Functional reconstitution of β-glucan elicitor-binding activity upon incorporation into lipid vesicles

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Abstract In temperature-induced Triton X-114 phase separation experiments the β-glucan elicitor-binding site from soybean (*Glycine max* L.) root membranes was identified as (a) hydrophobic membrane protein(s). The Zwittergent 3-12-solubilized β-glucan-binding proteins were incorporated into lipid vesicles by the detergent-dilution procedure. Reconstituted binding proteins were functional in that binding of the hepta-β-glucoside ligand was saturable, reversible and of high affinity (K_d = 6–7 nM). Competition studies using β-glucans with different degrees of polymerization (DP 7–15; DP 15–25) showed effective displacement of the radioligand from the binding site whereas β-glucan fragments with DP < 7 were ineffective. The total amount of reconstituted binding activity was dependent on the acyl chain length of the phospholipids used for the reconstitution with a preference for decanoic (C10) and dodecanoic (C12) chains. Restored ligand binding was maximally 37% as compared to the former detergent-solubilized binding activity. The presence of a lipid environment stabilized the purified β-glucan-binding proteins.

Key words: β-Glucan-binding protein; Reconstitution; Lipid; Soybean

1. Introduction

Reconstitution experiments have contributed to the understanding of receptor function and how both binding activity and subsequent transduction mechanisms can be influenced by various factors such as membrane lipid composition [1]. The involvement of lipids in receptor-ligand interactions has been suggested by the finding that membrane proteins respond differently to the various solubilization and/or reconstitution procedures and nearly every approach is empirical [2]. Thus, in order to study the requirement of specific lipids in receptor functions, it is necessary to reconstitute these proteins in vesicles of known lipid composition.

Defence reactions elicited in plants upon invasion by microorganisms or treatment with pathogen-derived elicitors comprise a variety of processes including the generation of reactive oxygen species and the accumulation of phytoalexins [3]. The specificity observed in several of the elicitor-plant cell interactions suggests the involvement of receptors in elicitor perception and subsequent signal transduction [3]. High-affinity binding sites for 1,3-1,6-β-glucans of oomycete origin have recently been demonstrated in the plasma membrane of soybean [4–6]. Ligand-binding studies and photoaffinity labelling experiments using an azido derivative as the photoreactive ligand revealed the existence of a low abundance 75-kDa protein as the main component of the β-glucan-binding activity in this system [7–9]. This β-glucan elicitor-binding protein of soybean was purified to apparent homogeneity by affinity chromatography [9,10] and its cDNA was cloned [10]. Moreover, the photoaffinity labelling technique enabled the identification of three polypeptides with relative molecular masses of 75 000, 100 000, and 150 000 which probably represent subunits of the native receptor complex with an apparent M_r of 240 000 [9].

Knowledge about the conditions for a functional reconstitution of the binding activity of the putative β-glucan elicitor receptor is a prerequisite for the reconstitution of the whole receptor activities. Here we report the reconstitution of the soybean hepta-β-glucoside-binding protein into proteoliposomes. The reconstituted binding proteins show similar binding characteristics as the microsomal and solubilized binding proteins, respectively.

2. Materials and methods

2.1. Chemicals

Zwittergent 3-12 (dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate) and all lipids used were purchased from Sigma (Munich, Germany); aprotinin and polyethylene glycol 4000 (PEG 4000) were obtained from Roth (Karlsruhe, Germany). Triton X-114 was supplied by Serva (Heidelberg, Germany). The hepta-1,3-1,6-β-glucoside was from Biocarb (Lund, Sweden).

2.2. Plant material, membrane protein preparation and solubilization

Soybean (*Glycine max* L. cv. 9007) seeds were kindly provided by Pioneer Seeds Ltd. (Buxtehude, Germany). Plants were grown in the greenhouse as described by Schmidt and Ebel [4]. Roots from 12-day-old plants were used to prepare a total membrane fraction as described previously [5].

Membrane proteins were solubilized according to Cosio et al. [11] in 25 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 2 mM DTT, and 20% (v/v) glycerol (buffer A), plus 1% (w/v) Zwittergent 3-12 (ZW 3-12). Fractions obtained after affinity chromatography were precipitated twice (1 h each) by adding PEG 4000 to a 20% (w/v) final concentration in the presence of 1 mg/ml aprotinin, to remove the bound β-glucan. Protein content was measured by the Bradford method [12] with bovine serum albumin as standard.

Triton X-114 phase partitioning was performed as described [13]. To a microsomal preparation 10 mM Tris-HCl, pH 7.4, plus 150 mM NaCl was added at 0°C resulting in a final detergent concentration of 1% (v/v) and 1 mg protein/ml. After 5 min of incubation a centrifugation step at 160 000 × *g* (4°C, 20 min) was carried out to separate a lipid-rich pellet which contains strongly hydrophobic membrane proteins according to Pryde and Phillips [14]. To induce phase separation the supernatant was warmed in a water bath at 30°C for 30 min and centrifuged for 3 min at 300 × *g*. The upper, detergent-poor phase was removed and washed twice as described [13]. The lower, detergent-rich phase was adjusted to the same volume as the detergent-poor phase and equal aliquots were analyzed.

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2.3. Reconstitution of β-glucan-binding proteins

ZW 3-12-solubilized soybean β-glucan-binding proteins were diluted with an equal volume of buffer A and lipids of desired composition and concentrations. The solution was incubated with gentle stirring for 20 min at 4°C, followed by the addition of 9 volumes of 50 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.8, over a period of 20 min. This solution was then stirred for 12 h at 4°C. For purified proteins, the pellet obtained after the second precipitation step was dissolved in buffer A plus 0.1% (w/v) ZW 3-12. In these experiments, the incubation for 12 h at 4°C followed directly after the addition of lipids. All lipids were dissolved in buffer A and sonicated for 10 min before they were added to the reconstitution mixture. The lipid:protein ratio was routinely 3:1. The lipid vesicles generated that way were precipitated by centrifugation at 200 000×*g* for 40 min at 4°C. The pellet was dissolved in buffer A and used immediately for binding analyses.

2.4. Hepta-β-glucoside-binding assay

Binding assays were performed as described by Cosio et al. [5]. Briefly, protein fractions were incubated (2 h at 4°C) in the presence of 3 nM ^{125}I-labeled 2-(4-aminophenyl)ethylamine conjugate of the hepta-β-glucoside (HG-APEA) in a final volume of 200 μl of a buffer consisting of 25 mM Tris-HCl, pH 8.0, plus 100 mM NaCl, 10 mM $MgCl_2$ and 5 mM D-gluconic acid lactone. Synthesis of the HG-APEA radioligand was described earlier [15]. Non-specific binding was determined in the presence of a 1000-fold excess of unlabeled ligand. Binding constants from ligand saturation experiments and values for half-maximal displacement (IC_{50}) were calculated from the data by non-linear regression using Sigmaplot 5.0 (Jandel Scientific).

3. Results and discussion

High-affinity, reversible, and specific β-glucan-binding activity is localized on soybean plasma membranes [4–6]. To analyze whether the corresponding binding site is an integral or peripheral membrane protein its partitioning behavior was investigated in Triton X-114 phase partitioning experiments that distinguish membrane proteins according to their hydrophobicity [13]. Depending on a temperature shift-induced separation hydrophilic (i.e. peripheral) proteins were found in the aqueous phase and hydrophobic proteins in the detergent phase. The modified phase separation procedure of Pryde and Phillips [14] allows a further separation of the detergent fraction into a fraction which contains Triton X-114-soluble membrane proteins and a lipid-rich detergent-insoluble phase known to contain residual, hydrophobic integral membrane proteins. As shown in Table 1, compared to the original microsomal β-glucan-binding activity 95% of the binding activity partitioned into the lipid-rich detergent-insoluble phase, indicating that the β-glucan-binding protein or the complex containing the binding protein behaves as an integral membrane protein.

Functional solubilization of the binding activity for the hepta-β-glucoside elicitor from soybean root membranes strongly depends on the type and size of the acyl chain of the chosen detergent, rather than on the hydrophilic moiety, with a preference for dodecanoid chains [6,11]. In order to investigate a possible role of specific phospholipids in supporting the binding activity, the binding protein was reconstituted separately with different lipids and assayed for specific hepta-β-glucoside binding. The most efficient method for the reconstitution was the detergent-dilution procedure. The optimal dilution of the reconstitution mixture was 5–10-fold using a constant lipid:protein ratio of 3:1. Under these conditions, nearly 40% of the solubilized binding activity was found in lipid vesicle pellets obtained after centrifugation at 200 000×*g* (Table 2). In the absence of lipids, binding activity in the pellet was less than 3%. Proteolipid vesicle generation with crude soybean phosphatidylcholine lipids (cL) restored a higher amount of binding activity than the usage of crude egg phosphatidylcholine lipids, indicating the presence of more reconstitution-promoting lipid species in the soybean lipids (Table 2).

Experiments with two different dodecanoyl-acyl chains carrying lipids showed that with pure dilauroylphosphatidylcholine (dilauroyl-PC) a twofold higher amount of total restored binding activity was obtained, as compared to the crude soy-

Table 1
Triton X-114 phase partitioning of microsomal hepta-β-glucoside-binding activity

Phase	Total HG-APEA binding activity	
	(pmol)	(%)
Microsomes	10.26	100
Detergent-poor phase	0.36	3.5
Detergent-rich phase	0.15	1.0
Lipid-rich phase	9.75	95.0

Binding activity was analyzed by determination of specific HG-APEA binding (total minus non-specific binding). The results are the average of two independent experiments with similar results.

Table 2
Reconstitution of solubilized hepta-β-glucoside-binding activity by different types of lipid

Lipid	Total reconstituted HG-APEA binding activity	
	(pmol)	(%)
Control	2.21	100
Crude soybean lipid	0.42	19.0
Egg yolk lipid	0.19	8.6
Dilauroyl-PC (C12:0/C12:0)	0.82	37.1
Lysolauroyl-PC (C12:0)	0.39	17.6

Binding activity was analyzed by determination of specific HG-APEA binding (total minus non-specific binding). The results presented are the average of two independent experiments with less than 10% variation between them.

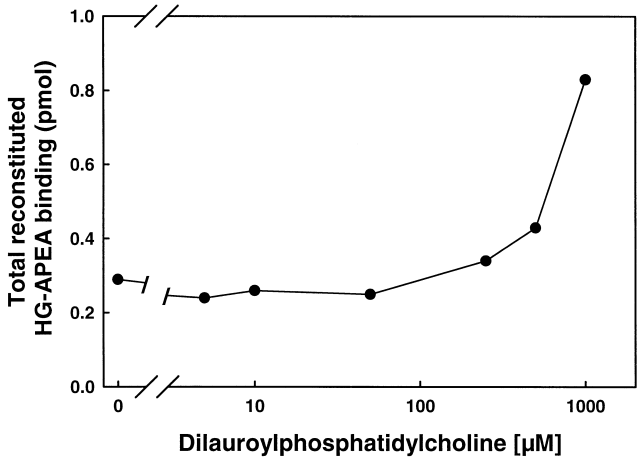

Fig. 1. Influence of increasing concentrations of dilauroyl-PC on the total amount of crude soybean lipid-reconstituted specific ^{125}I-labeled HG-APEA binding. Non-specific binding was determined in the presence of a 1000-fold excess of unlabeled ligand. Data points show specific binding (total minus non-specific binding). The data presented are the average of two independent experiments with similar results.

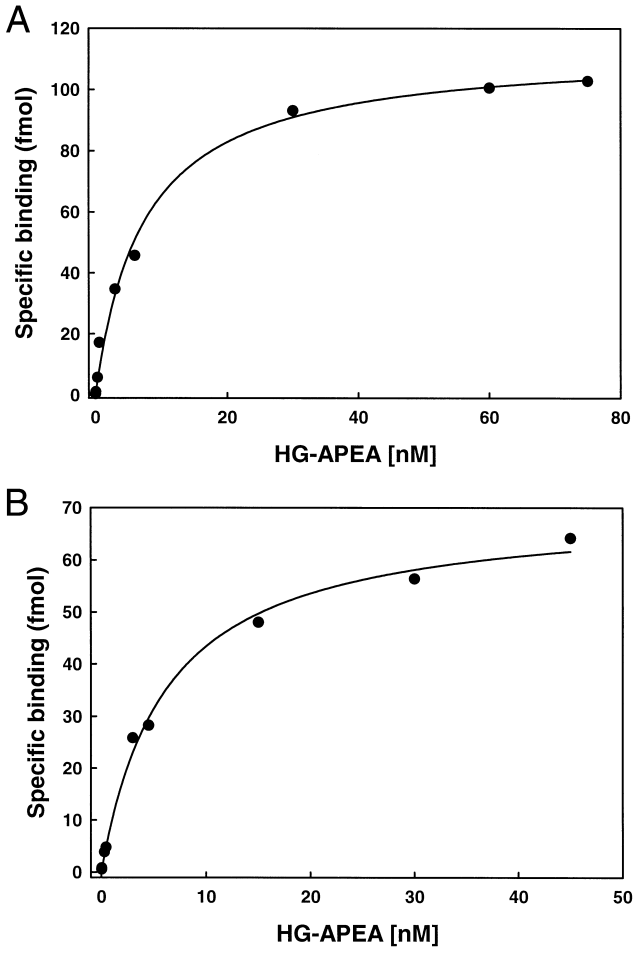

Fig. 2. Concentration dependence of specific ^{125}I-labeled HG-APEA binding reconstituted by (A) crude phosphatidylcholine or (B) dilauroyl-PC. Non-specific binding for each concentration was determined in the presence of a 1000-fold excess of unlabeled ligand. Data points show specific binding (total minus non-specific binding). Curve fitting and calculation of the binding constant were performed by non-linear regression. The data points represent the average of two independent experiments with less than 10% variation between them.

bean lipids (Table 2). Moreover, total crude soybean lipid-reconstituted hepta-β-glucoside binding was further increased by adding dilauroyl-PC (Fig. 1), supporting the observation that dilauroyl-PC increases the efficiency of the reconstitution process.

To compare hepta-β-glucoside-binding affinity of the membrane-bound, detergent-solubilized, and lipid-reconstituted proteins, binding affinities were determined for binding proteins incorporated into both dilauroyl-PC and cL (Fig. 2A,B). Binding of the hepta-β-glucoside ligand was saturable and the analyses of the data by non-linear regression yielded an apparent dissociation constant (K_d) of 7.3 ± 1.0 nM for cL and 6.1 ± 0.6 nM for dilauroyl-PC. These data indicate a slight decrease in binding affinity upon reconstitution when compared to the results obtained for the membrane-bound binding proteins (K_d: 0.8–3.0 nM [15,16]) or solubilized preparations (K_d: 1.8–5.5 nM [6–8]). Although the reasons for this affinity decrease are not clear, a change in the affinity of ligands has been reported for several receptor systems reconstituted in lipid vesicles [17,18]. One parameter that might

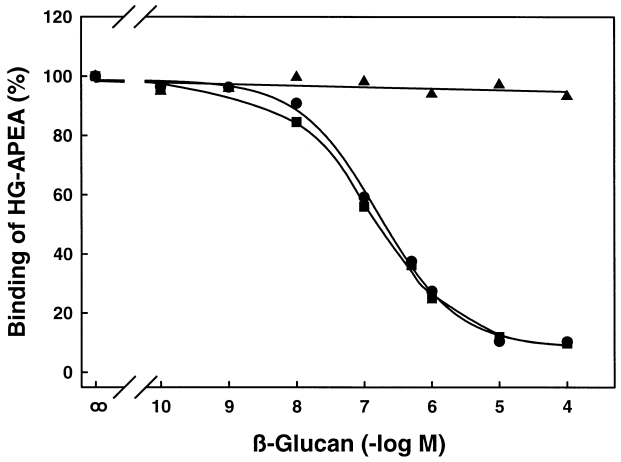

Fig. 3. Competition for binding sites between ^{125}I-labeled HG-APEA (3 nM) and β-glucans with different degrees of polymerization (DP 7–15, ●; DP 15–25, ■; DP < 7, ▲). Curve fitting was performed by non-linear regression. The data points represent the average from duplicate determinations with less than 10% variation between them.

have caused changes in ligand affinity was the ratio of lipid to protein concentration used in the present experiments to achieve the most efficient reconstitution of the binding proteins.

In order to investigate the specificity of the reconstituted binding activity, radioligand displacement experiments using increasing concentrations of β-glucans with different degrees of polymerization (DP) as competitors were performed. The results clearly showed that the β-glucan fractions with DP 7–15 and 15–25 effectively displace the radiolabeled hepta-β-glucoside (Fig. 3). The concentrations of the competing β-glucans giving 50% inhibition of binding of the radioligand (IC_{50}) were 128 nM (DP 7–15) and 161 nM (DP 15–25). These IC_{50} values agree with data obtained from experiments done with β-glucans of similar sizes (DP 10 and DP 16–20) as competitors. In that study, the IC_{50} values were determined to be 410 and 100 nM, respectively, using microsomal fractions [15]. No displacement activity was detected for a β-glucan fraction with a DP < 7, either in reconstituted binding sites (Fig. 3) or in microsomes [15].

To determine the optimum size of acyl chains for efficient reconstitution of the binding protein, various pure lipids with different chain lengths were tested. The highest amount of total restored binding activity was found in lipid vesicles con-

Table 3
Reconstitution of solubilized hepta-β-glucoside-binding activity by phosphatidylcholine lipids of different acyl chain length

Lipid	Total reconstituted HG-APEA binding activity	
	(pmol)	(%)
Control	2.94	100
Diheptanoyl-PC (C7:0/C7:0)	0.82	27.9
Didecanoyl-PC (C10:0/C10:0)	1.13	38.4
Dilauroyl-PC (C12:0/C12:0)	1.10	37.4
Dimyristoyl-PC (C14:0/C14:0)	0.44	14.9
Dipalmitoyl-PC (C16:0/C16:0)	0.26	8.8

Binding activity was analyzed by determination of specific HG-APEA binding (total minus non-specific binding). The results presented are the average of two independent experiments with less than 10% variation between them.

taining didecanoyl-PC and dilauroyl-PC, respectively. Dimyristoyl-PC and dipalmitoyl-PC were around threefold less efficient and likewise diheptanoyl-PC was less effective (Table 3). In contrast to the recovery of total β-glucan-binding activities, the specific binding activity (pmol bound ligand/mg protein) was very similar (between 1.1 for dipalmitoyl-PC and 1.7 for diheptanoyl-PC) over all tested lipids. While the reconstitution data may suggest a superior role for specific lipids in the efficiency of the incorporation of binding proteins into the proteolipid vesicles, their influence on specific binding parameters of the putative β-glucan elicitor receptor from soybean is not fully resolved. Although little is known about the molecular interactions between lipids and proteins during reconstitution to form vesicles, there could be several possibilities for the differential effects of lipids. For instance, different lipids may either associate to the same extent with the receptor or the conformation formed by such an association may differentially be suitable for ligand binding.

To investigate the effects of lipid on pure β-glucan-binding proteins, an affinity purified fraction was reconstituted in lipid vesicles using cL. Hepta-β-glucoside-binding activity determined for the cL-reconstituted β-glucan binding proteins was approximately 10-fold higher than for a non-cL-treated control. Because the amount of protein obtained after affinity purification and lipid treatment was too low for an exact calculation of specific binding activity, the protein concentration determined after the affinity chromatography was used for the calculation of the apparent specific binding activity The apparent specific binding activity for the cL-reconstituted β-glucan-binding proteins was 58.8 pmol/mg protein and 5.5 pmol/mg protein in the control sample ($n = 2$). These values demonstrate that the presence of lipid strongly affected the stability of the purified β-glucan-binding activity during the long-lasting treatments of the reconstitution procedure. This result therefore confirmed the observation by Frey et al. [8] that β-glucan-binding activity in purified fractions without stabilizing agent was rather unstable over longer periods.

The reconstitution data described in our study suggest a specific role for lipids in preserving the binding properties of the β-glucan-binding protein from soybean and should prove useful in studies on functional reconstitution of the whole receptor complex.

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