Elicitor Actions of N-Acetylchitoooligosaccharides and Laminarioligosaccharides for Chitinase and L-Phenylalanine Ammonia-lyase Induction in Rice Suspension Culture

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When a series of oligosaccharides was added into a rice suspension culture, N-acetylchito-hexaose, N-acetylchito-pentaose, and N-acetylchito-tetraose caused an increase in extracellular chitinase activity, mainly due to induction of a class III chitinase. In the case of N-acetylchito-hexaose, a substantial increase in the chitinase activity was observed at a concentration higher than 0.01 µg/ml, and a maximum effect was reached at 1 µg/ml. In contrast, N-acetylchito-triose, N-acetylchito-biose, N-acetyl-d-glucosamine, and chito-hexaose (a chitosan oligosaccharide) were not very effective. Chitinase induction was also observed with laminarihexaose (a β-1,3-glucan oligosaccharide), but about a 10-fold higher concentration, compared with N-acetylchito-hexaose, was needed to get the maximum effect. β-1,3-Glucanase activity was found in cells (but not in medium), and the activity was increased by neither N-acetylchito-hexaose nor laminarihexaose. When cells were incubated with N-acetylchito-hexaose, l-phenylalanine ammonia-lyase (PAL) activity increased promptly. A biphasic profile was obtained when a dose-dependent effect of the elicitor on the PAL induction was examined; the first phase was observed in a range from 0.01 to 1 µg/ml and the second phase from 3 to 300 µg/ml. Laminarihexaose also acted as an elicitor for PAL induction.

Key words: chitinase; l-phenylalanine ammonia-lyase; N-acetylchitoooligosaccharide; laminarioligosaccharide; rice (Oryza sativa L. var. Japonica)

Chitinases [EC 3.2.1.14] and β-1,3-glucanases [EC 3.2.1.39] are widely distributed in higher plants, and are proposed to participate in defense mechanisms against pathogen infection by directly attacking the cell walls of phytopathogenic fungi, in which chitin and β-1,3-glucan are important constituents.¹ ¹ Further, oligosaccharides that are released from the fungal cell walls by the actions of chitinases and β-1,3-glucanases are thought to act as signals (elicitors) for further induction of these enzymes and for activation of other defense mechanisms such as phytoalexin production and lignification.¹ ³ ⁶ High affinity binding sites for chitin oligosaccharides⁴ ⁸ or for β-1,3-glucan oligosaccharides⁵ have been found in certain plant cells.

l-Phenylalanine ammonia-lyase (PAL) [EC 4.3.1.5], which catalyzes the conversion from l-phenylalanine to trans-cinnamic acid, is a key regulatory enzyme of phenylpropanoid metabolism to form a number of potentially protective compounds, such as flavonoids, furanoocumarin and isoflavonoid phytoalexins, and the cell wall structural component lignin, in higher plants.⁹ Thus, PAL activity is increased when plant cells are treated with fungal cell wall elicitors.⁹ ¹¹

In a previous paper,¹² we have reported that rice cells in suspension culture expressed two extracellular class Ib chitinases, which are capable of actively degrading insoluble chitin with high affinities, in a normal state. These enzymes are suggested to participate in early defense mechanisms against phytopathogenic fungi by direct attack on fungal cell wall chitin in rice plants. It has also been shown that an additional extracellular enzyme involved in class III chitinases appears when cells are incubated with N-acetylchitoooligosaccharides (chitin oligosaccharides), which may be released from fungal cell walls by the actions of the two class Ib chitinases. On the basis of catalytic properties, this class III chitinase is thought to function in the acceleration of further hydrolysis of fungal cell wall chitin that has been degraded partially by the two class Ib chitinases (rather than direct attack on fungal cell walls). In this paper, we report elicitor actions of N-acetylchitoooligosaccharides and laminarioligosaccharides (β-1,3-glucan oligosaccharides) for chitinase and PAL induction in rice suspension culture.

Materials and Methods

Materials. N-Acetylchitoooligosaccharides (biose, triose, tetraose, pentose, and hexose), chito-hopexaose, and laminarioligosaccharides (biose, triose, tetraose, pentose, hexose, and heptose) were products of Seikakaku Kogyo Co., Ltd. (Tokyo, Japan). N-Acetylchitosan (a regenerated chitin) was prepared from chitosan, kindly donated from Katoichi Co., Ltd. (Takamatsu, Japan), with acetic anhydride¹³ (degree of N-acetylation was confirmed to be 1.0 by elemental analysis). 6-O-Hydroxyethylchitin (HE-chitin, glycolchitin) was synthesized from the regenerated chitin according to a published method¹⁴ (degree of substitution for 6-O-hydroxyl group was estimated to be 0.8 by elemental analysis), and 6-O-hydroxyethylchitosan (HE-chitosan, glycolchitosan) was prepared by the N-deacetylation of HE-chitin in 20% NaOH at 100°C (degree of N-deacetylation was 0.95 when calculated from elemental analysis). Curdlan was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and laminarin from Nacalai Tesque Inc. (Kyoto, Japan).

Cell culture. Cells, derived from a caryopsis of rice (Oryza sativa L. var. japonica cv. Koshikihari), were cultured in a modified B5 medium with

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Abbreviations: DP, degree of polymerization; HE-chitin, 6-O-hydroxyethylchitin; HE-chitosan, 6-O-hydroxyethylchitosan; PAL, l-phenylalanine ammonia-lyase.
rotary shaking (120 rpm) at 25°C in the dark for 14 days to the late logarithmic phase of growth, and used for experiments.

Enzyme assay. Cells (about 0.5 g) suspended in 20 ml of medium were incubated with an elicitor at 25°C. After incubation for 6 or 3 days, these cells were harvested by filtration, washed three times with 25 mM imidazole/HCl buffer, pH 6.8, containing 1 mM ethylenediamine tetracacetate, and disrupted with a Physcion homogenizer in the same buffer (4 ml) at 4°C. After centrifugation at 20,000 × g for 20 min, the supernatant obtained was used as a crude enzyme solution for measuring the intracellular chitinase and β-1,3-glucanase activities. The filtrate obtained after removal of cells was dialyzed against the imidazole/HCl buffer at 4°C, and used as a crude enzyme solution for assay of the extracellular chitinase and β-1,3-glucanase activities. To measure PAL activity, cells obtained by filtration were homogenized in 4 ml of 100 mM sodium borate buffer, pH 8.7, containing 10 mM 2-mercaptoethanol, at 4°C, and the supernatant obtained by centrifugation (20,000 × g, 20 min) was used as a crude enzyme solution. Protein content in these enzyme solutions was measured according to Lowry et al. using bovine serum albumin as a standard.

Chitinase was assayed using a suspension, containing 20 mg N-acetyltetraxosan, 30 mM Na₂HPO₄/25 mM citric acid buffer, pH 5.5, and crude enzyme, in a total volume of 2 ml, and β-1,3-glucanase using a suspension, containing 20 mg curdlan, 51 mM Na₂HPO₄/24 mM citric acid buffer, pH 5.0, and crude enzyme, in a total volume of 2 ml. These enzyme reactions were done with shaking (150 strokes/min) at 37°C for 60 min, and stopped by adding 1 ml of 67 mg/ml Na₂WO₄ in 0.33 mM H₂SO₄. After centrifugation (1500 × g, 5 min) to remove the insoluble substrates, reducing sugars in the supernatant were measured by a ferri-ferro-cyanide method using N-acetylglucosamine in the chitinase assay and trehalose in the β-1,3-glucanase assay as standards to measure these enzyme activities. The PAL reaction was done at 30°C using a reaction mixture containing 6.7 mM L-phenylalanine, 33 mM sodium borate buffer, pH 8.7, and crude enzyme, in a total volume of 3 ml. The activity was measured by following the increase in absorbance at 290 nm owing to formation of trans-cinnamic acid. One unit (U) is defined as the amount of enzyme that produces 1 µmol of reducing sugars per 1 min in chitinase and β-1,3-glucanase or converts 1 µmol of L-phenylalanine to trans-cinnamic acid per 1 min in PAL.

Results

Chitinase induction by N-acetyltetraxosan and laminariligosaccharides in rice suspension culture

When intracellular and extracellular chitinase activity were examined with N-acetyltetraxosan as a substrate in rice suspension culture, most of the chitinase activity was found in medium (Table). When N-acetyltetraxosan (a chitin oligosaccharide) was added to the culture at 10 µg/ml and incubated for 3 days, an increase in the extracellular chitinase activity occurred after a lag time (6 h), and after 3 days the activity reached 3-fold over the control (without the oligosaccharide). The intracellular chitinase activity also increased, but the ratio of intracellular to extracellular chitinase activities was barely changed during the incubation. These results confirm our previous report in which a mixture of chitin oligosaccharides (composed of tetraose, pentaose, and hexaose) was used. CH-heptin (a water-soluble chitin derivative) and laminarihexaoe (a β-1,3-glucan oligosaccharide) also acted as potent elicitors to induce an increase in the extracellular chitinase activity; however, chitohexaoe (a chitosan oligosaccharide), HE-chitosan (a water-soluble chitosan derivative), and laminar-in (a water-soluble β-1,3-glucan) were not very effective (Table).

When the amount of N-acetyltetraxosan added to cell suspension was varied, a substantial increase in the extracellular chitinase activity was observed at concentrations higher than 0.01 µg/ml, and a maximum effect was reached at 1 µg/ml (Fig. 1). When laminarihexaoe was used, about 10-fold higher concentration, compared with N-acetyltetraxosan, was needed to reach the maximum effect. Elicitor activity to induce an increase in extracellular chitinase activity was examined in N-acetyltetraxosan oligosaccharides with different degrees of polymerization (DP). As shown in Fig. 2, N-acetyltetraxosan, as well as N-acetyltetraxosan, showed a potent elicitor activity. N-Acetyltetraxosan also caused a substantial increase in the activity, but the extent was obviously less than that with N-acetyltetraxosan. In contrast, N-acetyltetraxosan, N-acetyltetraxosan, and N-acetyltetraxosan were barely active. Similar size-dependency was shown when elicitor activity was examined with laminariligosaccharides with different DP.

| Table | Intracellular and Extracellular Chitinase Activities in Rice Suspension Culture after Incubation with Chitin- and β-1,3-Glucan-related Compounds
<table>
<thead>
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<tr>
<td></td>
<td>Chitinase activity (mU/ml culture)</td>
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<tr>
<td></td>
<td>Intracellular</td>
</tr>
<tr>
<td>Before incubation</td>
<td>6.7 ± 1.21</td>
</tr>
<tr>
<td>Incubated with</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>8.3 ± 0.82</td>
</tr>
<tr>
<td>N-Acetyltetraxosan (10 µg/ml)</td>
<td>23.3 ± 3.85</td>
</tr>
<tr>
<td>HE-Chitin (100 µg/ml)</td>
<td>—</td>
</tr>
<tr>
<td>Chitohexaoe (10 µg/ml)</td>
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<tr>
<td>HE-Chitosan (100 µg/ml)</td>
<td>—</td>
</tr>
<tr>
<td>Laminarihexaoe (100 µg/ml)</td>
<td>20.3 ± 2.13</td>
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<tr>
<td>Laminarin (100 µg/ml)</td>
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a Rice cells in suspension culture were incubated with a chitin- or β-1,3-glucan-related compound at a concentration cited in parenthesis for 3 days, and intracellular and extracellular chitinase activities were measured as described in Materials and Methods (n = 4; ± SE).
b — not measured.

Fig. 1. Dose-dependent Effect of N-Acetyltetraxosan or Laminarihexaoe on the Level of Extracellular Chitinase Activity.

Rice cells were incubated with N-acetyltetraxosan (○) or laminarihexaoe (●) at a concentration cited (0-100 µg/ml) for 3 days, and extracellular chitinase activity was measured as described in Materials and Methods. Chitinase activity without elicitors was 57 ± 7.2 mU/ml medium. Data are presented as the mean of four experiments (±SE).
Elicitor Actions of Chitin and β-1,3-Glucan Oligomers

Fig. 2. Increase in Extracellular Chitinase Activity Induced by N-Acetylglucosaminidase or Laminarioligosaccharides with Different DP.

Rice cells were incubated with N-acetylglucosaminidase (○) or laminarioligosaccharides (△) with different DP at 100 μg/ml for 3 days, and extracellular chitinase activity was measured as described in Materials and Methods. Data are presented as the mean of four experiments (±SE). The result obtained without elicitors is plotted at the position of DP of "0" for convenience.

Fig. 3. Change in PAL Activity after Incubation with N-Acetylglucosaminidase.

Rice cells were incubated with N-acetylglucosaminidase at 100 μg/ml, and PAL activity in these cells was followed for 24 h as described in Materials and Methods. Data are presented as the mean of four measurements (±SE).

β-1,3-Glucanase activity in rice suspension culture

Intracellular and extracellular β-1,3-glucanase activities were measured with curdland as a substrate in rice suspension culture. In contrast to chitinase activity, β-1,3-glucanase activity was found in cells (42 ± 0.7 mU/mg protein), but was little detected in medium (data not shown). The intracellular β-1,3-glucanase activity was barely increased even when cells were incubated with N-acetylglucosaminidase, HE-chitin, chitohexaose, HE-chitosan, laminarihexaose or laminarin for 3 days. In addition, the incubation did not result in an appearance of β-1,3-glucanase activity in the medium.

PAL induction by N-acetylglucosaminidase and laminarioligosaccharides

When rice cells in suspension culture were incubated with N-acetylglucosaminidase at 100 μg/ml, a prompt increase in PAL activity in these cells occurred with a short lag time (about 1 h) (Fig. 3). The activity reached a maximum level 6 h after the incubation, and then decreased gradually. Cells were incubated with N-acetylglucosaminidase at various concentrations for 6 h, and PAL activity in these cells was examined. As shown in Fig. 4, a biphasic increase in the PAL activity was observed by increasing the elicitor concentration in a range examined (0 to 300 μg/ml); the first phase increase occurred in a range from 0.01 to 1 μg/ml and the second phase increase from 3 to 300 μg/ml. The level of the activity at 1 μg/ml was about half of that at 300 μg/ml.

Laminarihexaose also acted as an elicitor to induce an increase in PAL activity in rice cells (Fig. 4). When the concentration of the oligosaccharide was varied, the PAL activity increased in response to the increase in the concentration showing a monophasic profile in a range examined (0 to 300 μg/ml), and the profile was similar to a dose-dependent curve observed when chitinase activity was examined (see Fig. 1). The level of the PAL activity in cells treated with laminarihexaose at 300 μg/ml was about half of that with N-acetylglucosaminidase at the same concentration.

Discussion

We have reported that rice cells in suspension culture express two extracellular class Ib chitinases in a normal state, and proposed that in this plant these enzymes participate in early defense mechanisms to prevent pathogen infection by directly attacking the cell walls of phytopathogenic fungi. It has also been shown that N-acetylglucosaminidase...
saccharides, which may be released from fungal cell walls by the actions of these class Ib chitinases, induce an increase in extracellular chitinase activity, mainly due to induction of an additional enzyme involved in a family of class III chitinases.

Data obtained in this paper (Figs. 1 and 2) suggest that the induction of the class III chitinase by N-acetylchitoooligosaccharides is mediated through a specific receptor with a high affinity on the surface of rice cells, and this receptor requires 4 or more N-acetyl-d-glucosamine units for recognition. Further, the N-acetyl groups in the oligosaccharides are indicated to be important for binding, because chitohexaose was barely effective for the chitinase induction (Table). Shibuya et al. found a high affinity binding site for N-acetylchitoooligosaccharides in the membrane fraction of rice cells, and proposed that the binding site was a putative receptor to induce the formation of diterpene phytoalexins in response to the oligosaccharides. Since dose- and size-dependencies observed in the chitinase induction by N-acetylchitoooligosaccharides (Figs. 1 and 2) are similar to those reported in the induction of the phytoalexin formation, it is reasonable to postulate that the high affinity binding site also functions as a receptor for the chitinase induction. HE-chitin, a water-soluble chitin derivative which is used as a substrate for the two class Ib chitinases, induced an increase in extracellular chitinase activity (Table). It is thought that the chitin derivative was once degraded by the action of these class Ib chitinases, and produced oligosaccharides acted as elicitors to induce the class III chitinase through the receptor for N-acetylchitoooligosaccharides.

A prompt increase in PAL activity was observed when rice cells were incubated with N-acetylchitohexaose (Fig. 3), supporting a previous report showing that the transcriptional level of a gene encoding PAL increased after treatment with a fungal cell wall elicitor. The induction of PAL may result in lignification in rice cells. When the concentration of N-acetylchitohexaose was increased, a biphasic increase in the PAL activity was observed in response to an increase in the elicitor concentration (Fig. 4). Since it has been reported that rice cells have multiple genes encoding PAL, it is thought that different kinds of PAL isozymes are induced in response to N-acetylchitoooligosaccharides by two distinct signaling mechanisms showing different dose-dependencies. This notion is supported by previous reports that multiple PAL isozymes are found in one species of plants and their expression levels are differentially regulated during plant development and by environmental cues.

Laminarioligosaccharides acted as elicitors for inducing increases in extracellular chitinase activity (Table, and Figs. 1 and 2) and PAL activity (Figs. 3 and 4). The increase in the chitinase activity was mainly due to induction of the class III chitinase (data not shown), as observed with chitoooligosaccharides. These results suggest that a receptor specific for laminarioligosaccharides with DP higher than 4 occurs on the surface of rice cells. In other plants, it has been proposed that β-1,3-glucan oligomers are released from fungal cell walls by β-1,3-glucanases located in the intercellular space, and recognized by a specific receptor to activate plant defense mechanisms.