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Identification of a novel high-affinity binding site for *N*-acetylchitooligosaccharide elicitor in the membrane fraction from suspension-cultured rice cells

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Binding experiments using a ^{125}I-labeled tyramine conjugate of *N*-acetylchitooctaose, a highly potent elicitor for the induction of phytoalexin production in rice cells, and a microsomal membrane preparation from suspension-cultured rice cells showed the presence of a novel high-affinity binding site for this oligosaccharide. The binding of the ligand was saturable and the Scatchard plot analysis of the results indicated the presence of a single class of binding site with a K_d of 5.4 nM which is comparable with that reported for the binding of the hepta-β-glucoside elicitor in soybean membrane. The ligand binding was inhibited by unlabeled *N*-acetylchitoheptaose but not by its deacetylated form. These characteristics of this binding site coincide well with the specificity and sensitivity for the elicitor in several assay systems, suggesting the possible involvement of this binding site in the recognition of the elicitor in vivo.

N-Acetylchitooligosaccharide; Elicitor; Host–pathogen interaction; Receptor; Rice suspension culture

1. INTRODUCTION

Higher plants initiate various defense reactions when they are attacked by pathogens such as fungi, bacteria and viruses. These defense responses include the production of phytoalexins, proteinase inhibitors, hydrolases such as chitinase and β-glucanase, wall glycoproteins and liginification [1–3]. These responses can be triggered by oligo-/polysaccharide elicitors derived from the cell surface of pathogenic microbes as well as host plants. Some of these elicitors could trigger these responses in plants at a very low concentration and their recognition by host plants seems to be very strictly related to their structure [1]. These results strongly indicate the presence of specific recognition systems in the host cells.

So far, the detailed information on such recognition systems, especially on putative receptors, is only available for soybean-pathogen interaction where the fragment of β-1,3-, 1,6-glucan from *Phytophthora megasperma* [4,5] and α-1,4-linked oligogalacturonides of a certain size can serve as active elicitors [6,7]. Several papers reported the presence of a specific, high-affinity binding site for the former elicitor [8–13], though none of them has succeeded in purifying the binding protein.

To our knowledge, no information is available on the receptor(s) which recognizes elicitors derived from different classes of cell surface carbohydrates. Chitin is a major constituent of the cell wall of most fungi and its fragments have been shown to induce defense responses in several plants [14–16], suggesting the presence of another class of receptors in plants. We recently showed that a very low concentration (as low as 10^{-9} M) of *N*-acetylchitooligosaccharides of a specific size could induce phytoalexin production [17] as well as a rapid and transient membrane depolarization [18] in suspension-cultured rice cells. These observations suggested the presence of a recognition system in the rice cells which could be completely different from the soybean system. We here report the presence of a new class of high-affinity binding site for oligosaccharide elicitor in rice cells which specifically recognizes *N*-acetylchitooligosaccharides.

2. MATERIALS AND METHODS

2.1. *Plant materials*

Suspension-cultured cells of *Oryza sativa* L. cv. Nipponbare were obtained from Dr. Yoshiaki Ohtsuki of National Agriculture Research Center, Tsukuba, Japan and maintained using a modified N-6 medium as described previously [18].

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Abbreviations: PBS, phosphate-buffered saline; FAB-MS, fast-atom-bombardment mass spectrometry; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPLC, high-performance liquid chromatography.

2.2. *Chemicals*

Preparations of chitoheptaose and chitooctaose were kindly supplied by Yaizu Suisankagaku Industry Co., Ltd. (Shizuoka, Japan) and re-N-acetylated in our laboratory. Purity of these *N*-acetylchitooligosaccharides was shown to be 80–85% by reverse-phase HPLC on an Inertsil ODS column (4 × 250 mm, GL Sciences Inc., Tokyo, Japan) with a linear gradient elution of 0–10% methanol. The $Na^{125}I$ solution was purchased from Amersham Japan (Tokyo, Japan).

2.3. *General analytical procedures*

Protein was determined by the method of Bradford [19]. Hexosamine was determined by a modified method of Elson and Morgan [20]. FAB-MS spectra were taken with a Finnigan MAT mass spectrometer, model MAT 90.

2.4. *Preparation of the tyramine conjugate of* N*-acetylchitooctaose and its radioiodination*

The coupling of *N*-acetylchitooctaose, $(GlcNAc)_8$, with tyramine was conducted by reductive amination using modified conditions of Wang et al. [21]. Briefly, 1 mg of $(GlcNAc)_8$ and 20 mg of tyramine-HCl was dissolved in 50 mM PBS and the pH of the solution was adjusted to 7.2 with NaOH. Sodium cyanoborohydride (10 mg/ml) was added to this solution and incubated at 37°C for 2 days then at 80°C for 1 h. After degassing and centrifugation, the reaction mixture was applied to a Bio-Gel P-2 gel-filtration column (0.9 × 60 cm) equilibrated with 0.1 M NH_4HCO_3 and eluted with the same solution to remove excess reagent. The elution was monitored by UV absorption at 274 nm and those fractions containing the product were combined and lyophilized. The tyramine conjugate, $(GlcNAc)_8$-Tyr, was further purified by HPLC using an Inertsil PREP-ODS column (20 × 250 mm, GL Sciences Inc., Tokyo, Japan) which was eluted with 25% methanol. The $(GlcNAc)_8$-Tyr was rechromatographed on the same column to obtain the final preparation. UV absorption spectrum and Elson-Morgan colorimetric assay indicated the presence of both tyramine and hexosamine units in this preparation. FAB-MS analysis showed the presence of a major peak corresponding to the parent ion (m/z = 1765). The concentration of the $(GlcNAc)_8$-Tyr solution was determined from UV absorption at 274 nm using the molar extinction coefficient, 1420 $M^{-1}\cdot cm^{-1}$ [5].

The tyramine conjugate was radioiodinated using iodogen as the oxidizing agent [11,22]. Typically, 1 nmol of $(GlcNAc)_8$-Tyr solution (5–25 ml) and 0.5 M sodium phosphate buffer (pH7.5, 5 ml) were put into a test tube that was precoated with iodogen. $Na^{125}I$ (18.5 MBq) was added to this solution and allowed to react for 1 h at room temperature in the sealed tube. Five ml of 0.1 M NaI solution was added to the reaction mixture and allowed to react for 5 min. The reaction mixture was diluted with a small amount of distilled water and applied to a PolyPrep AG1-X8 column (Bio-Rad Laboratories, Tokyo, Japan) [11] and eluted with distilled water. Radioactive fractions were combined and used for the binding assay. Specific activity of the labeled ligand could be controlled by changing the ratio of ^{125}I and the tyramine conjugate as well as the reaction time.

2.5. *Preparation of the microsomal membrane fraction*

The cells were harvested by filtration, washed and then suspended in the homogenization buffer (25 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$, 2 mM DTT, 2 mM EGTA and 1 mM phenylmethanesulfonyl fluoride). They were homogenized with a mortar and pestle and then with a glass homogenizer for 20 strokes. The homogenate was centrifuged at 1,500 × g for 10 min, and the supernatant was further centrifuged at 10,000 × g for 10 min. The resulting supernatant was centrifuged at 100,000 × g for 20 min. The pellet was resuspended in buffer A containing 20% (v/v) glycerol, and stored in liquid nitrogen.

2.6. *Binding experiments*

An aliquot of the labeled ligand (5–10 pmol) was mixed with the microsomal membrane preparation (200 mg protein) and adjusted to a total volume of 400 ml with cold 25 mM Tris-HCl buffer (pH 7.0) containing 1 M NaCl, 1 mM $MgCl_2$ and 2 mM DTT. For inhibition analysis, an appropriate amount of unlabeled ligand was added to this reaction mixture. After incubation for 1 h in an ice-cold bath, a 300 ml portion was taken from the reaction mixture and directly applied on to a glass fiber filter (GC50, ADVANTEC TOYO KAISHA Ltd., Tokyo, Japan) kept on a vacuum filtration apparatus. The glass fiber filter was then rinsed with 10 ml of cold rinsing buffer in which NaCl was omitted from the binding buffer. The radioactivity retained on the glass fiber filter was directly analyzed using a gamma counter. A reaction mixture without the membrane preparation was processed similarly and the radioactivity on the filter from this 'blank' experiment was subtracted from those containing the membrane preparation.

3. RESULTS

N-Acetylchitooctaose, a potent elicitor for phytoalexin formation in suspension-cultured rice cells, was conjugated with tyramine by reductive amination according to modified conditions of Wang et al. [21]. The tyramine derivative was then purified by successive gel filtration and reversed phase HPLC (Fig. 1). The structure of the purified product, designated as $(GlcNAc)_8$-Tyr, was confirmed by FAB-MS, UV-spectrum and colorimetric assay for hexosamine. The $(GlcNAc)_8$-Tyr, which contains intact *N*-acetylchitoheptaose structure, showed the elicitor activity between those of *N*-acetylchitoheptaose and *N*-acetylchitooctaose, inducing the phytoalexin formation at the level of 10^{-9} M (Table I). The $(GlcNAc)_8$-Tyr was then radioiodinated using iodogen as the oxidizing agent to get a labeled ligand for binding studies. Specific activity of the labeled ligand

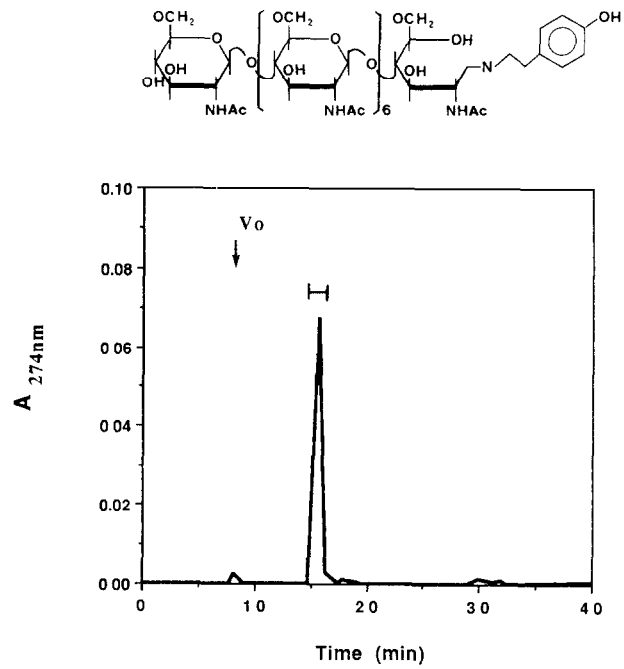

Fig. 1. Purification of the tyramine conjugate of *N*-acetylchitooctaose by HPLC. The reaction product was first separated by gel filtration on Bio-Gel P-2 and then applied to a reverse-phase HPLC column. The bar indicates the fractions collected for further characterization. The structure of the tyramine conjugate is also illustrated.

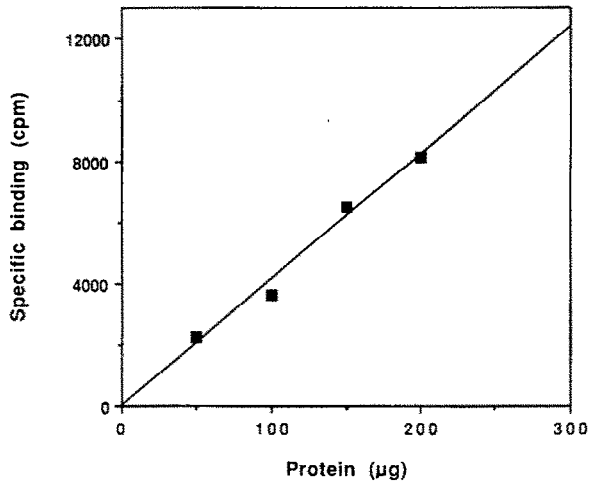

Fig. 2. Relationship between the amount of specifically bound ligand and membrane protein. Varying amounts of the microsomal membrane preparation were reacted with 10 pmol of [^{125}I](GlcNAc)$_8$-Tyr. The difference between the bound ligand in the presence or the absence of 25 mM unlabeled (GlcNAc)$_7$ was taken as specific binding.

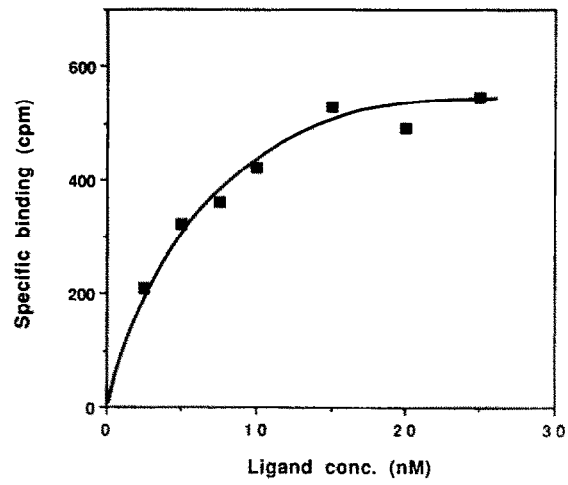

Fig. 3. Saturability of the binding of [^{125}I](GlcNAc)$_8$-Tyr to the microsomal membrane. Varying amounts of [^{125}I](GlcNAc)8-Tyr were reacted with 200 mg of microsomal membrane preparation. Specific binding was calculated as described in Fig. 2.

varied depending on the reaction conditions but typically it was around 100 mCi/mmol (3.7×10^9 Bq/mmol).

Binding experiments were conducted using a filtration method [9,11]. The labeled ligand was reacted with the microsomal membrane preparation obtained from suspension-cultured rice cells at ice-cold temperature. The membrane fraction was recovered on a glass fiber filter after the reaction, and the amount of bound ligand was determined from the radioactivity associated with the filter. The binding experiments were conducted either in the absence or the presence of competitive sugar, 25 mM *N*-acetylchitoheptaose, to see the level of total binding or non-specific binding, respectively. Specific binding of the labeled ligand to the membrane preparation was defined as the difference between the total and non-specific binding. Under these conditions, there was a linear relationship between the amount of specifically bound ligand and the amount of membrane protein within a certain protein concentration range (Fig. 2). Saturability of the binding site with the labeled ligand was examined by changing the ligand concentration against a constant membrane protein. The results obtained from these experiments clearly demonstrated the saturable nature of the binding (Fig. 3). The level of non-specific binding was less than 20% of total binding in most cases. Scatchard plot analysis of these binding data (Fig. 4) indicated the presence of a single class of binding site with a K_d of 5.4 nM. The concentration of such a binding site in the microsomal membrane preparation was also estimated to be 0.3 pmol/mg protein.

Fig. 5 shows the inhibition curve of the ligand binding by unlabeled *N*-acetylchitoheptaose. The binding of the

Table I

Elicitor activity of (GlcNAc)$_8$-Tyr[a]

Substance	(ng/ml)	Momilactone A formation (mg/ml)[b]
(GlcNAc)$_8$-Tyr	(5)	122
	(20)	157
(GlcNAc)$_8$	(5)	125
	(20)	175
(GlcNAc)$_7$	(5)	91
	(20)	103
Control (without elicitor)		15

[a] Elicitation of phytoalexin formation in suspension-cultured rice cells was analyzed as described in a previous paper [17].
[b] Average of triplicate analyses.

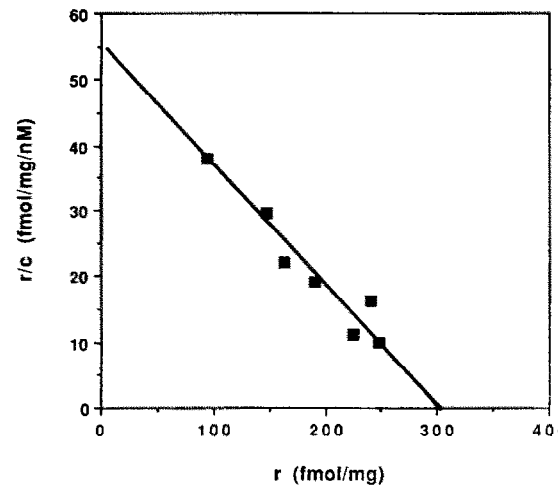

Fig. 4. Scatchard plot of the binding data. r = amount of bound ligand; c = concentration of free ligand.

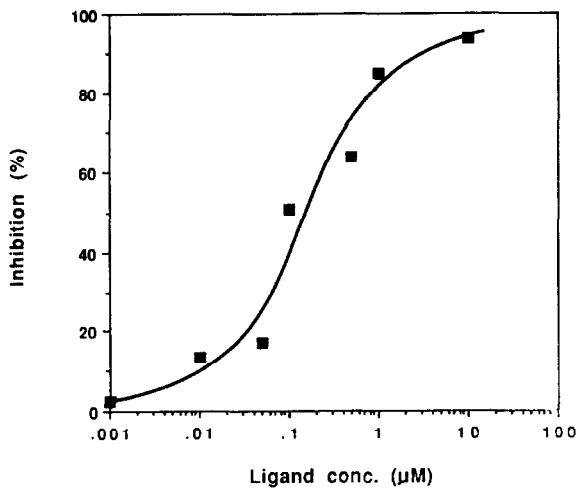

Fig. 5. Inhibition of the binding of [^{125}I](GlcNAc)$_8$-Tyr by unlabeled (GlcNAc)$_7$. Five pmol of [^{125}I](GlcNAc)$_8$-Tyr was reacted with 200 mg of microsomal membrane preparation in the presence of varying amounts of unlabeled (GlcNAc)$_7$.

labeled ligand was almost completely inhibited by the *N*-acetylchitoheptaose at a concentration range above 10 mM. On the contrary, chitoheptaose, the deacetylated form of the same size, did not show significant inhibition even at 25 mM. Protease treatment of the membrane preparation prior to the binding experiment completely abolished the binding of the labeled ligand, suggesting the proteinaceous nature of the binding site (data not shown).

4. DISCUSSION

The present study clearly demonstrates the presence of a new class of high-affinity binding site which is specific to *N*-acetylchitooligosaccharides in the microsomal membrane preparation of suspension-cultured rice cells. The ligand used for radiolabeling, *N*-acetylchitooctaose, is a potent elicitor which could induce the production of phytoalexins in the rice cells at very low concentrations such as 10^{-9} M [17]. Derivatization of the reducing end of this oligosaccharide yielded a product in which the *N*-acetylchitoheptaose unit remained intact and still maintained very high elicitor activity. This ligand could bind to the microsomal membrane preparation from suspension-cultured rice cells with a very high affinity and also in a saturable manner. The K_d value, 5.4 nM, is comparable with those reported for the hepta-β-glucoside in soybean system [10,11] and seems to be reasonable considering that the nM level of *N*-acetylchitoheptaose could induce the formation of phytoalexins [17] as well as transient membrane depolarization [18] in suspension-cultured rice cells. The binding of the labeled ligand was inhibited by the unlabeled *N*-acetylchitoheptaose but not by the deacetylated form, indicating that the specificity of the binding strongly correlates to the structure of the oligosaccharide moiety. These characteristics of this binding site coincide well with the specificity and sensitivity for this elicitor in several assay systems and indicate the possible involvement of this binding site in the recognition of the elicitor in vivo, though more information is necessary to confirm this.

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