A Minimum Essential Structure of LN-3 Elicitor Activity in Bean Cotyledons

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A great deal of an elicitor-active oligosaccharide (1.47 g) was obtained from 40 g of poly-saccharide laminaran with the guidance of elicitor activity in the bean cotyledon assay. Physicochemical means suggested that the oligosaccharide was a β-1,3- and β-1,6-linked tri-glucoside. This was a partial structure of LN-3, having elicitor activity to alfalfa and bean. The triglucoside exhibited a specific elicitor activity to bean, not to alfalfa and pea. The highly digested hydrolysate containing monomer and dimers as major components did not show elicitor activity in the bean cotyledon assay. The structural feature was essential for elicitation of phytoalexin accumulation in bean cotyledons. These results suggested that this triglucoside had a specific elicitor activity and a minimum elicitor-active entity to bean cotyledons.

Introduction

We have developed a convenient method for preparation of a pure elicitor-active oligosaccha-ride, LN-3, from a naturally occurring polysaccha-ride (Kobayashi *et al.*, 1993, 1995). The enzymatic hydrolysate of an algal laminaran showed a signifi-cant elicitor activity to alfalfa cotyledons. Intro-duction of the pyridylamino (PA) group into an elicitor-active oligosaccharide enhanced the origi-nal activity. LN-3 was shown to be a linear pyridy-laminated hepta-β-glucoside (Fig. 1). The mini-mum effective concentration of LN-3 was 650 nM in the alfalfa cotyledon assay.

LN-3 was also examined for phytoalexin-induc-ing activity in the pea epicotyl and the bean cotyle-don assays (unpublished data). LN-3 did not show (+)-pisatin (6a-hydroxy-3-methoxy-8,9-methylene-dioxypterocarpan)-inducing activity to pea epicot-yls. In the bean cotyledon assay, the (±)-kievitone [5,7,2′,4′-tetrahydroxy-8-(3″,3″-dimethylallyl)-iso-flavanone] content gradually increased with ele-vating concentration of LN-3, and reached a maxi-mum (*ca.* 17 μg/g fresh wt) at 100 μg/ml. A half-maximum elicitor activity was observed at *ca.* 16 μM.

In preliminary experiments, a sugar fraction with degrees of polymerization <7 from laminaran hydrolysate showed elicitor activity to bean cotyle-dons. This paper will demonstrate a minimum essential structure of LN-3 elicitor to bean cotyledons.
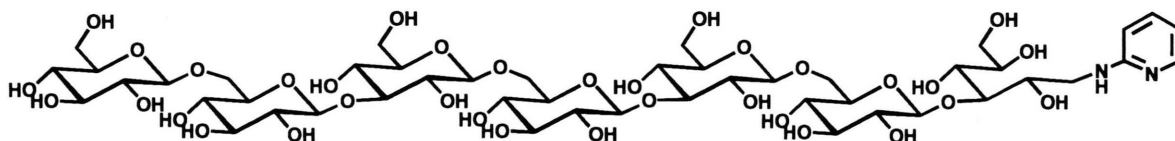

Fig. 1. The structure of LN-3, acting as elicitor in alfalfa and bean cotyledons.

Materials and Methods

Isolation of an elicitor-active trisaccharide

By assaying elicitor activity with the bean coty-ledon assay, preparation of an elicitor-active oligo-saccharide from the laminaran hydrolysate was carried out as follows. Laminaran (40.0 g; Tokyo

Kasei Kogyo Co., Ltd., Tokyo) was dissolved in 0.1 M Na phosphate buffer (4.0 l ; pH 7.0) and a β-1,3-glucanase, TUNICASE R70 (1.0 g; Daiwa Kasei Co., Osaka) was added to the solution. The reaction mixture was shaken at 37 °C for 24 h. The resulting solution was concentrated to 300 ml total volume, to which 2.7 l of methanol was added at 0 °C, and then centrifuged. The supernatant was concentrated to dryness *in vacuo*. The dried residue (16.9 g) was subjected to charcoal (Nacalai Tesque, Inc., Kyoto; 60–150 mesh) column (∅ 6.5×27 cm) chromatography eluted stepwise with solvents from H_2O through 80% EtOH-H_2O. The 30% EtOH-H_2O eluate (3.6 g) was chromatographed on a TSKgel TOYOPEARL HW-40F (TOSOH Co., Ltd., Tokyo) column (∅ 4.0×50 cm). An elicitor-active oligosaccharide (1.47 g) was obtained as a single compound. Complete hydrolysis of the oligosaccharide gave a single product which was identified as D-glucose. Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) MS were measured on a Finnigan MAT Vision 2000 with 10 mg/ml 2,5-dihydroxybenzoic acid in 30% CH_3CN-H_2O (0.1% trifluoroacetic acid) as a matrix solution. Ions were accelerated to an energy of 6 keV before entering the TOF mass spectrometer. Analysis of the oligosaccharide by MALDI TOF MS gave a $[M+Na]^+$ ion peak at m/z 527.4.

Identification of phytoalexin (±)-kievitone

(±)-Kievitone was isolated from bean cotyledons treated with a partially N-deacetylated chitin (Kobayashi *et al.*, 1994). ¹H 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): 227 sh (4.37), 291 (4.27); λ_{max} (MeOH+NaOH) nm (log ε): 331 (4.45). ¹H 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). 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 intensively with sterile distilled water. The seeds were transferred onto a germination medium containing 0.1% $MgCl_2$ and 0.2% gellan gum (San-Ei Gen F.F.I., Inc., Osaka) in test tubes (∅ 25×130 mm) and incubated in the dark at 25 °C for 6 days. Six-day old cotyledons were collected and longitudinally cut in half. A half of cotyledon was placed in 1 ml of the test solution in a test tube (∅ 18×130 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 into 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 (∅ 4.6×250 mm, 5 μm, GL Sciences Inc., Tokyo) and a flow rate of 0.8 ml/min. The elution was performed by a linear gradient system with two solvents (solvent A: 1% acetic acid in 30% MeOH/H_2O, v/v, solvent B: 1% acetic acid in 90% MeOH/H_2O, v/v). The gradient was achieved within 35 min. Absorbance at 285 nm was monitored. Retention time for (±)-kievitone under this condition was 35.5 min. The (±)-kievitone content was determined quantitatively by the peak area of the sample with reference to calibration with authentic (±)-kievitone.

Reduction of an elicitor-active triglucoside

A triglucoside (20 mg) was deuterioreduced for 3 h at room temperature with 2 ml of sodium borodeuteride solution (10 mg of $NaBD_4$/ml). To the reaction mixture acetic acid and methanol were added, and then concentrated to dryness. The re-

duced trisaccharide (20 mg) was purified on a TSKgel TOYOPEARL HW-40S (TOSOH Co., Ltd.) column (∅ 1.0×27.3 cm). The diglucosyl-glucitol was dissolved in D_2O. ^1H NMR spectra were recorded with a Varian VXR-500 Instrument. Chemical shifts were given relative to internal acetone (δ 2.225). Two anomeric protons were observed at δ 4.49 (1H, d, *J*=7.9 Hz) and 4.66 (1H, d, *J*=7.9 Hz).

Methylation of the deuterioreduced triglucoside

The diglucosyl-glucitol (3.0 mg) was lyophilized overnight *in vacuo* at −58 °C. The sample was dissolved in dry dimethyl sulfoxide (345 µl), and 3.6 M potassium dimethylsulfinyl anion (55 µl) was slowly added. The mixture was stirred for 2 h at room temperature. Methyliodide (12.3 µl) was added under ice-bath condition, and then the solution was stirred for 1 h at room temperature. The addition of potassium dimethylsulfinyl anion (55 µl) and methyliodide (100 µl) was repeated. The reaction was continued overnight. The reaction mixture was diluted with water (2.0 ml), and extracted twice with chloroform (2.0 ml). The extracts were combined, washed six times with water (2.0 ml), and concentrated to dryness under a stream of nitrogen. The per-O-methylated diglucosyl-glucitol (2.9 mg) was purified by preparative TLC (Kieselgel 60 F_{254}; MERCK; 7:2:1, v/v, toluene:acetone:methanol).

Glycosyl-linkage analysis

The per-O-methylated diglucosyl-glucitol (100 µg) was hydrolyzed with 2 M TFA (100 µl) for 1 h at 100 °C. The mixture was dried under a stream of nitrogen. Methanol was added to the dried residue, and then concentrated to dryness. This procedure was repeated five times. The resulting partially O-methylated monosaccharide derivatives were reduced for 3 h at room temperature with a 50-µl aliquot of an ethanol solution of sodium borohydride (10 µg of $NaBH_4$/µl) containing 1 M ammonia. Acetic acid and methanol were added, and the solution was concentrated to dryness. The residue was acetylated with acetic anhydride and pyridine for 12 h at room temperature. The partially O-methylated alditol acetates were dissolved in acetone, and injected into the GLC-MS (JEOL Automass 20 system equipped with a Hewlett-

Packard model 5890 gas chromatograph). A DB-1 (J and W Scientific, California) capillary column (30 m×0.25 mm i.d.; 0.4 µm) was used for the analysis. The temperature program consisted of holding for 3 min at the injection temperature of 150 °C and then raising the temperature at 6 °C/min to 240 °C.

Results

Isolation of an elicitor-active trisaccharide

With the guidance of elicitor activity to bean cotyledons, the preparation of an elicitor-active oligosaccharide from laminaran hydrolysate was carried out. Laminaran (40.0 g) was hydrolyzed with a β-1,3-glucanase. After hydrolysis, the solution was concentrated to 300 ml total volume. To the solution, 2.7 l of methanol was added, the resulting supernatant was collected by centrifugation, then concentrated to dryness *in vacuo*. The supernatant fraction (16.9 g) was subjected to charcoal column chromatography. The 30% EtOH-H_2O eluate (3.6 g) was chromatographed on a gel filtration column yielding 1.47 g of an elicitor-active oligosaccharide. Complete hydrolysis of the oligosaccharide gave a single product which was identified as D-glucose. Analysis of the oligosaccharide by MALDI TOF MS gave a $[M+Na]^+$ ion peak at *m/z* 527.4. These data indicate that the elicitor-active oligosaccharide was a triglucoside.

The structure of an elicitor-active trisaccharide

A triglucoside was reduced with sodium borodeuteride. The reduced trisaccharide was purified on a TSKgel TOYOPEARL HW-40S column. ^1H NMR analysis of the sample showed two anomeric protons at δ 4.49 (1H, d, *J*=7.9 Hz) and 4.66 (1H, d, *J*=7.9 Hz).

The reduced trisaccharide was lyophilized and permethylated with dry dimethyl sulfoxide, 3.6 M potassium dimethylsulfinyl anion and methyl iodide. The per-O-methylated diglucosyl-glucitol was hydrolyzed with TFA, and the resulting partially O-methylated monosaccharide derivatives were reduced with sodium borohydride. The sample was acetylated with acetic anhydride and pyridine. The resulting partially O-methylated alditol acetates were subjected to GLC-MS analysis.
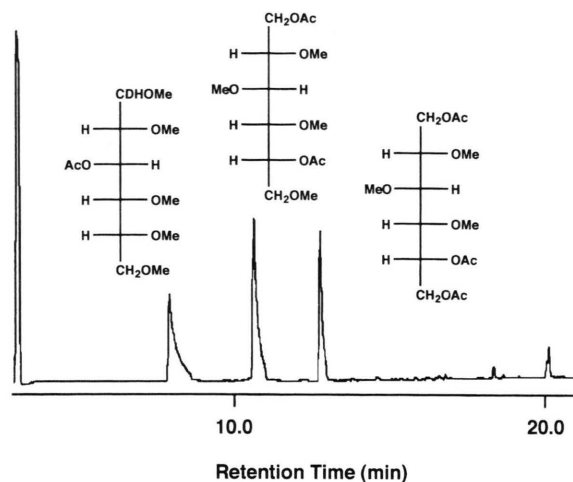
Fig. 2. A total-ion chromatographic trace of partially O-methylated alditol acetates from a per-O-methylated trisaccharide. On the basis of EI-MS data, the peaks at *Rt* 8.0, 10.6 and 12.8 were identified as 3-O-acetyl-1-deuterio-1,2,4,5,6-penta-O-methylglucitol derived from the reducing terminal, 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol from the nonreducing terminal, and 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol from 6-linked glucosyl residues, respectively.

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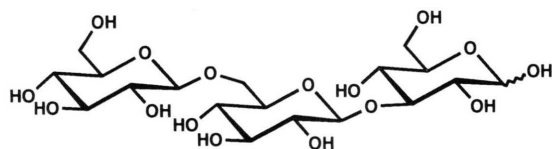These results showed that an elicitor-active trisaccharide was a β-1,3- and β-1,6-linked triglucoside (Fig. 3).

Fig. 3. The structure of an elicitor-active trisaccharide.

Elicitor activity of the triglucoside to three leguminous plants

The triglucoside was examined for elicitor activity in the alfalfa cotyledon, pea epicotyl and bean cotyledon assays. The triglucoside showed elicitor activity to bean, not to alfalfa and pea. The triglu-
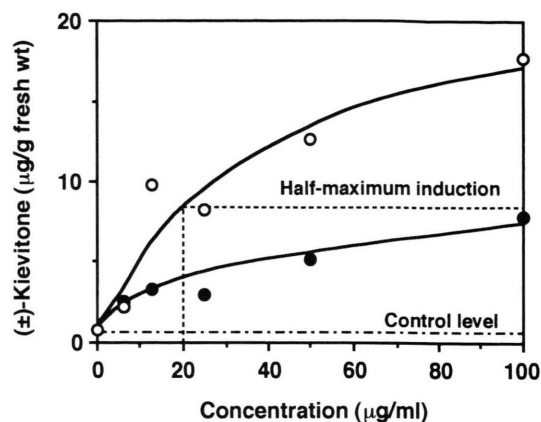

Fig. 4. The kievitone-inducing activity of a purified triglucoside (●) and LN-3 (○).

coside and LN-3, an elicitor-active pyridylaminated hepta-β-glucoside, were tested for the (±)-kievitone-inducing activity in bean cotyledons at the concentrations ranging from 6.3 to 100 µg/ml (Fig. 4). The kievitone-inducing activity of the triglucoside is lower than that of LN-3 at the entire concentration range. The kievitone induction of the triglucoside at 100 µg/ml was about 8 µg/g fresh wt, whereas LN-3 induced approximately 17 µg/g fresh wt. This induction was less than the half-maximum kievitone induction of LN-3. However, the highly digested hydrolysate containing monomer and dimers as major components did not show elicitor activity in the bean cotyledon assay. So, the triglucoside had a specific elicitor activity and a minimum elicitor-active entity to bean cotyledons.

Discussion

The triglucoside exhibited a specific elicitor activity to bean, not to alfalfa and pea. The kievitone-inducing activity of this compound is lower than that of LN-3 at the concentration range tested (Fig. 4). The induction level of kievitone at 100 µg/ml (198 µM) of the triglucoside was less than the half-maximum of LN-3 at 20 µg/ml (16.2 µM). Thus, the triglucoside is *ca.* 10-fold less active than LN-3 in terms of elicitor activity for bean cotyledons. However, the highly digested hydrolysate containing monomer and dimers as major components did not show elicitor activity in the bean cotyledon assay. Neither laminaribiose

(β-1,3-linked diglucoside) nor gentiobiose (β-1,6-linked diglucoside) was not a bean elicitor. Molecular size-activity relationships of β-glucan oligomers as elicitors are little examined. The branched hepta-β-glucoside from *Phytophthora megasperma* f. sp. *glycinea* is an only β-glucan elicitor whose structure is fully elucidated (Sharp *et al.*, 1984a, b). Recently, the activity of a family of chemically synthesized oligo-β-glucosides was examined in soybean cotyledon assay, and their structural elements for elicitor activity of the oligoglucosides were determined (Cheong *et al.*, 1991). The penta- and triglucosides were 3000-fold and 6000-fold, respectively, less effective than the hepta-β-glucoside (Darvill *et al.*, 1992). Removing glucosyl residues from the hepta-β-glucoside results in significant reduction of elicitor activity. The marked decrease of elicitor activity was seen in the smaller units originating from the hepta-β-glucoside but the magnitude of the decrease was not significant in the laminaran triglucoside. This result allows us to propose that the trisaccharide unit was the minimum essential structure to exert elicitor activity to bean cotyledons.

Pea plant was not elicited by the triglucoside, LN-3 and the original laminaran, although a partially N-deacetylated chitin oligomer is a potent elicitor (Akiyama *et al.*, 1994, 1995; Kobayashi *et al.*, 1994). Alfalfa plant showed a strong response to LN-3, but not to the triglucoside, although the triglucoside has the same structural unit found in the LN-3 molecule. In contrast, the bean plant responded to both elicitors. Such significant difference in elicitor responses suggests that these two elicitors may exhibit species-specificity to other legumes. It is also suggested that each leguminous plant has its own specific receptors capable of accepting elicitor molecules different in size and structure of the sugar units.

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