# Elicitor-Active Oligosaccharides from Algal Laminaran Stimulate the Production of Antifungal Compounds in Alfalfa

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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 liquid chromatography mass spectrometry (LC-MS) analysis revealed that the elicitor-active principles form two ion clusters with the same molecular weights, *m/z* 1070 and 1232. Their high performance liquid chromatography (HPLC) analysis showed three main peaks. The individual peaks (LN-1, 2, 3) were collected and subjected to the alfalfa cotyledon assay. LN-3 showed the highest activity (minimum effective concentration, 0.8 μg/ml).

## 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 [1–4]. The structure-activity relationship of chemically synthesized oligo-β-glucosides as elicitors has been determined in soybeans [5].

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 study, we 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.

## Materials and Methods

$^1$H NMR spectra were recorded with a Varian VXR-500 Instrument. Mass spectra were measured with a JEOL JMS-D 300. UV spectra were obtained on a Shimadzu UV -3000 spectrophotometer. Optical rotation was measured with a Jasco DIP-360. LC-MS were taken on a Perkin-Elmer API III.

### *Plant material*

Alfalfa seeds were surface-sterilized with 70% ethanol for 10 min and 8% $H_2O_2$ for 20 min, and then washed extensively 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, Osaka) in a test tube. Plants were grown at 25 °C for 10 days in the light.

### *Chemicals*

Acacia, Tragacanth Gum, Inulin (Nacalai tesque, Kyoto), Curdlan, Pectic acid (Wako Pure Chemical, Osaka), and Laminaran from *Eisenia bicyclis* (Lot. FCY01; Tokyo Kasei Kogyo, Tokyo) 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:

Medicarpin. $^1$H NMR δ (500 MHz, $CDCl_3$): 3.51 (1 H, ddd, $J$ = 4.8, 6.7, 11.1 Hz), 3.60 (1 H, t, $J$ = 11.1 Hz), 3.75 (3 H, s), 4.22 (1 H, dd, $J$ = 4.8, 11.1 Hz), 5.48 (1 H, d, $J$ = 6.7 Hz), 6.39 (1 H, d, $J$ = 2.4 Hz), 6.43 (1 H, d, $J$ = 2.5 Hz), 6.44 (1 H, dd, $J$ = 2.4, 8.8 Hz), 6.53 (1 H, dd, $J$ = 2.5, 8.4 Hz), 7.11 (1 H, d, $J$ = 8.8 Hz),

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7.37 (1 H, d, $J$ = 8.4 Hz); EI-MS *m/z:* 270 ($M^+$), 269 ($M^+$–H), 255 ($M^+$–$CH_3$); UV $\lambda_{max}$ (EtOH) nm (log ε): 283 sh, 287 (3.93); $[\alpha]_D^{21}$ –188.3 (MeOH, $c$ = 0.6).

Sativan. $^1$H NMR δ (500 MHz, $CDCl_3$): 2.84 (1 H, ddd, $J$ = 1.9, 5.3, 15.6 Hz), 2.95 (1 H, dd, $J$ = 10.9, 15.6 Hz), 3.53 (1 H, m), 3.78 (3 H, s), 3.79 (3 H, s), 3.97 (1 H, t, $J$ = 10.1 Hz), 4.27 (1 H, ddd, $J$ = 1.9, 3.4, 10.1 Hz), 4.63 (1 H, s), 6.33 (1 H, d, $J$ = 2.6 Hz), 6.36 (1 H, dd, $J$ = 2.6, 8.1 Hz), 6.44 (1 H, dd, $J$ = 2.5, 8.3 Hz), 6.46 (1 H, d, $J$ = 2.5 Hz), 6.92 (1 H, d, $J$ = 8.1 Hz), 7.00 (1 H, d, $J$ = 8.3 Hz); EI-MS *m/z:* 286 ($M^+$), 164, 152, 151, 149, 121; UV $\lambda_{max}$ (EtOH) nm (log ε): 281 (3.86), 284 (3.86).

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

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

*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 (∅ 18 mm × 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 (∅ 4.6 × 250 mm, 5 µm; GL Sciences, Tokyo) and a flow rate of 0.8 ml/min. A linear gradient of 30% MeOHaq. in 1% AcOH to 90% MeOHaq. in 1% AcOH in 35 min was employed. Four flavonoids were identified. Medicarpin, sativan, known phytoalexins in alfalfa [6, 7], 4′,7-dihydroxyflavanone and 4′,7-dihydroxyflavone were induced by elicitor treatment (Fig. 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.

*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 R 70 (500 mg; Daiwa Kasei, Osaka) 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.

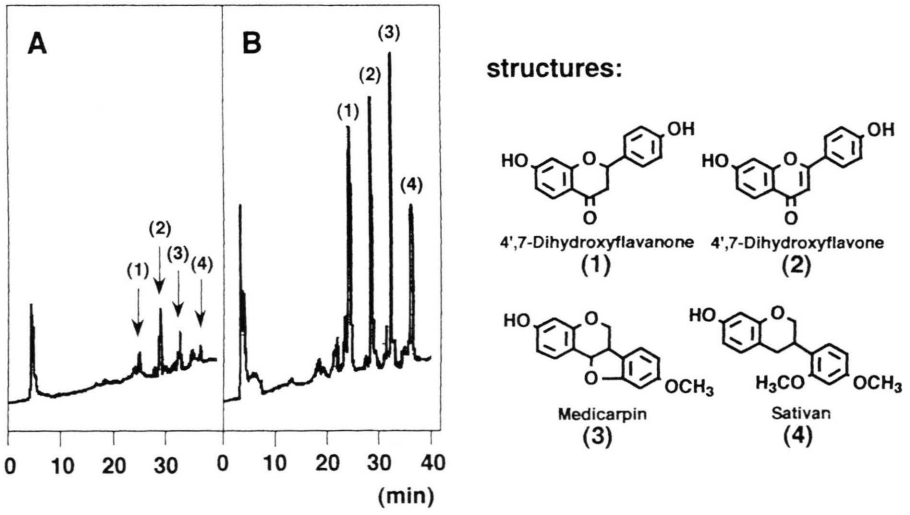


Fig. 1. HPLC profiles of flavonoids accumulated in elicitor-treated alfalfa cotyledons. A, untreated; B, treated (see Materials and Methods).

*Purification of elicitor-active oligosaccharides*

Laminaran hydrolysate (5.5 g) was subjected to charcoal column (∅ 2.0 × 60 cm) chromatography using a linear gradient of $H_2O$ (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 (∅ 1.6 × 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 (∅ 1.6 × 72 cm, 200–400 mesh). Finally, elicitor-active oligosaccharides (151 mg) were obtained.

*Pyridylamination* [8] *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 was a coupling reagent 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 $NaBH_3CN$ 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 AGl-X8 acetate form (200–400 mesh; BIO-RAD) and then eluted with water. The eluate was concentrated and further purified by a TOYO-PEARL HW-40S (TOSOH) column (with 0.1 M $NH_4HCO_3$). Pyridylamino derivatives (140 mg) thus prepared were subjected to LC-MS analysis as well as to the elicitor assay.

*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 (∅ 4.6 × 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 wavelength of 320 nm and an emission wavelength of 380 nm were used.

## Results and Discussion

*Investigations of natural polysaccharides possessing elicitor activity*

Natural polysaccharides (500 μg/ml) such as pullulan, inulin, acacia, tragacanth gum, laminaran, pectic acid [9, 10] 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 (Fig. 2). The elicitor activities of laminaran and pectic acid were evaluated in different concentrations. Laminaran showed a pronounced activity at a concentration of 100 μg/ml and the highest activity was seen between 200 and 400 μg/ml. In contrast, the elicitor activity of pectic acid increased

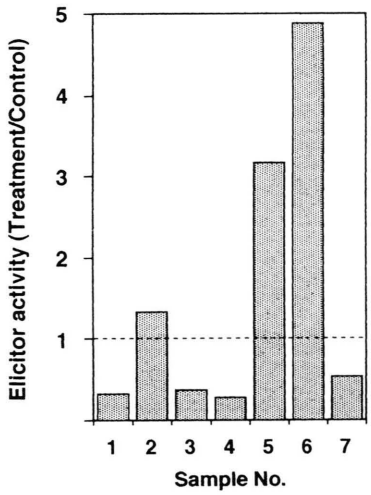


Fig. 2. Elicitor activity of natural polysaccharides at 500 μg/ml. Samples No. **1–7** are pullulan, inulin, acacia, tragacanth gum, laminaran, pectic acid and curdlan, respectively.

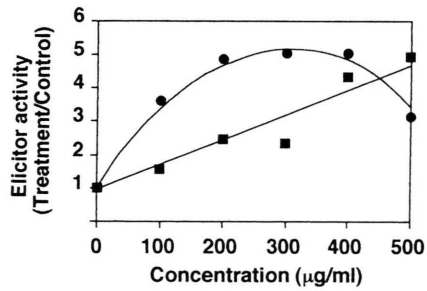


Fig. 3. Comparison of the elicitor activities of laminaran (●) and pectic acid (■) as a function of their concentration.

proportionally to its concentration (Fig. 3). The isolation of an elicitor-active oligosaccharide from the laminaran digest was attempted next.

*Preparation of elicitor-active fragments from laminaran by tunicase*

Among the tested enzymes a β-1,3-glucanase, TUNICASE R 70, 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 (Fig. 4). Therefore, the 2 h enzyme reaction was adopted for preparation of oligosaccharides with elicitor activity.

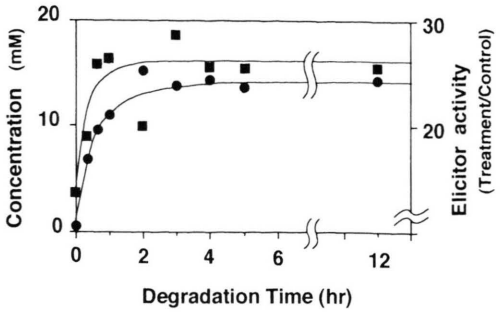


Fig. 4. The amount of carbohydrate (●) and the elicitor activity (■) of laminaran hydrolysate produced by Tunicase treatment. The amount of carbohydrate was determined by a modification of Schales method [11] as glucose equivalent. Elicitor activity of the hydrolysate (500 μg/ml, calculated for sugar) was evaluated without removing buffer and enzyme.

*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 (Fig. 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 (Fig. 6). This also suggested that the degrees of polymerization of the two clusters of the oligosaccharides were 6 and 7. Their

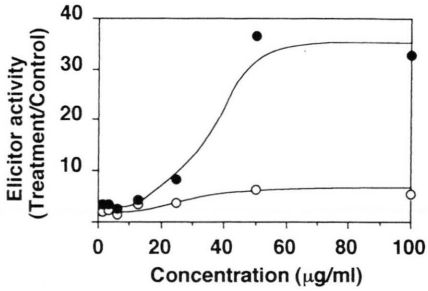


Fig. 5. Comparison of the elicitor activities of pyridylamino derivatives (●) and of the original oligosaccharides (○).

HPLC analysis showed main peaks at *Rt* 18.1 (LN-1), 21.5 (LN-2) and 27.6 min (LN-3) (Fig. 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

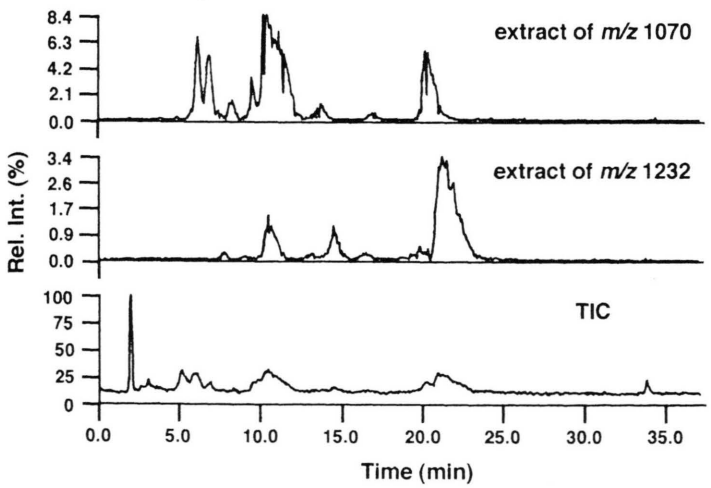


Fig. 6. LC-MS total ion chromatograms of pyridylamino derivatives.

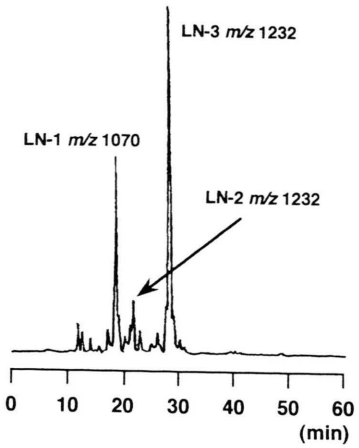


Fig. 7. Separation of pyridylamino derivatives by HPLC (see Materials and Methods).

determined as shown in Table I. LN-3 revealed the highest activity. Laminaran from sea weed is known to have a linear polysaccharide structure with β-1,3 and 1,6 linkages [12, 13]. 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.* [2]. The anomeric proton signals of its $^1$H 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 μg/ml. However, no marked activity was seen in the three components.

Using PA derivatization techniques we 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.

*Acknowledgements*

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

| PA oligosaccharide | Concentration [μg/ml] 50 | 25 | 12.5 | 6.3 | 3.2 | 1.6 | 0.8 | 0.4 |
|---|---|---|---|---|---|---|---|---|
| LN-1 | + | + | – | – | – | – | – | – |
| LN-2 | + | + | + | + | + | – | – | – |
| LN-3 | + | + | + | + | + | + | + | – |

+, active; –, inactive.

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