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A Specific, High-Affinity Binding Site for the Hepta-β-glucoside Elicitor Exists in Soybean Membranes

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The presence of a specific binding site for a hepta-β-glucoside elicitor of phytoalexin accumulation has been demonstrated in soybean microsomal membranes. A tyramine conjugate of the elicitor-active hepta-β-glucoside was prepared and radiolabeled with ^{125}I. The labeled hepta-β-glucoside-tyramine conjugate was used as a ligand in binding assays with a total membrane fraction prepared from soybean roots. Binding of the radiolabeled hepta-β-glucoside elicitor was saturable, reversible, and with an affinity (apparent $K_d = 7.5 \times 10^{-10}$ M) comparable with the concentration of hepta-β-glucoside required for biological activity. A single class of hepta-β-glucoside binding sites was found. The binding site was inactivated by proteolysis and by heat treatment, suggesting that the binding site is a protein or glycoprotein. Competitive inhibition of binding of the radiolabeled hepta-β-glucoside elicitor by a number of structurally related oligoglucosides demonstrated a direct correlation between the binding affinities and the elicitor activities of these oligoglucosides. Thus, the hepta-β-glucoside-binding protein fulfills criteria expected of a bona fide receptor for the elicitor-active oligosaccharin.

INTRODUCTION

Little is known about the molecular mechanisms by which plant cells perceive and transmit extracellular signals. This stands in contrast to the understanding of signal transduction in animal cells. A number of animal receptors have been purified and the genes encoding many of these receptors have been isolated and sequenced (for examples, see Hahn, 1989). Animal receptors can be grouped into families based on common functional domains. Examples of such receptor families include steroid hormone receptors and G-protein coupled receptors. Steroid hormone receptors are hormone-dependent transcription factors with conserved DNA binding domains (Evans, 1988). G-protein coupled receptors transmit their signal to the cell interior by way of the action of guanine nucleotide-binding proteins (Stryer and Bourne, 1986; Gilman, 1987; Birnbaumer et al., 1990) and appear to have seven conserved membrane-spanning domains (Lefkowitz and Caron, 1988). The purification and characterization of animal receptors have been key steps in elucidating the role of the receptors in signal transduction pathways in animals.

The induced biosynthesis of phytoalexins, an important plant defense response, constitutes a good model system for the study of signal transduction mechanisms in plants (Dixon, 1986; Lamb et al., 1989). The biosynthetic pathways of phenylpropanoid phytoalexins of several legumes have been elucidated (Ebel, 1986; Templeton and Lamb, 1988; Grisebach et al., 1989; Hahlbrock and Scheel, 1989). The enzymes involved have been identified, a number of the enzymes purified, and some genes encoding those enzymes have been cloned. This has allowed the analysis of promoter regions (*cis*-acting elements) and the search for *trans*-acting factors (Lamb et al., 1989). In addition, the structures of molecules (elicitors) that induce phytoalexin synthesis, in particular certain oligosaccharides derived from fungal and plant cell wall polysaccharides, have been determined. The smallest elicitor-active β-glucan fragment isolated from fungal cell walls is a branched hepta-β-glucoside (Sharp et al., 1984a, 1984b, 1984c). This hepta-β-glucoside was purified from a complex mixture of oligosaccharides released by partial acid hydrolysis from the mycelial walls of the soybean pathogen *Phytophthora megasperma* f sp *glycinea*. Structure-activity studies using a number of oligoglucosides structurally related to the elicitor-active hepta-β-glucoside have identified the structural requirements for effective induction of phytoalexin accumulation in soybean (Cheong et al., 1991). Because information is available about both ends of the phytoalexin elicitation signal transduction pathway (i.e., signals and genes), this is an attractive system for elucidating the intermediate steps in the signal cascade, that is, perception of the inducer molecule (elicitor) by the plant cell and transmittal of the signal to the cell nucleus. We demonstrate here that a binding site for a hepta-β-glucoside elicitor exists in soybean microsomal membranes and that

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this binding site has properties characteristic of a physiologically effective receptor.

RESULTS

Preparation of Radiolabeled Elicitor

Previous work had shown that the presence of an iodophenoxyl group conjugated to the reducing end of the elicitor-active hepta-β-glucoside did not alter significantly the elicitor activity of the oligosaccharide (Cheong et al., 1991). Thus, radio-iodination of the phenoxyl ring of a tyramine conjugate of the hepta-β-glucoside elicitor, using Iodogen (Fraker and Speck, 1978) as the oxidizing agent, was chosen as the method for the preparation of the radiolabeled elicitor ligand, whose structure is shown in Figure 1. A molar ratio of ^{125}I to tyramine conjugate of 1:10 typically yielded radio-iodinated oligosaccharides with specific radioactivities of ~100 Ci/mmol. Under these conditions, ~70% of the ^{125}I was incorporated into the hepta-β-glucoside-tyramine conjugate. The specific radioactivity of the radiolabeled elicitor could be adjusted by varying the ratio of iodine to elicitor.

Binding of Radiolabeled Hepta-β-glucoside Elicitor to Membranes from Different Soybean Tissues

The presence of hepta-β-glucoside elicitor binding sites in total membrane fractions isolated from different parts of soybean seedlings (root, hypocotyl, cotyledon, and leaf) was detected by incubating increasing amounts of each membrane preparation with radio-iodinated hepta-β-glucoside-tyramine conjugate. The data in Figure 2 show that each membrane preparation had some ability to bind the radiolabeled elicitor; the amount of elicitor bound correlated with the amount of each type of membrane (measured as milligrams of protein) present. Membranes prepared from soybean roots bound the greatest amount of hepta-β-glucoside elicitor per milligram of membrane protein. Thus, further characterization of the binding site was carried out with membranes isolated from root tissue.

Characterization of the Binding of Radiolabeled Hepta-β-glucoside Elicitor to Soybean Root Membranes

The pH dependence of the binding to root membranes of radiolabeled hepta-β-glucoside elicitor was determined. Optimum binding was observed from pH 6.5 to 8 (data not shown). Binding of elicitor to the membranes dropped off rapidly at pH values less than 6 and greater than 8. Based on these results, binding assays were routinely carried out in 10 mM Tris-HCl, pH 7.0.

Binding of radiolabeled hepta-β-glucoside elicitor to soybean root membranes was inhibited either by preincubation of the membranes at 60°C for 10 min or by treatment with a proteolytic enzyme, as shown in Table 1. The binding site was stable to preincubation at 30°C for 30 min in the absence of protease. Heat-inactivated protease reduced the ability of the soybean membranes to bind hepta-β-glucoside by less than 20%.

The saturability of the binding of hepta-β-glucoside to the membrane binding sites was examined by incubating root membranes with increasing concentrations of the radiolabeled hepta-β-glucoside. The results of this experiment, shown in Figure 3A, demonstrate that the binding was saturable and nonspecific binding was relatively low (<3% of specific binding at an initial ligand concentration of 5 nM). No further increase in specifically bound ligand was observed at higher initial ligand concentrations, whereas a linear increase in nonspecific binding was observed that reached 22% of specific binding at the highest initial ligand concentration used (16 nM) (data not shown). Analysis of the specific binding data by the methods of Scatchard (Scatchard, 1949) or Woolf (Haldane, 1957; Keightley and Cressie, 1980) indicated the presence of a single class of binding sites having an apparent K_d of 7.5 $\times 10^{-10}$ M and an apparent site concentration of 1.2 pmol/mg of protein (Figures 3B and 3C). The linearity of the Hill plot (Hill, 1913; Wold, 1971) of the specific binding data (Figure 3D) also indicates the presence of a single class of hepta-β-glucoside binding sites, and the Hill coefficient of ≈1 suggests that there is no cooperativity in the binding.

Figure 4 shows the kinetics of binding and dissociation of the radiolabeled hepta-β-glucoside with the membrane-localized binding site. Association of the labeled hepta-β-glucoside to its binding site was more rapid than dissociation; half-maximum binding was achieved at 0°C within 20 min after adding the ligand. In contrast, dissociation of the radiolabeled hepta-β-glucoside from the binding site,

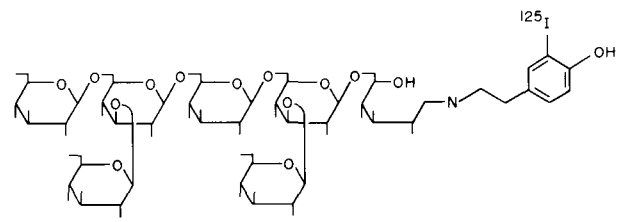


Figure 1. Structure of the Radio-iodinated Tyramine Conjugate of Hepta-β-glucoside 1 Used in Ligand Binding Assays.

Hydroxyl groups not involved in glycosidic linkages have been omitted from the structure for clarity.

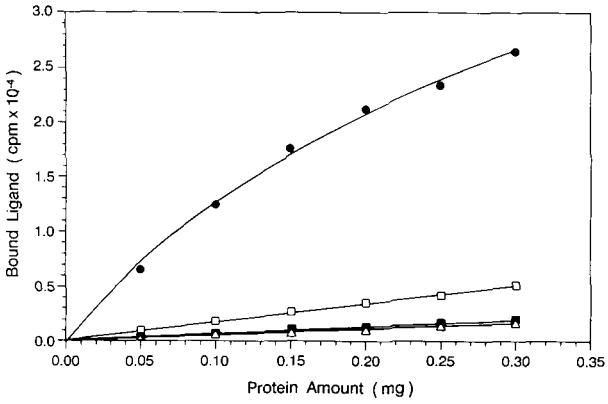


Figure 2. Binding of ^{125}I-Hepta-β-glucoside-Tyramine Conjugate to Total Microsomal Membranes Prepared from Various Soybean Tissues.

Increasing amounts of membrane prepared from root (●), hypocotyl (□), cotyledon (■), and leaf (△), measured as milligrams of protein, were incubated with ^{125}I-labeled hepta-β-glucoside (about 100,000 cpm; 0.56 pmol) in a total volume of 0.4 mL. The binding assays were carried out as described in Methods. Each data point represents the average of three replicates.

in the presence of excess unlabeled ligand, was half complete after 2.5 hr at 0°C. Performing the binding experiments at room temperature (22°C) yielded more rapid kinetics for both association ($t_{½} \approx 4$ min) and dissociation ($t_{½} \approx 6$ min) (data not shown).

Specificity of the Hepta-β-glucoside Binding Site

The structural specificity of the binding site was examined by testing several synthetic oligoglucosides structurally related to the hepta-β-glucoside elicitor for their ability to inhibit binding of the ^{125}I-hepta-β-glucoside-tyramine conjugate to the membranes. The results, shown in Figure 5A, demonstrate that the membrane-localized binding site for the hepta-β-glucoside elicitor exhibits strong selectivity in terms of the oligoglucosides that are recognized efficiently. Those oligoglucosides (1, 2, 3, and 4, see Figure 5B for structures) in which the arrangement of glucosyl residues is identical to that found in the radiolabeled hepta-β-glucoside-tyramine conjugate are equally effective inhibitors of ligand binding, requiring concentrations of 10 nM to 20 nM to achieve 50% inhibition of binding. Other oligoglucosides that either differed in the arrangement of glucosyl residues (9 and 11) or were substituted with *N*-acetyl groups (5 and 6) or amino groups (7 and 8) required higher concentrations (10-fold to 10,000-fold) to achieve 50% inhibition of binding of the radiolabeled ligand. A mixture of heptaglucosides released from *P. megasperma* mycelial walls and a linear β-(1 → 6)-heptaglucoside (derived from pustulan) required 100-fold and 1000-fold higher concentrations, respectively, to reduce ligand binding by 50%, as shown in Table 2. Diglucosides and triglucosides, including maltose [α-(1 → 4)-diglucoside], a disaccharide structurally unrelated to the hepta-β-glucoside elicitor, were also able to inhibit binding of radiolabeled hepta-β-glucoside, although concentrations as high as 0.5 mM were required (Table 2).

Table 2 shows the correlation between the effectiveness of oligosaccharides as competitors in the ligand binding assay and the ability of the oligosaccharides to induce phytoalexin accumulation. Those oligoglucosides having a high elicitor activity were efficient competitors of the radiolabeled ligand, whereas biologically less active oligoglucosides were less efficient. Specifically, hexa-β-glucoside 4, hepta-β-glucoside 1, nona-β-glucoside 2, and deca-β-glucoside 3 that have similar biological activities are equally efficient inhibitors of the binding of the radiolabeled hepta-β-glucoside. An amino-substituted hexaglucoside (7), that had eightfold lower elicitor activity than oligoglucosides 1 through 4, was about sixfold less efficient in the ligand competitions assay. A heptaglucoside (9) and an *N*-acetyl substituted hexaglucoside (5) that have ~800-fold

Table 1. Effects of Proteolytic Cleavage and Heat Treatment on the Ability of Soybean Root Membranes To Bind ^{125}I-labeled Hepta-β-glucoside-Tyramine Conjugate

Treatment	Binding Activity,[a] %
Control	100
Pronase[b]	
0 μg/mL	93.5
5 μg/mL	81.2
50 μg/mL	32.5
500 μg/mL	4.1
Heat-inactivated Pronase[c]	
5 μg/mL	95.3
50 μg/mL	85.2
500 μg/mL	80.6
Heat[d]	0.2

[a] Radiolabeled hepta-β-glucoside tyramine conjugate (~1 nM) was incubated with soybean root membranes (0.15 mg) as described in Methods. The amount of radiolabeled elicitor bound to the membranes was normalized to the amount bound to untreated membranes (control). Each datum is the average of three replicates.

[b] Soybean root membranes were incubated with Pronase at the final concentrations indicated for 30 min at 30°C and then cooled to 0°C before the addition of radiolabeled elicitor.

[c] Pronase was heated at 100°C for 10 min before incubation with the soybean root membranes.

[d] Soybean root membranes were heated at 60°C for 10 min and then cooled to 0°C before the addition of radiolabeled elicitor.

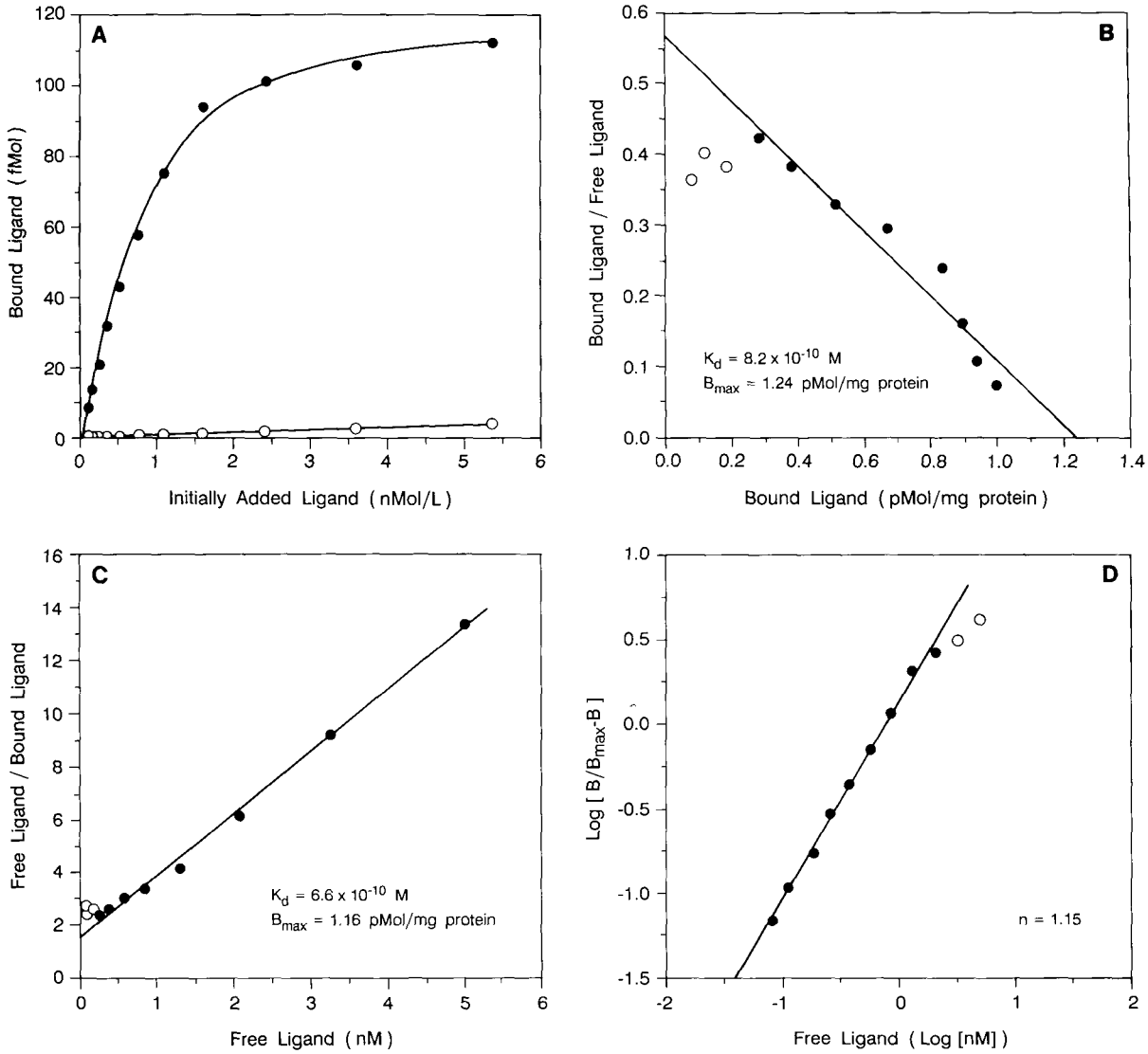


Figure 3. Saturability of the Binding of ^{125}I-Labeled Hepta-β-glucoside-Tyramine Conjugate to Soybean Root Membranes.

(A) Direct plot. Increasing amounts (0.1 nM to 5.4 nM) of radiolabeled hepta-β-glucoside were added to membranes (0.15 mg of protein) and incubated as described in Methods. Nonspecific binding (○) was determined in the presence of 10 μM unlabeled hepta-β-glucoside. The amount of specific binding (●) was determined by subtracting nonspecific binding from total binding. Each data point represents the average of three replicates.

(B) Scatchard plot.

(C) Woolf plot.

(D) Hill plot.

The specific binding data from **(A)** were used to calculate the plots shown in **(B)** through **(D)**. The apparent dissociation constants (K_d) shown in **(B)** and **(C)** and the Hill coefficient (n) shown in **(D)** were calculated from each of these plots by least-squares analysis. (Data points shown as open symbols were not included in the least-squares analyses.)

lower activity as elicitors were less efficient as inhibitors of binding of the radiolabeled ligand, requiring 760 and 260 times higher concentrations, respectively. Two other oligoglucosides (6 and 10) that are even less effective elicitors are also less efficient inhibitors of binding of the labeled hepta-β-glucoside. Thus, there is a good correlation between the concentrations of oligoglucosides required to inhibit binding of the radiolabeled hepta-β-glucoside to

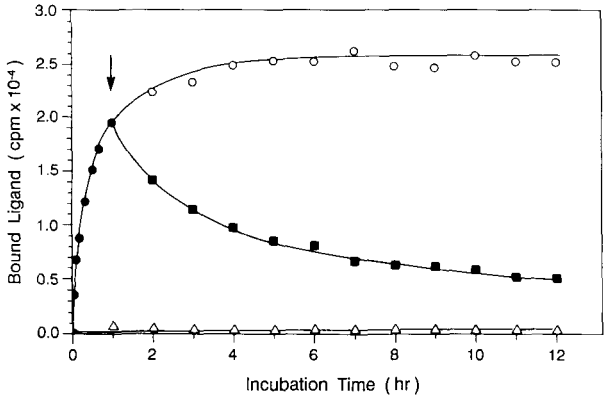


Figure 4. Kinetics of Binding and Dissociation of ^{125}I-Labeled Hepta-β-glucoside to Total Microsomal Membranes from Soybean Roots.

Binding assays were carried out as described in Methods. Association kinetics (●,○) were determined by adding labeled hepta-β-glucoside (about 0.59 pmol) at time 0 to root membranes (0.15 mg of protein) and incubating the samples for increasing amounts of time. Dissociation kinetics (■) were determined by adding a 1000-fold excess of unlabeled hepta-β-glucoside 1 (0.68 nmol in 20 μL of binding assay buffer) 1 hr (arrow) after starting the incubation with labeled ligand and incubating the samples further for the times indicated. Controls (○) received 20 μL of binding assay buffer. Nonspecific binding (△) was measured by adding a 1000-fold excess of unlabeled hepta-β-glucoside 1 (0.68 nmol) simultaneously with the labeled ligand at time 0. Each data point represents the average of two replicates.

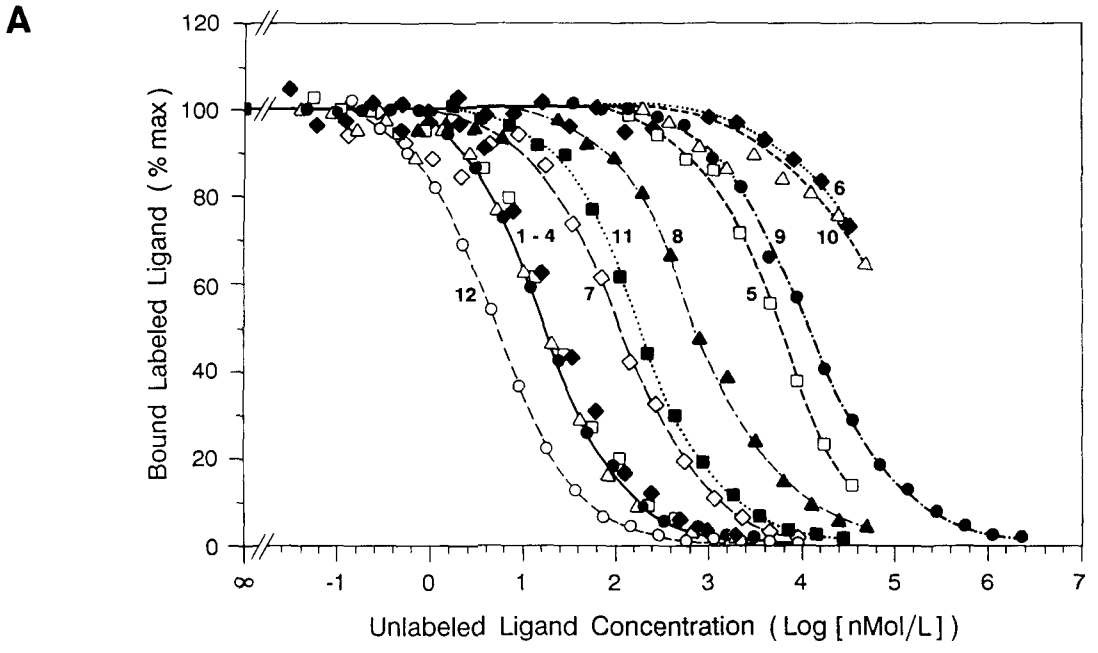
soybean membranes and the ability of the oligoglucosides to elicit phytoalexin accumulation.

DISCUSSION

A large number of studies have demonstrated that fragments of fungal cell walls induce plant defense responses in plant tissues (reviewed in West, 1981; Darvill and Albersheim, 1984; Ryan, 1987; Anderson, 1989). The first step in the signal transduction leading to the activation of defense responses is the recognition of the fungal wall fragments by receptive cells, presumably by way of specific receptors for the active wall fragments. Several previous studies utilizing heterogeneous mixtures of fungal glucan fragments have suggested that binding sites for such fragments exist in soybean membranes. Peters et al. (1978) reported that a crude preparation of fungal glucans agglutinated potato protoplasts. Yoshikawa et al. (1983) described a binding site in soybean for radiolabeled mycolaminarin [a β(1 → 3)-linked glucan having a small number of 6-linked branches] having an apparent $K_d \approx 10^{-6}$ M. Ebel and coworkers, using a somewhat purified elicitor-active mixture of fungal glucan fragments, have reported the existence of membrane-localized binding sites ($K_d \approx 10^{-8}$ M) for these glucan fragments in soybean membranes (Schmidt and Ebel, 1987; Cosio et al., 1988). The heterogeneous nature of the glucan fragments used (i.e., both active and inactive fragments are present in these mixtures) raises questions concerning the connection between the binding sites identified in those studies and the activation of a specific plant defense response.

We have demonstrated here that soybean membranes bind a homogeneous, radiolabeled hepta-β-glucoside phytoalexin elicitor. The binding site, which appears to be detectable in membranes isolated from four major parts of the soybean plant (Figure 2), appears to be a heat-labile protein or glycoprotein (Table 1). Binding of radiolabeled hepta-β-glucoside to its binding site in root membranes is saturable over a ligand concentration range of 0.1 nM to 5 nM (Figure 3A). This is comparable with, albeit somewhat lower than, the range of hepta-β-glucoside concentrations (6 nM to 200 nM) required to saturate the bioassay for phytoalexin accumulation (Sharp et al., 1984b, 1984c; Cheong et al., 1991). A single class of binding sites, with a high affinity for the hepta-β-glucoside elicitor (apparent $K_d = 7.5 \times 10^{-10}$ M; Figures 3B and 3C), was present in root membranes. Binding of the hepta-β-glucoside elicitor to the binding protein was reversible (Figure 4), which suggests that the elicitor does not become covalently attached to the binding protein. The elicitor-binding protein exhibited a high degree of specificity with respect to the oligosaccharides that it binds (Figure 5A). Furthermore, there is a high degree of correlation between the ability of an oligoglucoside to induce phytoalexin accumulation and the ability to bind to soybean root membranes (Table 2). Thus, the binding site for the hepta-β-glucoside elicitor fulfills criteria expected of a bona fide receptor for this ligand (Venis, 1985).

The results of the present study differ in several notable respects from other work on glucan binding sites in soybean. This study was carried out with a homogeneous elicitor ligand having high biological activity ($EC_{50} \approx 10^{-8}$ M), whereas other studies used either a ligand, mycolaminarin, having very low elicitor activity (Yoshikawa et al., 1983), or a high-activity ligand ($EC_{50} \approx 10^{-7}$ M) that was heterogeneous (Schmidt and Ebel, 1987; Cosio et al., 1988). Nonspecific binding of the labeled hepta-β-glucoside to soybean microsomal membranes was much lower than observed in other studies (Yoshikawa et al., 1983; Cosio et al., 1988), less than 5% of maximum binding at saturation (Figure 3A). The association and dissociation kinetics for binding of the hepta-β-glucoside to its binding site determined in the present study are more rapid and showed more complete reversibility than those observed previously (Schmidt and Ebel, 1987; Cosio et al., 1988). Whether these observed differences reflect differences in the binding sites identified in the different studies or are solely a consequence of the chemical nature of the radio-



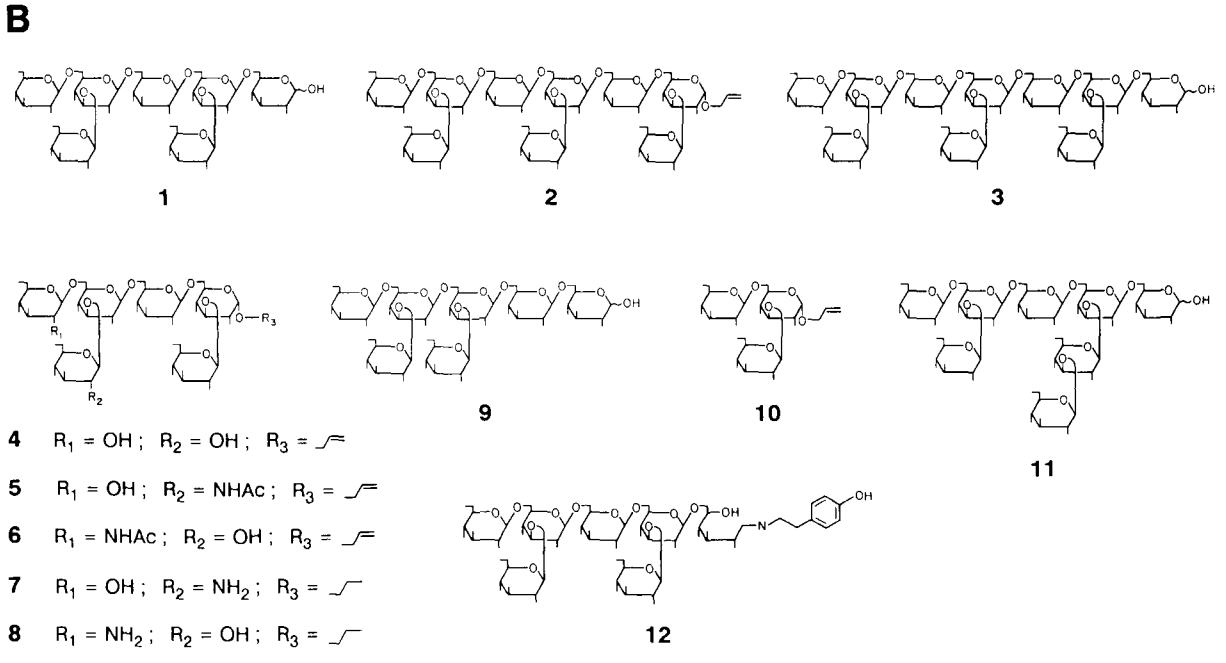


Figure 5. Competitive Inhibition of Binding of ^{125}I-Hepta-β-glucoside-Tyramine Conjugate to Soybean Root Membranes by Unlabeled Oligoglucosides.

(A) Competitive inhibition assays. Labeled hepta-β-glucoside tyramine conjugate (1.06 nM) was incubated with soybean membranes (0.15 mg of protein) in the presence of increasing amounts of hepta-β-glucoside 1 (—●—), α-allyl-nona-β-glucoside 2 (—◆—), deca-β-glucoside 3 (—△—), α-allyl-hexa-β-glucoside 4 (—□—), α-allyl-*N*-acetylglucosaminyl-penta-β-glucoside 5 (– –□– –), α-allyl-*N*-acetylglucosaminyl-penta-β-glucoside 6 (· ·◆· ·) α-propyl-glucosaminyl-penta-β-glucoside 7 (– –◇– –), α-propyl-glucosaminyl-penta-β-glucoside 8 (– – ·▲– – ·), hepta-β-glucoside 9 (– ·●· –), α-allyl-tri-β-glucoside 10 (– –△– –), octa-β-glucoside 11 (· ·■· ·), and hepta-β-glucoside tyramine conjugate 12 (– –○– –). The numbers adjacent to the curves refer to the structures of the oligoglucosides shown in **(B)**. Binding assays were carried out as described in Methods. The amount of radiolabeled elicitor remaining bound to the membranes was normalized to the amount bound in the absence of any unlabeled oligoglucoside. Each data point is the average of two replicates.

(B) Structures of synthetic oligoglucosides. Hydroxyl groups not involved in glycosidic linkages or located at the reducing end of the oligosaccharides have been omitted from the structures for clarity.

Table 2. Comparison of the Relative Abilities of Oligosaccharides To Induce Phytoalexin Accumulation in Soybean Cotyledons and To Inhibit Binding of ^{125}I-labeled Hepta-β-glucoside-Tyramine Conjugate to Soybean Root Membranes

Oligosaccharide[a]	Elicitor Activity[b] (EC_{50}, nM)	Binding Activity[c] (IC_{50}, nM)
Hepta-β-glucoside 1	8.0	20.7
Reduced hepta-β-glucoside 1	3.9	8.04
Hepta-β-glucoside-tyramine conjugate 12	11	5.76
α-Allyl-hexa-β-glucoside 4	11	21.5
α-Allyl-nona-β-glucoside 2	14	23.0
Deca-β-glucoside 3	19	15.0
Octa-β-glucoside 11	30	223
α-Propyl-glucosaminyl-penta-β-glucoside 7	60	116
α-Propyl-glucosaminyl-penta-β-glucoside 8	1810	934
Hepta-β-glucoside 9	6880	15700
α-Allyl-*N*-acetylglucosaminyl-penta-β-glucoside 5	6480	5460
α-Allyl-*N*-acetylglucosaminyl-penta-β-glucoside 6	$>10^5$	9.28×10^4
Crude *Phytophthora* heptaglucosides	4480	1590
β-(1 → 6)-Heptaglucoside	ND[d]	1.63×10^4
Maltoheptaose	ND	7.83×10^7
α-Allyl-tri-β-glucoside 10	5.1×10^4	17.6×10^4
Gentiobiose	48.8×10^5	4.60×10^5
Laminaribiose	$>10^7$	7.64×10^4
Maltose	NA[e]	3.29×10^6

[a] Structures of oligosaccharides 1 to 12 are shown in Figure 5B.
[b] Elicitor activities were determined using the soybean cotyledon bioassay as described in Methods. The relative elicitor activity (EC_{50}) is defined as the concentration of an oligosaccharide required to give half-maximum induction of phytoalexin accumulation ($A/A_{std} = 0.5$) in the cotyledon bioassay corrected to the standard curve for hepta-β-glucoside 1. The 95% confidence interval for the elicitor activity of hepta-β-glucoside 1 is 2.5 nM to 28 nM (Cheong et al., 1991).
[c] Concentration of oligosaccharide (IC_{50}) required to give 50% inhibition of the binding of radiolabeled hepta-β-glucoside 1 to its binding site in soybean root membranes (see Figure 5A).
[d] ND, no activity detected.
[e] NA, not assayed.

labeled ligands used in these studies must await purification and characterization of the receptor molecule(s).

The hepta-β-glucoside binding site in soybean shows a remarkable specificity for elicitor-active oligoglucosides. Four oligoglucosides ranging in size from hexaglucoside to decaglucoside that are indistinguishable on the basis of their abilities to induce phytoalexin accumulation (Cheong et al., 1991) are equally effective competitive inhibitors of binding of the radiolabeled hepta-β-glucoside to its binding site (Figure 5A; Table 2). Interestingly, the tyramine conjugate of hepta-β-glucoside 1 is somewhat more efficient (threefold) as a competitive inhibitor in the binding assays than the underivatized hepta-β-glucoside, suggesting that the tyramine moiety may contribute to the binding of the derivatized hepta-β-glucoside. Two other structurally related oligoglucosides, a hexasaccharide and a heptasaccharide, that have several-hundredfold lower biological activity, are also several-hundredfold less efficient as competitive inhibitors in the binding studies (Figure 5A; Table 2). Thus, there is no clear correlation between the binding affinity of these oligoglucosides and their degree of polymerization as observed by others (Cosio et al., 1988), whereas there is a strict correlation with the ability of an oligoglucoside to induce phytoalexin accumulation (Table 2). Indeed, the results of our binding studies confirm previous conclusions that a hexasaccharide structure is the minimum structure recognized by the binding site (Cheong et al., 1991). This conclusion is in accord with a number of studies on oligosaccharide binding sites of carbohydrate-binding proteins (e.g., antibodies and enzymes) that showed that a maximum of six or seven sugars can be accommodated in a binding site (Kabat, 1966; Kelly et al., 1979; Goldsmith and Fletterick, 1983; Young et al., 1983; Matsuura et al., 1984; Quiocho, 1986; Kabat et al., 1988; Glaudemans et al., 1989; Rouvinen et al., 1990).

Structure-activity studies described in the preceding paper (Cheong et al., 1991) identified structural elements of the hepta-β-glucoside elicitor that are essential for maximum biological activity. These structural elements include the branched trisaccharide at the nonreducing end of the hepta-β-glucoside and the attachment pattern of the side-chain glucosyl residues to the β-(1 → 6)-linked backbone. The results of the binding studies reported here suggest that these structural elements are also essential for effi-

cient binding of the hepta-β-glucoside elicitor to the binding protein present in soybean membranes. Substitution of the terminal glucosyl residues in the nonreducing terminal trisaccharide with either glucosaminyl or *N*-acetylglucosaminyl residues reduced the ability of the oligosaccharides to competitively inhibit binding of the radiolabeled hepta-β-glucoside fivefold to 4500-fold (Figure 5A; Table 2), with the *N*-acetylglucosaminyl substitution having a greater effect. The more dramatic effect of the *N*-acetylglucosaminyl substitutions is probably attributable to greater steric hindrance caused by the bulk of the acetyl group. The reduced ability of hepta-β-glucoside 9 to compete with radiolabeled elicitor suggests that the altered arrangement of side-chain glucosyl residues results in a conformation of the oligoglucoside that is no longer able to bind efficiently to the binding protein. In summary, the results of the biological assays reported in the preceding paper (Cheong et al., 1991) and the binding studies described here suggest that the binding protein recognizes all or parts of the three terminal nonreducing glucosyl residues present in hepta-β-glucoside 1. The precise identification of which atoms in the hepta-β-glucoside elicitor interact directly with the binding protein must await isolation of the binding protein(s) and functional dissection of the binding site.

The hepta-β-glucoside elicitor binding site described in this study is the first putative receptor for a defined "oligosaccharin" [oligosaccharide with biological regulatory properties (Albersheim et al., 1983; Albersheim and Darvill, 1985)]. A number of oligosaccharins have been identified, including those derived from fungal (Kendra and Hadwiger, 1984; Sharp et al., 1984c; Barber et al., 1989), plant (Hahn et al., 1981; Nothnagel et al., 1983; York et al., 1984; McDougall and Fry, 1988, 1989a), and, most recently, bacterial (Lerouge et al., 1990) glycoconjugates. These oligosaccharins regulate such diverse processes as the induction of plant defense responses (Darvill and Albersheim, 1984; Ryan, 1987; Hahn et al., 1989), hormone responses (York et al., 1984; Branca et al., 1988; McDougall and Fry, 1988, 1989b), and plant development (Tran Thanh Van et al., 1985; Eberhard et al., 1989; Lerouge et al., 1990). Thus, oligosaccharins constitute signals that trigger cellular responses, presumably by interacting with specific receptors to activate a signal transduction cascade. Purification and characterization of physiologically important oligosaccharin receptors is a first step toward elucidating at the molecular level how this new class of regulatory molecules induces changes in cellular metabolism.

METHODS

Elicitors and Other Chemicals

The structures of the oligoglucosides used in this study are shown in Figure 5B. Chemically synthesized hepta-β-glucosides 1 and 9 [each a mixture of the reduced and reducing oligosaccharides, which have indistinguishable elicitor activities (Sharp et al., 1984b, 1984c; Cheong et al., 1991)], deca-β-glucoside 3, and octa-β-glucoside 11 were obtained from Per Garegg (University of Stockholm, Sweden); α-allyl-nona-β-glucoside 2, α-allyl-hexa-β-glucoside 4, α-allyl-*N*-acetylglucosaminyl-penta-β-glucosides 5 and 6, α-propyl-glucosaminyl-penta-β-glucosides 7 and 8, and α-allyl-tri-β-glucoside 10 were from Tomoya Ogawa (RIKEN, Saitama, Japan). Void glucan elicitor and a mixture of hepta-β-glucosides were prepared from mycelial walls of *Phytophthora megasperma* f sp *glycinea* as described (Sharp et al., 1984c; Hahn et al., 1990). Pustulan [(1 → 6)-linked β-D-glucan] was purified from a crude commercial preparation (Calbiochem, San Diego, CA) as described (Reese et al., 1962). A heptaglucoside derived from pustulan was prepared as described (Cheong et al., 1991). Tyramine, maltoheptaose, laminaribiose, gentiobiose, maltose, β-D-thioglucose, and D-gluconic acid lactone were purchased from Sigma; carrier-free $Na^{125}I$ (13 to 16 mCi/μg of iodine) in dilute NaOH was from Amersham; Iodogen was from Pierce; Pronase (nonspecific protease from *Streptomyces griseus*) was from Calbiochem.

Plant Material and Growth Conditions

Foundation quality soybean (*Glycine max* L cv Williams 82) was obtained from Illinois Foundation Seeds, Inc. (Champaign, IL) and stored at room temperature. Seed was from the newest crop available and was less than 12 months old. Soybean seedlings were grown as described (Cheong et al., 1991).

Colorimetric Assays

Concentrations of oligosaccharide solutions were determined as glucose equivalents with the anthrone assay (Dische, 1962; Hahn et al., 1991), using glucose as the standard. Where appropriate, glucose equivalent concentrations were corrected for the presence of unreactive glycosyl residues in the oligosaccharides (e.g., *N*-acetyl-glucosaminyl or glucitol residues) as described (Sharp et al., 1984b, 1984c). Protein concentrations of the membrane preparations were estimated by the Bio-Rad Protein Assay based on the method of Bradford (Bradford, 1976) using bovine γ-globulin as the standard.

Soybean Cotyledon Bioassay

The ability of various oligosaccharides to induce the accumulation of phytoalexins in soybean tissue was determined using the cotyledon bioassay as described (Cheong et al., 1991; Hahn et al., 1991).

Preparation of Soybean Root Membranes

Crude total microsomal membranes were prepared from the roots of 9-day-old soybean seedlings. Seedlings were carefully removed from the soil/vermiculite, washed extensively under cold tap water to remove residual soil/vermiculite, and placed on ice. Roots were excised and kept on ice until homogenized. All subsequent steps

were performed at 4°C. Soybean roots (~190 g, wet weight) were placed in a chilled Waring Blendor together with ~250 mL of cold homogenization buffer (25 mM Tris-HCl, pH 7.0, containing 30 mM $MgCl_2$ and 2 mM dithiothreitol). Roots were homogenized three times for 10 sec each. The root slurry was filtered through a double layer of Miracloth (Chicopee Mills, Inc., Milltown, NJ) and the filtrate centrifuged at 10,000*g* for 15 min. The supernatant was collected and microsomal membranes pelleted by centrifugation at 100,000*g* for 20 min. The surfaces of the membrane pellets were briefly rinsed with 0.5 mL of homogenization buffer and the pellets were frozen and stored at −80°C until used. Membranes from hypocotyl, cotyledon, and leaves of soybean seedlings were prepared by the same procedures.

Preparation of Radiolabeled Hepta-β-glucoside Elicitor

The tyramine conjugate of hepta-β-glucoside 1 was prepared by reductive amination as described (Wang et al., 1984; Cheong et al., 1991). The conjugate was purified by chromatography on Bio-Gel P-2 as described (Cheong et al., 1991).

Radiolabeling of the hepta-β-glucoside-tyramine conjugate was accomplished as follows: Iodogen-coated test tubes were prepared as described in the preceding paper (Cheong et al., 1991). An Iodogen-coated test tube was rinsed with 0.5 mL of 250 mM sodium phosphate, pH 7.5, and the test tube was drained. Hepta-β-glucoside-tyramine conjugate (3 nmol in 5 μL of 250 mM sodium phosphate, pH 7.5) was added to the bottom of the rinsed Iodogen-coated test tube, followed by the addition of ~0.5 mCi of $Na^{125}I$, and the solution was allowed to stand at room temperature for 15 min. The radio-iodinated product was purified by chromatography on a disposable AG 1-X8 (200 to 400 mesh, Cl^- form) anion-exchange column (0.5 mL bed volume, Poly-Prep, Bio-Rad). The column had been washed with 5 mL of deionized water, blocked with 1 mL of maltoheptaose (10 mg/mL), and washed again with 5 mL of deionized water before loading the radio-iodination reaction mixture onto the column. The column was eluted with 4 mL of deionized water and 0.5-mL fractions were collected in 1.5-mL Eppendorf tubes. The first three fractions eluting from the column, containing ~90% of the radiolabeled oligosaccharide, were pooled and stored at room temperature in a lead container for no longer than 2 weeks.

Binding Assays

The assays to determine binding of radiolabeled hepta-β-glucoside to microsomal membranes were carried out in binding assay buffer (10 mM Tris-HCl, pH 7.0, containing 1 M NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 7.5 mM each β-D-thioglucose and D-gluconic acid lactone) as follows. Microsomal membrane pellets were suspended in ice-cold binding assay buffer and briefly homogenized using a Teflon tissue homogenizer. Unless otherwise indicated, incubation mixtures contained microsomal membranes (~0.15 mg of protein) and ^{125}I-labeled hepta-β-glucoside (~100,000 cpm; 0.5 pmol to 0.6 pmol) in a total volume of 0.4 mL of binding assay buffer in 1.5-mL Eppendorf tubes. Samples were incubated in an ice-water bath for 2 hr. Incubations were terminated by adding a 300-μL aliquot of the assay mixture to 10 mL of ice-cold washing buffer (10 mM Tris-HCl, pH 7.0, containing 1 M NaCl and 10 mM $MgCl_2$) on a glass fiber filter (GF/B, Whatman, 2.5 cm) in a filtration manifold (Millipore, 12-unit) and rapidly filtering the solution with vacuum. The filters were washed three times with 10 mL of ice-cold washing buffer, and the amount of radioactivity remaining on the filters was measured directly using a γ counter.

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NOTE ADDED IN PROOF

After this article was submitted for publication, a paper appeared reporting high-affinity binding of a 2-(4-aminophenyl)ethylamine conjugate of the hepta-β-glucoside elicitor to soybean membranes. [**Cosio, E.G., Frey, T., Verduyn, R., van Boom, J., and Ebel, J.** (1990). High-affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors to soybean membranes. FEBS Lett. **271,** 223–226.]

REFERENCES

Albersheim, P., and Darvill, A.G. (1985). Oligosaccharins. Sci. Am. **253,** 58–64.

Albersheim, P., Darvill, A.G., McNeil, M., Valent, B.S., Sharp, J.K., Nothnagel, E.A., Davis, K.R., Yamazaki, N., Gollin, D.J., York, W.S., Dudman, W.F., Darvill, J.E., and Dell, A. (1983). Oligosaccharins: Naturally occurring carbohydrates with biological regulatory functions. In Structure and Function of Plant Genomes, O. Ciferri and L. Dure, III, eds (New York: Plenum Publishing Corp.), pp. 293–312.

Anderson, A.J. (1989). The biology of glycoproteins as elicitors. In Plant-Microbe Interactions. Molecular and Genetic Perspectives, Vol. 3, T. Kosuge and E. Nester, eds (New York, NY: McGraw Hill Inc.), pp. 87–130.

Barber, M.S., Bertram, R.E., and Ride, J.P. (1989). Chitin oligosaccharides elicit lignification in wounded wheat leaves. Physiol. Mol. Plant Pathol. **34,** 3–12.

Birnbaumer, L., Abramowitz, J., and Brown, A.M. (1990). Receptor-effector coupling by G proteins. Biochim. Biophys. Acta Rev. Biomembr. **1031,** 163–224.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Branca, C., De Lorenzo, G., and Cervone, F. (1988). Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. Physiol. Plant. **72,** 499–504.

Cheong, J.-J., Birberg, W., Fügedi, P., Pilotti, Å., Garegg, P.J., Hong, N., Ogawa, T., and Hahn, M.G. (1991). Structure-activity relationships of oligo-β-glucoside elicitors of phytoalexin accumulation in soybean. Plant Cell **3,** 127–136.

Cosio, E.G., Pöpperl, H., Schmidt, W.E., and Ebel, J. (1988). High-affinity binding of fungal β-glucan fragments to soybean (*Glycine max* L.) microsomal fractions and protoplasts. Eur. J. Biochem. **175,** 309–315.

Darvill, A.G., and Albersheim, P. (1984). Phytoalexins and their elicitors: A defense against microbial infection in plants. Annu. Rev. Plant Physiol. **35,** 243–275.

Dische, Z. (1962). Color reactions of carbohydrates. In Methods in Carbohydrate Chemistry, Vol. 1, R.L. Whistler and M.L. Wolfrom, eds (New York, NY: Academic Press Inc.), pp. 478–481.

Dixon, R.A. (1986). The phytoalexin response: Elicitation, signalling and control of host gene expression. Biol. Rev. **61,** 239–291.

Ebel, J. (1986). Phytoalexin synthesis: The biochemical analysis of the induction process. Annu. Rev. Phytopathol. **24,** 235–264.

Eberhard, S., Doubrava, N., Marfà, V., Mohnen, D., Southwick, A., Darvill, A., and Albersheim, P. (1989). Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. Plant Cell **1,** 747–755.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science **240,** 889–895.

Fraker, P.J., and Speck, J.C., Jr. (1978). Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. **80,** 849–857.

Gilman, A.G. (1987). G proteins: Transducers of receptor-generated signals. Annu. Rev. Biochem. **56,** 615–649.

Glaudemans, C.P.J., Kovác, P., and Rao, A.S. (1989). The subsites of monoclonal anti-dextran IgA W3129. Carbohydr. Res. **190,** 267–277.

Goldsmith, E., and Fletterick, R.J. (1983). Oligosaccharide conformation and protein-saccharide interactions in solution. Pure Appl. Chem. **55,** 577–588.

Grisebach, H., Edelmann, L., Fischer, D., Kochs, G., and Welle, R. (1989). Biosynthesis of phytoalexins and nod-gene inducing isoflavones in soybean. In Signal Molecules in Plants and Plant-Microbe Interactions. NATO ASI Series, Vol. H36, B.J.J. Lugtenberg, ed (Berlin: Springer-Verlag), pp. 57–64.

Hahlbrock, K., and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 347–369.

Hahn, M.G. (1989). Animal receptors: Examples of cellular signal perception molecules. In Signal Molecules in Plants and Plant-Microbe Interactions. NATO ASI Series, Vol. H36, B.J.J. Lugtenberg, ed (Berlin: Springer-Verlag), pp. 1–26.

Hahn, M.G., Darvill, A.G., and Albersheim, P. (1981). Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Plant Physiol. **68,** 1161–1169.

Hahn, M.G., Bucheli, P., Cervone, F., Doares, S.H., O'Neill, R.A., Darvill, A., and Albersheim, P. (1989). The roles of cell wall constituents in plant-pathogen interactions. In Plant-Microbe Interactions. Molecular and Genetic Perspectives, Vol. 3, T. Kosuge and E.W. Nester, eds (New York, NY: McGraw Hill Publishing Co.), pp. 131–181.

Hahn, M.G., Darvill, A., Albersheim, P., Bergmann, C., Cheong, J.-J., Koller, A., and Lò, V.-M. (1991). Preparation and characterization of oligosaccharide elicitors of phytoalexin accumulation. In Molecular Plant Pathology: A Practical Approach, D.J. Bowles, S. Gurr, and M. McPherson, eds (Oxford, UK: IRL Press, Inc.), in press.

Haldane, J.B.S. (1957). Graphical methods in enzyme chemistry. Nature **179,** 832.

Hill, A.V. (1913). The combination of haemoglobin with oxygen and with carbon monoxide. I. Biochem. J. **7,** 471–480.

Kabat, E.A. (1966). The nature of an antigenic determinant. J. Immunol. **97,** 1–11.

Kabat, E.A., Liao, J., Osserman, E.F., Gamian, A., Michon, F., and Jennings, H.J. (1988). The epitope associated with the binding of the capsular polysaccharide of the group B Meningococcus and of *Escherichia coli* K1 to a human monoclonal macroglobulin, IgM^{NOV}. J. Exp. Med. **168,** 699–711.

Keightley, D.D., and Cressie, N.A.C. (1980). The Woolf plot is more reliable than the Scatchard plot in analysing data from hormone receptor assays. J. Steroid Biochem. **13,** 1317–1323.

Kelly, J.A., Sielecki, A.A., Sykes, B.D., James, M.N.G., and Phillips, D.C. (1979). X-ray crystallography of the binding of the bacterial cell wall trisaccharide NAM-NAG-NAM to lysozyme. Nature **282,** 875–878.

Kendra, D.F., and Hadwiger, L.A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. Exp. Mycol. **8,** 276–281.

Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56,** 215–224.

Lefkowitz, R.J., and Caron, M.G. (1988). Adrenergic receptors. Models for the study of receptors coupled to guanine nucleotide regulatory proteins. J. Biol. Chem. **263,** 4993–4996.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344,** 781–784.

Matsuura, Y., Kusunoki, M., Harada, W., and Kakudo, M. (1984). Structure and possible catalytic residues of Taka-amylase A. J. Biochem. **95,** 697–702.

McDougall, G.J., and Fry, S.C. (1988). Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan. Planta **175,** 412–416.

McDougall, G.J., and Fry, S.C. (1989a). Anti-auxin activity of

xyloglucan oligosaccharides: The role of groups other than the terminal α-L-fucose residue. J. Exp. Bot. **40,** 233–238.

McDougall, G.J., and Fry, S.C. (1989b). Structure-activity relationships for xyloglucan oligosaccharides with antiauxin activity. Plant Physiol. **89,** 883–887.

Nothnagel, E.A., McNeil, M., Albersheim, P., and Dell, A. (1983). Host-pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. Plant Physiol. **71,** 916–926.

Peters, B.M., Cribbs, D.H., and Stelzig, D.A. (1978). Agglutination of plant protoplasts by fungal cell wall glucans. Science **201,** 364–365.

Quiocho, F.A. (1986). Carbohydrate-binding proteins: Tertiary structures and protein-sugar interactions. Annu. Rev. Biochem. **55,** 287–315.

Reese, E.T., Parrish, F.W., and Mandels, M. (1962). β-D-1,6-Glucanases in fungi. Can. J. Microbiol. **8,** 327–334.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C., and Jones, T.A. (1990). Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science **249,** 380–386.

Ryan, C.A. (1987). Oligosaccharide signaling in plants. Annu. Rev. Cell Biol. **3,** 295–317.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51,** 660–672.

Schmidt, W.E., and Ebel, J. (1987). Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean *Glycine max*. Proc. Natl. Acad. Sci. USA **84,** 4117–4121.

Sharp, J.K., McNeil, M., and Albersheim, P. (1984a). The primary structures of one elicitor-active and seven elicitor-inactive hexa(β-D-glucopyranosyl)-D-glucitols isolated from the mycelial walls of *Phytophthora megasperma* f. sp. *glycinea*. J. Biol. Chem. **259,** 11321–11336.

Sharp, J.K., Albersheim, P., Ossowski, P., Pilotti, Å., Garegg, P.J., and Lindberg, B. (1984b). Comparison of the structures and elicitor activities of a synthetic and a mycelial-wall-derived hexa(β-D-glucopyranosyl)-D-glucitol. J. Biol. Chem. **259,** 11341–11345.

Sharp, J.K., Valent, B., and Albersheim, P. (1984c). Purification and partial characterization of a β-glucan fragment that elicits phytoalexin accumulation in soybean. J. Biol. Chem. **259,** 11312–11320.

Stryer, L., and Bourne, H.R. (1986). G proteins: A family of signal transducers. Annu. Rev. Cell Biol. **2,** 391–419.

Templeton, M.D., and Lamb, C.J. (1988). Elicitors and defense gene activation. Plant Cell Environ. **11,** 395–401.

Tran Thanh Van, K., Toubart, P., Cousson, A., Darvill, A.G., Gollin, D.J., Chelf, P., and Albersheim, P. (1985). Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. Nature **314,** 615–617.

Venis, M. (1985). Hormone Binding Sites in Plants. (New York, NY: Longman), pp. 1–189.

Wang, W.T., LeDonne, N.C., Jr., Ackerman, B., and Sweeley, C.C. (1984). Structural characterisation of oligosaccharides by high-performance liquid chromatography, fast-atom bombardment-mass spectrometry, and exoglycosidase digestion. Anal. Biochem. **141,** 366–381.

West, C.A. (1981). Fungal elicitors of the phytoalexin response in higher plants. Naturwissenschaften **68,** 447–457.

Wold, F. (1971). Macromolecules: Structure and Function. (Englewood Cliffs, NJ: Prentice-Hall, Inc.), pp. 21–29.

York, W.S., Darvill, A.G., and Albersheim, P. (1984). Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol. **75,** 295–297.

Yoshikawa, M., Keen, N.T., and Wang, M.-C. (1983). A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. Plant Physiol. **73,** 497–506.

Young, W.W., Jr., Johnson, H.S., Tamura, Y., Karlsson, K.-A., Larson, G., Parker, J.M.R., Khare, D.P., Spohr, U., Baker, D.A., Hindsgaul, O., and Lemieux, R.U. (1983). Characterization of monoclonal antibodies specific for the Lewis a human blood group determinant. J. Biol. Chem. **258,** 4890–4894.