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Chemical Studies on Elicitor-active Oligosaccharides
from a Naturally Occurring Polysaccharide,
Laminaran

(天然多糖ラミナラン由来エリシター活性オリゴ糖の化学的研究)

AKIHIRO TAI

1996

Chemical Studies on Elicitor-active Oligosaccharides from a Naturally Occurring Polysaccharide, Laminaran

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General introduction

Plants are equipped with a wide array of constitutive deterrents against pathogen and herbivore attacks. At least, there are two types of responses induced by different types of attack. The first type is brought about by mechanical injury and involves a systemic accumulation of proteinase inhibitors and additional toxic metabolites. This defense response is probably aimed at herbivores. The initial finding was that both insect damage and wounding cause the systemic induction of protease inhibitor in tomato and potato (Green and Ryan 1972), and in a range of other plant genera (Walker-Simmons and Ryan 1977). The second type of response involves the recognition by plant cells of a potential pathogen. The accumulation of antimicrobial phytoalexins (Figure 1) as the main event is observed at the infection site. The plant defense mechanisms, in addition to phytoalexin synthesis, that are caused by the pathogen recognition are hypersensitive cell death (Ricci *et al.*, 1989; Culver and Dawson, 1989; Chen and Heath, 1990; Keen *et al.*, 1990), lignification (Kogel *et al.*, 1988), the synthesis of cell wall extensins (HRGPs) (Bolwell and Dixon, 1986), β -endoglucanases and chitinases (Hedrick *et al.*, 1988), proteins that induce lysis in fungal sporangia (Woloshuk *et al.*, 1991).

The extracellular signal compounds involved in triggering defense reactions are commonly known as elicitors. Originally this name was applied exclusively to those capable of inducing the synthesis of phytoalexins in plant tissues (West, 1981; Darvill and Albersheim, 1984). Lately, however, the name has been used to describe factors capable of inducing many types of physiological responses and is not restricted to plants.

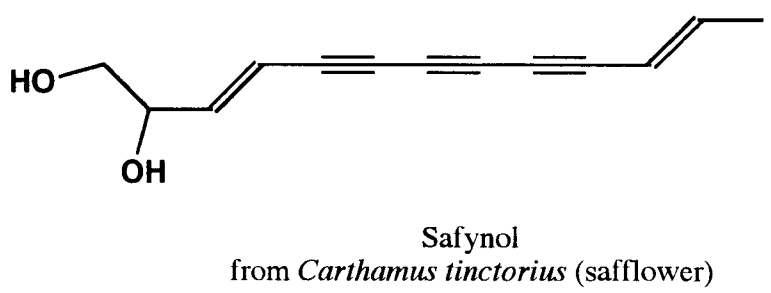
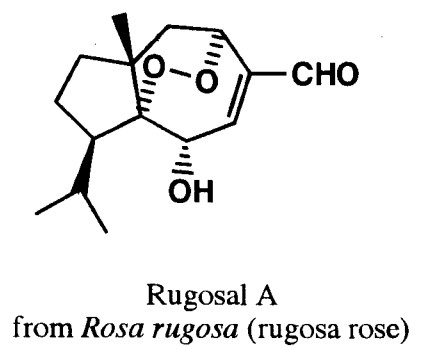
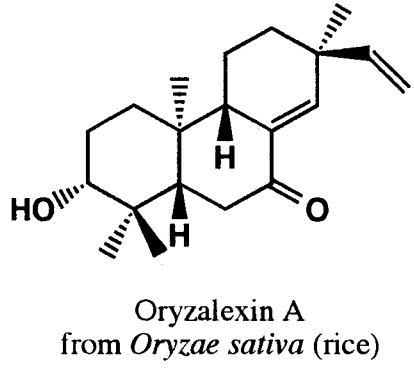
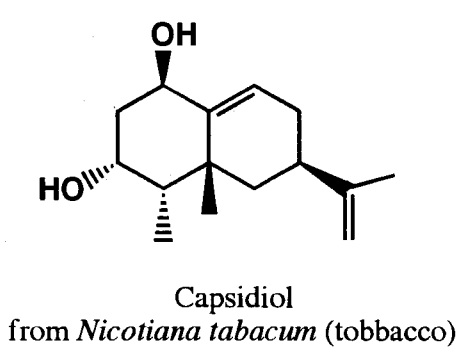
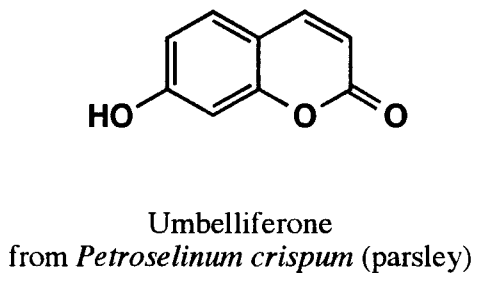
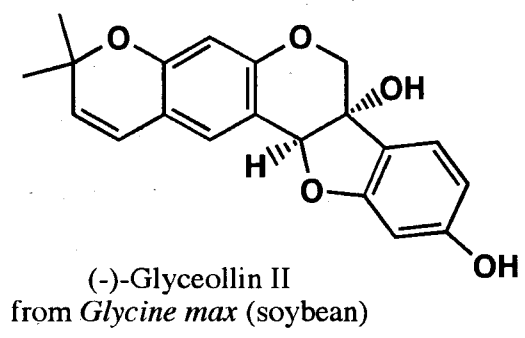
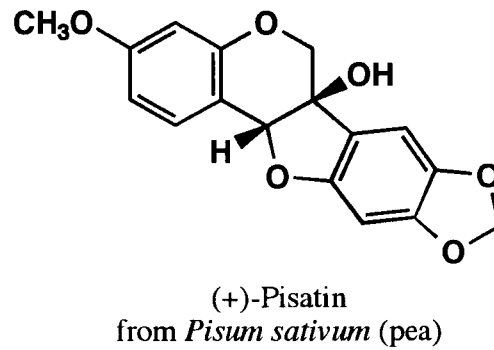
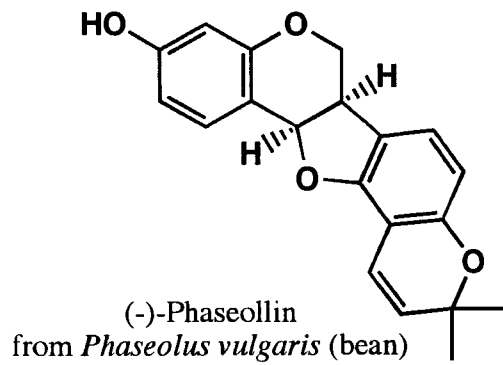


Figure 1. Phytoalexins

Many elicitors have been described, including various polysaccharides, oligosaccharide fragments, proteins, glycoproteins, and fatty acids. In a general model, elicitors of plant defense responses fall into two categories depending on their sources: exogenous and endogenous signals (Table I). Exogenous elicitors can be considered the primary signals in plant-pathogen interactions. They originate in the pathogen or aggressor itself, appear to have a limited mobility within plant tissues, and evoke a response in cells in the immediate vicinity to the pathogen. Endogenous elicitors, on the other hand, are of plant origin and arise as a result of the interaction with the aggressor. Most appear to be apoplastic and their function may be to modulate the extent of the response in the surrounding tissue. This modulation can be exerted independent of the presence of exogenous elicitors or in a synergistic manner (Darvill and Albersheim, 1984). The known endogenous elicitors vary widely in their mobility within plant tissues and, depending on their type, they can mediate localized or systemic responses (Farmer and Ryan, 1992). Among those best characterized are the oligogalacturonides derived from the pectic components of cell walls. Other, more recently identified, endogenous signals include the 18-amino acid polypeptide systemin (Figure 2, Pearce *et al.*, 1991), methyl jasmonate and jasmonic acid (Farmer and Ryan, 1990; Farmer *et al.*, 1992), which induce proteinase inhibitor proteins in tomato.

Oligosaccharide fragments from cell walls of fungi and plants including chitin, chitosan, β -glucan, and pectic acid elicit a wide variety of defence responses in plants. Preparation and identification of homogenous oligosaccharides from the hydrolysates of chitin, chitosan and pectic acid are simpler than

Table I. Elicitors of plant defense responses

Source	Elicitor	Biological effect	Reference
Exogenous			
<i>Cladosporium fulvum</i>	Protein (M_r 3,120)	Necrosis in tomato leaves	Scholtens-Toma and de Wit (1988)
<i>Colletotrichum lindemuthianum</i>	Glycoprotein (M_r 28,000)	Phaseollin* in bean	Coleman <i>et al.</i> (1992)
<i>Erwinia amylovora</i>	Protein (M_r 44,000)	Necrosis in tobacco leaves	Wei <i>et al.</i> (1992)
<i>Fusarium moniliforme</i>	Chitin oligomers**	Phytoalexins in rice*	Ren and West (1992)
<i>Fusarium solani</i> f.sp. <i>phaseoli</i>	Chitosan oligomers**	Pisatin* in pea	Hadwiger and Beckman (1980)
<i>Phytophthora capsici</i>	Protein (M_r 10,155)	Systemic necrosis in tobacco	Ricci <i>et al.</i> (1989)
<i>Phytophthora cryptogea</i>	Protein (M_r 10,323)	Systemic necrosis in tobacco	Ricci <i>et al.</i> (1989)
<i>Phytophthora megasperma</i> f.sp. <i>glycinea</i>	Hepta- β -glucoside**	Glyceollins* in soybean	Sharp <i>et al.</i> (1984a,b)
<i>Phytophthora megasperma</i> f.sp. <i>glycinea</i>	Glycoprotein (M_r 42,000)	Furanocoumarins* in parsley	Scheel <i>et al.</i> (1989)
<i>Phytophthora parasitica</i> var. <i>nicotianae</i>	Protein (M_r 46,000)	Capsidiol* in tobacco	Farmer and Helgeson (1987)
<i>Puccinia graminis</i> f.sp. <i>tritici</i>	Peptidoglycan (M_r 67,000)	Lignification in wheat leaves	Kogel <i>et al.</i> (1988)
Tobacco mosaic virus	Protein (M_r 17,500)	Necrosis in tobacco leaves	Culver and Dawson (1989)
Endogenous			
<i>Glycine max</i>	Oligogalacturonides**	Glyceollins*	Nothnagel <i>et al.</i> (1983)
<i>Lycopersicon esculentum</i>	Polypeptide, systemin**	Proteinase inhibitor in leaves	Pearce <i>et al.</i> (1991)
<i>Ricinus communis</i>	Oligogalacturonides**	Lignin synthesis	Bruce and West (1989)
<i>Vigna unguiculata</i>	Oligogalacturonides**	Necrosis in leaves	Cervone <i>et al.</i> (1987)

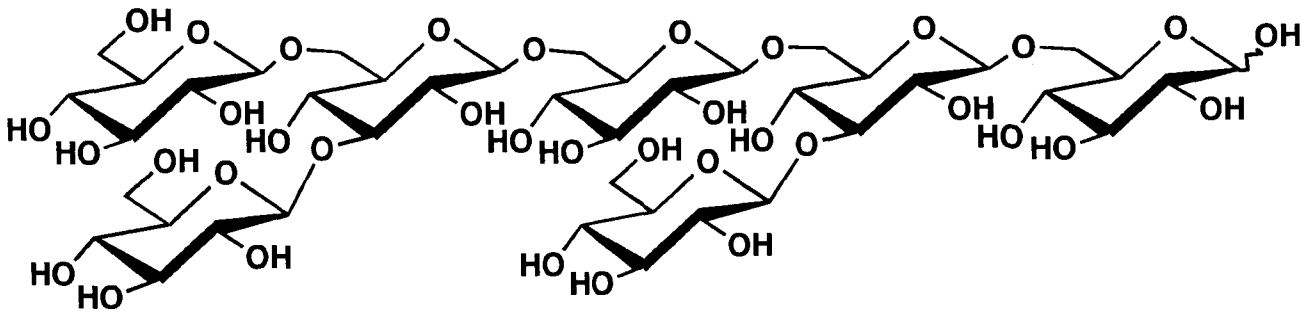
*; Figure 1, **; Figure 2.

those of heterogeneous oligosaccharides from β -glucan, and such simple oligosaccharides have been investigated for elicitor-activity in detail.

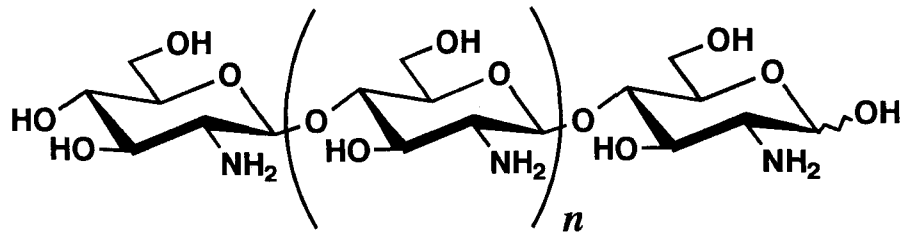
Chitin- and chitosan-derived oligosaccharides (Figure 2) generally must have a degree of polymerization (DP) >4 to induce a biological response (Darvill *et al.*, 1992). Chitin oligomers with DP >6 induce phytoalexin formation in suspension-cultured rice cells (Yamada *et al.*, 1993). Chitosan oligosaccharides with DP >7 elicit pisatin accumulation in pea (Kendra and Hadwiger, 1984; Kendra *et al.*, 1989). Kobayashi *et al.* (1994) and Akiyama *et al.* (1994, 1995) have 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 for the induction of (+)-pisatin.

Oligogalacturonides (Figure 2) elicit various defence responses including the accumulation of phytoalexins, the induction of wall-degrading enzymes, the synthesis of proteinase inhibitor proteins, and the induction of lignin biosynthesis (Ryan, 1988; Darvill *et al.*, 1992). The size range of oligogalacturonides that activate defence responses is usually quite narrow, generally with DPs between 10 and 15 (Darvill *et al.*, 1992). However, the proteinase inhibitor proteins are induced by diuronides as well as larger oligomers in tomato leaves (Bishop *et al.*, 1984).

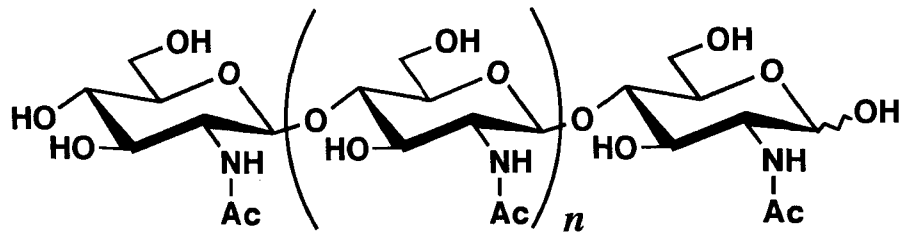
In contrast, molecular size-activity relationships of β -glucan oligomers are little examined. A branched hepta- β -D-glucoside from the mycelial walls of a soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* is the only heterogeneous elicitor whose structure is fully understood (Figure 2, Sharp *et al.*, 1984a, b).



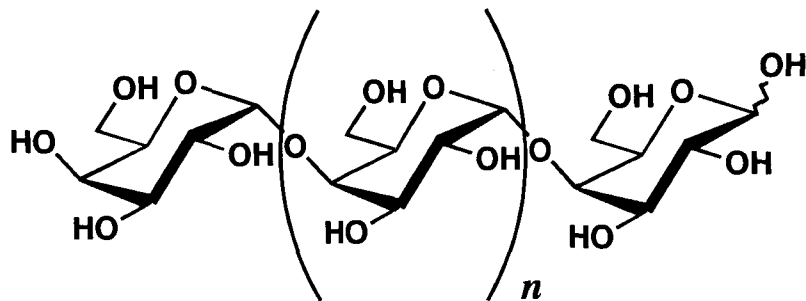
Hepta- β -glucoside



Chitosan oligomer



Chitin oligomer



Oligogalacturonide



Systemin

Figure 2. Exogenous and endogenous elicitors

Recently, the activities as elicitors of a family of chemically synthesized oligo- β -glucosides are examined in soybean cotyledon assay, and the structural elements for elicitor activity of the oligoglucosides were determined (Cheong *et al.*, 1991a). In most of the elicitor studies, crude elicitor fractions have been used because of the difficulty in preparing pure elicitors. However, for the study on the plant species-specificity of elicitors, the purity and identity of elicitors must be primarily considered. So the author attempts to prepare massive elicitor-active oligosaccharides from new sources other than cell walls of fungi or plants. The resulting elicitors should help to get a clue to the plant species-specificity of the elicitors.

This study deals with the massive preparation of elicitor-active oligosaccharides from a naturally occurring polysaccharide, laminaran together with their structural elucidation and specific elicitor activities to three legumes.

Chapter I

Elicitor-active oligosaccharides from algal laminaran stimulate the production of antifungal compounds in alfalfa

I-1. Introduction

One of the most studied defense mechanisms of plants against pathogens is the accumulation of phytoalexins at the site of infection. The biosynthesis of phytoalexins is induced by molecules called elicitors, which may be of abiotic or biotic origin. A hepta- β -glucoside from the mycelial walls of the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* is the first biotic elicitor whose chemical structure is fully understood (Sharp *et al.*, 1984a, b, c; Ossowski *et al.*, 1984). The structure-activity relationship of chemically synthesized oligo- β -glucosides as elicitors has been determined in soybeans (Cheong *et al.*, 1991a).

In most elicitor studies, crude elicitor fractions have been used because of the limited supply of the authentic pure elicitors. However, for the study on the species specificity pure elicitors are definitely needed.

In this chapter, the author attempted to obtain pure biotic elicitors from natural polysaccharides in order to make them available to scientists upon request, who could apply them to a variety of plants in order to get a clue to the plant species specificity of the elicitors.

I-2. Alfalfa cotyledon assay for elicitor activity

Ten-day-old alfalfa cotyledons were collected aseptically in a 9 cm Petri dish. Five cotyledons were placed in 1 ml of the test solution in a test tube and then incubated at 25 °C in the dark for

48 h. After incubation, the test solution was filtered, the filtrate was concentrated *in vacuo*, and 500 μ l of MeOH was added. One hundred μ l of the methanolic solution was subjected to HPLC analysis. Medicarpin, sativan, known phytoalexins in alfalfa (Smith *et al.*, 1971; Dewick and Martin, 1979), 4',7-dihydroxyflavanone and 4',7-dihydroxyflavone were induced by elicitor-treatment (Figure I-1). The elicitor activity was judged positive only when the total peak area corresponding to these 4 compounds exceeded that of the 4 compounds in the control by a factor of 2.

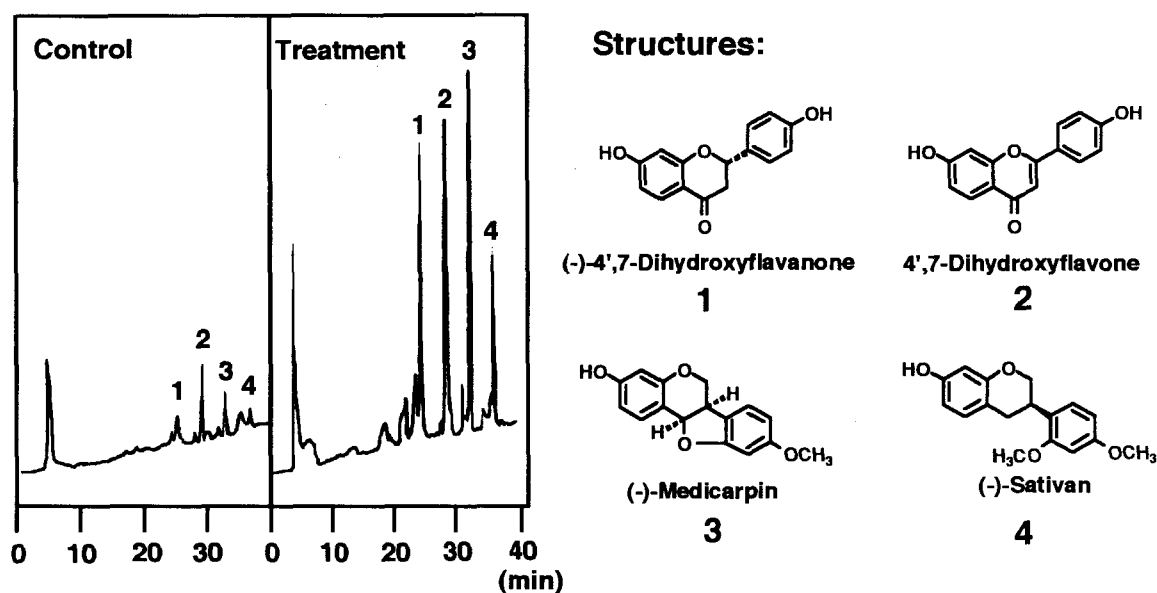


Figure I-1. HPLC profiles of flavonoids accumulated in elicitor-treated alfalfa cotyledons.

I-3. Investigations of natural polysaccharides possessing elicitor activity

Natural polysaccharides (500 μ g/ml) such as pullulan, inulin, acacia, tragacanth gum, laminaran, pectic acid (Nothnagel *et al.*, 1983; Ryan, 1988) and curdlan were examined for elicitor activity

in the alfalfa cotyledon assay. Only two polysaccharides, laminaran and pectic acid, had elicitor activities. Pullulan, acacia, tragacanth gum and curdlan were found to suppress flavonoid accumulation (Figure I-2). The elicitor activities of laminaran and pectic acid were evaluated in different concentrations. Laminaran showed a pronounced activity at a concentration of 100 $\mu\text{g}/\text{ml}$ and the highest activity was seen between 200 and 400 $\mu\text{g}/\text{ml}$. In contrast, the elicitor activity of pectic acid increased proportionally to its concentration (Figure I-3). The isolation of an elicitor-active oligosaccharide from the laminaran digest was attempted next.

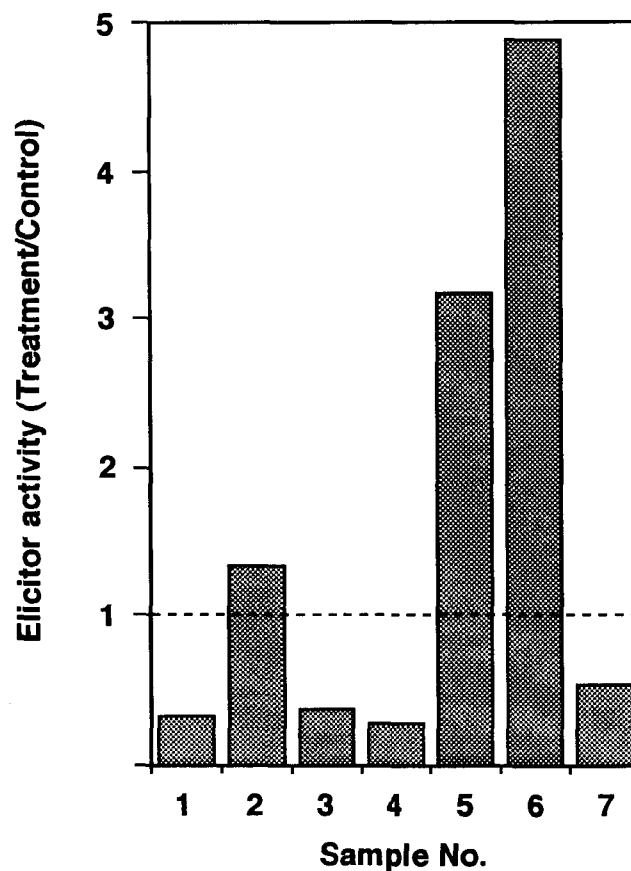


Figure I-2. Elicitor activity of natural polysaccharides at 500 $\mu\text{g}/\text{ml}$. Samples No. 1-7 are pullulan, inulin, acacia, tragacanth gum, laminaran, pectic acid and curdlan, respectively.

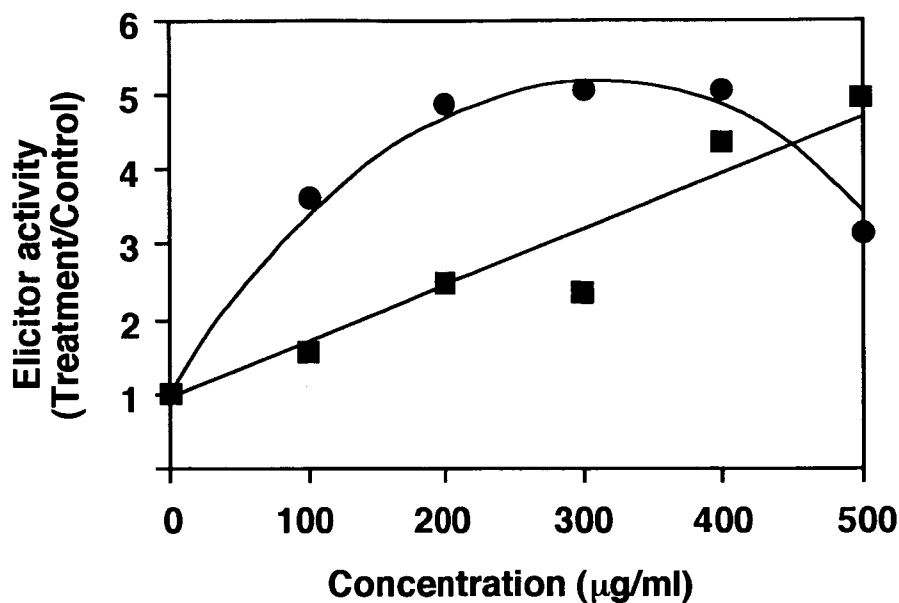


Figure I-3. Comparison of the elicitor activities of laminaran (●) and pectic acid (■) as a function of their concentration.

I-4. Preparation of elicitor-active fragments from laminaran by tunicase

Among the tested enzymes a β -1,3-glucanase, TUNICASE R70, produced the most promising result. After 1 h incubation, the elicitor activity reached the maximum level which was maintained in the following incubation period. The enzyme reaction also reached the plateau 2 h after the reaction started (Figure I-4). Therefore, the 2 h enzyme reaction was adopted for preparation of oligosaccharides with elicitor activity.

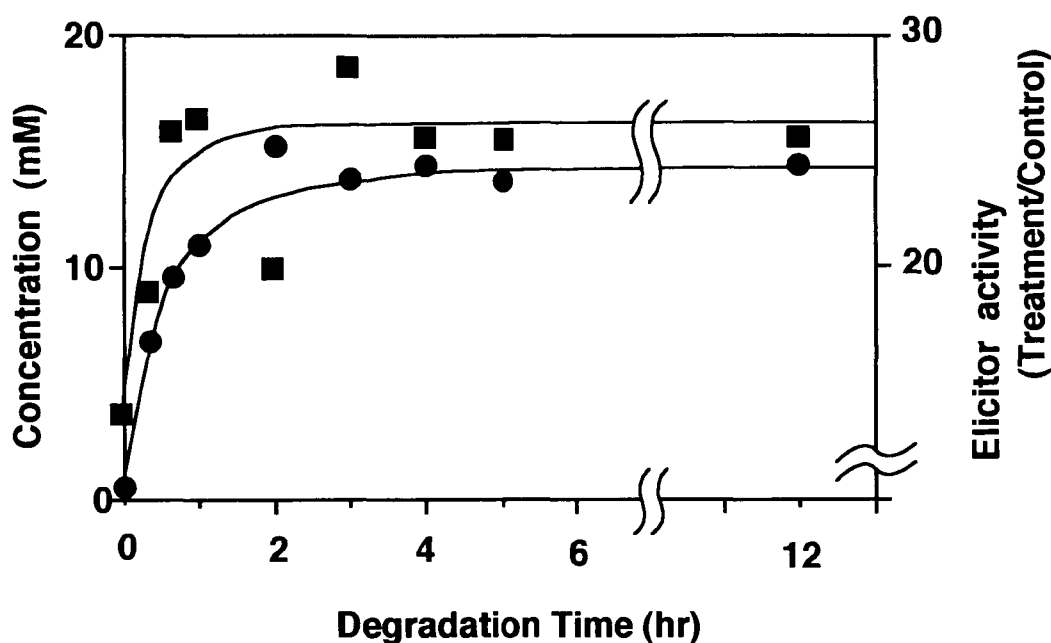


Figure I-4. The amount of carbohydrate (●) and the elicitor activity (■) of laminaran hydrolysate produced by Tunicase treatment. The amount of carbohydrate was determined by a modification of Schales method (Imoto and Yagishita, 1971) as glucose equivalent. Elicitor activity of the hydrolysate (500 $\mu\text{g/ml}$, calculated for sugar) was evaluated without removing buffer and enzyme.

I-5. Purification of elicitor-active oligosaccharides from laminaran hydrolysate

Laminaran hydrolysate was subjected to charcoal and gel filtration column chromatography. Elicitor-active oligosaccharides (151 mg) were obtained and identified in the alfalfa cotyledon assay. Introduction of a fluorescence probe, the pyridylamino (PA) group, into the elicitor-active molecules was attempted in order to facilitate the isolation procedure. The PA derivatives were subjected to the assay and found to exhibit higher activity than the original oligosaccharides (Figure I-5). LC-MS of the PA derivatives showed that the elicitor-active principles form two ion clusters with the same molecular weights, m/z 1070 and 1232 (Figure I-6). This

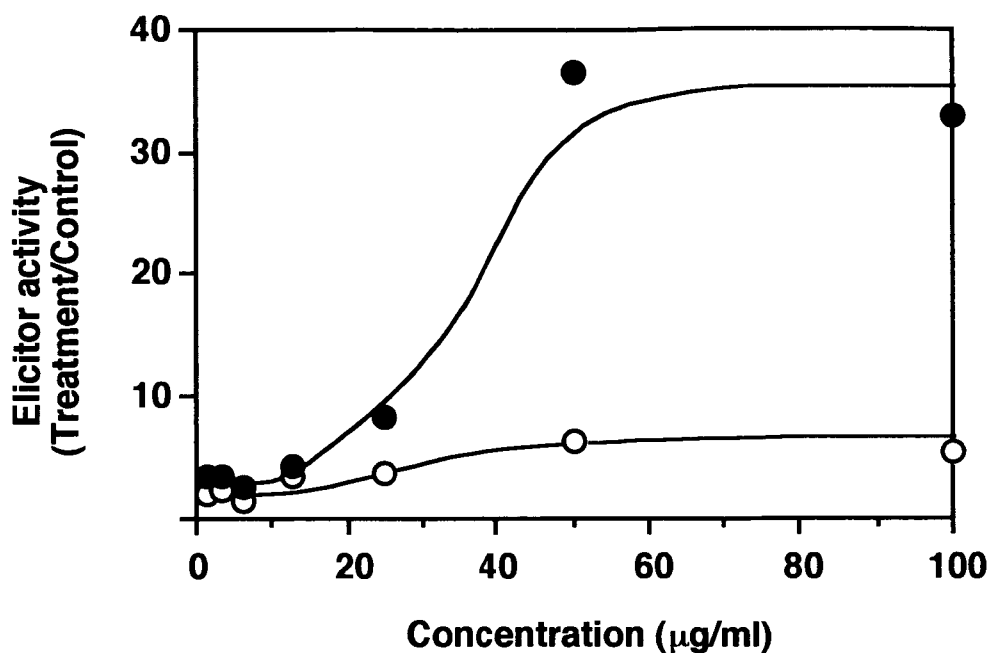


Figure I-5. Comparison of the elicitor activities of pyridylamino derivatives (●) and of the original oligosaccharides (○).

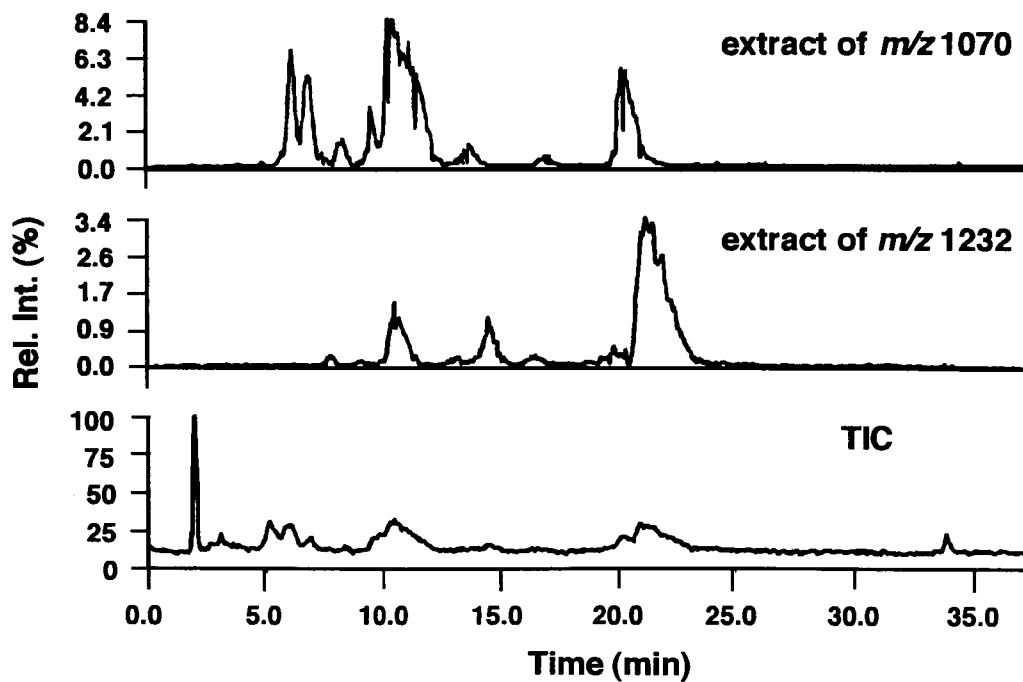


Figure I-6. LC-MS total ion chromatograms of pyridylamino derivatives.

also suggested that the degrees of polymerization of the two clusters of the oligosaccharides were 6 and 7. Their HPLC analysis showed main peaks at *Rt* 18.1 (LN-1), 21.5 (LN-2) and 27.6 min (LN-3) (Figure I-7). The individual peaks were then subjected to both LC-MS and the alfalfa assay. All peaks were active and their minimum effective concentrations were determined as shown in Table I-1. LN-3 revealed the highest activity.

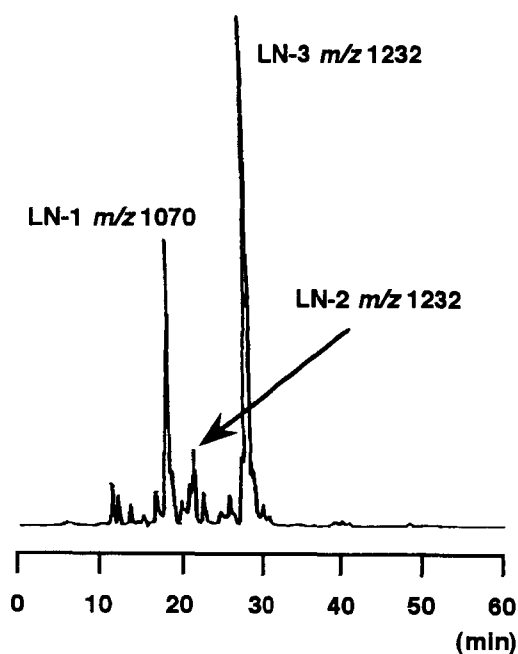


Figure I-7. Separation of pyridylamino derivatives by HPLC.

Table I-1. Elicitor activity of pyridylamino (PA) oligosaccharides, LN-1, 2 and 3.

PA-oligosaccharide	Concentration ($\mu\text{g/ml}$)							
	50	25	12.5	6.3	3.2	1.6	0.8	0.4
LN - 1	+	+	-	-	-	-	-	-
LN - 2	+	+	+	+	+	-	-	-
LN - 3	+	+	+	+	+	+	+	-

+ : active, - : inactive

I-6. Discussion

Laminaran from sea weed is known to have a linear polysaccharide structure with β -1,3- and 1,6-linkages (Peat *et al.*, 1958a, b). This suggests that the structure of the mother heptaglucoside of LN-3 is different from that of the Pmg elicitor, reported by Sharp *et al.* (1984a, b). The anomeric proton signals of its ^1H NMR spectrum also supported the dissimilarity (data not shown).

In this experiment, PA derivatives not only sustained but rather enhanced the elicitor activity. The six sugar unit in the oligosaccharides of the laminaran hydrolysate appears to have the optimal elicitor activity. Preliminary elicitor assays with pea seedlings and kidney bean cotyledons were carried out in the concentration range of 25 to 100 $\mu\text{g}/\text{ml}$. However, no marked activity was seen in the three components. Using PA-derivatization techniques the author will now try to isolate elicitor-active oligosaccharides from various polysaccharide hydrolysates, which would effectively induce new secondary metabolites in a variety of intact plants as well as in calli.

The structural elucidation of LN-1, 2 and 3, as well as the determination of their plant species specificity are in progress.

I-7. Materials and Methods

Plant material

Alfalfa seeds were surface-sterilized with 70% ethanol for 10 min and 8% H_2O_2 for 20 min, and then washed intensively with sterile distilled water. About ten seeds were transferred onto a germination medium containing 0.1% MgCl_2 (w/v) and 0.2%

GELRITE (w/v, San-Ei Gen F.F.I., Inc.) in a test tube. Plants were grown at 25 °C for 10 days in the light.

Chemicals

Acacia, Tragacanth Gum, Inulin (Nacalai Tesque, Inc.), Curdlan, Pectic acid (Wako Pure Chemical Industries, Ltd.), and Laminaran from *Eisenia bicyclis* (Lot. FCY01; Tokyo Kasei Kogyo Co., Ltd.) were commercial samples. Pullulan (MW 12,200) was a gift from Hayashibara Biochemical Laboratories (Okayama). Medicarpin, sativan, 4',7-dihydroxyflavanone, and 4',7-dihydroxyflavone were isolated from alfalfa treated with yeast extract and identified. ¹H NMR spectra were recorded with a Varian VXR-500 Instrument. Mass spectra were measured with a JEOL JMS-D300. UV spectra were obtained on a Shimadzu UV-3000 spectrophotometer. Optical rotation was measured with a Jasco DIP-360.

Medicarpin. ¹H NMR δ (500 MHz, CDC1₃): 3.51 (1H, ddd, J = 4.8, 6.7, 11.1 Hz), 3.60 (1H, t, J = 11.1 Hz), 3.75 (3H, s), 4.22 (1H, dd, J = 4.8, 11.1 Hz), 5.48 (1H, d, J = 6.7 Hz), 6.39 (1H, d, J = 2.4 Hz), 6.43 (1H, d, J = 2.5 Hz), 6.44 (1H, dd, J = 2.4, 8.8 Hz), 6.53 (1H, dd, J = 2.5, 8.4 Hz), 7.11 (1H, d, J = 8.8 Hz), 7.37 (1H, d, J = 8.4 Hz); EI-MS m/z : 270(M⁺), 269(M⁺-H), 255(M⁺-CH₃); UV λ_{\max} (EtOH) nm (log ϵ): 283sh, 287 (3.93); $[\alpha]_{\text{D}}^{21}$ -188.3 (MeOH, c 0.6)

Sativan. ¹H NMR δ (500 MHz, CDC1₃): 2.84 (1H, ddd, J = 1.9, 5.3, 15.6 Hz), 2.95 (1H, dd, J = 10.9, 15.6 Hz), 3.53 (1H, m), 3.78 (3H, s), 3.79 (3H, s), 3.97 (1H, t, J = 10.1 Hz), 4.27 (1H, ddd, J = 1.9, 3.4, 10.1 Hz), 4.63 (1H, s), 6.33 (1H, d, J = 2.6 Hz), 6.36 (1H, dd, J = 2.6, 8.1 Hz), 6.44 (1H, dd, J = 2.5, 8.3 Hz), 6.46 (1H, d, J = 2.5 Hz), 6.92 (1H, d, J = 8.1 Hz), 7.00 (1H, d, J = 8.3 Hz); EI-MS m/z : 286(M⁺), 164, 152, 151, 149, 121; UV λ_{\max} (EtOH) nm (log ϵ): 281(3.86), 284 (3.86)

4',7-Dihydroxyflavanone. ^1H NMR δ (500 MHz, acetone- d_6 + CDCl_3): 2.65 (1H, d, $J= 16.5$ Hz), 3.00 (1H, dd, $J= 13.1, 16.5$ Hz), 5.40 (1H, d, $J= 13.1$ Hz), 6.40 (1H, s), 6.53 (1H, d, $J= 8.9$ Hz), 6.86 (2H, d, $J= 7.6$ Hz), 7.35 (2H, d, $J= 7.6$ Hz), 7.71 (1H, d, $J= 8.9$ Hz), 8.30 (1H, s), 9.18 (1H, s); EI-MS m/z : 256(M^+); UV λ_{max} (MeOH) nm (log ϵ): 275 (4.16), 310 (3.84)

4',7-Dihydroxyflavone. ^1H NMR δ (500 MHz, acetone- d_6 + CDCl_3): 6.58 (1H, s), 6.93 (1H, dd, $J= 2.2, 8.6$ Hz), 6.99 (2H, d, $J= 8.8$ Hz), 6.99 (1H, d, $J= 2.2$ Hz), 7.87 (2H, d, $J= 8.8$ Hz), 7.96 (1H, d, $J= 8.6$ Hz), 9.05 (1H, s), 9.50 (1H, s); UV λ_{max} (MeOH) nm: 253sh, 312sh, 328

Alfalfa cotyledon assay for elicitor activity (Figure I-8)

Ten-day-old alfalfa cotyledons were collected aseptically in a 9 cm Petri dish. Five cotyledons were placed in 1 ml of the test solution in a test tube (ϕ 18 mm x 13 cm) and then incubated at 25 °C in the dark for 48 h. After incubation, the test solution was filtered, the filtrate was concentrated *in vacuo*, and 500 μl of MeOH was added. One hundred μl of the methanolic solution was subjected to HPLC analysis (L-6200 Intelligent Pump; L-4200 UV-VIS Detector; AS-2000 Autosampler; D-2500 Chromato-Integrator; HITACHI, Tokyo) using an Inertsil ODS (ϕ 4.6 x 250 mm, 5 μm ; GL Sciences Inc.) and a flow rate of 0.8 ml/min. A linear gradient of 30% MeOH_{aq.} in 1% AcOH to 90% MeOH_{aq.} in 1% AcOH in 35 min was employed. Four flavonoids were identified. Medicarpin, sativan, known phytoalexins in alfalfa, 4',7-dihydroxyflavanone and 4',7-dihydroxyflavone were induced by elicitor-treatment. The elicitor activity was judged positive only when the total peak area

corresponding to these 4 compounds exceeded that of the 4 compounds in the control by a factor of 2.

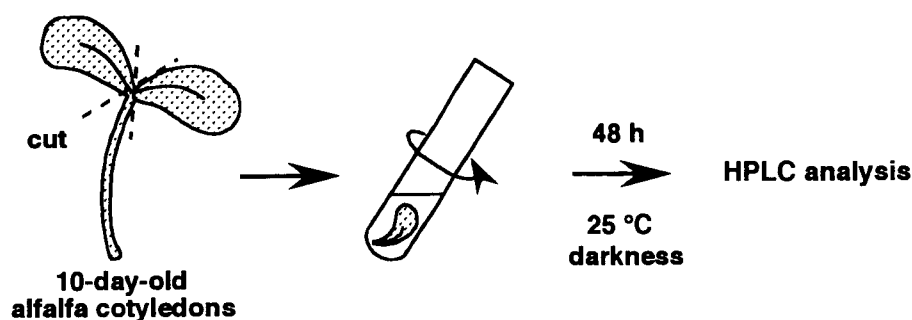


Figure I-8. Alfalfa cotyledon elicitor assay

Hydrolysis of laminaran by tunicase

Laminaran (5.5 g) was dissolved in 0.1 M Na phosphate buffer (500 ml; pH 7.0) and a hydrolytic enzyme, TUNICASE R70 (500 mg; Daiwa Kasei Co.) was added to the solution. The reaction mixture was shaken at 37 °C for 4 h. The reaction was stopped by immersing the vessel in boiling water for 5 min and the reaction mixture was then centrifuged at 12,000 *g* for 20 min. The supernatant was used in the following experiments.

Purification of elicitor-active oligosaccharides

Laminaran hydrolysate (5.5 g) was subjected to charcoal column (ϕ 2.0 x 60 cm) chromatography using a linear gradient of H₂O (800 ml) to EtOH (800 ml) and 31 fractions (50 ml each) were collected. The elicitor-active oligosaccharides (687 mg) were obtained after concentrating fractions # 21- 31. The compounds were chromatographed on a Bio-Gel P-2 column (ϕ 1.6 x 72 cm, 200-400 mesh; BIO-RAD). The active fractions were combined and concentrated *in vacuo* to give oligosaccharides (440 mg), which

were further purified on the Bio-Gel P-2 column (ϕ 1.6 x 72 cm, 200-400 mesh). Finally, elicitor-active oligosaccharides (151 mg) were obtained.

Pyridylamination (Hase et al., 1981) of elicitor-active fractions

The elicitor-active oligosaccharide concentrate (120 mg) was placed at the bottom of a 30 ml-flask and dried. To the residue a coupling reagent was added prepared by mixing 1.08 g 2-aminopyridine, 0.7 ml of HCl, and 6.3 ml of distilled water. The flask was sealed and heated at 80 °C for 1 h. Subsequently, 350 mg of NaBH₃CN was added. The flask was re-sealed and heated at 80 °C for 3 h. The reaction mixture was subjected to a column packed with AG1-X8 acetate form (200-400 mesh; BIO-RAD) and then eluted with water. The eluate was concentrated and further purified by a TOYOPEARL HW-40S (TOSOH Co., Ltd.) column (with 0.1M NH₄HCO₃). Pyridylamino derivatives (140 mg) thus prepared were subjected to LC-MS analysis as well as to the elicitor assay. LC-MS were taken on a Perkin Elmer API III.

HPLC analysis of pyridylamino derivatives

HPLC separation was carried out with a HITACHI chromatograph (L-6200 Intelligent Pump; F-1000 Fluorescence Spectrophotometer; L-5020 Column Oven; D-2500 Chromato-Integrator). The sample was separated using a DAISOPAK SP-120-5-ODS B type column (ϕ 4.6 x 250 mm, DAISO, Osaka) at 30 °C. The flow rate was 0.8 ml/min and a linear gradient of 5% MeOHaq. in 1% AcOH to 12.5% MeOHaq. in 1% AcOH in 60 min was applied. For detecting pyridylamino derivatives, an excitation wave length of 320 nm and an emission wave length of 380 nm were used.

Chapter II

Structural elucidation of an elicitor-active oligosaccharide, LN-3, prepared from algal laminaran

II-1. Introduction

Poly- and oligosaccharides from cell walls of fungi and plants including β -glucans, chitin, chitosan, and pectin, induce various defense responses in plants (Keen *et al.*, 1983; Ryan, 1988; Parker *et al.*, 1988; MacDougall *et al.*, 1992; Kobayashi *et al.*, 1994; Akiyama *et al.*, 1994). Such molecules that stimulate any plant defense mechanism are commonly called "elicitors" (Dixon, 1986). A hepta- β -D-glucoside from the mycelial walls of a soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* (Pmg) is the only elicitor whose structure is fully understood (Sharp *et al.*, 1984a, b). Recently, the activity as elicitors of a family of chemically synthesized oligo- β -glucosides was examined in soybean cotyledon assay, and the structural elements for elicitor activity of the oligoglucosides were determined (Cheong *et al.*, 1991a). Furthermore, the plant receptor that was presumably responsible for the specific recognition of Pmg elicitor was also found in soybean root (Cheong *et al.*, 1991b).

In most elicitor studies, crude elicitor fractions have been used, because of the limited supply of authentic pure samples. However, for the study of specificity of elicitors on the plant species, the purity of elicitors must be primarily argued.

The author has developed a convenient procedure to obtain pure biotic elicitors from naturally occurring polysaccharides (Kobayashi *et al.*, 1993). Natural polysaccharides were examined for their activity as elicitors for flavonoid accumulation in alfalfa

cotyledons. Laminaran from *Eisenia bicyclis*, which had a prominent elicitor-activity, was hydrolyzed with β -1,3-glucanase and the resulting hydrolysate was subjected to chromatography on charcoal and gel filtration columns. Introduction of the pyridylamino (PA) group into the elicitor-active oligosaccharides was attempted in order to facilitate the isolation. These oligosaccharides were separated on HPLC. Three PA-oligosaccharides (LN-1, 2 and 3) were collected each in a pure form and subjected to the alfalfa cotyledon assay. LN-3 showed the highest activity (the minimum effective concentration, 0.8 μ g/ml). ^1H NMR and LC-MS analyses suggested that LN-3 was a PA-hepta- β -glucoside (Kobayashi *et al.*, 1993).

In this chapter, the author demonstrates the structural elucidation of the elicitor-active PA-oligosaccharide, LN-3.

II-2. Glycosyl-linkage analysis

The elicitor-active oligosaccharide, LN-3, prepared from brown-algal laminaran by the action of β -1,3-glucanase, followed by pyridylation, was a pyridylaminated (PA) hepta- β -glucoside (Kobayashi *et al.*, 1993). The Pmg elicitor was a branched oligosaccharide (Sharp *et al.*, 1984a, b). However, a preliminary structural elucidation study suggested that LN-3 could have a linear chain. This prompted the author to elucidate its structure.

First, glycosyl-linkage analysis was carried out. The per-*O*-methylated LN-3 was hydrolyzed with TFA, reduced with sodium borohydride, and acetylated. The resulting partially *O*-methylated alditol acetates were subjected to GLC-MS.

On the basis of the GLC-MS data, the peaks at *Rt* 11.1, 12.7 and 13.2 were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-

methylglucitol derived from the nonreducing terminal, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol from 3-linked glucosyl residues, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol from 6-linked glucosyl residues, respectively (peak ratio; 1:2:3, Figure II-1). The peak ratios show that LN-3 is a linear hepta- β -D-glucoside with two 1,3- and three 1,6-linkages, in addition to glucosyl-linkage at the reducing end. The peak at *Rt* 19.3 affords a prominent fragment (*m/z* 121), which is assignable to a daughter ion from a PA-sugar derivative.

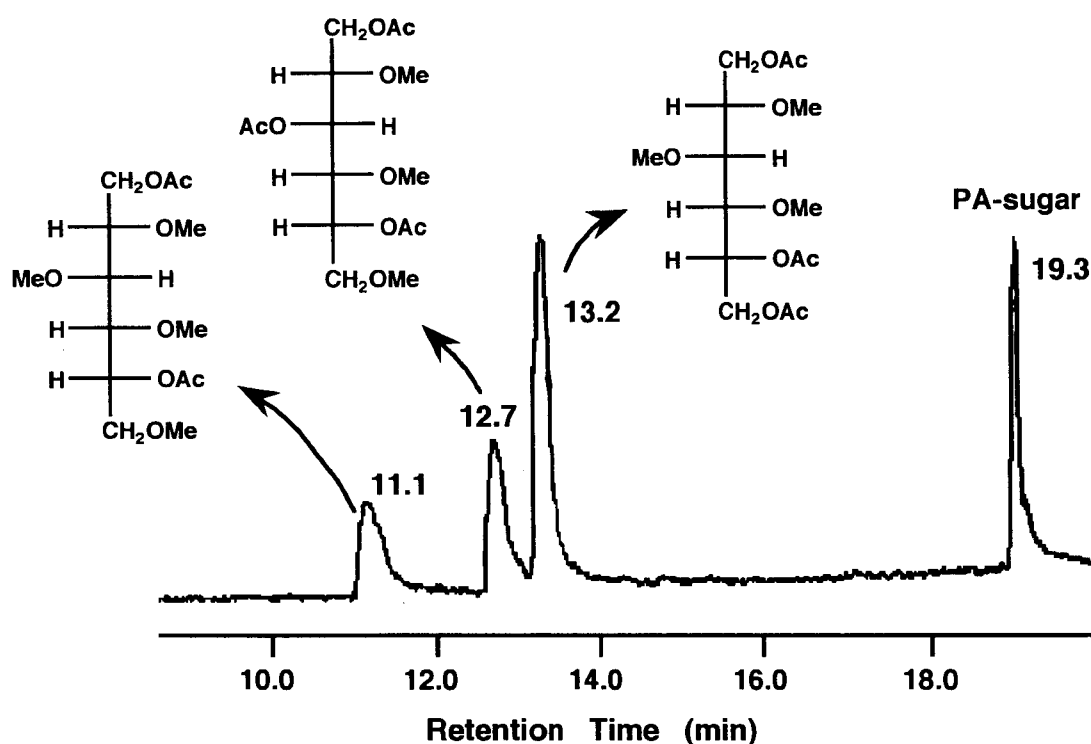


Figure II-1. A total-ion chromatographic trace of partially *O*-methylated alditol acetates from per-*O*-methylated LN-3. On the basis of EI-MS data, the peaks at *Rt* 11.1, 12.7 and 13.2 were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, respectively.

II-3. Analysis of fragments by acetolysis

On acid hydrolysis, 1,6-glycosidic bonds are relatively stable, whereas acetolysis can preferentially cleave 1,6-bonds (Matsuda *et al.*, 1961; Rosenfeld and Ballou, 1974).

A systematic analysis of the structure of LN-3 by acetolysis is summarized in Figure II-2. LN-3 was first acetylated and then acetolyzed. The reaction mixture was divided into two groups (experiments A and B).

In procedure A, the sample was *N,O*-deacetylated with hydrazine. Only PA-linked fragments from the reducing end were detected by fluorescence spectrophotometry. These PA-fragments obtained by acetolysis were identical to those of the authentic PA-Glc and PA-laminaribiose (Figure II-3A). The ratio of PA-Glc to PA-laminaribiose increased with the time of acetolysis (data not shown), indicating that PA-laminaribiose was further degraded to give PA-Glc. Therefore, it is suggested that the reducing end of LN-3 must be a PA-laminaribiose unit.

In procedure B, complete *O*-deacetylation was achieved by sodium methoxide, but the *N*-acetyl group was retained. The reaction mixture was pyridylaminated. The *N*-acetyl-PA-derivatives have no fluorescence. The PA-derivatives from the nonreducing end are readily detected by fluorescence spectrophotometry (Figure II-2). By HPLC analysis, only two fragments, PA-Glc and PA-laminaribiose were detected (Figure II-3B). This analysis as well as the glycosyl-linkage analysis described above suggests that LN-3 possesses a glucose and two laminaribiose units besides a PA-laminaribiose unit at the reducing end.

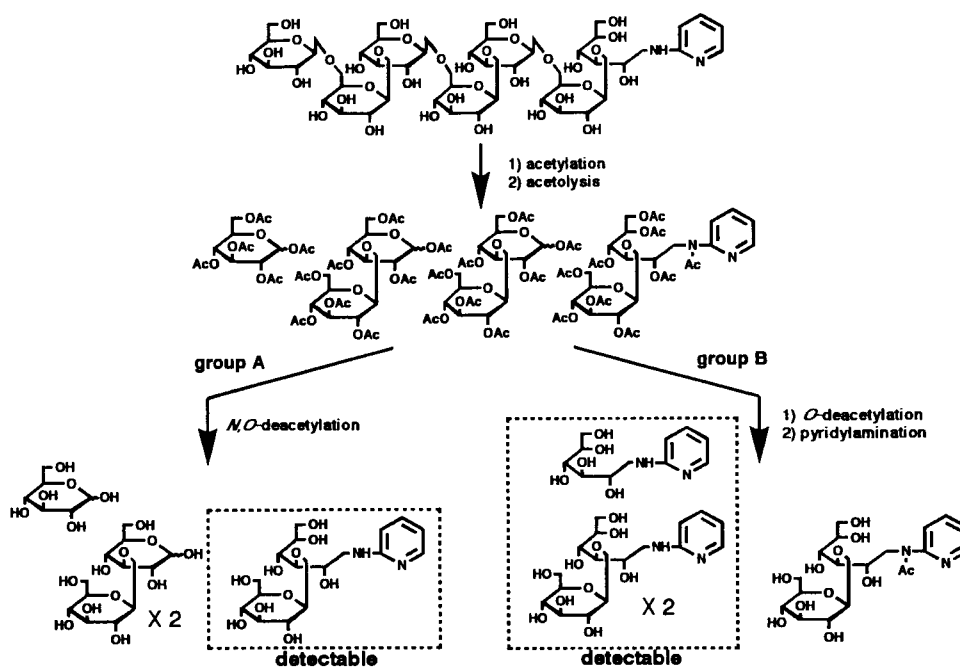


Figure II-2. Principle of the analysis of the structure of LN-3 by acetolysis

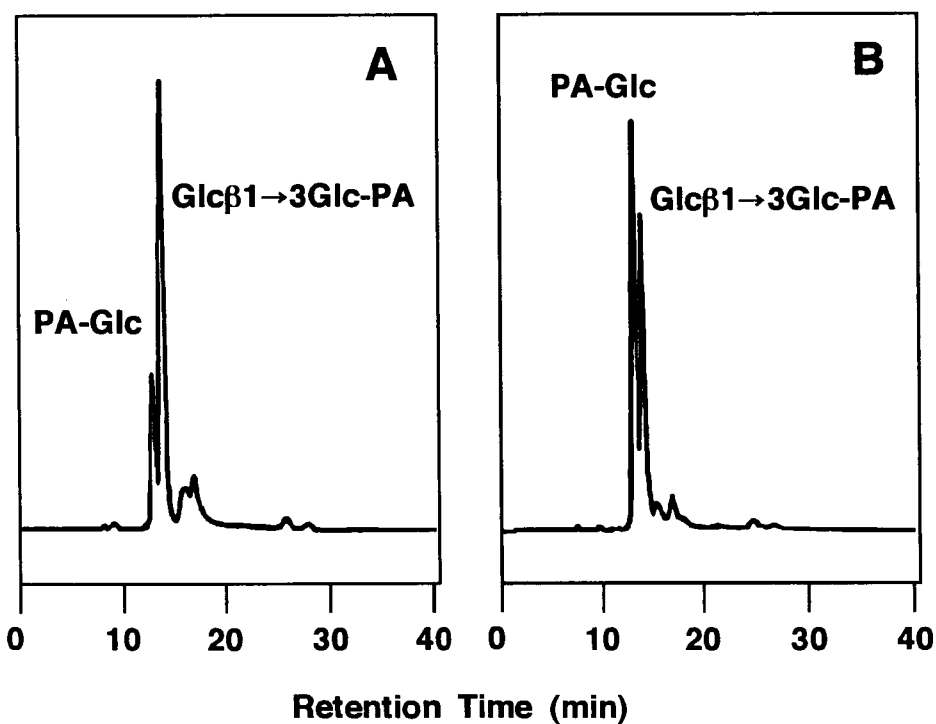


Figure II-3. The reversed-phase HPLC elution profiles of A) *N,O*-deacetylated and B) *O*-deacetylated and pyridylaminated acetolysis product mixtures of peracetylated LN-3.

II-4. Glycosyl-sequence analysis

The glycosyl sequence of the per-*O*-alkylated oligosaccharide-alditols has been analyzed by GLC-MS (Kováčik *et al.*, 1968; Kärkkäinen, 1970; Valent *et al.*, 1980; McNeil *et al.*, 1981; Sharp and Albersheim, 1984). Per-*O*-methylated LN-3 was partially hydrolyzed to give several products, which were reduced and then ethylated. The resulting per-*O*-alkylated oligosaccharide-alditols were subjected to GLC-MS. The total ion chromatogram showed five prominent peaks (Figure II-4). Selected-ion monitoring at m/z 121, 219, and 233 indicated the presence of a characteristic fragment of PA-sugar at R_t 7.6 and per-*O*-alkylated oligosaccharide-alditols at R_t 9.3, 10.1, and 10.5.

In the EI mass spectrum of a PA-per-*O*-alkylated alditol at R_t 7.6, the molecular ion peak at m/z 356 was observed and assigned to fragment [a] (Figure II-5). The cleavages between C-1 and C-2, and between C-3 and C-4 of the PA-per-*O*-alkylated alditol, reported by Hase *et al.* (1978), were clearly detected in EI mass spectra.

A peak, fragment [b] at R_t 9.3 shows characteristic ions at m/z 88, 101, 201, 233, 263, 323, 377, and 409 (Figure II-6). The fragment ion at m/z 233 agrees with an A_1 ion and indicates that the nonreducing glycosyl group bears an *O*-ethyl group. A_1 ions from partially methylated, partially ethylated oligosaccharide-alditols can lose methanol or ethanol to yield A_2 ions. The occurrence of A_2 ion at m/z 201 and H_1 ion at m/z 88 indicates that an ethyl group is substituted at O-6 of the glycosyl residue and not at O-3. The fact that the per-*O*-alkylated alditol residue bears two *O*-ethyl groups is verified by the occurrence of the J_2 ion at m/z 263. The ion at m/z 409 is yielded by cleaving the C-C bonds of the

alditol portion between C-2 and C-3, and C-4 and C-5. There are two ways to give the ion peak at m/z 409. However, the evidence obtained by glycosyl-linkage analysis aforementioned excludes the possibility of the presence of a 1,4-linked residue. Thus the per-*O*-alkylated disaccharide-alditol is 1,3-linked.

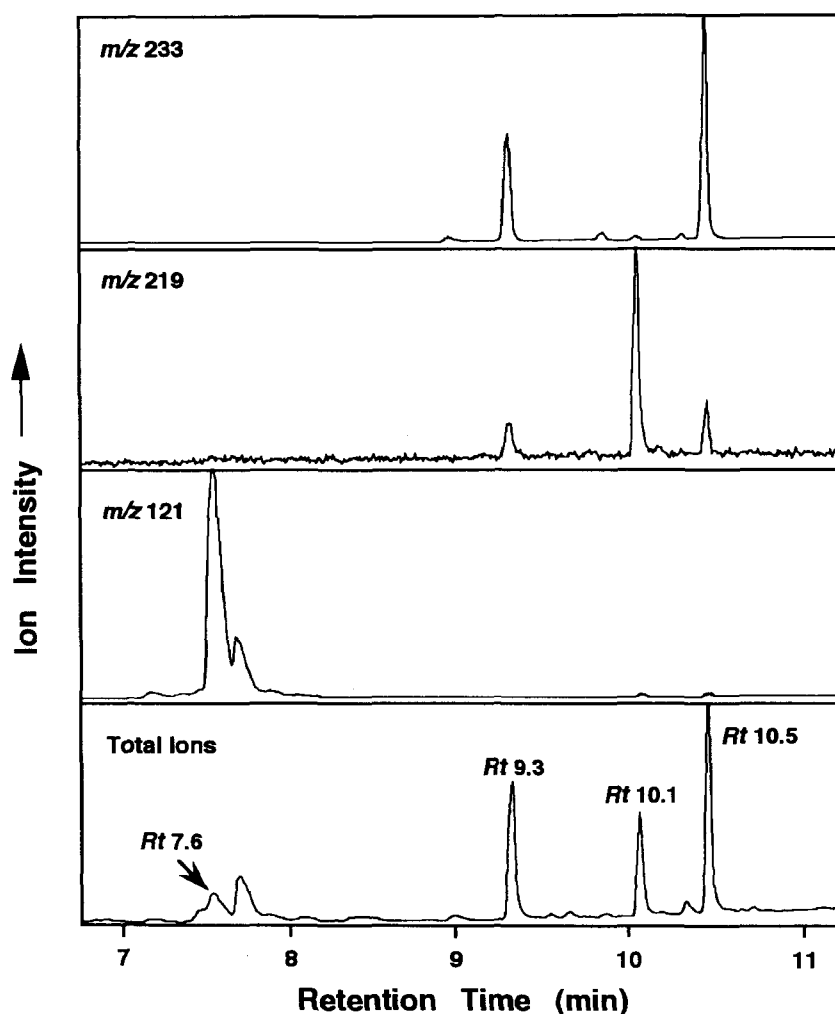


Figure II-4. Total-ion and selected-ion chromatographic traces of per-*O*-alkylated oligosaccharide-alditols from reduced and ethylated hydrolysis products of permethylated LN-3.

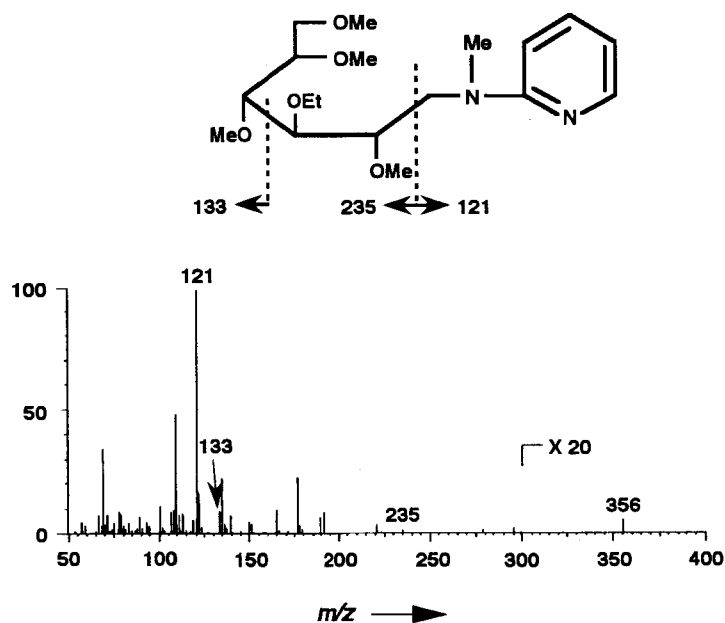


Figure II-5. EI mass spectrum of a PA-per-*O*-alkylated alditol, fragment [a] at *Rt* 7.6.

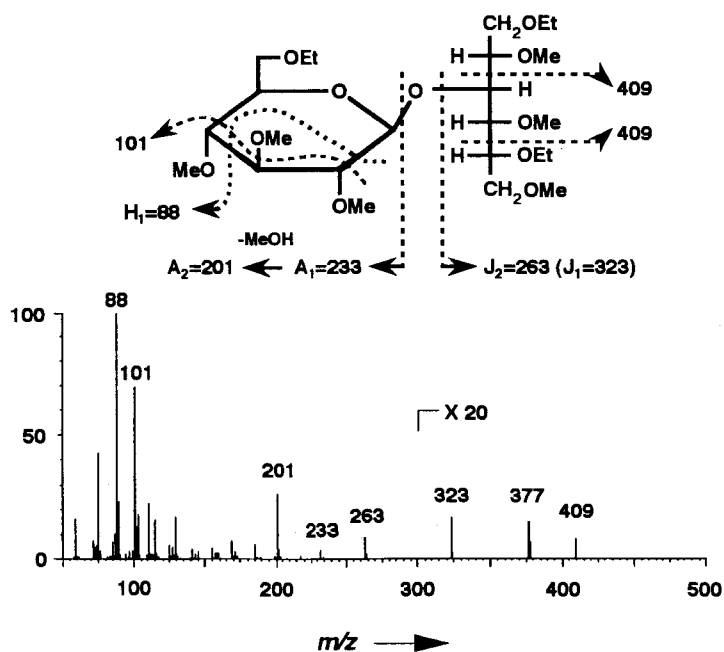


Figure II-6. EI mass spectrum of a per-*O*-alkylated disaccharide-alditol, fragment [b] at *Rt* 9.3.

A peak, fragment [c] at *Rt* 10.1 shows characteristic ions at *m/z* 88, 101, 159, 187, 191, 219, 249, 263, 323 and 351 (Figure II-7). The fragment ion at *m/z* 219 agrees with an A₁ ion, and corresponds to the nonreducing glycosyl terminal of LN-3 due to the absence of ethyl groups in this fragment. The A₂ ion at *m/z* 187 and the H₁ ion at *m/z* 88 are yielded as shown in Figure II-7. The fragment ion at *m/z* 263 is assigned to a J₂ fragment. Ions at *m/z* 191, 249, and 351 are rationally assignable to the fragments from the alditol chain. These ions establish that the per-*O*-alkylated disaccharide-alditol is 1,6-linked.

A peak, fragment [d] at *Rt* 10.5 shows characteristic ions at *m/z* 101, 102, 159, 187, 191, 201, 233, 263, 337 and 351 (Figure II-8). The fragment ion at *m/z* 233 agrees with an A₁ ion, and indicates that the nonreducing glycosyl group bears an *O*-ethyl group. The formation of the predominant A₂ ion at *m/z* 187 from the A₁ ion at *m/z* 233 indicates that O-3 of the glycosyl residue is substituted with an ethyl group. This is further supported by the presence of the abundant H₁ ion at *m/z* 102. The fragment ion at *m/z* 263 is assigned to a J₂ fragment. The presence of an ethyl group on O-3 of the nonreducing glycosyl group is also confirmed by the mass of the J₁ ion (Sharp and Albersheim, 1984) at *m/z* 337. Ions at *m/z* 191, 263 and 365 are also rationally assignable to the fragments from the alditol chain. These ions establish that the per-*O*-alkylated disaccharide-alditol is 1,6-linked.

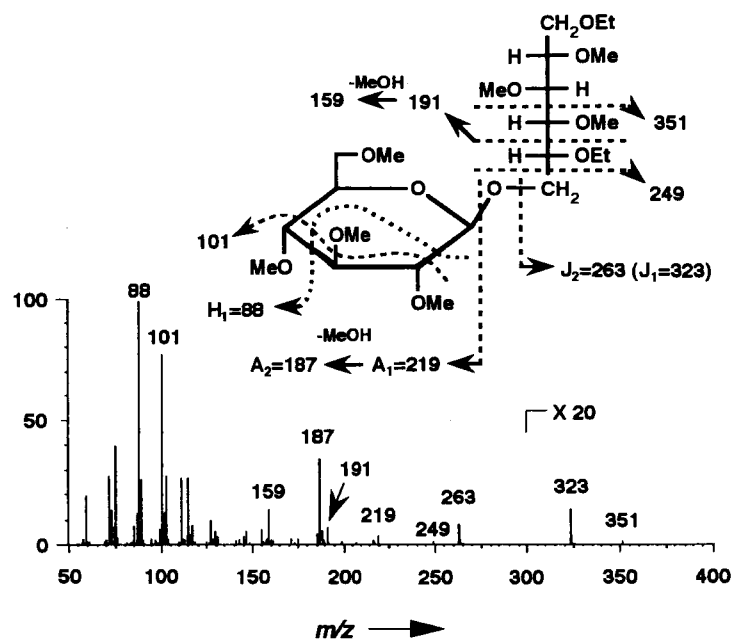


Figure II-7. EI mass spectrum of a per-*O*-alkylated disaccharide-alditol, fragment [c] at *Rt* 10.1.

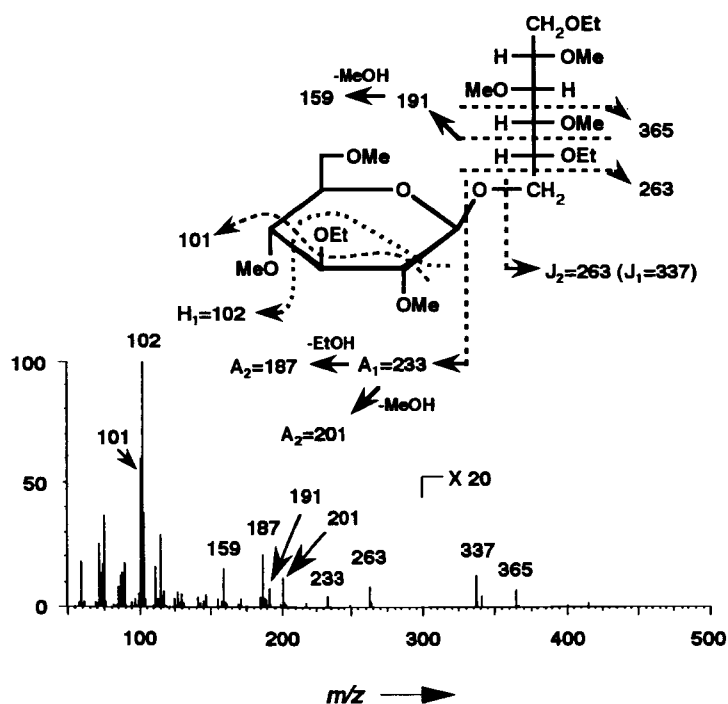


Figure II-8. EI mass spectrum of a per-*O*-alkylated disaccharide-alditol, fragment [d] at *Rt* 10.5.

II-5. The structure of LN-3

The elicitor-active oligosaccharide, LN-3 was a PA- hepta- β -D-glucoside (Kobayashi *et al.*, 1993). Based on the analyses of glycosyl-linkage and fragmentation by acetolysis, LN-3 is a linear pyridylaminated hepta- β -glucoside which consists of a PA-laminaribiose unit at the reducing end, and a glucose and two laminaribiose units with three β -1,6-linkages.

In the glycosyl-sequence analysis, the fragment [c] clearly shows that the glycosyl-linkage at the nonreducing end is 6-linked. This allows the author to depict the structure of LN-3 (Figure II-9). The fragments, [a], [b] and [d] further support this structure.

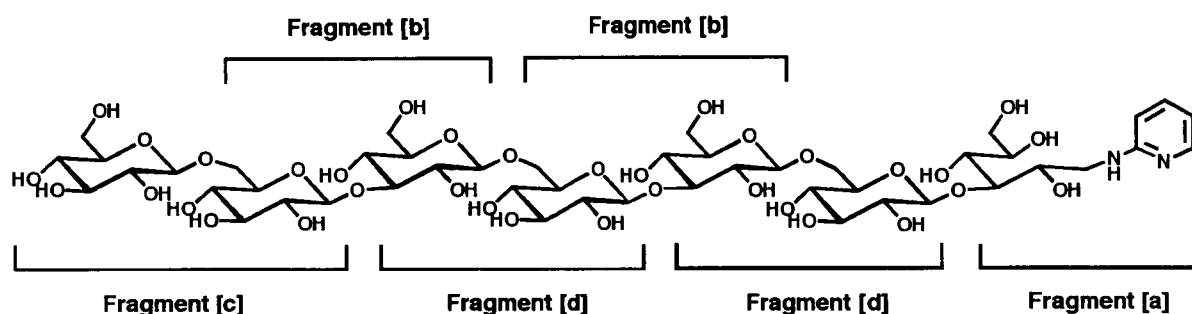


Figure II-9. A primary structure of an elicitor-active oligosaccharide, LN-3.

LN-3 is the first elicitor prepared from a neutral polysaccharide, which occurs widely in nature. Introduction of the pyridylamino group into the elicitor-active oligosaccharides not only sustained the elicitor activity but enhanced it (Kobayashi *et al.*, 1993). The minimum sugar unit required for the exertion of elicitor activity is being examined in our laboratory.

II-6. Experimental

Preparation of elicitor-active oligosaccharide, LN-3 from algal laminaran

Laminaran from *Eisenia bicyclis* (Tokyo Kasei Kogyo Co., Tokyo) was commercially available. Laminaran was hydrolyzed with β -1,3-glucanase. With the guidance of elicitor activity in alfalfa cotyledons, the hydrolysate was fractionated by means of charcoal and gel-filtration column chromatography. The pyridylamino group was introduced to the elicitor-active oligosaccharides in order to facilitate isolation. An elicitor-active oligosaccharide, LN-3 was purified by ODS-HPLC as described (Kobayashi *et al.*, 1993).

Methylation of elicitor-active oligosaccharide, LN-3

Per-*O*-methylation was accomplished by a modified Hakomori procedure (Phillips and Fraser, 1981). LN-3 (3.0 mg) was lyophilized overnight *in vacuo* at -58 °C. The LN-3 was dissolved in dry dimethyl sulfoxide (345 μ l), and 3.6 M potassium dimethylsulfinyl anion (55 μ l) was slowly added. The mixture was stirred for 2 h at room temperature. Methyl iodide (12.3 μ l; equimolar to the potassium dimethylsulfinyl anion) was added at ice-bath temperature, and then the solution was stirred for 1 h at room temperature. The addition of potassium dimethylsulfinyl anion and methyl iodide was repeated twice in the same way, except that the last addition of methyl iodide (100 μ l). The sample was stirred 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 LN-3 (1.9 mg) was purified by preparative TLC (Kieselgel 60 F₂₅₄; MERCK; 7:2:1 v/v benzene : acetone : methanol).

Glycosyl-linkage analysis

The per-*O*-methylated LN-3 (100 µg) was hydrolyzed with 2 M TFA (75 µl) for 1 h at 120 °C. The mixture was dried under a stream of nitrogen. Methanol was added and the solution was 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 NaBH₄/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. A JEOL Automass 20 system equipped with a Hewlett-Packard model 5890 gas chromatograph was used for GLC-MS analysis. A DB-1 (J and W Scientific) capillary column (30 m x 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. The gas-chromatographic effluent was ionized by electron impact at 70 eV.

Analysis of fragments by acetolysis

A procedure analogous to that reported (Hase, 1990) was employed for the fragment analysis. LN-3 (25 µg) was first acetylated with pyridine and acetic anhydride (40 µl each) for 15 min at 100 °C. Toluene was added to the reaction mixture and then

the solution was concentrated to dryness. The acetylated LN-3 was treated with 40 μl of a mixture of acetic anhydride-acetic acid-sulfuric acid (10:10:1; v/v) for 10 h at 37 °C. Then, 8 μl of pyridine was added. The dried residue was dissolved in 800 μl of water, saturated with sodium hydrogen carbonate. The solution was extracted with 1 ml of chloroform (x 3). The extracts were combined and dried over sodium sulfate. The sample was divided into two groups (experiments A and B).

Procedure A was employed to verify the fragments from the reducing end. An aliquot of the sample was placed in a glass tube (ϕ 10 x 100 mm) and 250 μl of anhydrous hydrazine was added. The tube was sealed under vacuum, and heated at 100 °C for 22 h. After *N,O*-deacetylation, hydrazine was removed *in vacuo*. The residue was dissolved in 500 μl of NaHCO_3 -saturated water and the pH was adjusted to 3 by adding a Dowex 50W-X8 (100-200 mesh; The Dow Chemical Co.). The resin was transferred to a minicolumn and then washed with ten bed volumes of water. The sugar fragments were eluted with six bed volumes of 1.4 M aqueous ammonia, and the eluate was concentrated to dryness. The residue was dissolved in 100 μl of water for HPLC analysis.

Procedure B was used to verify the fragments from the nonreducing end. An aliquot of the sample was placed in a 0.3-ml Mini-Vial (GL Sciences Inc.). The sample was dissolved in 50 μl dry 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 (Hase *et al.*, 1981; Suzuki *et al.*, 1991). The reaction mixture was chromatographed on a Dowex 50W-X8 column as described above.

The eluate was further purified on a TSKgel TOYOPEARL HW-40S (TOSOH Co., Ltd.) column (ϕ 1.0 x 30 cm). The flow rate was 0.4 ml/min and 0.1 M 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 100 μ l of water.

The sample (5 μ l each) was subjected to HPLC analysis and identified by co-chromatography with authentic PA-glucose and PA-laminaribiose, prepared from D-glucose and laminaribiose (Seikagakukogyo Co.). HPLC conditions were as follows: HITACHI, L-6200 Intelligent Pump; L-1000 Fluorescence Spectrophotometer (excitation: 320 nm, emission: 400 nm); L-5020 Column Oven (at 30 °C); D-2500 Chromato-Integrate; Inertsil ODS (ϕ 4.6 x 250 mm, 5 μ m; GL Sciences Inc.); flow rate, 0.5 ml/min; eluent, 1% acetic acid in water (v/v).

Glycosyl-sequence analysis

The per-O-methylated LN-3 (600 μ g) was partially hydrolyzed with 90% formic acid (200 μ l) for 1 h at 80 °C. The formic acid was evaporated under a stream of nitrogen. The sample was then reduced with sodium borohydride. The resulting partially O-methylated oligoglucosyl-alditols were desalted on a Sep-Pak C18 cartridge (MILLIPORE). The cartridge was washed with 10 ml of water and eluted with 5 ml of 70% methanol in water. The dried sample was dissolved in dry dimethyl sulfoxide (345 μ l), and 3.6 M potassium dimethylsulfinyl anion (55 μ l) was slowly added. The mixture was stirred for 2 h at room temperature. Ethyl iodide (100 μ l) was added, and the solution was stirred for 4 h at room temperature. The mixture was extracted

with chloroform as described in the methylation procedure. The solution was concentrated and subjected to preparative TLC (Kieselgel 60 F₂₅₄; MERCK; 7:2:1 v/v benzene : acetone : methanol). The per-*O*-alkylated oligosaccharide-alditol fraction was dissolved in acetone. The GLC-MS analysis was performed under the previous condition except for the column temperature. The temperature was programmed to remain at 160 °C for 2 min, and then rise from 160 to 220 °C at 30 °C/min, and finally from 220 to 300 °C at 8 °C/min.

Chapter III

Species-specificity of an elicitor-active oligosaccharide, LN-3, to leguminous plants

III-1. Introduction

Oligosaccharide fragments from cell walls of fungi and plants including chitin, chitosan, β -glucan, and pectic acid elicit a wide variety of defence responses in plants. Preparation and identification of homogenous oligosaccharides from the hydrolysate of chitin, chitosan and pectic acid are simpler than those of heterogeneous oligosaccharides originated from β -glucan, and such simple oligosaccharides have been investigated for elicitor-activity in detail (see General introduction).

The author has developed a convenient method for preparation of a pure elicitor-active oligosaccharide, LN-3, from a naturally occurring polysaccharide (Kobayashi *et al.*, 1993, 1995). The enzymatic hydrolysate of a seaweed polysaccharide laminaran showed a significant elicitor activity to alfalfa cotyledons. Introduction of the pyridylamino (PA) group into an elicitor-active oligosaccharide enhanced activity by several times of the original activity. LN-3 was shown to be a linear pyridylaminated hepta- β -glucoside which consists of a PA-laminaribiose unit at the reducing end, and a glucose and two laminaribiose units with three β -1,6-linkages (Chapter II, Figure II-9). The minimum effective concentration of LN-3 was 650 nM to alfalfa cotyledons.

In this chapter, the author demonstrates phytoalexin-inducing activities of LN-3 to pea and bean together with the fate of LN-3 during the elicitor assays.

III-2. Establishment of elicitor bioassay system with bean cotyledon

An optimum condition for the elicitor assay in bean cotyledon was established. The time course for (\pm)-kievitone induction after treatment of the bean cotyledons with laminaran (500 $\mu\text{g}/\text{ml}$) was taken (Figure III-1). Various elicitor-exposure time points were set up. (\pm)-Kievitone induction was in low level during 24 h and reached a maximum 48 h after the elicitor treatment. Besides (\pm)-kievitone as a major component, several unidentified compounds were induced by the elicitor treatment. Browning at the surface of the cotyledon cross-sections treated with the elicitor started at 24 h and was gradually intensified with the passage of incubation time. Figure III-2 shows a HPLC chromatographic trace of MeOH- H_2O extract of the cotyledons 48 h after the elicitor treatment. Therefore, the 48-h incubation period was adopted for evaluation of elicitor activity.

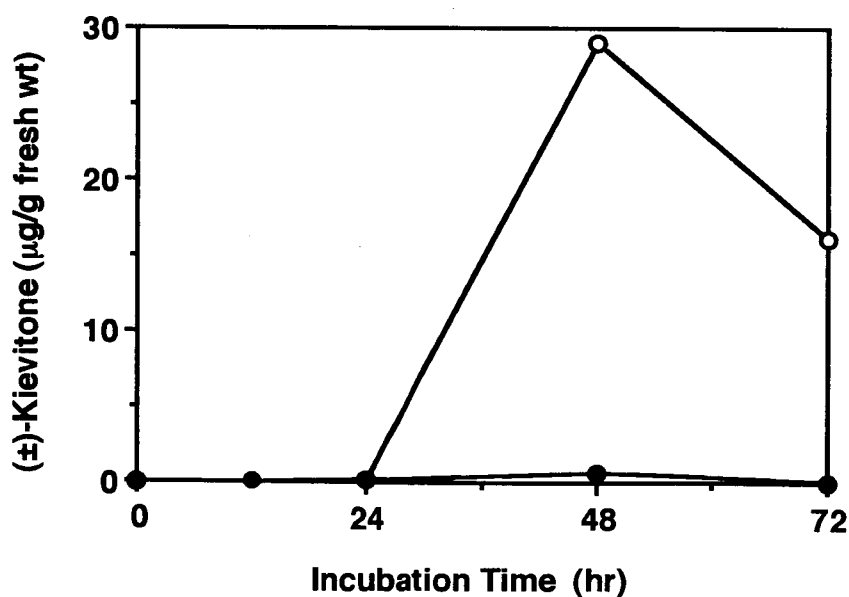


Figure III-1. Time course of the accumulation of phytoalexin (\pm)-kievitone. Laminaran 500 $\mu\text{g}/\text{ml}$ (○), control (●).

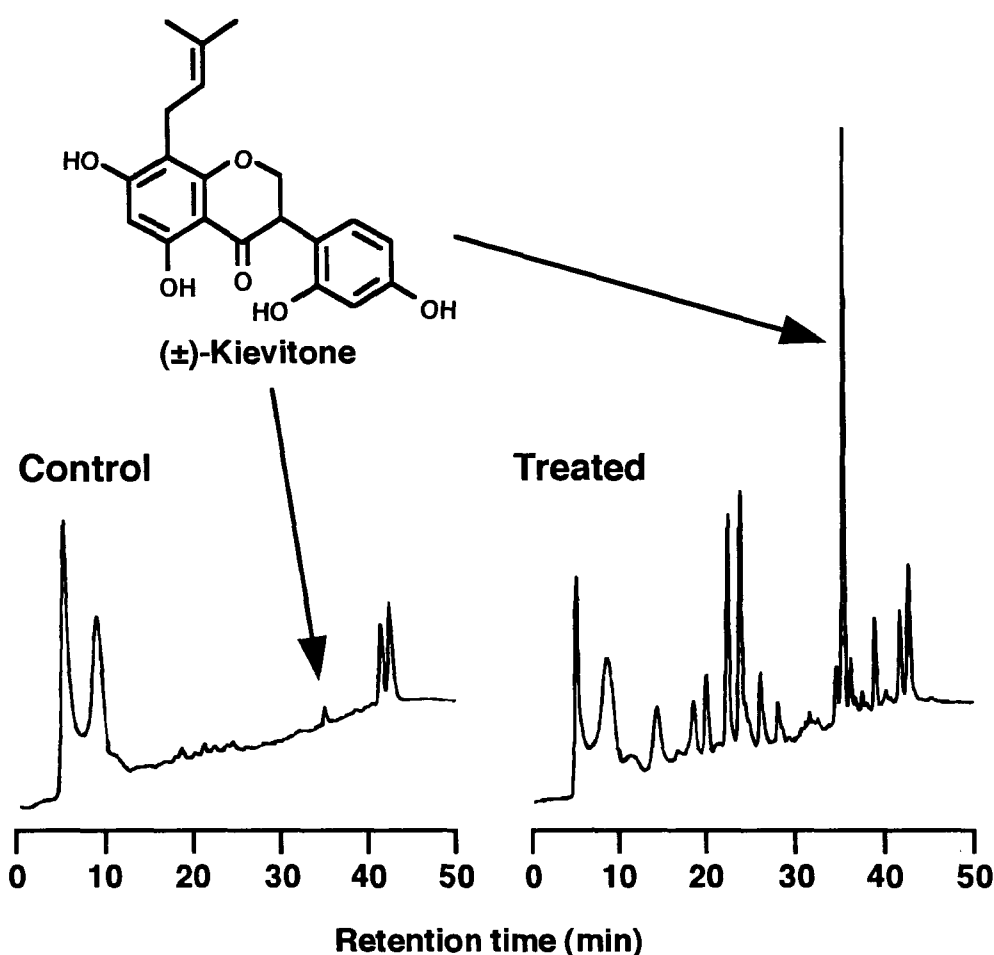


Figure III-2. HPLC profiles of (±)-kievitone and other unidentified compounds accumulated in bean cotyledon treated with laminaran (500 $\mu\text{g/ml}$) for 48 h.

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

LN-3 was tested for the (+)-pisatin-inducing activity in pea epicotyls at the concentrations ranging from 12.5 to 100 $\mu\text{g/ml}$ (Figure III-3A). LN-3 had no significant activity such as (+)-pisatin-inducing activity and no browning at the above concentration range.

LN-3 was also examined for the (±)-kievitone-inducing activity in bean cotyledons at the concentrations ranging from 0.8

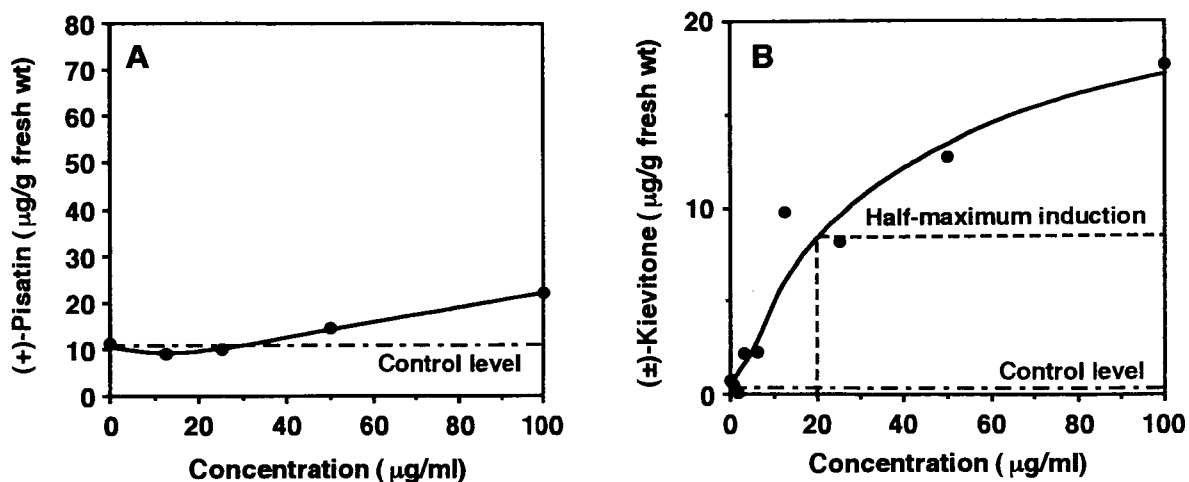


Figure III-3. Phytoalexin-inducing activity of LN-3 in pea and bean.

A: (+)-Pisatin-inducing activity of LN-3 in pea epicotyl assay.

B: (±)-Kievitone-inducing activity of LN-3 in bean cotyledon assay.

to 100 µg/ml (Figure III-3B). The (±)-kievitone content gradually increased with elevating concentration of LN-3 and reached a maximum (ca. 17 µg/g fresh wt) at 100 µg/ml. LN-3 exhibited a half-maximal elicitor activity at approximately 20 µg/ml (16.2 µM) in the bean cotyledon assay (Figure III-3B). The concentration of LN-3 more than 12.5 µg/ml also caused browning at the cotyledon surface.

III-4. Fate of LN-3 after the 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. 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 III-1). Almost 100% of the fluorescent entities from LN-3 was recovered from the elicitor-treated pea experiment. In contrast, alfalfa and bean experiments showed 63.8 and 38.1 recovery %, respectively.

Table III-1. The recovery % 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

Characterization of the fluorescent entities was carried out. The PA-linked fragments were only detectable in the HPLC analysis using a fluorescence spectrophotometer as a detector. The elicitor-treated solutions of alfalfa and bean afforded only a single peak which appeared at *Rt* 6.0 (Figure III-4A, C). In the LC-MS experiments, the single peak at *Rt* 6.0 in alfalfa gave two [M+H]⁺ ions at *m/z* 259 and 421 and in bean gave a [M+H]⁺ ion at *m/z* 259. This result suggested that LN-3, a PA-heptagluco-side, was hydrolyzed to give PA-Glc and PA-digluco-side in alfalfa and to give PA-Glc in bean. In the elicitor-treated solution of pea, ca. 30% of LN-3 remained intact. In addition to LN-3, five peaks were observed (Figure III-4B) and each peak 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 LN-3 was hydrolyzed to afford a variety of PA-sugars with a different degree of polymerization <7.

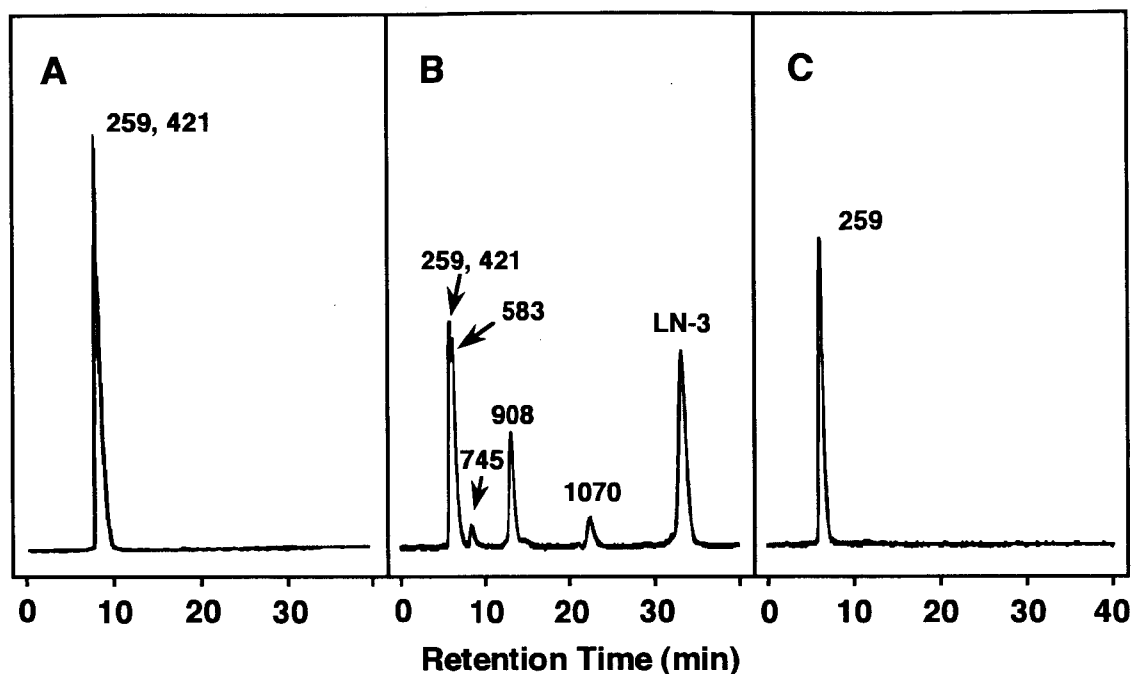


Figure III-4. LC-MS analysis of fragments from the reducing end of LN-3 by plant hydrolytic enzymes.

In the elicitor-treated solutions of alfalfa (A), pea (B) and bean (C), pyridylamino-linked fragments from the reducing end were analyzed by HPLC. Numbers in this figure show $[M+H]^+$ ions in LC-MS analysis.

Furthermore, analyses of the fragments from the nonreducing end of LN-3 were also carried out. The 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 a HPLC experiment, in which the

newly PA-introduced derivatives were detectable by a fluorescence spectrophotometer. In the three plants, a peak corresponding to PA-glucose (*Rt* 6.0) was always observed (Figure III-5). Therefore, this result suggested that a sugar released from the nonreducing end of LN-3 was D-glucose.

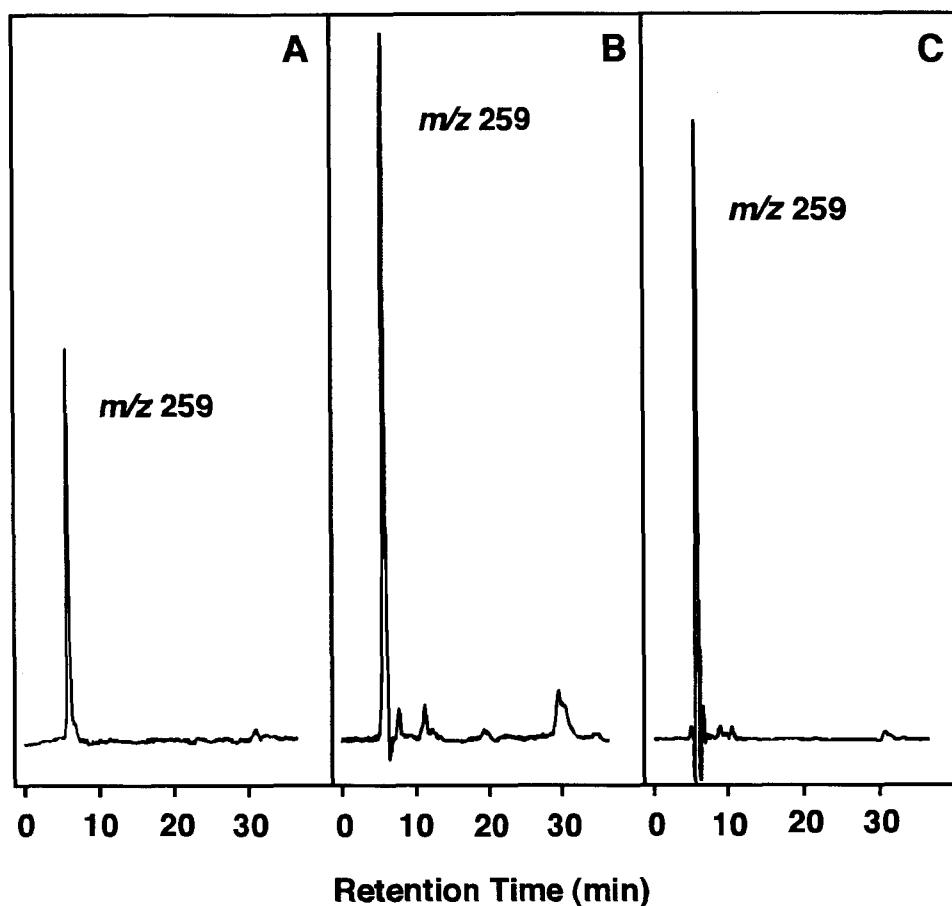


Figure III-5. LC-MS analysis of fragments from the nonreducing end of LN-3 by plant hydrolytic enzymes.

After acetylation, *O*-deacetylation and pyridylation of the elicitor-treated solutions, newly pyridylaminated fragments were analyzed by HPLC. These HPLC profiles were expressed by subtracting chromatogram of control from that of treatment (A: alfalfa, B: pea, C: bean). Numbers in this figure show $[M+H]^+$ ions.

III-5. Discussion

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

The author attempted to obtain a pure elicitor-active oligosaccharide, LN-3, which stimulated phytoalexin induction in alfalfa, and the structure was elucidated by physicochemical means (Kobayashi *et al.*, 1993, 1995). 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 μ g/ml) of LN-3 tested (Figure III-

3A). Hundred $\mu\text{g}/\text{ml}$ of NaNO_2 -degraded chitosan induces ca. 80 $\mu\text{g}/\text{g}$ fresh wt of (+)-pisatin (Kobayashi *et al.*, 1994). The pisatin-inducing activity of LN-3 is lower than that of partially *N*-deacetylated chitin at the entire concentration range (Kobayashi *et al.*, 1994; Akiyama *et al.*, 1995). These results indicate that LN-3 is inactive to pea epicotyls. In bean cotyledons, the (\pm)-kievitone content gradually increased with elevating concentration of LN-3, and reached a maximum (ca. 17 $\mu\text{g}/\text{g}$ fresh wt) at 100 $\mu\text{g}/\text{ml}$ (Figure III-3B). LN-3 also exhibited a half-maximal elicitor activity 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 lindemuthianus*. Therefore, LN-3 exhibits specific activity for alfalfa and bean, not for pea. LN-3 is also a first elicitor, structurally characterized, 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 1991b; 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 may be a favorable process for elicitors to exert the activity. The possession of a large amount of a pure biotic elicitor let the author examine fate of LN-3 in intact tissues. The author analyzed the sugars originated from LN-3 in

the test solutions after LN-3 feeding. After the three legumes were treated with 100 $\mu\text{g}/\text{ml}$ 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 from the pea experiment (Table III-1). These findings suggest that the different recovery % 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 uptake of LN-3 into the cells and also to attachment to the epidermis. These experiments suggest that LN-3 may also bind to the 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, and that in the pea a part of LN-3 was hydrolyzed into a variety of sugars with a different degree of polymerization <7 (Figure III-4 and 5). 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 the 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, the author found that some part of LN-3 was hydrolyzed by constitutive hydrolytic enzymes secreted from pea epicotyl segments. It is suggested that the newly

induced enzymes by LN-3 can hydrolyze the excess of LN-3 molecules remaining in the alfalfa and bean test solution to give mono- and/or diglucoside(s).

In further experiments, the author aims at preparing the massive pure elicitor-active oligosaccharides with high activity and specificity from other sources, and this may help to clarify the elicitor recognition system in plants.

III-6. Materials and Methods

Preparation of an elicitor-active oligosaccharide, LN-3

Laminaran was purchased from Tokyo Kasei Kogyo Co., Ltd.. Laminaran was hydrolyzed with β -1,3-glucanase. With the guidance of elicitor activity in alfalfa cotyledons, the hydrolysate was fractionated by means of charcoal and gel-filtration column chromatography. The pyridylamino group was introduced to 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 (\pm)-kievitone

(\pm)-Kievitone was induced by partially *N*-deacetylated chitin (Kobayashi *et al.*, 1994) treatment of bean cotyledons. Elicitor-treated bean cotyledons of 330 plants were extracted with MeOH. The extract was concentrated to give a thick aqueous solution, and then partitioned with EtOAc. 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.)] column eluted stepwise with solvents of increasing polarity from *n*-hexane through EtOAc. 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 employing gradient elution from H₂O through 70% MeOH-H₂O. The fractions eluted with 60% MeOH-H₂O, 10.48 mg of kievitone was obtained.

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). [α]_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). ¹H NMR (500 MHz, CD₃OD) 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 (Figure III-6). Bean seeds (*Phaseolus vulgaris* L.) obtained from Takii & Company, Ltd. were surface-sterilized with 70% EtOH for 5 min and 5% H₂O₂ for 30 min, and then washed extensively with sterile distilled water. The seeds were transferred onto a germination medium containing 0.1% MgCl₂ and 0.2% GELRITE (San-Ei Gen F.F.I., Inc.) 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 longitudinally cut in half. A half of 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 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 μ l of the methanolic solution was subjected to HPLC analysis using an Inertsil ODS column (ϕ 4.6 x 250 mm, 5 μ m, GL Sciences Inc.) 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/H₂O, solvent B: 1% acetic acid in 90% MeOH/H₂O). The gradient was achieved within 35 min. Absorbance at 285 nm was monitored. Retention time for (\pm)-kievitone under this condition was 35.5 min. For quantification of (\pm)-kievitone, the (\pm)-kievitone content was determined from the peak area of the sample with reference to the calibration of the authentic (\pm)-kievitone.

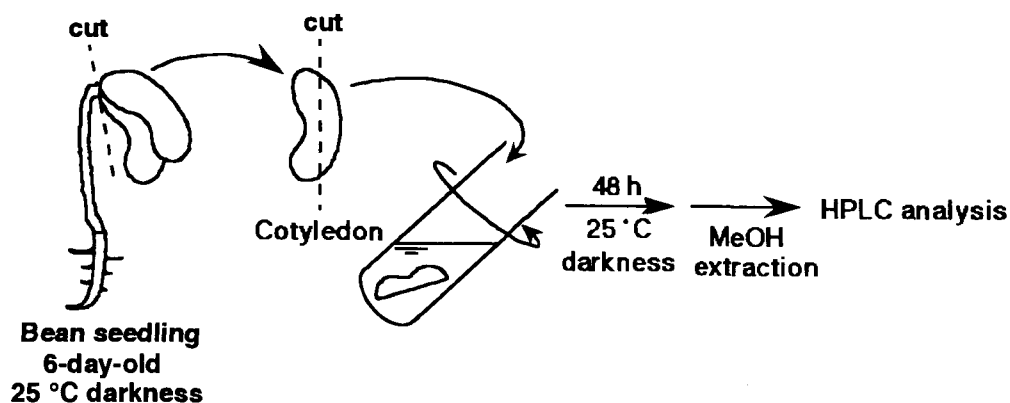


Figure III-6. Bean cotyledon elicitor assay

The analysis of fragments from LN-3 by plant hydrolytic enzymes

Fragment analyses from the reducing end were carried out as follows. 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/H₂O, solvent B: 1% acetic acid in 10% MeOH/H₂O). 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.

The 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.) column (ϕ 1.0 x 27 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 μ l of water. The aqueous solution was subjected to HPLC analysis as mentioned above. Further, the analyses of the fragments from the reducing end and the nonreducing end were performed by LC-MS. LC-MS analyses were taken on a Perkin-Elmer API *III*.

Chapter IV

A minimum essential structure of LN-3 elicitor activity in bean cotyledons

IV-1. Introduction

The author has developed a convenient method for preparation of a pure elicitor-active oligosaccharide, LN-3, from a naturally occurring polysaccharide (Kobayashi *et al.*, 1993, 1995). The enzymatic hydrolysate of an algal laminaran showed a significant elicitor activity to alfalfa cotyledons. Introduction of the pyridylamino (PA) group into an elicitor-active oligosaccharide enhanced the original activity. LN-3 was shown to be a linear pyridylaminated hepta- β -glucoside (Chapter II, Figure II-9). The minimum effective concentration of LN-3 was 650 nM in the alfalfa cotyledon assay.

LN-3 was also examined for phytoalexin-inducing activity in the pea epicotyl and the bean cotyledon assays (Chapter III). LN-3 did not show (+)-pisatin-inducing activity to pea epicotyls. In the bean cotyledon assay, the (\pm)-kievitone content gradually increased with elevating concentration of LN-3, and reached a maximum (ca. 17 $\mu\text{g/g}$ fresh wt) at 100 $\mu\text{g/ml}$. 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 cotyledons. This chapter will demonstrate a minimum essential structure of LN-3 elicitor to bean cotyledons.

IV-2. 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-H₂O 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.

IV-3. 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.

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 (peak ratio; 1:1:1, Figure IV-1).

These results showed that an elicitor-active trisaccharide was a β -1,3- and β -1,6-linked triglucoside (Figure IV-2).

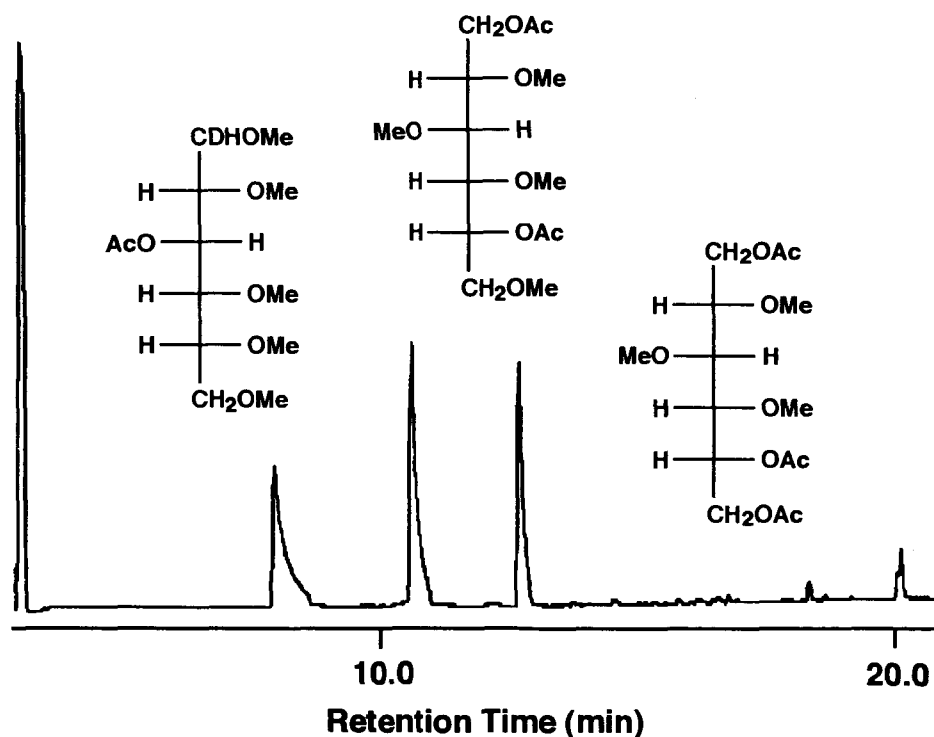


Figure IV-1. A total-ion chromatographic trace of partially *O*-methylated alditol acetates from a per-*O*-methylated trisaccharide. On the basis of EI-MS data, the peaks at *Rt* 8.0, 10.6 and 12.8 were identified as 3-*O*-acetyl-1-deuterio-1,2,4,5,6-penta-*O*-methylglucitol derived from the reducing 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.

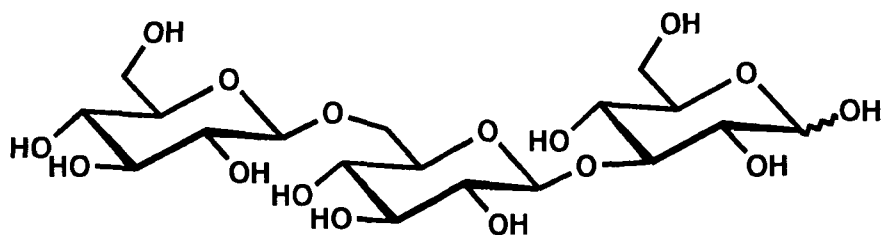


Figure IV-2. The structure of an elicitor-active trisaccharide.

IV-4. 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 triglucoside and LN-3, an elicitor-active pyridylaminated hepta- β -glucoside, were tested for the (\pm)-kievitone-inducing activity in bean cotyledons at the concentrations ranging from 6.3 to 100 $\mu\text{g/ml}$ (Figure IV-3). 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 $\mu\text{g/ml}$ was about 8 $\mu\text{g/g}$ fresh wt, whereas LN-3 induced approximately 17 $\mu\text{g/g}$ 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.

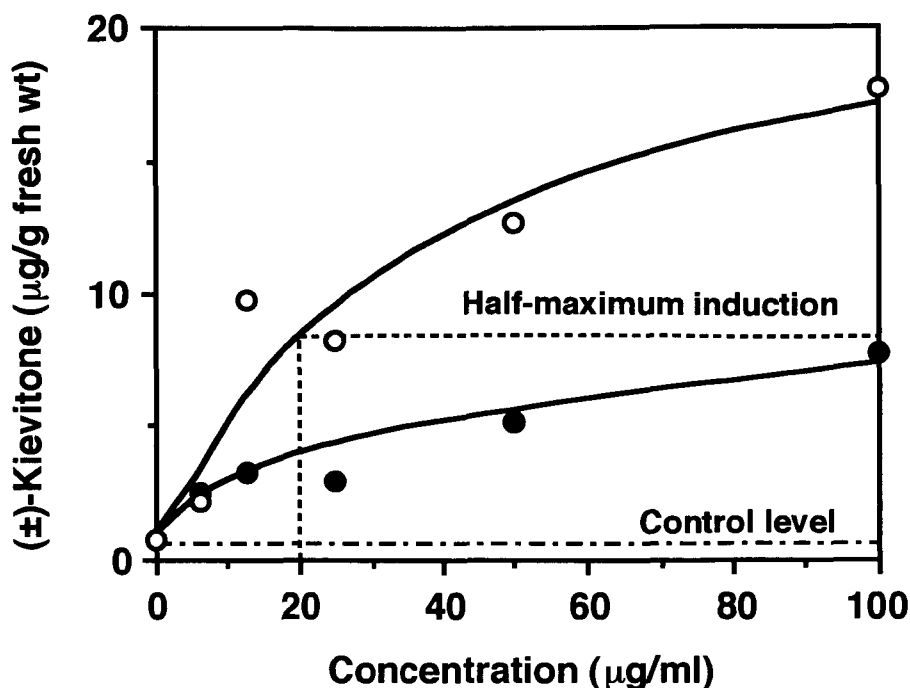


Figure IV-3. The kievitone-inducing activity of a purified triglucoside (●) and LN-3 (○).

IV-5. Discussion

Our previous study on the preparation of elicitor-active oligosaccharides from naturally occurring polysaccharides aimed at the massive preparation of a pure elicitor LN-3 (Kobayashi *et al.*, 1993). The author found that this elicitor was highly active to alfalfa and bean. In this study, the author succeeded in obtaining 1.47 g of an elicitor-active oligosaccharide from 40 g of polysaccharide laminaran. The oligosaccharide was elucidated to be β -1,3- and β -1,6-linked triglucoside by means of physicochemical analyses (Figure IV-2). This unit was found in LN-3 molecule.

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 (Figure IV-3). The induction level of kievitone at 100 μ g/ml

(198 μM) of the triglucoside was less than the half-maximum of LN-3 at 20 $\mu\text{g/ml}$ (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 triglucoside 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 the author 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.

IV-6. Materials and Methods

Isolation of an elicitor-active trisaccharide

By assaying elicitor activity with the bean cotyledon assay, preparation of an elicitor-active oligosaccharide from the laminaran hydrolysate was carried out as follows. Laminaran (40.0 g; Tokyo Kasei Kogyo Co., Ltd.) 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.) 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.; 60-150 mesh) column (ϕ 6.5 x 27 cm) chromatography eluted stepwise with solvents from H₂O through 80% EtOH-H₂O. The 30% EtOH-H₂O eluate (3.6 g) was chromatographed on a TSKgel TOYOPEARL HW-40F (TOSOH Co., Ltd.) column (ϕ 4.0 x 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. MALDI TOF MS spectra were measured on a Finnigan MAT Vision 2000 with 10 mg/ml 2,5-dihydroxybenzoic 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. Analysis of the oligosaccharide by MALDI TOF MS gave a $[M+Na]^+$ ion peak at m/z 527.4.

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. were surface-sterilized with 70% EtOH for 5 min and 5% H₂O₂ for 30 min, and then washed intensively with sterile distilled water. The seeds were transferred onto a germination medium containing 0.1% MgCl₂ and 0.2% GELRITE (San-Ei Gen F.F.I., Inc.) 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 longitudinally cut in half. A half of 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 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 μ l of the methanolic solution was subjected to HPLC analysis using an Inertsil ODS column (ϕ 4.6 x 250 mm, 5 μ m, GL Sciences Inc.) 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/H₂O, solvent B: 1% acetic acid in 90% MeOH/H₂O). The gradient was achieved within 35 min. Absorbance at 285 nm was monitored. Retention time for (\pm)-kievitone under this condition

was 35.5 min. The (\pm)-kievitone content was determined quantitatively by the peak area of the sample with reference to calibration with authentic (\pm)-kievitone.

Reduction of an elicitor-active triglucoside

A triglucoside (20 mg) was deuterio-reduced for 3 h at room temperature with 2 ml of sodium borodeuteride solution (10 mg of NaBD₄/ml). To the reaction mixture acetic acid and methanol were added, and then concentrated to dryness. The reduced trisaccharide (20 mg) was purified on a TSKgel TOYOPEARL HW-40S (TOSOH Co., Ltd.) column (ϕ 1.0 x 27.3 cm). The diglucosyl-glucitol was dissolved in D₂O. ¹H NMR spectra were recorded with a Varian VXR-500 Instrument. Chemical shifts are 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 deuterio-reduced triglucoside

The diglucosyl-glucitol (3.0 mg) was lyophilized overnight *in vacuo* at -58 °C. The sample was dissolved in dry dimethyl sulfoxide (345 μ l), and 3.6 M potassium dimethylsulfinyl anion (55 μ l) was slowly added. The mixture was stirred for 2 h at room temperature. Methyl iodide (12.3 μ l) 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 μ l) and methyl iodide (100 μ l) 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 F₂₅₄; 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 NaBH₄/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) capillary column (30 m x 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.

Conclusion

In this study, the author developed a convenient procedure to obtain pure biotic elicitors from naturally occurring polysaccharides and succeeded in preparation of the pure elicitors from a polysaccharide laminaran. Their phytoalexin-inducing activities were investigated and the following results were obtained.

In chapter I, natural polysaccharides were examined for their activity as elicitors of phytoalexins and flavonoids in alfalfa cotyledons. Only two polysaccharides, laminaran and pectic acid, had elicitor activity. Laminaran, which was more active than pectic acid, was hydrolyzed by tunicase and the hydrolysate was subjected to charcoal and gel filtration columns. Introduction of the pyridylamino group into the elicitor-active oligosaccharides was attempted in order to facilitate isolation. The pyridylamino derivatives were found to exhibit higher activity than the original oligosaccharides. Their LC-MS analysis revealed that the elicitor-active principles form two ion clusters with the same molecular weights, m/z 1070 and 1232. Their HPLC analysis showed three main peaks. The individual peaks (LN-1, 2 and 3) were collected and subjected to the alfalfa cotyledon assay. LN-3 showed the highest activity (minimum effective concentration, 0.8 $\mu\text{g}/\text{ml}$).

In chapter II, the primary structure of an elicitor-active oligosaccharide, LN-3, prepared from partially hydrolyzed algal laminaran was determined by means of the analyses of glycosyl-linkage, fragments by acetolysis, and glycosyl-sequence. The elicitor-active oligosaccharide, LN-3, is a pyridylaminated hepta- β -D-glucoside which was shown to have the following linear structure:

β -D-Glcp(1,6)- β -D-Glcp(1,3)- β -D-Glcp(1,6)- β -D-Glcp(1,3)- β -D-Glcp(1,6)- β -D-Glcp(1,3)-Glc-PA.

In chapter III, LN-3, a linear pyridylaminated hepta- β -glucoside having an elicitor activity in alfalfa cotyledon, was examined for phytoalexin-inducing activity in the pea epicotyl and bean cotyledon assays. LN-3 did not show (+)-pisatin-inducing activity to the pea epicotyls. In the bean cotyledon assay, (\pm)-kievitone content gradually increased with elevating concentration of LN-3, and reached a maximum (ca. 17 μ g/g fresh wt) at 100 μ g/ml. A half-maximal elicitor activity was seen at ca. 16.2 μ M. 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, the recovery % of the solutions of alfalfa and bean were 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, and that in the pea experiment a small portion of LN-3 was hydrolyzed to give sugar fragments with a different degree of polymerization <7.

In chapter IV, a great deal of an elicitor-active oligosaccharide (1.47 g) was obtained from 40 g of polysaccharide 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 triglucoside. 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.

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.

In further experiments, the author aims at preparing the massive pure elicitor-active oligosaccharides with high activity and specificity from other sources, and this may help to clarify the elicitor recognition system in plants.

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List of publications

(concerning this thesis)

1. Kobayashi, A., Tai, A., Kanzaki, H. and Kawazu, K. (1993)
Elicitor-active oligosaccharides from algal laminaran stimulate the production of antifungal compounds in alfalfa.
Z. Naturforsch., **48c**, 575-579.
2. Kobayashi, A., Tai, A. and Kawazu, K. (1995)
Structural elucidation of an elicitor-active oligosaccharide, LN-3, prepared from algal laminaran.
J. Carbohydr. Chem., **14**, 819-832.
3. Tai, A., Kawazu, K. and Kobayashi, A. (submitted)
Species-specificity of an elicitor-active oligosaccharide, LN-3, to leguminous plants.
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4. Tai, A., Ohsawa, E., Kawazu, K. and Kobayashi, A. (in press)
A minimum essential structure of LN-3 elicitor activity in bean cotyledons.
Z. Naturforsch.

(the other publication)

1. Okamoto, G., Fujii, Y., Hirano, K., Tai, A. and Kobayashi, A. (1995)
Pollen tube growth inhibitors from 'Pione' grape pistils.
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