Solubilization of Functional Plasma Membrane-Localized Hepta-β-Glucoside Elicitor-Binding Proteins from Soybean

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Total membranes prepared from roots of soybean (Glycine max L.) seedlings have previously been shown to contain proteinaceous binding site(s) for a hepta-β-glucoside elicitor of phytoalexin accumulation. The hepta-β-glucoside elicitor-binding proteins have now been shown to co-migrate with a plasma membrane marker enzyme (vanadate-sensitive H-ATPase) on linear sucrose density gradients. With the use of detergents, the elicitor-binding proteins have been solubilized in functional form from soybean root membranes. The nonionic detergents n-dodecylsucrose, n-dodecylmalto-side, and Triton X-114, at concentrations of 5 to 10 mg/mL, each solubilizes between 50 and 60% of the elicitor-binding activity in a single extraction of the membranes. A zwitterionic detergent, n-dodecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (ZW 3–12), also solubilizes about 40% of the total binding activity at detergent concentrations between 1 and 2 mg/mL, but the total binding activity recovered is only approximately 50% of that recovered with the nonionic detergents. The elicitor-binding proteins solubilized with either n-dodecylsucrose or ZW 3–12 retain the high affinity for radiolabeled hepta-β-glucoside elicitor (apparent dissociation constant \( K_d \) = 1.8 nM and 1.4 nM, respectively) that was observed with the membrane-localized binding proteins (apparent \( K_d \) = 1 nM). Competitive ligand-binding experiments with several structurally related synthetic oligoglucosides demonstrate that the solubilized binding proteins retain specificity for elicitor-active oligosaccharides, irrespective of the detergent used for solubilization. Moreover, the binding affinities of the oligoglucosides for the solubilized binding proteins correlate well with their abilities to induce phytoalexin accumulation in soybean cotyledon tissue. Gel-permeation chromatography of n-dodecysucrose-solubilized elicitor-binding proteins demonstrate that the bulk of the elicitor-binding activity is associated with large detergent-protein micelles (relative molecular weight > 400,000). Our results suggest that n-dodecysucrose is a suitable detergent for solubilizing elicitor-binding proteins from soybean root membranes with minimal losses of binding activity. More importantly, we demonstrate that solubilization does not significantly alter the binding properties of the proteins for elicitor-active oligoglucosides.

The elicitation of antimicrobial phytoalexins in plants constitutes a useful model system for molecular studies of signal-transduction mechanisms in plant cells (Dixon, 1986; Lamb et al., 1989). This important plant defense response is induced by defined signal molecules, called elicitors, that are released from the cell walls of invading pathogens (Darvill and Albersheim, 1984; Kendra and Hadwiger, 1984; Barber et al., 1989) or of their host plants (Hahn et al., 1981; Nothnagel et al., 1983; Jin and West, 1984). The best-studied elicitors are β-glucan fragments derived from the mycelial walls of plant pathogenic fungi (Darvill and Albersheim, 1984; Ebel, 1986). One of the first steps of the signaling process leading to phytoalexin biosynthesis is probably the recognition of the mycelial wall fragments by plant cells. Several studies have suggested that binding sites for such fragments exist in plant membranes (Yoshikawa et al., 1983; Schmidt and Ebel, 1987; Cosio et al., 1988) and that those binding sites are located on the plasma membrane (Peters et al., 1978; Schmidt and Ebel, 1987; Cosio et al., 1988).

A branched hepta-β-glucoside is the smallest elicitor-active oligoglucoside that could be isolated from the mycelial walls of Phytophthora sojae (syn. Phytophthora megasperma f. sp. glycinea [Faris et al., 1989]) in sufficient quantities to fully characterize its structure (Sharp et al., 1984a, 1984b, 1984c). Subsequently, it was shown that a hexaglucoside having the same overall structure but lacking the terminal reducing end glucosyl residue constituted the minimum structural unit required for maximum elicitor activity (Cheong et al., 1991). A structure-activity study using synthetic oligoglucosides structurally related to the hepta-β-glucoside elicitor revealed that specific structural elements of oligo-β-glucosides were required for effective induction of phytoalexin accumulation in soybean (Glycine max L.) (Cheong et al., 1991). Moreover, the existence, in soybean tissues, of membrane-bound proteins that specifically recognize the elicitor-active hepta-β-glucoside has been demonstrated using ligand-binding assays (Cosio et al., 1990b; Cheong and Hahn, 1991). Recent photo-affinity labeling experiments have identified a 70-kD protein

Abbreviations: bis-Tris, bis[2-hydroxy-ethyl]limino-tris[hydroxy-methyl]methane; Brij 35, polyoxyl-20-ethyl-N-dodecyl ether; Brij 58, polyoxyl-20-ethyl-N-dodecyl ether; PEG-20-monoethyl ether; BTP, 1,3-bis[(tris(hydroxymethyl)amino)methyl] propane; Hecameg, methyl-6-O-[(N-hepta-carbamoyl)-N-N-glucopyranoside; MEGA-10, n-decanoyl-N-methyl-glucamide; Thesit, dodecylpolyethylene-glycol ether; ZW 3–12, N-dodecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate.
in solubilized soybean membrane preparations whose labeling characteristics suggest that it is an elicitor-binding protein (Cosio et al., 1992).

Binding characteristics of the membrane-localized elicitor-binding proteins, such as saturaibility, high affinity, and reversibility of ligand-binding, together with a direct correlation between the binding affinities and the elicitor activities of oligo-β-glucosides, provide strong evidence that the elicitor-binding proteins are physiological receptors for the hepta-β-glucoside elicitor (Cheong and Hahn, 1991). Confirmation of the physiological and functional significance of this putative receptor as part of the signal-transduction pathway leading to phytoalexin biosynthesis would be facilitated by the characterization of the binding proteins.

The solubilization of the elicitor-binding proteins from the membranes in a functional form is a major step toward purification of these proteins. A β-glucan-binding activity was solubilized from soybean root membranes using the zwitterionic detergent ZW 3–12 (Cosio et al., 1990a). However, these authors reported significant losses of the detergent-solubilized binding activity in subsequent chromatographic purification steps (Cosio et al., 1990a; Frey et al., 1993). Only about 30% and 2% of the binding activity, initially solubilized with ZW 3–12, was recovered after Q-Sepharose (Cosio et al., 1990a) and subsequent glucan-affinity chromatography (Frey et al., 1993), respectively. These losses have hampered efforts to obtain sufficient amounts of the elicitor-binding proteins for further characterization.

This paper reports additional characterization of the behavior and properties of hepta-β-glucoside elicitor-binding proteins in soybean root membranes, and a detailed investigation of the solubilization of the binding proteins from the membranes. Specifically, we have localized the hepta-β-glucoside elicitor-binding proteins in subcellular membrane fractions of soybean root cells. We have reinvestigated the detergent-assisted solubilization of hepta-β-glucoside elicitor-binding activity from soybean root membranes and provide evidence that the nonionic detergent n-dodecylsucrose is suitable for further purification of the hepta-β-glucoside elicitor-binding proteins. Finally, we have determined the binding affinity and specificity of the solubilized elicitor-binding proteins.

**MATERIALS AND METHODS**

**Chemicals**

Tyramine, ATP (Tris salt), IDP (sodium salt), Cyt c (type II, from horse heart), and sodium orthovanadate were obtained from Sigma; β-NADH (dissodium salt) was from ICN (Costa Mesa, CA); carrier-free Na<sup>125</sup>I (13–16 mCi/mg of iodine) in dilute NaOH was from Amersham; iodogen was from Pierce. n-Dodecylglucoside, n-dodecylmaltoside, MEGA-10, Triton X-114, Thesit, Brij 35, and ZW 3–12 were purchased from Boehringer Mannheim (Indianapolis, IN), Digitonin, Hecameg, and n-dodecylsucrose were from Calbiochem (San Diego, CA); Brij 58 was from Aldrich (Milwaukee, WI).

**Colorimetric Assays**

Concentrations of oligoglucoside solutions were determined as Glc equivalents with the anthrone assay (Dische, 1962; Hahn et al., 1992), using d-Glc and d-Gal as standards. Molar concentrations of the oligoglucoside solutions were calculated taking into account the presence of unreactive glycosyl (e.g., xylopyranosyl, glucosaminyl, and glucitol residues) or partially reactive (e.g., galactosyl) residues in the oligoglucosides as described (Sharp et al., 1984a, 1984c).

Protein concentrations of membrane preparations and detergent-solubilized fractions were estimated by the Bio-Rad protein assay based on the method of Bradford (1976) using bovine γ-globulin as standard. The protein standard was dissolved in the appropriate detergent (25 μg/mL for all detergents except ZW 3–12 [5 μg/mL]) for preparation of the standard curve when determining the protein concentration of solubilized membrane proteins.

**Cotyledon Bioassay**

The abilities of various oligoglucosides to induce the biosynthesis of phytoalexins were determined using the soybean cotyledon bioassay as described (Cheong et al., 1991; Hahn et al., 1992).

**Preparation of Soybean Root Membranes**

Foundation quality soybean (*Glycine max* L. cv Williams 82) was obtained from Illinois Foundation Seeds, Inc. (Champaign, IL), and the seedlings were grown as described (Cheong et al., 1991). Unless otherwise indicated, crude total cellular membranes were prepared from the roots of 9-d-old soybean seedlings as described (Cheong and Hahn, 1991), except that 25 mM bis-Tris, pH 7.0, containing 30 mM MgCl<sub>2</sub> and 2 mM DTT was used as the homogenization buffer.

**Fractionation of Soybean Root Membranes on Suc Density Gradients**

Membrane preparation was as above except for the following modifications. Prior to homogenization, excised roots were washed three times in root wash buffer consisting of 2.5 mM bis-Tris, pH 7.0, containing 0.1 mM MgCl<sub>2</sub> and 1 mM EDTA. Roots were then minced thoroughly in 25 mM bis-Tris, pH 7.0, containing 2 mM MgCl<sub>2</sub>, 2 mM DTT, 10 mM KCl, 10 mM EDTA, and 8% (w/w) Suc (1 mL buffer/g fresh weight) using a razor blade. The minced root tissue was homogenized three times for 60 s each (speed = 8) using a Polytron homogenizer (Brinkman Instruments Co., Westbury, NY), and the homogenate was strained through two layers of Miracloth.

The supernatant from an initial rate zonal centrifugation, 4,000g at 4°C for 10 min, was loaded on top of 26-mL linear Suc density gradients consisting of 2.5 mM bis-Tris, pH 7.0, containing 0.5 mM EDTA, 1 mM DTT, and 20 to 45% (w/w) Suc for isopycnic centrifugation at 100,000g at 4°C for 3 h (Beckman L8–70M, SW-28 rotor). Gradients were fractionated into approximately 1.7-mL fractions, from the bottom up, using an LKB peristaltic pump (model 2232; Pharmacia LKB Biotechnology, Uppsala, Sweden) and an LKB fraction.
Solubilization of Glucan Elicitor-Binding Proteins

Soybean root membrane pellets from the high-speed ultracentrifugation were suspended in solubilization buffer (25 mM bis-Tris, pH 7.0, containing 100 mM NaCl, 2 mM DTT, and 10 mM MgCl₂). The membrane suspensions (5 mg/mL of protein) were mixed with aliquots of detergent stock solutions (100 mg/mL) in a final volume of 0.6 mL, stirred slowly for 30 min in an ice-water bath, and then centrifuged at 105,000g for 1 h at 4°C. The supernatants were removed and the pellets were rehomogenized in 0.6 mL of solubilization buffer. Unless otherwise indicated, the samples were used immediately in binding assays without storage.

For sequential solubilizations, the soybean root membranes were extracted with n-dodecylsucrose (10 mg/mL) as described above in a final volume of 0.6 mL, and at a final concentration of 600 mM NaCl. After ultracentrifugation as above, the supernatants were removed and kept on ice. The pellet was rehomogenized with solubilization buffer and then diluted to a final NaCl concentration of 600 mM. A second solubilization was performed, and the supernatant and the pellet were treated as described above. All the different fractions were analyzed immediately in binding and protein assays and compared with the original membrane suspension.

Stability of Elicitor-Binding Activity after Membrane Solubilization

Soybean root membranes were prepared as described above, and aliquots of the membrane preparation were stored at −80°C. The remainder of the membrane preparation was solubilized with either n-dodecysucrose or n-dodecylmalto-side. The solubilized membrane proteins were then stored at 4°C. Binding assays were done either immediately following solubilization or at different times thereafter (1, 2, 3, 7, 14, and 21 d). The binding activities of solubilized membrane preparations were expressed as a percentage of the binding activity of freshly thawed membrane suspensions aliquoted from the original membrane preparation to account for decay of the radioligand over the time course of the experiment. The binding activity of membranes stored at −80°C did not change over the time span of this experiment.

Binding Assays

Preparation of radio-iodinated tyraminylated hepta-β-glucoside elicitor was as described previously (Cheong and Hahn, 1991). The assays to determine binding of radiolabeled hepta-β-glucoside to intact membranes were carried out as described previously (Cheong and Hahn, 1991) except that the binding assay buffer was 10 mM bis-Tris, pH 7.0, containing 100 mM NaCl, 2 mM DTT, 5 mM MgCl₂, 7.5 mM β-thiogluconoside, and 7.5 mM d-gluconic acid lactone.

The binding assays for detergent-solubilized fractions were similar except that the assays contained detergent at a final concentration of 1 mg/mL. Inclusion of n-dodecysucrose at concentrations between 0.2 and 5 mg/mL had no effect on the binding assays (data not shown). The glass fiber filters (GF/B, Whatman, 2.5 cm) used to retain ligand-protein complexes were pretreated with 0.3% (w/v) polyethyleneimine.

Membrane Marker Enzyme Assays

Cyt c oxidase was assayed spectrophotometrically at 550 nm by measuring the oxidation of reduced Cyt c using a SLT ELISA plate reader (model EAR 400; SLT-Lab Instruments, Salzburg, Austria). The 0.3-mL reaction was carried out at room temperature and contained 10 to 50 µg of membrane protein, 30 mM K₂HPO₄, pH 7.2, 1 mM EDTA, 1 mg/mL reduced Cyt c, and 0.2% Triton X-100 (modified from the method of Hodges and Leonard [1974]). The specific activity of Cyt c oxidase was calculated from the initial linear rate of Cyt c oxidation (decline in A₅₅₀) using an extinction coefficient of 18.5 mM⁻¹ cm⁻¹. NADH-Cyt reductase was assayed exactly as above, except that the reaction was started with 0.5 mM NADH and contained 1 mg/mL oxidized Cyt c and 1 µM antimycin A (modified from the method of Hodges and Leonard [1974]). The specific activity of NADH-Cyt reductase was calculated as above except that the initial linear increase in A₆₉₀ was used.

Inosine diphosphatase activity was determined spectrophotometrically by following the release of Pi from IDP (modified from the methods of Hodges and Leonard [1974] and Gallagher and Leonard [1982]). Nitrate-sensitive ATPase assays were carried out using 20 to 50 µg/mL of membrane protein at a final concentration of 1 mg/mL. Inclusion of n-dodecylsucrose at concentrations between 0.2 and 5 mg/mL had no effect on the activity present after 4 to 5 d of storage of the membranes at 4°C. Vanadate-sensitive ATPase assays were carried out exactly as above except that the assays were conducted immediately after membrane fractionation and were done in 40 mM Tris-Mes, pH 6.5, containing 3 mM MgSO₄, 50 mM KCl, 2 mM NaN₃, 1 mM NaN₃MoO₄, 3 mM ATP, 0.2% Triton X-100, and with or without 50 µM NaN₃VO₄ (modified from the methods of Hodges and Leonard [1974] and Gallagher and Leonard [1982]). Nitrate-sensitive ATPase assays were carried out using 20 to 50 µg/mL of membrane protein in 25 mM BTP-Mes, pH 7.5, containing 3.75 mM MgSO₄, 1 mM NaN₃, 0.1 mM NaN₃MoO₄, 3.75 mM ATP, 250 mM sorbitol, and 0.02% Brij 58, with either 100 mM KNO₃ or 100 mM KCl (Giannini et al., 1991). Vanadate- and nitrate-sensitive ATPase activities were calculated as the difference between the activities with and without the respective inhibitor. Specific ATPase activity was calculated as above. All membrane marker experiments were repeated at least three times.

collector (model 2112). Suc density was determined using an Abbe refractometer (model 3L; Milton Roy, Rochester, NY). Gradient fractions were assayed for membrane marker enzyme activities (see below) or diluted 20-fold in gradient buffer containing 0% Suc, repelleted at 100,000g at 4°C for 30 to 45 min, and resuspended in homogenization buffer containing no Suc for use in binding assays.
membranes (Vac); vanadate-sensitive ATPase (---) = plasma membranes (PM); Cyt c oxidase (---) = mitochondrial membranes (Mit). All membrane marker experiments were repeated at least three times, and a representative data set is shown. b, Distribution of hepta-β-glucoside elicitor-binding activity in linear Suc density gradients (20-45%, w/w) of soybean root homogenates. Centrifugation conditions are as above. The binding activity was determined as cpm of 125I-labeled tyraminylated hepta-β-glucoside elicitor bound to the membranes. Data from four independent gradients are shown.

Fast Protein Liquid Chromatography

Detergent-solubilized (n-dodecylsucrose) soybean root membranes were filtered through 0.2-μm Nylon-66 membrane filters (Microfilterfuge tubes, Rainin Instrument Co., Woburn, MA) using a table-top centrifuge (Microfuge 12, Beckman) at full speed. Little if any binding activity was retained by the filters. An aliquot of the solubilized membrane protein solution (0.2 mL containing 0.6 mg of protein) was loaded onto a Superose 12 (Pharmacia LKB) column (1 cm × 30 cm) equilibrated in 25 mM bis-Tris, pH 7.0, containing 2 mM DTT, 10 mM MgCl2, 100 mM NaCl, and 0.2 mg/mL n-dodecylsucrose (see Fig. 6). The column was eluted at room temperature with the same buffer at a flow rate of 0.4 mL/min. Fractions (0.4 mL) were analyzed for hepta-β-glucoside elicitor-binding activity and protein as described above.

The column was calibrated with a mixture of molecular mass markers that included blue dextran (2000 kD), bovine thyroglobulin (669 kD), horse spleen apoferritin (443 kD), sweet potato β-amylase (200 kD), yeast alcohol dehydrogenase (150 kD), BSA (66 kD), bovine erythrocyte carbonic anhydrase (29 kD), and horse heart Cyt c (12.4 kD). All markers were purchased from Sigma.

RESULTS

Membrane Fractionation and Subcellular Localization of Hepta-β-Glucoside Elicitor-Binding Proteins

The subcellular localization of elicitor-binding proteins was determined after separating soybean root membranes by isopycnic centrifugation on linear 20 to 45% (w/w) Suc density gradients. The distribution of five membrane marker enzymes separated by such gradients is shown in Figure 1a. The location of the five enzyme activities coincided with Suc densities previously published for these plant membrane markers (Leonard and Vanderwoude, 1976; Chanson and Pilet, 1987; Morré et al., 1987; Widell and Larsson, 1990; Giannini et al., 1991). The distribution of elicitor-binding activity after isopycnic centrifugation of soybean root cell homogenate on linear 20 to 45% (w/w) Suc density gradients is shown in Figure 1b. The elicitor-binding activity peaked at a density of 37% Suc, which coincided with the peak of vanadate-sensitive H+-ATPase activity (Fig. 1a), a marker enzyme for the plant plasma membrane.

Solubilization of the Hepta-β-Glucoside Elicitor-Binding Activity from Soybean Root Membranes

A number of nonionic and a zwitterionic detergents were tested for their ability to solubilize hepta-β-glucoside elicitor-binding activity from soybean root membranes (Fig. 2). Solubilization buffer containing 1 mM NaCl, but no detergent, was unable to solubilize hepta-β-glucoside elicitor-binding activity from soybean root membranes, although 10 to 30% of the total protein was released. The nonionic detergents n-dodecylsucrose, n-dodecylmaltoside, and Triton X-114 were the three most effective detergents tested. n-Dodecylsucrose and n-dodecylmaltoside solubilized increasing amounts of elicitor-binding activity up to a detergent concentration of 5 mg/mL. Further increases in detergent concentration only mar-
activity was recovered in the supernatant and in the pellet after high-speed centrifugation of detergent-treated membranes over all detergent concentrations examined (Fig. 2). The single exception to this pattern was the zwitterionic detergent ZW 3–12, which showed a markedly different solubilization profile from the nonionic detergents. At low concentrations (<2 mg/mL), ZW 3–12 solubilized up to 38% of the elicitor-binding activity and 74% of the total protein present in the membranes. However, higher detergent concentrations reduced the binding activity recovered in the supernatant. Furthermore, little or no elicitor-binding activity could be recovered in the residual membrane pellets after solubilization with ZW 3–12 at concentrations higher than 2 mg/mL. Thus, ZW 3–12 showed a tendency to inactivate the elicitor-binding proteins.

A second extraction of the soybean root membranes with nonionic detergents yielded only a 10 to 20% improvement in the yield of solubilized elicitor-binding activity (Table I). Although there was no detectable loss of protein throughout the detergent extractions, the second detergent extraction resulted in a loss of about 30% of the elicitor activity found in the original untreated membranes. In total, two successive extractions of soybean root membrane suspension with 10 mg/mL n-dodecylsucrose solubilized a total of 60% of the initial binding activity. Essentially similar results were obtained with n-dodecylmaltoside (data not shown).

**Stability of Hepta-β-Glucoside Elicitor-Binding Activity after Solubilization**

The activity of the elicitor-binding proteins, solubilized with either n-dodecylsucrose or n-dodecylmaltoside, declined markedly (about 40%) during the first 2 d of storage in solubilization buffer at 4°C (Fig. 3). Only 10% of the original

| Table I. Recovery of hepta-β-glucoside elicitor-binding activity and protein after two successive solubilizations of soybean root membranes |
|---------------------------------|-----------------|----------------|
| Percentage of Original Membrane Suspension |
| Binding activity | Protein |
| --- | --- | --- | --- |
| **First solubilization** | | |
| Supernatant (S1) | 54.5 ± 5.5 | 84.7 ± 8.4 |
| Pellet (P1) | 51.2 ± 15.2 | 19.5 ± 0.8 |
| **Second solubilization** | | |
| Supernatant (S2) | 5.9 ± 1.6 | 13.0 ± 0.7 |
| Pellet (P2) | 10.5 ± 3.6 | 4.3 ± 0.7 |
| **Total solubilized (S1 + S2)** | 60.4 | 97.7 |
binding activity was lost in storage during the succeeding 10 d, after which the activity appeared to remain stable.

Characterization of the Binding of Radiolabeled Hepta-β-Glucoside Elicitor to Detergent-Solubilized Binding Proteins

Experiments were performed to ascertain whether detergent solubilization altered the properties of the elicitor-binding proteins. These experiments were carried out using n-dodecysucrose and ZW 3–12 to determine whether the type of detergent used had any effect on the elicitor-binding properties of the solubilized proteins.

The saturability of the binding of radiolabeled hepta-β-glucoside elicitor to the elicitor-binding proteins solubilized from soybean membranes with 10 mg/mL n-dodecysucrose was examined by incubating the solubilized fraction with increasing concentrations of the radiolabeled ligand (Fig. 4, inset). Specific binding was saturable, and nonspecific binding was less than 5% of specific binding at an initial ligand concentration of 1.0 pmol/mg protein (data not shown), in agreement with published results (Cosio et al., 1992). The Hill plot was linear and the Hill coefficient was 1.03 (data not shown). Nonspecific binding was less than 5% of specific binding at an initial ligand concentration of 5.5 nM.

Ligand Specificity of Detergent-Solubilized Hepta-β-Glucoside Elicitor-Binding Proteins

The selectivity of solubilized elicitor-binding proteins for various oligoglucosides structurally related to the hepta-β-glucoside elicitor was tested in ligand competition assays to determine whether the solubilized binding proteins retained the specificity exhibited by the membrane-localized binding proteins. The results of these assays, shown in Figure 5a and Table II, demonstrate that the binding proteins solubilized from membranes with n-dodecysucrose or with ZW 3–12 exhibit strong selectivity in terms of the oligoglucosides that are recognized efficiently. Furthermore, the specificity of the detergent-solubilized binding proteins was essentially the same as that of the membrane-localized binding proteins.
Correlation between the Binding Affinities and the Elicitor Activities of Oligosaccharides

The abilities of oligogluicosides structurally related to the hepta-β-glucoside elicitor to induce phytoalexin accumulation in soybean cotyledon tissue was compared with the effectiveness of the oligosaccharides as competitors in the ligand-binding assay. The ligand competition assays employed preparations of either membrane-localized binding proteins or binding proteins solubilized with n-dodecylsucrose or ZW 3-12. The results of these assays, shown in Table II, demonstrate a strict correlation between the abilities of the oligogluicosides to induce phytoalexin accumulation and their abilities to bind to the elicitor-binding proteins. Those oligosaccharides having a high elicitor activity were efficient competitors of the radiolabeled hepta-β-glucoside elicitor, whereas biologically less active oligosaccharides were correspondingly less efficient.

Gel-Permeation Chromatography of n-Dodecylsucrose-Solubilized Hepta-β-Glucoside Elicitor-Binding Proteins

Gel-permeation chromatography was carried out with the n-dodecylsucrose-solubilized membrane proteins in an attempt to determine the approximate size of the solubilized hepta-β-glucoside elicitor-binding proteins and to ascertain whether such chromatography could be used as an initial purification step. Since the detergent concentrations used for membrane solubilization and subsequent column chromatography were above the critical micelle concentration for n-

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**Table II. Comparison of the abilities of oligosaccharides to induce phytoalexin accumulation in soybean cotyledons and to inhibit the binding of radiolabeled hepta-β-glucoside elicitor to intact and detergent-solubilized soybean root membranes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elicitor Activity (EC50, nM)</th>
<th>Membranes</th>
<th>Solubilized Dodecylsucrose ZW 3-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>21</td>
<td>5.8</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
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<td>950</td>
</tr>
<tr>
<td>6</td>
<td>1,390</td>
<td>930</td>
<td>870</td>
</tr>
<tr>
<td>7</td>
<td>6,900</td>
<td>16,000</td>
<td>8,000</td>
</tr>
<tr>
<td>8</td>
<td>26,000</td>
<td>17,000</td>
<td>21,000</td>
</tr>
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</table>

* Structures of oligosaccharides 1 to 8 are depicted in Figure 5b.  
** Elicitor activities were determined using the soybean cotyledon bioassay as described (Cheong et al., 1991). The relative elicitor activity (EC50) is defined as the concentration of an oligosaccharide required to give half-maximum induction of phytoalexin accumulation (A/Ao = 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 to 28 nM (Cheong et al., 1991). The relative binding activity (IC50) is defined as the concentration of an oligogluicoside required to give 50% inhibition of the binding of radiolabeled hepta-β-glucoside 1 to its binding site in detergent-solubilized and intact soybean root membranes (see Fig. 5a).  
* n.d., Not determined.
dodecylsucrose (0.16 mg/mL), we presume that the solubilized membrane proteins are in the form of protein-detergent micelles. Slightly over half of the elictor-binding activity (60%) recovered from the column eluted in the void volume, in which only 3.7% of the protein eluted (Fig. 6). Most of the remaining elictor-binding activity (30%) eluted with micelles having molecular masses between 200 and 670 kD. Only 10% of the elictor-binding activity eluted with the smallest micelles, which contained 70% of the eluted protein. The chromatography also removed UV-absorbing material that eluted after the included volume of the column and contained no elictor-binding activity or protein (data not shown). Overall recovery of elictor-binding activity from the column was 49%, and recovery of the applied protein was 97%. The specific elictor-binding activity of the largest micelles was about 9500 cpm/µg protein, about 150-fold higher than the specific binding activity of the smallest micelles. Overall, gel-permeation chromatography of the detergent-solubilized soybean root membrane proteins yielded an 8-fold purification of the hepta-β-glucoside elictor-binding proteins in the pooled fractions eluting in the void volume of the column when compared with the crude solubilized membrane proteins.

**DISCUSSION**

Previous studies have demonstrated that specific, high-affinity binding proteins for the hepta-β-glucoside elictor exist in soybean membranes (Cosio et al., 1990b, 1992; Cheong and Hahn, 1991). We have shown here that the majority of the hepta-β-glucoside elictor-binding activity co-sediments with a plasma membrane marker (vanadate-sensitive H+-ATPase) on linear Suc density gradients (Fig. 1), providing evidence that the elictor-binding proteins are plasma membrane localized. Our results are consistent with previous reports of subcellular localization of glucan elictor-binding sites. For example, Schmidt and Ebel (1987), using a partially purified elictor-active mixture of 3H-labeled glucan fragments, reported that β-glucan binding sites were associated with plasma membrane-enriched fractions of soybean membranes in Suc density gradients. The presence on soybean protoplasts of binding sites for a partially purified elictor-active mixture of 125I-labeled oligoglucosides has also been reported (Cosio et al., 1988). Our localization data obtained using the homogeneous hepta-β-glucoside elictor provide additional evidence that the binding sites first identified using partially purified glucan elictor preparations (Schmidt and Ebel, 1987; Cosio et al., 1988) are indeed hepta-β-glucoside elictor-binding sites (Cosio et al., 1990b, 1992; Cheong and Hahn, 1991).

The inclusion, in the ligand competition assays reported here (Fig. 5, Table II), of additional synthetic oligosaccharides that were not available in previous studies (Cheong et al., 1991; Cheong and Hahn, 1991), provides additional information about which structural features of the hepta-β-glucoside elictor are important for recognition by the binding proteins. The new oligosaccharides (Fig. 5b, compounds 3, 5, and 8) further demonstrate the importance of the nonreducing terminal backbone glucosyl residue for both the biological activity and the binding affinity of elictor-active oligoglucosides. Pentaglucoside 8, which lacks the nonreducing terminal glucosyl residue, is about 1000-fold less active in both the elictor and binding assays. The relative activities of the hexa- and hepta-oligosaccharides, in which the nonreducing terminal glucosyl residue has been substituted with xylosyl- (compound 3), galactosyl- (compound 5), or glucosaminyl- (compound 6) residues, provide information about the relative importance of the hydroxyl groups of this backbone glucosyl residue for the interactions of oligoglucoside elictors with their binding site. Thus, OH-6 (absent in the xylosyl-substituted hexaglucoside) appears to be least important, OH-4 (epimerized in the galactosyl-substituted hexaglucoside) is of intermediate importance, and OH-2 (amino-substituted in compound 6) is the most important. Whether these data identify functional groups on the hepta-β-glucoside elictor that interact directly with the binding protein or whether the substituted oligoglucosides adopt a different conformation that fits less efficiently into the binding site cannot be determined until the binding protein has been purified.

Purification of the hepta-β-glucoside elictor-binding proteins will require the removal of the binding proteins from the membranes in a form that retains their elictor-binding properties. No hepta-β-glucoside elictor-binding activity was released from soybean root membranes by 1 M NaCl (Fig. 2), confirming previous results (Cosio et al., 1990a) and suggesting that the elictor-binding proteins are integral membrane proteins. We have shown here that hepta-β-glucoside elictor-binding proteins can be solubilized in reasonable yield and
with minimal losses in binding activity using the three non-ionic detergents n-dodecylmaltoside, n-dodecylsucrose, and Triton X-114. As reported previously (Cosio et al., 1990a), the zwitterionic detergent ZW 3–12 also solubilizes up to 40% of the membrane-localized elicitor-binding activity at low detergent concentrations, but shows a tendency to inactivate the elicitor-binding proteins at higher detergent concentrations (Fig. 2). These results confirm to a large extent the overall pattern observed in the earlier solubilization study carried out by Cosio et al. (1990a), except that we achieved maximal solubilization of elicitor-binding activity from the membranes using 3- to 4-fold lower detergent concentrations than were utilized previously.

We have demonstrated here that detergent-solubilized elicitor-binding proteins fully retain their affinity (Fig. 4) and, more importantly, their binding specificity for homogeneous elicitor-active oligoglucosides (Fig. 5). Furthermore, the correlation, originally observed with intact soybean root membranes (Cheong and Hahn, 1991), between the elicitor activity of an oligosaccharide and the ability of an oligosaccharide to be recognized by the elicitor-binding proteins was also observed for the detergent-solubilized binding proteins (Table II). That is, biologically active oligoglucosides (e.g. hepta-β-glucoside 1 and hexa-β-glucoside 2) are efficient competitors of the radiolabeled hepta-β-glucoside elicitor, whereas biologically less active oligosaccharides (compounds 3-8) are correspondingly less efficient competitors in the binding assays. Thus, the elicitor-binding proteins can be solubilized from soybean root membranes in fully functional form using either nonionic detergents (e.g. n-dodecylsucrose) or the zwitterionic detergent ZW 3–12. This is an essential prerequisite for the purification of these binding proteins, which will undoubtedly require affinity methods (Jacobs and Cuatrecasas, 1981).

The binding characteristics of the elicitor-binding proteins are not affected by the detergent used for their solubilization. The apparent Kₐ obtained from ligand saturation plots using n-dodecylsucrose- and ZW 3–12-solubilized elicitor-binding proteins are the same (Kₐ approximately 1–2 nM). The specificity of the elicitor-binding proteins for various oligoglucosides is also similar for binding proteins solubilized with either detergent (Table II). These results suggest that the losses in elicitor-binding activity observed with higher concentrations of ZW 3–12 are not due to a reduction in affinity of the detergent-treated binding proteins for the hepta-β-glucoside elicitor, but rather result from an inactivation (e.g. denaturation) of the binding proteins.

The elicitor-binding activity is somewhat labile once extracted from the membranes by the nonionic detergents n-dodecylsucrose and n-dodecylmaltoside (Fig. 3). About half of the solubilized hepta-β-glucoside elicitor-binding activity is lost within the first 48 h of storage at 4°C (Fig. 3), although the remaining elicitor-binding activity appears to be reasonably stable over a 2-week time period. Separation of the elicitor-binding proteins from the bulk of the membrane proteins by gel-permeation chromatography (Fig. 6) does not improve the stability of the elicitor-binding activity (data not shown). However, inclusion of the protease inhibitor leupeptin in the solubilization buffer reduces the loss of activity of the solubilized binding proteins after 48 h of storage from 27 to 2%. Other protease inhibitors are not effective in stabilizing the solubilized elicitor-binding activity (data not shown). These results suggest that most of the observed lability of the solubilized binding proteins is due to the presence of a low level of protease activity in the extracts.

Cosio et al. (1990a) were unable to obtain any chromatographic separation of elicitor-binding activity using n-dodecylmaltoside-solubilized membrane proteins and achieved only partial separation of ZW 3–12-solubilized elicitor-binding proteins on a gel-permeation column. In contrast, we obtain a clear separation of the bulk of the n-dodecylsucrose-solubilized elicitor-binding activity from the bulk of the membrane proteins on a gel-permeation column (Fig. 6). An 8-fold purification was achieved for the elicitor-binding proteins eluting in the void volume of the column when compared with the crude solubilized membrane proteins, and about half of the elicitor-binding activity applied to the column was recovered. Thus, a degree of purification of the elicitor-binding proteins was achieved in a single step equivalent to that reported earlier after three steps (ion exchange, PEG precipitation, gel permeation) (Cosio et al., 1990a). The reason(s) for the differing success in achieving chromatographic separation of elicitor-binding activity with n-dodecylsucrose and n-dodecylmaltoside are not clear, particularly since both detergents have comparable structures and properties (e.g. critical micelle concentration) and the gel-permeation matrices used in the two studies have similar size-fractionation ranges. Based on their results, Cosio et al. (1990a) concluded that the zwitterionic detergent ZW 3–12 was most suitable for elicitor-binding protein purification and that the nonionic detergent n-dodecylmaltoside was unsuitable. The data reported here on the efficiency of solubilization and subsequent behavior of the solubilized binding proteins on a gel-permeation column lead us to suggest that the nonionic detergent n-dodecylsucrose merits serious consideration as an alternative detergent to the zwitterionic ZW 3–12 for the solubilization and purification of hepta-β-glucoside elicitor-binding proteins.

The results of the gel-permeation chromatography of n-dodecylsucrose-solubilized hepta-β-glucoside elicitor-binding proteins reported here (Fig. 6) and of ZW 3–12-solubilized binding proteins reported previously (Cosio et al., 1990a) indicate that elicitor-binding activity is associated primarily with large detergent-protein micelles (Mₙ > 200,000). Indeed, the highest specific elicitor-binding activity is observed in detergent-protein micelles having an Mₙ > 660,000 (Fig. 6). Little or no elicitor-binding activity is associated with the smallest detergent-protein micelles. These data suggest that the elicitor-binding activity consists of either a multimeric protein complex or a single large polypeptide (Mₙ > 200,000), although photoaffinity labeling studies suggest a smaller size (Cosio et al., 1992). Alternatively, the elicitor-binding activity could require a protein conformation preferentially stabilized in large detergent micelles. The available data suggest that successful purification of elicitor-binding proteins in sufficient quantities for characterization will require stabilization of detergent-protein micelles of sufficient size to retain elicitor-binding activity. The data reported here suggest that n-dodecylsucrose is a suitable detergent in this regard, since it
appears to favor the solubilization of elicitor-binding proteins in the form of large detergent-protein micelles.

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