High-affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors to soybean membranes

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Soybean membranes possess high-affinity binding sites for fungal β-glucans that elicit phytoalexin synthesis. The ability of 1,3-1,6-β-glucans, released by acid hydrolysis from mycelial walls of *Phytophthora megasperma* f.sp. *glycinea*, to compete for the putative phytoalexin elicitor receptors increases with their average degree of polymerization (DP). The results suggest a function where the probability for glucan fragments of containing a structural determinant that is optimal for binding approaches 1 as the DP tends to infinity. Ligand displacement data obtained against a ^{125}I-labeled glucan elicitor (average DP = 18) provided a theoretical minimum IC_{50} (50% inhibitory concentration) for 1,3-1,6-β-glucans of 3 nM. The IC_{50} value obtained for a synthetic hepta-β-glucoside having a known elicitor-active structure was 8 nM, remarkably close to the predicted value. Displacement of the ^{125}I-glucan of large DP was uniform and complete showing that the heptaglucoside had access, with similar affinity, to all sites available to the radioligand. Further analysis using a ^{125}I-labeled aminophenethylamine derivative of the heptaglucoside suggested that the putative glucan-elicitor receptors bind a basic structural determinant present in all elicitor-active glucans from the soybean pathogen *P. megasperma*.

Heptaglucoside; Membrane; Phytoalexin elicitor; Receptor

1. INTRODUCTION

The elicitation of the synthesis of isoflavonoid phytoalexins in soybean (*Glycine max.* L.) by fungal glucans is one of the best-studied cases of inducible plant defense responses [1]. The glucan elicitors from the fungus *Phytophthora megasperma* f.sp. *glycinea* (Pmg), a soybean pathogen, were among the first characterized [2,3]. The isolation from fungal cell wall hydrolysates of a branched 1,3-1,6-hepta-β-glucoside with high elicitor activity [4,5] provided initial information on the minimum structural requirements for elicitor activity in soybean. This was confirmed using a chemically synthesized heptaglucoside [6]. It is not clear whether the heptaglucoside is actually generated in in vivo interactions or if it functions as a structural determinant within larger glucans. In either case, the most widely proposed model for elicitor recognition would involve the existence of cell surface receptors for specific glucan elicitors [1], one of which may be the heptaglucoside.

The reports on the existence and characteristics of high-affinity binding sites for branched 1,3-1,6-β-glucans from Pmg cell walls [7–9] provided the initial evidence that elicitor recognition may be a receptor-mediated process. Glucan binding to soybean membranes fulfilled the criteria of saturability, reversibility, localization on the plasma membrane and high affinity (K_d = 10–30 nM). Binding studies, so far, employed glucan preparations consisting of a mixture of structural isomers of large DP within a narrow molecular size range [7,8]. The reasons for this were twofold. First, the use of larger glucans with a variety of branching patterns assured a broad enough approach to the identification of glucan-elicitor binding sites in soybean membranes, something a single structure might have omitted. Second, both the preparative isolation [4,5] and chemical synthesis [10] of 1,3-1,6-hepta-β-glucoside or other pure oligosaccharides were of limited applicability due to either extremely low yields or restricted access to synthetic expertise. The more recent availability of synthetic heptaglucoside elicitor obtained by refined and faster synthetic procedures [11] has made it possible to do comparative studies on the specificity of heptaglucoside binding to soybean membranes. In the present report we have analysed the binding characteristics of this synthetic elicitor and provide evidence that the binding of fungal glucans to the putative elicitor receptors of soybean may depend on a basic structural determinant, common to all elicitor-active glucans.

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Abbreviations: APEA, 2-(4-aminophenyl)ethylamine; DP, degree of polymerization; HG-APEA, 1-[2-(4-aminophenyl)ethyl]amino-1-[hexaglucosyl]deoxyglucitol; Pmg, *Phytophthora megasperma* f.sp. *glycinea*

2. MATERIALS AND METHODS

2.1. *Plant material and microsomal fractions*

Soybean seeds (*Glycine max* L. cv. Kalmit) were kindly provided by

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Kleinwanzlebener Saatzucht (Einbeck, FRG). Seven-day-old plants were grown as described by Schmidt and Ebel [7]. Microsomal fractions were prepared as previously described [8] and stored frozen at −70°C until needed.

2.2. *Preparation of glucan elicitors*

Raw glucan elicitor was prepared by acid hydrolysis of Pmg cell walls as described previously [7,8]. Size-fractionation of the glucan fragments was performed on a calibrated Bio-Gel P-4 (−400 mesh) gel permeation column (2.6 × 100 cm) at 50°C. Elution was performed with distilled water at a flow rate of 24 ml/h. The eluate was monitored using refractometric and ultraviolet (280 nm) detectors placed in series. The 1,3-1,6-hepta-β-glucoside was synthesized following the second synthetic route as reported by Fügedi et al. [11]. Most of the steps proceeded as described. However, the silver triflate-mediated glycosylation of the donor 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide with the acceptor methyl 4,6-benzylidene-1-thio-β-D-glucopyranoside did not proceed to completion due to anomerization of the acceptor into the corresponding methyl 1-thio-α-D-glucopyranoside. This resulted in a lower yield of the required dimer (i.e. 35%) than the one reported [11] (i.e. 55%). Nevertheless, the glucoheptaose was obtained in reasonable yield. Both ^{1}H and ^{13}C NMR-spectra obtained were in full accordance with the proposed structure.

2.3. *Synthesis of glucan derivatives*

The synthesis of the tyramine conjugate of the oligo-β-glucan (average DP = 18) and its radioiodination have been described previously [8]. The aminophenethylamine conjugate of the heptaglucoside (HG-APEA) was synthesized by reductive amination using 2-(4-aminophenyl)ethylamine (APEA) as described before [8], with minor modifications. Excess APEA was extracted after the reaction by addition of water and partition into ethyl acetate (10 times). The remainder was acidified to pH 5 with 1 N acetic acid and diluted 10-fold with 2 mM pyridinium acetate buffer, pH 5. HG-APEA was separated from unreacted carbohydrate and remaining APEA by cation exchange chromatography on S-Sepharose. After washing the column with pyridinium acetate buffer, bound HG-APEA was eluted with 0.1 N NH_4OH and monitored at 285 nm. The HG-APEA pool was taken to dryness in a centrifugal evaporator and redissolved in 1 ml of water.

2.4. *Radioiodination of HG-APEA*

Iodination of the aminophenyl moiety of HG-APEA was performed as described by Lowndes et al. [12]. The ^{125}I-labeled HG-APEA was purified by chromatography on S-Sepharose (1 ml column) as described above. Fractions containing the radioactive peak were pooled, neutralized with 1 N HCl and diluted to 8 ml with 0.1 M Tris-HCl, pH 7. Incorporation of iodine was about 60%. The specific radioactivity of the product was about 100 Ci/mmol (3.7 TBq/mmol).

2.5. *Glucan binding and biological activity assays*

The glucan binding assays contained 12 nM of either large-DP ^{125}I-glucan (120 Ci/mmol, 4.4 TBq/mmol) [8,9] or ^{125}I-labeled HG/APEA (3.7 TBq/mmol) and were performed as described previously [9] using 100–150 μg protein. Protein content in microsomal membranes was measured according to Bradford [13]. The ability of compounds to induce phytoalexin production was measured in a bioassay [2,14] using cotyledons from 7-day-old soybean seedlings.

3. RESULTS AND DISCUSSION

Glucan fractions of increasing average DP values were prepared by acid hydrolysis of Pmg mycelial walls followed by gel permeation chromatography. The resulting fractions consisted of mixtures of structural isomers of branched β-glucans with similar apparent molecular radii. The affinity of soybean β-glucan-binding sites for these fractions was analysed in ligand competition assays against a ^{125}I-labeled 1,3-1,6-β-glucan with an average DP of 18 [8]. The concentration of unlabeled glucan required for half-maximal displacement of the radioligand (IC_{50}) decreased by about two orders of magnitude when apparent DP values increased from 5 to 22 (not shown). The results of plotting $-\log[IC_{50}]$ against DP for each of the fractions are shown in Fig. 1. The curve fits a hyperbolic function as can be seen from the Hanes-Woolf linearization of the data (Fig. 1, inset). The hyperbola would be asymptotic to a $-\log[IC_{50}]$ value corresponding to a minimum theoretical IC_{50}, or maximum binding affinity, approached as the apparent DP tends to infinity. This value was calculated to be 3 nM. The higher apparent affinity of the soybean binding sites for the larger glucans was also observed in an earlier study [8] but was not further characterized. The most likely explanation for the pronounced dependence of ligand affinity on the apparent DP of the glucan fractions would be that the soybean binding sites recognize one or more 'basic' structural determinants within the glucans. The probability for a certain glucan of containing such structures increases with its size. The analysis of the binding data allowed us to make a prediction on the affinity for these putative 'structural determinants' The predicted value of 3 nM would represent a minimum average IC_{50} for these units in competition with our standard large-DP glucan.

A small elicitor-active oligoglucoside of known structure for soybean is the 1,3-1,6-hepta-β-glucoside

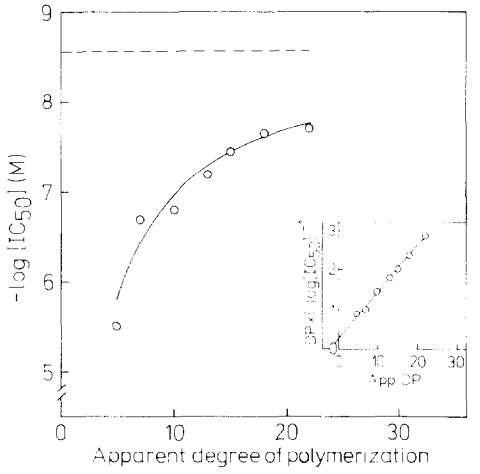

Fig. 1. Affinity of soybean membrane binding sites for *P. megasperma* 1,3-1,6-β-glucan fractions of varying size. Glucan fractions with increasing degrees of polymerization (DP), obtained by gel permeation chromatography of mycelial wall hydrolysates, were tested in radioligand displacement assays against an ^{125}I-labeled β-glucan elicitor (average DP = 18). Half-maximal displacement values (IC_{50}) are plotted in semilogarithmic form against the apparent DP of the fractions. The dashed line represents the minimum theoretical IC_{50} obtained from a Hanes-Woolf plot of the data (inset).

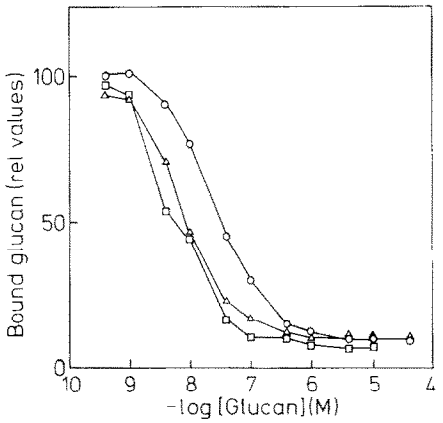

Fig. 2. Competitive displacement of 12 nM ^{125}I-labeled glucan (average DP = 18) from soybean membranes by increasing concentrations of a *P. megasperma* β-glucan fraction with an apparent DP of 22 (○), a synthetic 1,3-1,6-hepta-β-glucoside (△) and a 2-(4-aminophenyl)ethylamine derivative of the heptaglucoside (□).

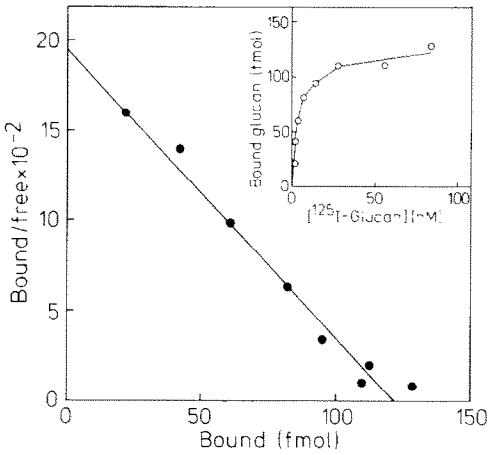

Fig. 3. Scatchard plot of ligand-binding data for the interaction of ^{125}I-labeled HG-APEA with soybean membranes. Specific binding was measured in a radioligand concentration range between 1 and 84 nM, using 120 μg of membrane protein per assay. Nonspecific binding was measured in the presence of 10 μM unlabeled heptaglucoside. The inset shows the ligand-saturation plot used for the analysis.

characterized by Sharp et al. [4,5]. This compound was analysed by ligand displacement against our large-DP ^{125}I-labeled glucan. The results, shown in Fig. 2, indicate that the glucan binding sites have a significantly higher affinity for the heptaglucoside than for a glucan fraction with an apparent DP of 22. The average IC_{50} value for heptaglucoside was 8 nM compared to 26 nM for the larger glucans. This value is the lowest of all glucan fractions analysed to date and very near to our theoretical prediction (see above). Additional features of this system are the complete displacement of radioligand by heptaglucoside at about 1 μM and the uniformity of the displacement pattern. This clearly shows that the heptaglucoside has complete access to all binding sites available to the large-DP glucan. If the binding sites recognize several distinct structural determinants within the Pmg cell wall glucans, then all of those present in our large-DP glucan fraction can be displaced by the single heptaglucoside tested. In addition to this, the apparent uniformity of the displacement pattern permits us to predict that should there be more than one 'receptor' or binding site for the soybean system, then all display very similar affinities for the heptaglucoside.

Further study of heptaglucoside binding to soybean membranes required the synthesis of a suitable radioactive derivative. The HG-APEA conjugate was prepared by a slight modification of a method used before for large-DP glucans [8]. This type of derivative was chosen because the aryl amino group offers a greater flexibility for future chemical modifications than the aryl hydroxy group of tyramine. HG-APEA showed no major loss in binding affinity when compared to underivatized heptaglucoside and to a glucan fraction with a DP of 22 (Fig. 2).

The K_d value for the interaction of soybean β-glucan-binding sites with ^{125}I-labeled HG-APEA was calculated from ligand saturation experiments (Fig. 3, inset). A Scatchard plot of this data provided a K_d value of about 3 nM for the complex (Fig. 3). This is the highest affinity for all glucans analyzed to date. The results suggest that the heptaglucoside may possibly be an optimal structural determinant for binding for this system. Ultimate identification of the structure with highest affinity for the putative soybean receptors will require further structure-affinity studies using a variety of alternative synthetic oligo-β-glucosides.

A comparison of the biological activities of the heptaglucoside and other glucan fractions tested is included in Table I along with the corresponding ligand displacement data obtained against ^{125}I-labeled HG-APEA. The data confirm the close correlation between the binding affinity for a glucan fraction (expressed as IC_{50}) and the corresponding phytoalexin elicitor activity as

Table I

Correlation between the binding affinity (IC_{50}) of soybean membranes for branched β-glucan fractions and the corresponding activity of the glucans as phytoalexin elicitors (EC_{50}) in a bioassay

β-Glucan fraction	IC_{50} (nM)	EC_{50}(μM)
App. DP = 5	12 000	200
App. DP = 10	410	12
App. DP = 16–20	100	0.6
Synthetic heptaglucoside	16	0.22

Binding affinity of the glucans is shown as the concentration necessary for half-maximal displacement of 12 nM ^{125}I-labeled HG-APEA from soybean membranes in ligand competition assays. Elicitor activity is shown as the concentration necessary for half-maximal response in a soybean cotyledon bioassay. Apparent DP values for each fraction were estimated using gel permeation chromatography. Results are averages of 2 (IC_{50}) or 3 (EC_{50}) independent experiments.

measured in a soybean cotyledon bioassay (shown as EC_{50}). The heptaglucoside not only displayed highest binding affinity (lowest IC_{50}) but had also the highest phytoalexin elicitor activity (lowest EC_{50}) in this study. These results support our interpretation of the binding data regarding the specificity of the recognition mechanism for β-glucan elicitors in soybean.

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