One-step purification of the β-glucan elicitor-binding protein from soybean (*Glycine max* L.) roots and characterization of an anti-peptide antiserum

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Abstract A low abundance β-glucan elicitor-binding protein from soybean was isolated by a rapid, simple and one-step purification method yielding about 9000-fold enrichment. The affinity-based purification technique was more efficient than a procedure that uses conventional methods and preserved the binding activity to a much larger extent. The final preparation consisted of one major protein with an apparent molecular mass of about 75 kDa. Electrophoretic analyses of the purified and photoaffinity-labeled binding protein showed that the native protein was an oligomer with apparent molecular mass of about 240 kDa. A polyclonal anti-peptide antiserum was raised against a synthetic 15-mer internal oligopeptide sequence derived from the 75-kDa protein. The antiserum recognized the purified binding protein in immunoblotting experiments and precipitated the affinity-labeled protein from a crude extract of the membrane fraction.

Key words: β-Glucan-binding protein; Glucan affinity purification; Peptide-specific antiserum; Soybean

1. Introduction

Plants possess the ability to recognize potential pathogens and to activate a successful resistance response. The spectrum of defense reactions elicited in plants upon invasion by microorganisms includes a variety of processes that require gene activation, such as phytoalexin production. Triggering of these defense responses is thought to require the ability to perceive signal compounds, commonly referred to as elicitors, on the plant plasma membrane and to transduce the signal, thereby initiating the response. The specificity observed in several of the elicitor–plant cell interactions suggests the involvement of receptors in elicitor perception and subsequent signal transduction [1].

High-affinity binding sites for purified carbohydrate and peptide elicitors of fungal origin have recently been demonstrated in the plasma membrane of soybean, tomato, parsley, and tobacco cells [2–6]. In most of the systems studied so far, the nature of the binding sites was not well characterized. Ligand-binding and photoaffinity-labeling experiments for a fungal hepta-β-glucoside phytoalexin elicitor in soybean tissues revealed the existence of a low abundance 70-kDa protein as the main component of the β-glucan-binding activity in this system [2,7]. The biochemical characteristics of the soybean β-glucan-binding protein, however, remained largely unknown [2,7]. This was presumably due to both the low abundance of the binding protein and the low recovery during purification [2,7,8].

We have recently introduced affinity chromatography as a method for partial purification of the β-glucan-binding protein from soybean [9]. Following refinement, this affinity method has now been used successfully to obtain highly enriched β-glucan-binding protein. In order to gain further insight into the role of the β-glucan-binding protein in the signaling process underlying defense gene activation, peptide-specific antisera were raised and their interaction with the β-glucan-binding protein was analyzed.

2. Materials and methods

2.1. Chemicals

Zwittergent 3-12 (Dodecyl-*N,N*-dimethyl-3-ammonio-1-propane sulfonate), anti-rabbit IgG alkaline phosphatase conjugate, and aminopropyl-derivatized controlled-pore glass beads were purchased from Sigma, München, Germany; aprotinin and polyethylene glycol 4000 (PEG 4000) were obtained from Roth, Karlsruhe, Germany.

2.2. Microsomal preparation and solubilization

Soybean (*Glycine max* L. cv 9007) seeds were kindly provided by Pioneer, Buxtehude, Germany. Plants were grown in the greenhouse as described by Schmidt and Ebel [10]. Roots from 12-day-old plants were used to prepare a total membrane fraction as described previously [11]. Membrane proteins were solubilized according to Cosio et al. [8] in 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM DTT, 20% (v/v) glycerol, and 1% (w/v) zwittergent 3-12 (buffer A). This procedure solubilized up to 90% of the microsomal β-glucan-binding activity.

2.3. Heptaglucoside-binding assay and photoaffinity labeling

Binding assays were performed as described by Cosio et al. [11]. Briefly, purified protein fractions were incubated 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, 100 mM NaCl, 10 mM $MgCl_2$ and 5 mM D-gluconic acid lactone at 4°C for 2 h. Synthesis of the HG-APEA was described earlier [2]. Non-specific binding was determined in the presence of a 1000-fold excess of unlabeled ligand. Fractions obtained after affinity chromatography were precipitated twice (1 h each) by adding PEG 4000 to 20% (w/v) final concentration in the presence of 1 $mg \cdot ml^{-1}$ aprotinin to remove the bound β-glucan.

Photoaffinity labeling of β-glucan-binding proteins was done according to Cosio et al. [7] using a ^{125}I-labeled 2-(4-azidophenyl)ethylamine conjugate of the hepta-β-glucoside (HG-AzPEA). Proteins were incubated with 3 nM HG-AzPEA followed by irradiation with UV light (366 nm). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were detected by autoradiography.

2.4. Synthesis of an affinity matrix with immobilized β-glucan

A β-glucan fraction from the cell walls of *Phytophthora sojae* was prepared as described previously [9]. β-Glucan fragments with a degree of polymerization of 15–25 were conjugated to controlled-pore glass beads derivatized with aminopropyl groups according to Frey et al. [9]. The content of bound carbohydrate was 10.2 µg/mg matrix.

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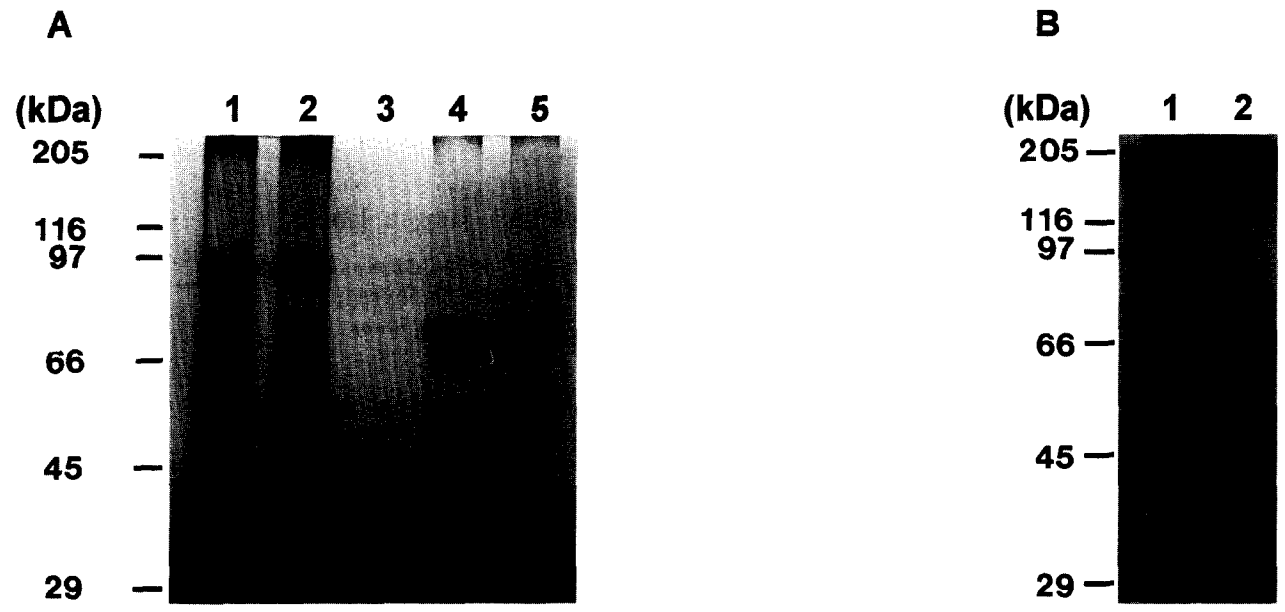

Fig. 1. Silver staining of proteins after SDS-PAGE of crude solubilized and affinity-purified soybean membrane fractions. (A) Aliquots of individual fractions obtained after affinity chromatography were precipitated and subsequently separated on SDS-PAGE (10% separating gel). Lanes correspond to (1) crude solubilized membrane proteins, (2) flow through, (3) last wash fraction, (4) first eluate fraction, (5) second eluate fraction. Samples of 5 μl (lanes 1,2) or 500 μl (lanes 3–5) per fraction were precipitated. (B) Aliquots of individual fractions obtained after affinity chromatography in the presence of 2 mg free β-glucan were analyzed. Lanes correspond to: (1) first eluate fraction, (2) second eluate fraction. 500 μl per fraction were precipitated. Binding proteins were eluted with 1 mg β-glucan per fraction (A, lane 4,5; B, lane 1,2).

2.5. Purification of the β-glucan binding protein by affinity chromatography

All steps were carried out at 4°C. Solubilized membrane proteins (35–40 mg) in buffer A containing 0.2 mM phenylmethanesulphonyl fluoride (PMSF) were loaded onto a β-glucan affinity column (2.5 ml bed volume). The protein sample was circulated through the matrix for at least 12 h at a flow rate of 1 $ml \cdot min^{-1}$ using a peristaltic pump. To remove non-specifically adsorbed proteins, the matrix was washed 4 times with 1.5 ml each of 50 mM Tris-HCl, pH 7.5, 0.1% (w/v) zwittergent 3-12, and 0.2 mM PMSF (buffer B). Glucan-binding proteins were eluted in two fractions by incubating the pre-washed matrix with 1.5 ml of buffer B containing 1 mg free β-glucan twice (2 h each).

2.6. Preparation of peptide-specific antiserum

Two different 15-mer oligopeptides derived from partial amino acid sequences of the binding protein were synthesized. One of the peptides (peptide a) was coupled to keyhole limpet hemocyanine and injected in rabbits to raise site-specific antisera (Eurogentec, Seraing, Belgium).

2.7. Immunoprecipitation

A protein fraction (75 μg), obtained after chromatography on Q-Sepharose and precipitation with PEG 4000, was labeled with the photoreactive radioligand HGAzPEA and incubated for 12 h at 4°C with anti-peptide a antiserum. After this treatment, 5 mg Protein A Sepharose was added and the mixture was further incubated at room temperature for 2 h to bind IgG antibodies. The sample was precipitated by centrifugation at 10,000×*g* for 15 min. Finally, the pellet was treated with SDS sample buffer and subjected to SDS-PAGE (10% separating gel), and proteins were detected by autoradiography.

2.8. Analytical methods

Protein content was measured according to Bradford [12] with bovine serum albumin as standard. SDS-PAGE was performed as described by Laemmli [13] using 10% separating and 3% stacking gels. Blue-Native polyacryamide gel electrophoresis was carried out as described [14] with gradient gels (3–15%) and a detergent/Coomassie G-250 ratio of 1 : 4. For the identification of antigen by immunoblotting, the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by the semi-dry electroblotting procedure. The membrane was blocked for 1 h with 1% (w/v) gelatine and then incubated with anti-peptide antiserum at 4°C for 12 h. Antigen–primary antibody complexes were detected by an alkaline phosphatase conjugated secondary antibody.

3. Results and discussion

The molecular analysis of elicitor-binding sites that show receptor-like properties was impossible until now, due to the lack of a reliable purification protocol suitable for use at a

Table 1
Purification of β-glucan-binding proteins after solubilization of soybean membrane proteins

Purification step	Protein (mg)	Total activity (pmol)	Specific activity (pmol/mg)	Purification (-fold)	Recovery (%)
Method A					
Solubilizate	350	91	0.26	1	100
Q-Sepharose/PEG	12.7	30	2.35	9.1	32.9
Heparin Sepharose	1.7	24	13.9	53.5	26.4
Mono P	0.06	0.07	1.2	4.6	0.1
Method B					
Solubilizate	37.5	22	0.58	1	100
Affinity Chromatography	0.0005	2.6	5200	8970	11.8

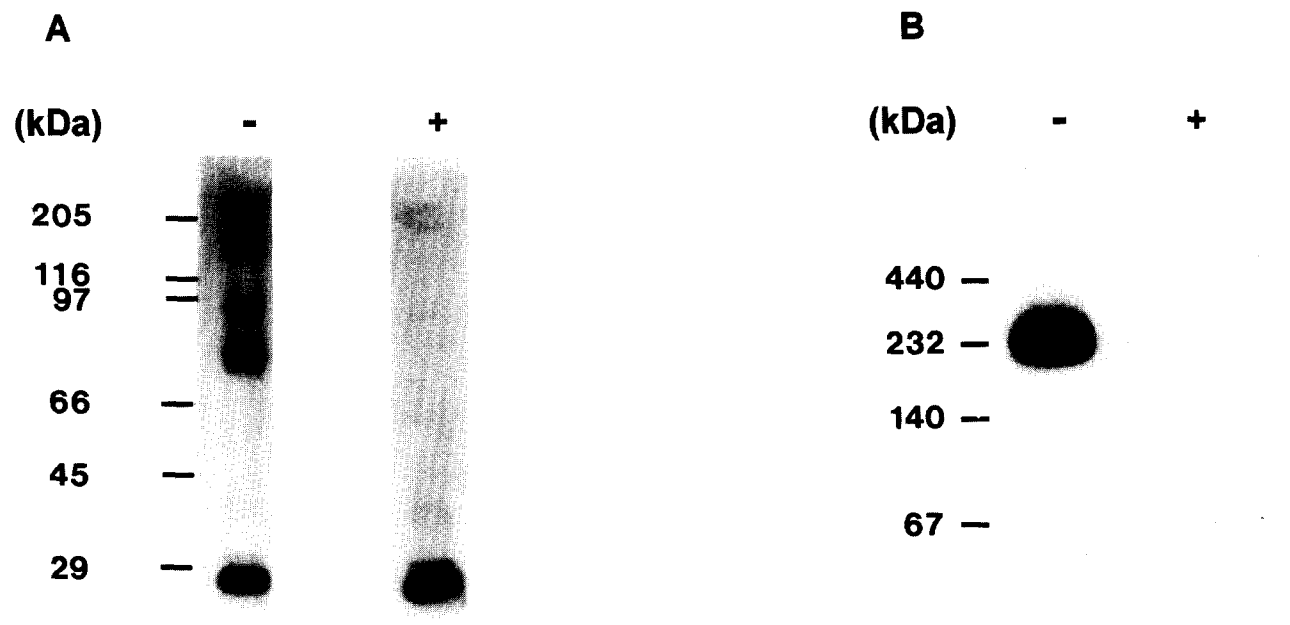

Fig. 2. Photoaffinity labeling of purified β-glucan-binding proteins. β-Glucan-binding proteins were purified by affinity chromatography and precipitated with PEG 4000 to remove bound β-glucan. The samples were photoaffinity labeled using the radioligand HGAzPEA in the presence (+) and absence (−) of unlabeled β-glucan, and finally subjected to (A) SDS-PAGE (10% separating gel) or (B) Blue-Native PAGE (3–15%).

preparative scale. For the β-glucan elicitor-binding proteins of soybean, a purification strategy was recently developed which combined anion exchange with affinity chromatography [9]. Unfortunately, the enrichment factor obtained after this procedure was very low (82-fold), indicating high losses of both binding protein and/or binding activity. For further detailed studies it was necessary to improve the yield of the purification procedures. An alternative method consisting of chromatography on Q-Sepharose, heparin-Sepharose, and Mono-P (Table 1) resulted in the isolation of too little material for further processing. Immunoblotting experiments indicated that a major fraction of the β-glucan-binding protein(s) was apparently degraded during the various purification steps (results not shown), as reflected by the rather low enrichment factors of the different steps (Table 1). In order to minimize loss of binding activity we then established a rapid one-step purification procedure by improving affinity chromatography and eliminating any other steps that might adversely affect yield (Fig. 1A). After specific elution of binding activity from the affinity matrix with an excess of free β-glucan in the buffer one prominent protein with an apparent M_r of about 75,000 was observed on a silver-stained SDS gel. The specific interaction of the binding activity with the glucan matrix was verified in a control experiment, where the presence of competing β-glucan during sample loading largely prevented binding of the 75-kDa protein to the affinity matrix (Fig. 1B vs. A). The yield of the one-step purification method was about 12% and the increase in specific binding activity was about 9000-fold (Table 1).

The apparent size of the purified binding protein appeared to be slightly larger than the size that was determined in earlier studies [7,9]. This discrepancy might have been caused by differences in experimental procedures used for size estimations.

Strong evidence that the affinity-purified protein of 75 kDa represented the β-glucan-binding protein of soybean was confirmed further by photoaffinity labeling (Fig. 2A,B). When the radiolabeled hepta-β-glucoside azido derivative was used as photoreactive ligand [7], the 75-kDa band was the main component in autoradiographs after separation of the products by SDS-PAGE. Minor labeled protein bands of 100 and 150 kDa that had been detected earlier [9] were again visible. The relative abundance of these components was too low to be detected by silver staining. While the relationship between the 75- and the 100-kDa protein was not clear, the 150-kDa protein might represent an aggregate. Aggregation of the 75-kDa protein was promoted in the absence of reducing agent, whereas in its presence it was largely prevented (results not shown). Aggregation of the β-glucan-binding protein might also occur in crude solubilizates, as indicated earlier by gel-permeation chromatography of the binding protein-containing micelles [8,15]. Cosio et al. [8] reported that β-glucan-binding activity was associated with detergent-protein micelles having a molecular mass of about 300 kDa, while Cheong et al. [15] suggested that the size of such micelles might be larger than 670 kDa. In this study, a different approach was used to analyze binding protein oligomerization. As reported by Schägger et al. [14], electrophoresis in the presence of Coomassie G-250 (Blue-Native PAGE) stabilizes the native state of proteins including protein complexes. When photoaffinity-labeled protein samples of the highly purified fraction were subjected to electrophoresis according to Schägger et al. [14], an apparent molecular mass of about 240 kDa was found (Fig. 2B). This result further supported the notion that oligomerization might represent an intrinsic property of the β-glucan-binding protein. Whether the oligomeric state results solely from self-association or requires any additional factor remains to be resolved. Following Blue-Native electrophoresis, the binding protein complex, unfortunately, could not be transferred by electroblotting for further analysis.

Several micrograms of the purified 75-kDa β-glucan-binding protein were subjected to microsequencing after digestion

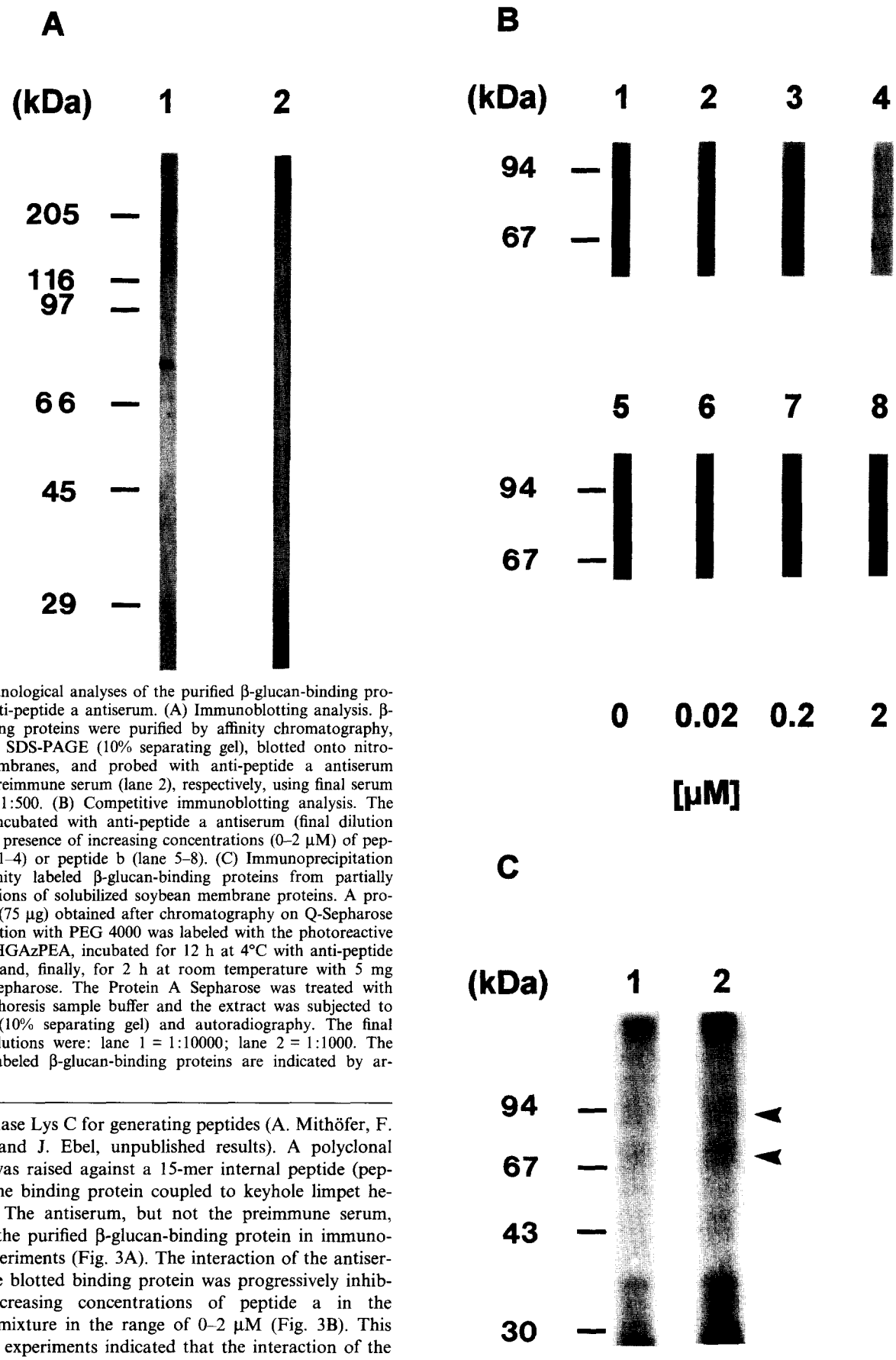

Fig. 3. Immunological analyses of the purified β-glucan-binding proteins with anti-peptide a antiserum. (A) Immunoblotting analysis. β-Glucan-binding proteins were purified by affinity chromatography, separated by SDS-PAGE (10% separating gel), blotted onto nitrocellulose membranes, and probed with anti-peptide a antiserum (lane 1) or preimmune serum (lane 2), respectively, using final serum dilutions of 1:500. (B) Competitive immunoblotting analysis. The blots were incubated with anti-peptide a antiserum (final dilution 1:500) in the presence of increasing concentrations (0–2 μM) of peptide a (lane 1–4) or peptide b (lane 5–8). (C) Immunoprecipitation of photoaffinity labeled β-glucan-binding proteins from partially purified fractions of solubilized soybean membrane proteins. A protein fraction (75 μg) obtained after chromatography on Q-Sepharose and precipitation with PEG 4000 was labeled with the photoreactive radioligand HGAzPEA, incubated for 12 h at 4°C with anti-peptide a antiserum and, finally, for 2 h at room temperature with 5 mg Protein A Sepharose. The Protein A Sepharose was treated with SDS-electrophoresis sample buffer and the extract was subjected to SDS-PAGE (10% separating gel) and autoradiography. The final antiserum dilutions were: lane 1 = 1:10000; lane 2 = 1:1000. The HGAzPEA-labeled β-glucan-binding proteins are indicated by arrows.

with proteinase Lys C for generating peptides (A. Mithöfer, F. Lottspeich and J. Ebel, unpublished results). A polyclonal antiserum was raised against a 15-mer internal peptide (peptide a) of the binding protein coupled to keyhole limpet hemocyanine. The antiserum, but not the preimmune serum, recognized the purified β-glucan-binding protein in immunoblotting experiments (Fig. 3A). The interaction of the antiserum with the blotted binding protein was progressively inhibited by increasing concentrations of peptide a in the incubation mixture in the range of 0–2 μM (Fig. 3B). This competition experiments indicated that the interaction of the antiserum with the binding protein was of high affinity. When a different peptide b was used in the competition experiment,

it did not have any effect on the interaction between the antiserum and the 75-kDa protein in the concentration range used (Fig. 3B). This result provided evidence that the antibody-antigen interaction was specific.

The antiserum also recognized the β-glucan-binding protein that was prelabeled with the photoaffinity reagent hepta-β-glucoside azido derivative (Fig. 3C). Precipitation of the radiolabeled antigen–antibody complex appeared to be, however, not very efficient. This problem has been encountered also in other studies when peptide-specific antisera were used. In spite of this disadvantage, the results shown in Fig. 3C added further support to the conclusion that the peptide-specific antiserum was directed against an epitope of the soybean β-glucan-binding protein.

The results reported here will enable us to generate probes that might be suitable for cloning the gene(s) encoding the β-glucan-binding protein. This is a prerequisite for obtaining further information on the molecular structure and the function of the binding protein. Analysis of its mode of action may significantly contribute to our understanding of signaling processes at the plant plasma membrane during the interaction of plants with their pathogens.

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