSKgel TOYOPEARL HW-40S (TOSOH Co., Ltd., Tokyo) column (ϕ 1.0x27 cm). The flow rate was 0.4 ml/min and 10 mM ammonium hydrogen carbonate was used as eluent. The pyridylamino derivatives were obtained by HPLC peak collection. The eluate was concentrated *in vacuo*, and then dissolved in 300 µl of water. The aqueous solution was subjected to HPLC analysis as above. Fragments from the reducing and nonreducing ends were analyzed by LC-MS, on a Perkin-Elmer API *III* system.

Results

Establishment of elicitor bioassay system with bean cotyledons

The optimum incubation period for assay of the elicitor in bean cotyledons was established. After treatment of the bean cotyledons with laminaran (500 µg/ml), the time course of (±)-kievitone induction was carried out. (±)-Kievitone induction was at a very low level during the first 24 h, and reached a maximum 48 h after the elicitor treatment. Besides (±)-kievitone as the major component, several unidentified compounds were induced. Fig. 1 shows a HPLC chromatographic trace of a MeOH-H_2O extract of the cotyledons 48 h after the elicitor treatment. Browning at the surface of the cotyledon cross-sections treated

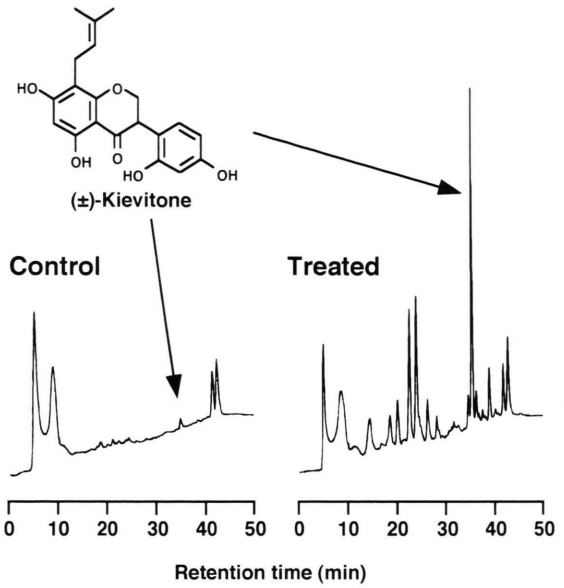

Fig. 1. HPLC profiles of (±)-kievitone and other unidentified compounds accumulated in bean cotyledon treated with laminaran (500 μg/ml) for 48 h.

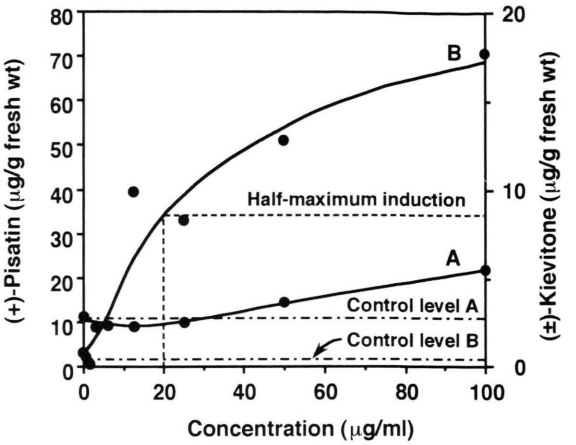

Fig. 2. Phytoalexin-inducing activity of LN-3 in pea and bean. A: (+)-Pisatin-inducing activity of LN-3 in pea epicotyl assay. B: (±)-Kievitone-inducing activity of LN-3 in bean cotyledon assay.

with the elicitor started at 24 h and was gradually intensified with the passage of incubation time. As a result of these findings, a 48-h incubation period was adopted for evaluating the elicitor activity.

Phytoalexin-inducing activity of LN-3 in pea epicotyl and bean cotyledon assays

LN-3 was tested for its (+)-pisatin-inducing activity in pea epicotyls at concentrations ranging from 12.5 to 100 μg/ml (Fig. 2A), but no significant activity (such as (+)-pisatin-inducing activity) and no browning, were evident in this concentration range.

LN-3 was also examined for its (±)-kievitone-inducing activity in bean cotyledons at concentrations ranging from 0.8 to 100 μg/ml (Fig. 2B). In this case, the (±)-kievitone content gradually increased with increasing LN-3, and reached a maximum (*ca.* 17 μg/g fresh wt) at 100 μg/ml. Half-maximal elicitor activity occurred at approximately 20 μg/ml (16.2 μM) (Fig. 2B). A concentration of LN-3 of more than 12.5 μg/ml also caused browning at the cotyledon surface.

Fate of LN-3 after elicitor treatment

After three legumes, alfalfa, pea and bean, were treated with 100 μg/ml of LN-3, the recovery% of fluorescent entities from LN-3 in the elicitor-treated solution was examined. The pyridylamino (PA) group has an intensive fluorescence and is used as a molecular probe in a quantitative analysis. The recovery% of the fluorescent entity was calculated as follows. The experimental fluorescence values were converted to the recovery% given by dividing the values by the value of the fluorescence intensity of LN-3 in the original solution (Table I). Almost 100% of the fluorescent entities from LN-3 was recovered from the elicitor-treated pea experiment. In contrast, alfalfa and bean experiments gave 63.8 and 38.1 recovery%, respectively.

Characterization of the fluorescent entities was also carried out. The PA-linked fragments were only detectable in the HPLC analysis using a fluorescence spectrophotometer as a detector. The

Table I. Recovery percentages of LN-3 from elicitor-treated alfalfa, pea and bean.

Plant	Recovery* (%)	Plant weight** [mg]
Alfalfa cotyledon	63.8 ± 6.2	40.4 ± 4.5
Pea epicotyl	99.1 ± 8.5	61.3 ± 12.0
Bean cotyledon	38.1 ± 6.5	132.7 ± 37.2

* The recovery % of the fluorescent entity was given by dividing the observed values by the value of the flurorescence intensity of LN-3 in the original solution.
** Fresh weight of elicitor-treated cotyledons or epicotyls.

elicitor-treated solutions of alfalfa and bean afforded only a single peak, which appeared at R_t 6.0 (Fig. 3A, C). In the LC-MS experiments, the single peak at R_t 6.0 for alfalfa gave two $[M+H]^+$ ions at *m/z* 259 and 421 and that for bean gave a $[M+H]^+$ ion at *m/z* 259. This result suggested that LN-3, a PA-heptaglucoside, was hydrolyzed to give PA-Glc and PA-diglucoside in alfalfa and to give PA-Glc in bean. In the elicitor-treated solution of pea, *ca.* 30% of the LN-3 remained intact. In addition to LN-3, five peaks were observed (Fig. 3B), each of which was subjected to LC-MS analysis. The prominent ion peaks ($[M+H]^+$ ions) at *m/z* 259, 421, 583, 745, 908, 1070, and 1232 indicated the presence of seven components corresponding to monomer to heptamer (LN-3). This showed that a part of the LN-3 was hydrolyzed to afford a variety of PA-sugars with different degrees of polymerization <7.

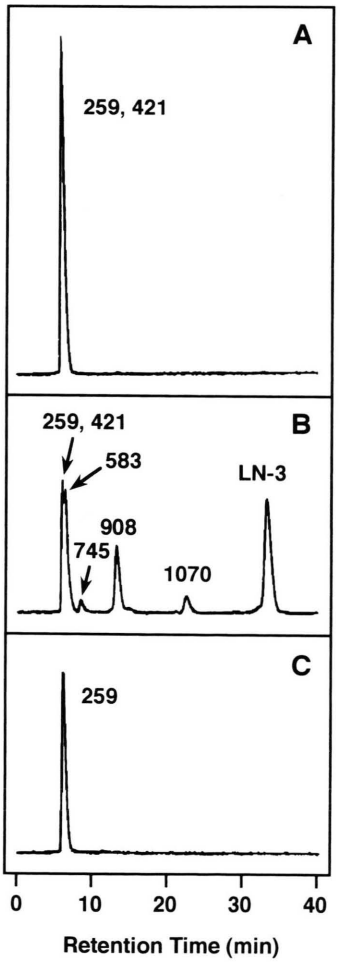

Fig. 3. LC-MS analysis of fragments from the reducing end of LN-3 hydrolyzed by plant hydrolytic enzymes. In elicitor-treated solutions of alfalfa (A), pea (B) and bean (C), pyridylamino-linked fragments from the reducing end were analyzed by HPLC. The numbers show $[M+H]^+$ ions in the LC-MS analysis.

Analyses of fragments from the nonreducing end of LN-3 were also carried out. Elicitor-treated solutions of the three plants were first subjected to acetylation and then to complete O-deacetylation. The N-acetyl group was still retained through this treatment, and the N-acetyl-PA-derivatives had no fluorescence. Therefore, the reaction mixture was pyridylaminated, and the product was subjected to HPLC in which the newly PA-introduced derivatives were detectable by a fluorescence spectrophotometer. In all three plants, a peak corresponding to PA-glucose (R_t 6.0) was observed (Fig. 4). This result suggested that the sugar released from the nonreducing end of LN-3 was D-glucose.

Discussion

In an early study of elicitor specificity, β-glucans from the mycelial walls of *Phytophthora megasperma* var. *sojae* and from yeast extract were found to stimulate the accumulation of phytoalexins in soybean, red kidney bean, and potato tubers (Cline *et al.*, 1978). However, because their structures have not been fully elucidated, it is not clear what structural elements are required for the exertion of elicitor activity in plants. Sharp *et al.* (1984a, b) set forth the entire structure of an elicitor-active hepta-β-D-glucoside from *P. m.* f. sp. *glycinea*. Parker *et al.* (1988) carried out a study on elicitor species-specificity with a chemically synthesized heptaglucoside elicitor – parsley cells and their protoplasts were tested up to the heptaglucoside, but phytoalexin was not induced in the culture media. Kopp *et al.* (1989) obtained another glucan preparation from the mycelial walls of the fungus *P. m. g.* and showed it to be a very efficient inducer of resistance against viruses in tobacco. However, infection caused by some bean and turnip viruses occurred after the elicitor treatment. These various observations indicate that elicitors may possess plant species-specificity, and it is therefore of interest to examine what specific structural elements are required for the exertion of elicitor activity in individual species.

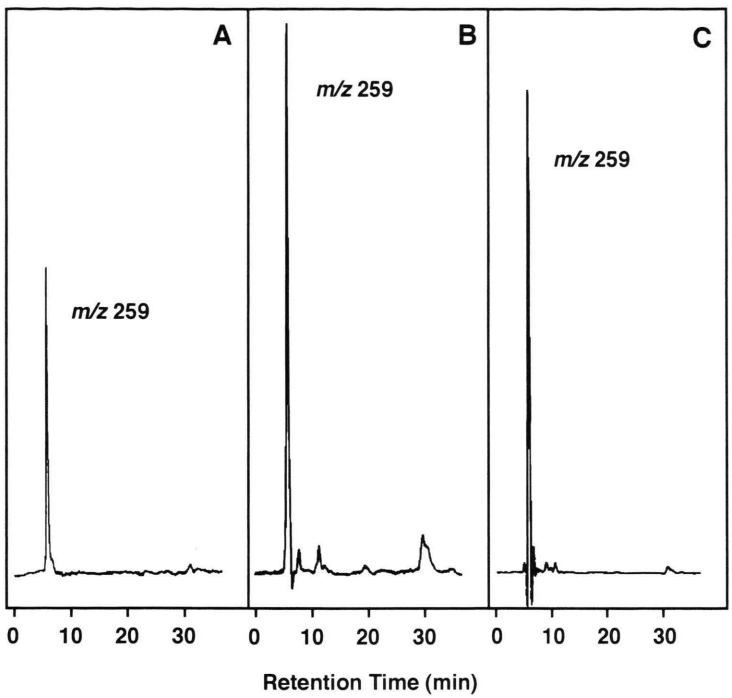

Fig. 4. LC-MS analysis of fragments from the nonreducing end of LN-3 hydrolyzed by plant hydrolytic enzymes. After acetylation, O-deacetylation, and pyridylamination of elicitor-treated solutions, newly pyridylaminated fragments were analyzed by HPLC. These HPLC profiles were obtained by subtracting the chromatogram of the control from that of the treatment (A: alfalfa, B: pea, C: bean). The numbers show $[M+H]^+$ ions.

We previously obtained a pure elicitor-active oligosaccharide, LN-3, which stimulated phytoalexin induction in alfalfa, and its structure was elucidated by physicochemical means (Kobayashi *et al.*, 1993, 1995). Here, LN-3 was tested for phytoalexin-inducing activity in pea epicotyls and bean cotyledons. In pea epicotyls, no significant increment of pisatin induction was seen at the highest concentration (100 μg/ml) used (Fig. 2A). One hundred μg/ml of $NaNO_2$-degraded chitosan induces *ca.* 80 μg/g fresh wt of (+)-pisatin (Kobayashi *et al.*, 1994). The pisatin-inducing activity of LN-3 is thus lower than that of partially N-deacetylated chitin in the entire concentration range (Kobayashi *et al.*, 1994; Akiyama *et al.*, 1995). These results indicate that LN-3 is inactive in pea epicotyls. In bean cotyledons, however, the (±)-kievitone content gradually increased with increasing LN-3 concentration, and reached a maximum (*ca.* 17 μg/g fresh wt) at 100 μg/ml (Fig. 2B). Half-maximal elicitor activity was exhibited at 16.2 μM. Bean seeds treated with $AgNO_3$ and exposed to the naturally occurring microflora accumulate 9.90 and 5.18 μg kievitone/g seed, respectively (Stössel and Magnolato, 1983). In elicitor-treated bean cells, the maximum induction of kievitone is *ca.* 14 μg (40 nmol)/g fresh wt (Robbins *et al.*, 1985). The induction level of kievitone by LN-3 is almost parallel to that by the cell wall elicitor of *Colletotrichum lindemuthianum*. It is therefore evident that LN-3 exhibits specific activity for alfalfa and bean, and not for pea. LN-3 is also the first structurally characterized elicitor which induces phytoalexin accumulation in bean cotyledons.

Recently, the presence of a specific binding site (receptor) for the heptaglucoside from *P. m. g.* has been indicated, and a high-affinity binding protein has been identified in soybean root membranes (Cheong and Hahn, 1991; Cosio *et al.*, 1992; Cheong *et al.*, 1993; Frey *et al.*, 1993). However, before exo-elicitors reach their specific binding sites the molecules may be processed to afford modified compounds. This process may be a favorable one for elicitors to exert their activity. In the present study, the possession of a large amount of the pure biotic elicitor LN-3 allowed us to examine its fate in intact tissues. We analyzed the sugars originating from LN-3 in the test solutions following LN-3 feeding. After the three legumes were treated with 100 μg/ml of LN-3, the recovery percentages of fluorescent entities from LN-3

were 63.8 and 38.1% in alfalfa and bean, respectively. In contrast, almost 100% of the fluorescence was recovered in the pea experiment (Table I). It is suggested that the different recovery percentages can be related to the elicitor activities of the test plants. The apparent fluorescence loss in the alfalfa and bean experiments may be due to the uptake of LN-3 into the cells and as well as to attachment to the epidermis. These experiments suggest that LN-3 may also bind to a specific site as does the heptaglucoside elicitor.

HPLC and LC-MS analyses of the elicitor-treated solutions indicated that LN-3 was hydrolyzed to give mono- and/or diglucoside(s) in the alfalfa and bean experiments, while in the pea experiment a part of the LN-3 was hydrolyzed into a variety of sugars with different degrees of polymerization < 7 (Figs 3 and 4). Plants possess a variety of constitutive and inducible hydrolytic enzymes. Most plant tissues have been shown to contain β-1,3-glucanases, which are capable of acting on fungal mycelial walls to release and modify the glucan elicitor or its precursors. Exo-β-glucanases present in plant cell walls are capable of processing larger inactive oligosaccharides into an active elicitor. These hydrolytic enzymes could also inactivate the elicitor, thereby restricting phytoalexin production to the site of infection (Hahn *et al.*, 1989). In bean, chitinase and β-1,3-glucanase have been induced by elicitor and ethylene treatments (Mauch and Staehelin, 1989; Hughes and Dickerson, 1991). In the pea system, we found that some part of the LN-3 was hydrolyzed by constitutive hydrolytic enzymes secreted from pea epicotyl segments. It is suggested that enzymes newly induced by LN-3 hydrolyzed the excess LN-3 molecules remaining in the alfalfa and bean test solutions to give inactive forms, mono- and/or diglucoside(s).

In further experiments, our aim will be to prepare a massive amount of pure elicitor-active oligosaccharides with high activity and specificity from other sources, in the belief that this may help to clarify the elicitor recognition system in plants.

Acknowledgements

This study was supported in part by a Grant-in-Aid to A. K. for Scientific Research (No. 06660136) from the Ministry of Education, Science and Culture of Japan. The authors thank Dr. H. Kanzaki, Dr. K. Akiyama, and Mr. S. Kajiyama for their valuable help and advice.

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