Solubilization of soybean membrane binding sites for fungal β-glucans that elicit phytoalexin accumulation

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Soybean membranes contain high-affinity binding sites for fungal β-glucans. These sites may play a role in the recognition by soybean tissues of fungal phytoalexin elicitors. We have solubilized β-glucan-binding activity from microsomal membranes using two C_{12}-alkyl zwitterionic detergents, Zwittergent 3-12 (ZW 3-12) and the lysolecithin analog 1-dodecyl-2-deoxy-phosphorylcholine (ES12H). The solubilized binding sites displayed identical affinity for β-glucans as that found in membranes ($K_D = 11$–34 nM). Detergent-protein micelles with glucan binding activity eluted with approximate M_r values of 300 000 in ZW 3-12 and 380 000 in ES12H in gel permeation chromatography. Maximal binding activity eluted from a chromatofocusing column in the pH range between 6.2 and 6.6 with both ES12H and ZW 3-12, suggesting an apparent p*I* close to neutral.

β-Glucan-binding protein; Phytoalexin-elicitor receptor; Solubilization

1. INTRODUCTION

Defense responses in plants are assumed to be triggered after specific recognition by plant cells of components from an incompatible pathogen. Both the signal molecules and the recognition mechanisms associated with this process most likely involve cell surface components of pathogen and host. The phytoalexin elicitors from *Phytophthora megasperma* f.sp. *glycinea* (Pmg), for the interaction with soybean (*Glycine max* L.), have been identified as glucans containing mainly 1,3-1,6-β-linkages [1–4]. Little, however, is known about the mechanisms underlying elicitor recognition by soybean cells.

Initial identification in soybean cell membranes of binding sites which might function as receptors for fungal β-glucans was obtained with mycolaminaran, a glucan with low elicitor activity [5]. More recently, ^{3}H- and ^{125}I-labeled Pmg 1,3-1,6-β-glucan derivatives with high elicitor activity and within a narrow molecular size range (18–22 glucose units) were used to show the existence of high-affinity binding to soybean membranes [6,7]. The observed binding was saturable, reversible, associated with the plasma membrane and accessible at the surface of intact protoplasts. Further characterization of these β-glucan-binding sites may provide information on the mechanisms involved in elicitor recognition for the soybean-Pmg system through the identification of a putative receptor for glucan elicitor. The present report describes the solubilization of glucan-binding activity from soybean membranes and discusses some of its properties.

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Abbreviations: CMC, critical micellar concentration; DM, dodecyl maltoside; ES12H, 1-dodecyl-2-deoxy-phosphorylcholine; PEG, polyethylene glycol; Pmg, *Phytophthora megasperma* f.sp. *glycinea*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ZW 3-12, dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate

2. MATERIALS AND METHODS

2.1. *Chemicals*

Dodecyl maltoside, hexyl and octyl glucoside and the Zwittergent series of detergents (*N*-alkyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate) were purchased from Calbiochem (Frankfurt, FRG) or Boehringer Mannheim (Mannheim, FRG); ES12H (1-dodecyl-2-deoxy-phosphorylcholine) was a gift from Dr W. Welte, (Freiburg, FRG); Na^{125}I (592 MBq/μg iodine) was from Amersham Buchler (Braunschweig, FRG); Sephacryl S-300 HR, Q-Sepharose, and Polybuffer 74 were from Pharmacia (Freiburg, FRG); all other reagents were obtained from Sigma Chemie (München, FRG).

2.2. *Plant material and microsomal fractions*

Soybean seeds (*Glycine max* L. Merr. cv Effi) were kindly provided by Kleinwanzlebener Saatzucht (Einbeck, FRG). Roots from 7-day-old seedlings were used as starting material. Microsomal fractions were prepared and stored frozen as previously described [7].

2.3. *Membrane solubilization*

Microsomal membranes were suspended in 25 mM Tris-HCl, pH 7, containing 2 mM DDT. The suspension was centrifuged at 100 000 × *g* for 30 min. The washed membranes were resuspended at a detergent/protein ratio of 2:1 in 1% ZW 3-12 in the same buffer. The suspension was stirred slowly for 30 min over ice and then centrifuged for 1 h at 100 000 × *g*. The supernatant was filtered through a 0.22 μm-pore membrane, taken to 20% (v/v) glycerol and stored at -70°C in aliquots until used. Prior to further analysis, the solubilized material was thawed and loaded onto a Q-Sepharose anion exchange column (20 cm long, 3 ml matrix/mg protein) equilibrated with 50 mM Tris-HCl, pH 7.5, 10% glycerol and 0.15% (w/v) ZW 3-12. After

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washing with 3 column vol of the same buffer, the proteins were eluted with buffer containing 0.22 M NaCl. This fraction was concentrated by taking it to 20% (w/v) PEG 6000 with slow stirring for 1 h over ice, and centrifugation at 100 000 × *g* for 1 h. The pellet was resuspended in a detergent-containing buffer appropriate for the analysis to be performed at a protein concentration of 2–4 mg/ml. This fraction is referred to as the 'PEG fraction' throughout the text.

2.4. *Glucan binding assay*

The synthesis of tyramine derivatives of the fungal glucans and their radioiodination have been described previously [7]. A modified assay for glucan binding contained 12 nM ^{125}I-glucan (120 Ci/mmol or 4.4 TBq/mmol), 10 mM Tris-HCl, pH 7, 0.1 M NaCl, 5 mM $MgCl_2$, 5 mM each of β-D-thioglucose and D-gluconic acid lactone and microsomes or solubilized protein (5–300 μg protein) in a final vol. of 200–400 μl. Nonspecific binding was determined in the presence of 10 μM unlabeled glucan. Binding assays with ZW 3-12-solubilized proteins contained 0.7 mg/ml ZW 3-12. Incubation was carried out for 2 h at 4°C. Assays were terminated by filtration under vacuum through polyethyleneimine-impregnated glass fiber filters [8]. The filters were then washed with 10 ml of 10 mM Tris-HCl, pH 7, and 0.1 M NaCl and radioactivity in them measured directly in a gamma counter.

2.5. *Analytical methods*

Protein concentration in detergent-solubilized fractions was determined according to Bradford [9], with appropriate blanks for each detergent and bovine serum albumin as standard. Protein in membrane fractions was measured after preincubation for 10 min at room temperature in 10% hexyl glucoside [10]. SDS-PAGE was performed as described by Laemmli [11]. Proteins were stained with silver [12].

3. RESULTS AND DISCUSSION

3.1. *Solubilization of β-glucan-binding activity*

Washing the soybean membranes with 1 M NaCl failed to remove β-glucan-binding activity from them. A number of detergents were then screened for their ability to solubilize the glucan-binding sites (Table I). DM, ZW 3-12 and the lysolecithin analog ES12H [13] were selected for further study. All three possess C_2 alkyl chains, the former being nonionic and the latter two zwitterionic detergents. They displayed the highest recoveries of binding activity in 100 000 × *g* supernatants. Two other detergents commonly used for membrane solubilization, CHAPS and octyl glucoside [14], proved unsuccessful. CHAPS was not effective in removing the binding activity from the membranes and octyl glucoside did not preserve glucan-binding activity in the solubilized fraction.

ZW 3-12 solubilized about 40% of the initial activity at concentrations between 3 and 12 mg/ml (2.5–10 times its CMC) (Fig. 1). Higher concentrations were inhibitory and almost no activity was observed in either supernatants or membrane pellets above 30 mg/ml. Significant solubilization of activity (40–70%) with ES12H was obtained between 10 and 40 mg/ml (60–250 times the CMC) and about 50% was obtained with DM in a broad range between 10 and 30 mg/ml (33–100 times the CMC). The addition of 2 mM DTT helped improve the recovery of binding activity in supernatants.

The use of ZW 3-12 at detergent/protein (w/w) ratios between 2 and 5, at constant 10 mg/ml ZW 3-12, did not significantly alter specific glucan binding on a per mg protein basis in solubilized preparations. Maximum recovery of binding activity was 40% and was obtained with ratios between 2 and 4 (not shown). Binding assays proved fairly sensitive to ZW 3-12 concentration. A 70% decrease in activity was observed by raising the ZW 3-12 concentration from 0.6 to 5 mg/ml (CMC = 1.2 mg/ml). Final ZW 3-12 concentration was kept at 0.7 mg/ml in all subsequent assays. The inhibitory effects of the other two detergents were less pronounced. A 70% inhibition of activity with ES12H was obtained only at 1.6 mg/ml or about 10 times its CMC.

Scatchard analysis of ligand-binding data with the solubilized fraction gave apparent K_D values between 11 and 34 nM (Fig. 2). These values were in the same range as those obtained for membranes [7], indicating no loss in ligand affinity through solubilization with any of the three detergents selected. The average maximal binding (B_{max}) determined for soluble fractions was 1.5 pmol/mg protein.

Table I

Solubilization of β-glucan-binding activity from soybean microsomal preparations with various detergents

Detergent	Concentration	Specific binding		Protein solubilized
	(%)	Soluble (%)	Pellet (%)	(%)
None	-	0	100	0
Lubrol PX	0.5(v/v)	6	104	38
Triton X-100	0.1(v/v)	20	76	43
Octyl glucoside	2.0(w/v)	0	43	82
Dodecyl maltoside	2.0(w/v)	54	61	47
CHAPS	2.0(w/v)	4	105	52
ES12H	2.0(w/v)	70	50	56
ZW 3-14	0.2(w/v)	20	14	33
ZW 3-12	1.0(w/v)	40	14	77
Sodium cholate	1.0(w/v)	14	62	77

Washed soybean membranes (1.8 mg protein) were suspended and stirred 1 h at 4°C in 25 mM Tris-HCl, pH 7, 0.1 M NaCl, 2 mM DTT and one of each of the detergents indicated below. After centrifugation at 100 000 × *g* for 1 h, glucan-binding activity and protein were measured in supernatants and resuspended pellets.

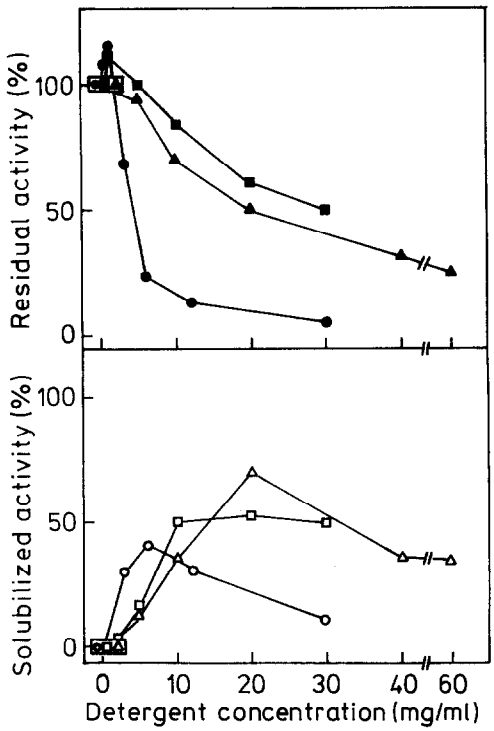

Fig. 1. Solubilization of ^{125}I-glucan-binding activity from soybean membranes with increasing concentrations of ZW 3-12 (○,●), DM (□, ■) and ES12H (△, ▲).

3.2. *Chromatographic analysis of the solubilized proteins*

ZW 3-12 was chosen as standard detergent for solubilization as it showed the best overall results based on good recovery of activity, a small micelle size which permitted dialysis and a low cost. ZW 3-12-solubilized binding activity was retained by Q-Sepharose at pH 7.5 and eluted at NaCl concentrations between 150 and 250 mM when using linear gradients from 0 to 500 mM salt (not shown). About 30% of the initial activity and 20% of the protein were recovered in this fraction. This step removed most of the UV-absorbing nonprotein material. For preparative purposes, the gradient elution of binding activity was replaced by a step elution with 0.22 M NaCl buffer. Proteins in the active fraction were concentrated by precipitation with 20% (w/v) PEG 6000. Phosphate determination in this 'PEG fraction' showed that it contained less than 13% of the phospholipids initially present in the raw solubilized fraction.

Gel permeation chromatography of the PEG fraction using ZW 3-12 yielded an apparent M_r of 300 kDa for the glucan-binding protein-detergent micelles (Fig. 3). The binding activity separated well from the main protein peak. This step yielded an increase in specific binding of almost 4 times that in microsomes.

The PEG fraction was also analyzed by ampholyte displacement chromatography using a Mono P column. Almost complete separation of the glucan binding activity from bulk protein was obtained when using a broad pH gradient from 7 to 4.5 (Fig. 4). Highest binding activity eluted between pH 6.8 and 6.2. This region contained 6% of the total protein and about 12% of the initial activity. The specific binding activity on a protein basis was 4.5-fold higher than in microsomes. The ma-

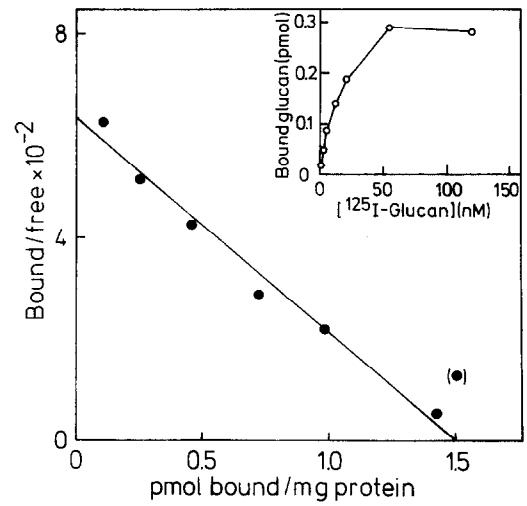

Fig. 2. Scatchard plot of ligand-binding data obtained with ZW 3-12-solubilized proteins. Specific binding was measured in an ^{125}I-glucan concentration range between 1 and 120 nM, using 200 μg of protein per assay. Nonspecific binding was measured in the presence of 10 μM unlabeled glucan (inset: ligand saturation curve used to obtain the data for the analysis).

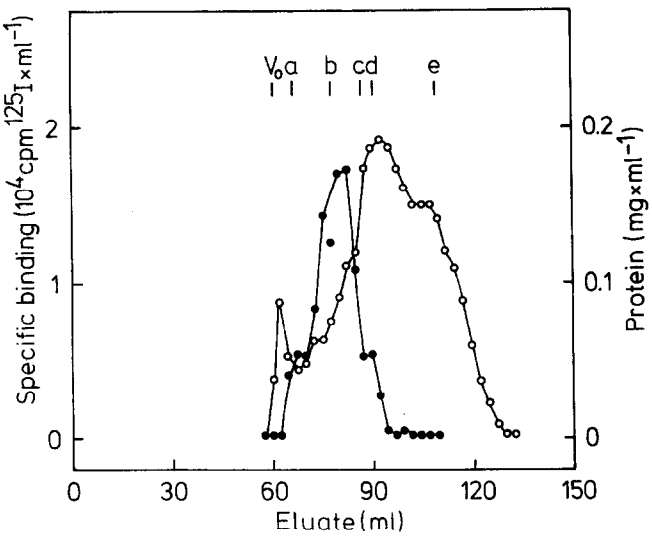

Fig. 3. Gel permeation chromatography of ZW 3-12-solubilized proteins. Fourteen mg protein from the PEG fraction were loaded onto a Sephacryl S-300 HR column (1.6 × 70 cm) equilibrated in 25 mM Na phosphate buffer, pH 7, 50 mM NaCl, 10% glycerol and 15 mg/ml ZW 3-12. Two-ml fractions were collected and assayed for glucan-binding activity (●) and protein (○). Molecular weight markers were: thyroglobulin (a, $M_r = 67 \times 10^4$), apoferritin (b, 44×10^4), β-amylase (c, 22×10^4), alcohol dehydrogenase (d, 15×10^4) and carbonic anhydrase (e, 3×10^4).

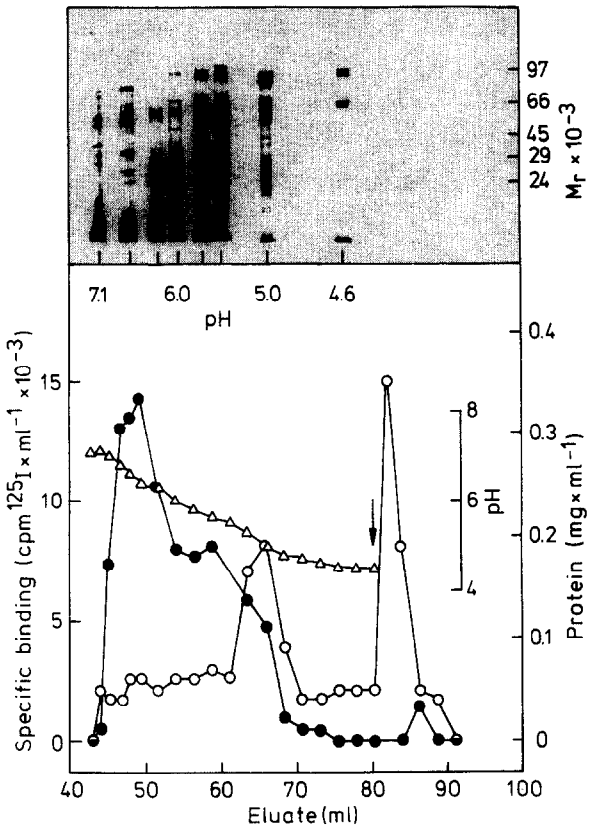

Fig. 4. Chromatofocusing of ZW 3-12-solubilized proteins. Five mg protein from the PEG fraction were loaded onto a Mono P HR 5/20 FPLC column equilibrated in 25 mM bis-Tris, pH 7.2, 10% glycerol and 15 mg/ml ZW 3-12. Bound proteins were eluted with 10% polybuffer 74, pH 4.5, 10% glycerol and detergent. One-ml fractions were collected and analyzed for glucan-binding activity (●), protein (○), pH (△) and by SDS-PAGE (top figure). The column was then washed with 1 M NaCl in starting buffer (arrow).

jority of unrelated proteins eluted below pH 5.0, as seen on SDS-PAGE. The low 'purification factor' achieved in this step does not reflect the excellent separation of binding activity from bulk protein. Total recovery of binding activity from the column was only 19%, that is, almost two-thirds of the recoverable activity eluted between pH 6.8 and 6.2.

Chromatofocusing and gel permeation chromatography of the PEG fraction in the presence of ES12H gave similar results to those with ZW 3-12. Glucan-binding activity displayed a somewhat higher apparent M_r, 380 instead of 300 kD. Separation from bulk protein and enrichment factors for activity were essentially similar (not shown). Chromatofocusing in ES12H resulted in maximal binding activity eluting 0.4 pH units lower than in ZW 3-12. On the other hand, the nonionic detergent DM was not suitable for chromatographic separation of glucan-binding activity. No clear separation of activity was obtained by using either anion exchange, gel permeation or any other method tested. Also, its large micelle molecular size and low CMC made ultrafiltration and dialysis difficult. Further use of this detergent was discarded.

3.3. *Conclusions*

The characteristics of the β-glucan-binding sites from soybean, encompassing ligand affinity, ligand specificity, number of sites and subcellular distribution [6,7], suggest a possible role as receptors for the glucan elicitor-mediated phytoalexin response in this plant. Confirmation of this possibility requires a better characterization of the binding sites. We now provide the first report on the solubilization of glucan-binding activity from soybean membranes. Our results demonstrate that two detergents, ZW 3-12 and ES12H, offer good preservation of activity and are suitable for use in chromatographic analyses of the glucan-binding protein(s). The methods which have been applied are suitable for partial purification, albeit with large loss of binding activity. Ligand-affinity techniques, which have been indispensable in the isolation of a number of less-abundant receptor proteins, are being presently developed to achieve a more efficient purification of the β-glucan-binding sites.

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