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A 55 kDa plasma membrane-associated polypeptide is involved in β-1,3-glucan synthase activity in pea tissue

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By glycerol gradient centrifugation of a detergent-solubilized plasma membrane fraction from pea tissue, we find a polypeptide of 55 kDa that copurifies with β-1,3-glucan synthase activity. An antiserum against this polypeptide adsorbs glucan synthase activity and the 55 kDa polypeptide from digitonin-solubilized plasma membrane. These results indicate that the 55 kDa polypeptide is involved in pea β-1,3-glucan synthase activity.

Cell wall synthesis, Polysaccharide synthase, Callose, Pea

1. INTRODUCTION

Although a number of membrane-associated plant cell wall polysaccharide biosynthetic enzymes have been biochemically characterized over the last several decades, thus far none of these enzymes has been conclusively identified at the polypeptide level [1]. The few detergents that solubilize these enzymes without complete inactivation, have not heretofore permitted a conclusive fractionation or isolation of any of them.

There are two sites of cell wall synthesis: the Golgi system, where matrix polysaccharides are synthesized, and the plasma membrane, the site of cellulose synthesis [2]. Callose, a β-1,3-glucan also synthesized at the plasma membrane, is not a component of ordinary cell walls, but occurs in the walls of some specialized cells or cell parts, e.g. pollen mother cells, sieve-tube members, and plasmodesmatal channels. However, the enzyme that synthesizes callose (β-1,3-glucan synthase, also called glucan synthase-II or GS-II [3]) occurs generally in higher plant plasma membranes, and is subject to activation by Ca^{2+} and other effectors [4], for example as a result of wounding or pathogen attack, when such effectors may be released. Because GS-II can be assayed in vitro, whereas cellulose synthase of higher plants (the polysaccharide synthase of greatest importance) thus far cannot be detected [5,6], several laboratories have concentrated on identifying GS-II [7,8]. Based on detergent solubilization and fractionation approaches, a wide variety of polypeptides ranging from 31 to 115 kDa has been suggested to correspond to GS-II activity [9–13]. Recent relatively persuasive results suggest that in red beet a 57 kDa polypeptide may be responsible for GS-II activity [8], but a definitive identification of this enzyme has still not been achieved. Fractionation of detergent-solubilized GS-II from pea tissue has led us to identify a plasma membrane-associated polypeptide of 55 kDa as responsible for GS-II activity. These results are reported in this communication.

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Abbreviations: Chaps, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate, DTT, dithiothreitol; GS-II, β-1,3-glucan synthase, Mes, (2-[*N*-morpholino]ethanesulfonic acid; Mops, morpholinopropanesulfonic acid; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1 *Membrane preparation*

Alaska pea (*Pisum sativum* L.) seedlings were grown in vermiculite under dim red light for 8 days. The upper two internodes were cut and ground in a pre-chilled mortar in a cold room (4°C) in 0.25 M sucrose, 10 mM KCl, 1 mM EDTA, 0.1 mM $MgSO_4$, and 80 mM Mops (pH 7.0). The homogenate was strained through nylon cloth and centrifuged at 1000 × *g* for 10 min. The filtrate was centrifuged at 104000 × *g* for 25 min. This crude membrane pellet was either fractionated directly, or else resuspended in 0.25 M sucrose, 4 mM KCl, 5 mM potassium phosphate, pH 7.5, and mixed with an aqueous 2 phase system [14]. The final composition of the mixture was 20% (w/w) crude membrane suspension, 6.5% (w/w) dextran T500 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), 6.5% (w/w) polyethylene glycol 3350 (Sigma Chemical Co., St. Louis, MO), 0.25 M sucrose, 4 mM KCl, and 5 mM potassium phosphate, pH 7.5. After stirring for 5 min, the mixture was centrifuged at 1000 × *g* for 5 min. The upper (plasma membrane) phase was removed, diluted 4 times with homogenization buffer without sucrose, and centrifuged at 104000 × *g* for 30 min. The resultant plasma membrane pellet was resuspended in complete homogenization buffer minus EDTA and stored in liquid nitrogen until used.

2.2 *Glycerol gradient centrifugation*

The membrane preparation was mixed to a final concentration of 4 mg protein/ml, with 20% (w/v) glycerol and 0.5% (w/v) detergent. The mixture was centrifuged at 150000 × *g* for 1 h, and 2 ml of the resulting supernatant were loaded onto a linear 10 ml 30–60% (w/w) glycerol gradient in 80 mM Mops (pH 7.0) and 0.25% (w/w) Chaps.

After centrifuging in a SW41 Rotor (Beckman Instruments, Inc., Palo Alto, CA) at 207000 × g for 15 h, the gradient was fractionated into 12 fractions. β-1,3-Glucan synthase (GS-II) activity was assayed according to Kauss and Jeblick [15]. Briefly, a total assay volume of 50 μl containing 0.5 mM UDP-[^{14}C]glucose (0.4 mCi/mmol), 10 mM cellobiose, 0.1 mM $CaCl_2$, 0.2 mM spermine, 0.01% digitonin, and 80 mM Mops (pH 7.0) was incubated at 25°C for 10 min. The reaction was stopped by adding 2 ml 70% ethanol, and radiolabel incorporation into polysaccharide product was measured as described by Ray [16].

2.3. *Gel electrophoresis*

SDS-PAGE was performed by the method of Laemmli [17] as described in Dhugga et al. [18]. Briefly, 50 μl of each fraction were mixed with an equal volume of buffer containing 10% SDS, 125 mM Tris-HCl (pH 6.8), and 0.2 M DTT, loaded onto the stacking portion of a 1.5 mm thick gel and run overnight into a 12.5–20% polyacrylamide gradient resolving gel. Gels were stained in Coomassie brilliant blue R250 (Serva Biochemicals, Westbury, NY).

2.4. *Antibody preparation*

The 55 kDa protein band from gel electrophoresis of the plasma membrane fraction was cut from the Coomassie blue-stained gels, and electroeluted. 100 μg of the eluted protein in 0.25 ml buffer were mixed with an equal volume of Freund's Complete Adjuvant (Sigma) and injected into a rabbit. Two additional, similar injections but instead using Incomplete Freund's Adjuvant (Sigma) were made at 3-week intervals. Antiserum was obtained three weeks after the last injection.

2.5. *Western blotting*

Western blotting was performed essentially as described in Harlow and Lane [19]. Briefly, after transferring the proteins from the gel on to nitrocellulose, the blot was either stained in India ink or blocked in 5% nonfat milk. The blocked blot was incubated with anti 55 kDa antiserum at a 20000 : 1 PBS : antiserum ratio for 2 h, followed by a further incubation for 2 h in alkaline phosphatase-coupled anti rabbit IgG goat antibody (Sigma). The blot was developed as described [19].

2.6. *Immunoadsorption*

Plasma membranes were suspended in the homogenization buffer at 2 mg protein/ml and mixed with an equal volume of 2% (w/v) digitonin solution. After centrifugation at 150000 × g for 1 h, the supernatant was incubated on ice with appropriately diluted anti-55 kDa antiserum overnight in a final volume of 80 μl. After adding 40 μl of protein A-Sepharose (Sigma) suspension (approx. 20 mg protein A-Sepharose), the resulting mixture was further incubated on ice for 1 h, and centrifuged at 16000 × g for 1 min. The supernatant was removed, and the pellet was washed 5 times with homogenization buffer containing 0.1% (w/v) digitonin, divided into 2 aliquots, and assayed for GS-II activity as above.

Radiolabeled membrane proteins for immunoadsorption were obtained by incubating 40 g of sub- 1 pea stem segments for 4 h at 25°C with 5 mCi of [^{35}S]methionine in 80 ml of 15 mM Mes, 25 mM sucrose, pH 6.5. After immunoadsorption of the solubilized labeled plasma membrane proteins and separation by SDS-PAGE as described above, the gel was impregnated with Amplify (Amersham Corp., Arlington Heights, IL) and fluorographed.

3. RESULTS AND DISCUSSION

The only detergents we have found that solubilize pea GS-II activity without virtually complete inactivation are digitonin and Chaps. When Chaps-solubilized crude membranes are fractionated by glycerol gradient centrifugation, a 55 kDa polypeptide correlates with the peak of GS-II activity (Fig. 1). Most other major

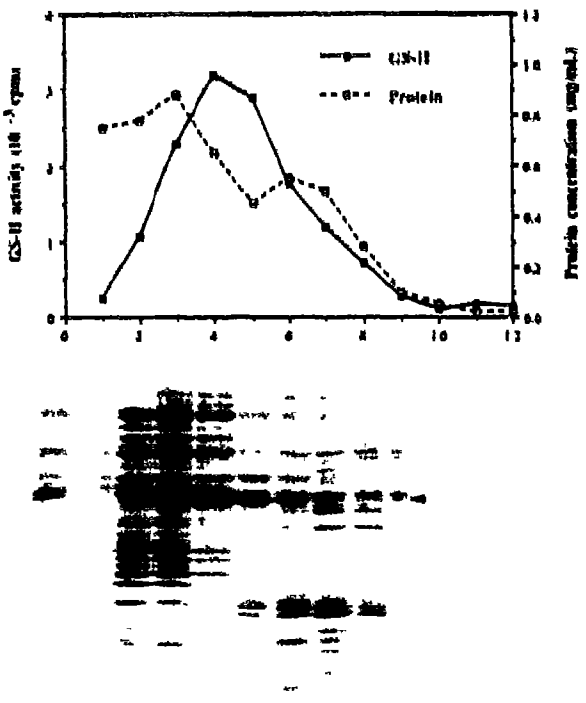

Fig. 1. Glycerol gradient fractionation of GS-II activity. The 150000 × g supernatant from Chaps solubilized crude membranes was layered onto a 30–60% glycerol gradient and centrifuged at 207000 × g for 15 h. Fraction 1 represents the top of the gradient. A (above) GS-II activity and total protein profiles. B (below) SDS-PAGE of gradient fractions. Left lane contains starting material; remaining lanes correspond to gradient fractions located directly above, in part A of the figure. Arrowhead points to 55 kDa band that correlates with GS-II activity peak.

polypeptides peak in the first three fractions and do not correlate with the GS-II activity peak, but a 68 kDa polypeptide, only faintly visible in Fig. 1B, also correlates with GS-II. A similar correlation between the 55 and 68 kDa bands and GS-II activity is found after glycerol gradient centrifugation of digitonin-solubilized crude membranes and of Chaps-solubilized plasma membranes (data not shown), even though digitonin-solubilized GS-II activity sediments considerably farther into the gradient than does Chaps-solubilized activity, presumably because of different composition of the respective activity-containing micelles.

Other fractionation techniques, notably product entrapment [20] and isoelectric focusing, which employ principles entirely different from those utilized in density gradient centrifugation, verified the correlation between the 55 kDa band GS-II activity, and allowed us to eliminate the above-mentioned 68 kDa polypeptide from further consideration as a candidate for GS-II activity (this evidence will be presented elsewhere). We therefore isolated the 55 kDa band from SDS gels of plasma membrane proteins, and raised an antiserum against it. This antiserum proved to be highly specific for the 55 kDa plasma membrane polypeptide (Fig. 2,

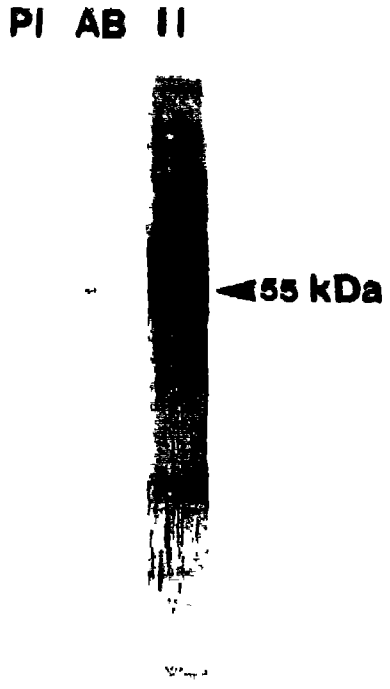

Fig 2 Western blots of SDS-PAGE-separated pea plasma membrane proteins, probed with: (lane PI) preimmune serum, (lane AB) anti 55 kDa antiserum, (lane II) India ink

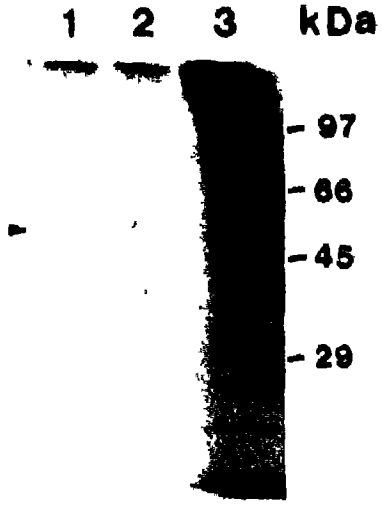

Fig. 3. Immunoadsorption, by anti-55 kDa antiserum, of digitonin-solubilized proteins from [^{35}S]methionine-labeled pea plasma membrane. Plasma membrane proteins (lane 3) were incubated either with antiserum (lane 1) or preimmune serum (lane 2), followed by protein A-Sepharose. Adsorbed proteins were separated by SDS-PAGE, and fluorographed. In lane 3, although the 55 kDa band is not visible (because of background darkening) in this reproduction, the band can be seen in the original fluorograph, from which it can be estimated that only about 1/4 of this polypeptide was immunoadsorbed (55 kDa band in lane 1).

lane AB), and to have very high titer, detecting its antigen even at 1:20000 dilution of the antiserum.

The anti-55 kDa antiserum adsorbs GS-II activity substantially, compared to preimmune serum (Table I). Since the mol wt of the 55 kDa polypeptide is the same as that of the heavy chain of IgG, the polypeptide could not be detected on Coomassie blue-stained gels run on antiserum-adsorbed enzyme. Therefore, we radiolabeled the membrane proteins in vivo with [^{35}S]methionine, and detected immunoadsorbed polypeptides by fluorography (Fig. 3). Since growing cells were used, most of their proteins should become labeled; lane 3 of Fig. 3 shows that numerous membrane proteins are indeed labeled. The antiserum adsorbs two polypeptides

Table I

Immunoadsorption of pea GS-II activity by anti-55 kDa antiserum

Treatment	GS-II activity (cpm)	% of GS-II adsorbed
Preimmune serum	298	4
Antiserum	1602	22

Digitonin-solubilized plasma membrane (5 parts) was incubated with either antiserum or preimmune serum (1 part), followed by an additional incubation with protein A-Sepharose beads, which were then washed. Both beads and unadsorbed proteins were assayed for GS-II activity. The values for % of activity adsorbed, given in the right hand column, were the same whether expressed relative to the initial, or to the total recovered, activity

of 80 and 55 kDa, whilst preimmune serum adsorbs the 80 kDa, but not the 55 kDa polypeptide (Fig. 3). Both antiserum and preimmune serum also weakly recognize an 80 kDa band on Western blots (barely visible in Fig. 2), indicating that some nonspecific IgGs against this polypeptide may be present in rabbit serum. These observations indicate that the 55 kDa polypeptide is responsible for GS-II activity. However, even at 1:5 dilution only about 20% of the total GS-II activity is immunoadsorbed by this antiserum, despite its high titer mentioned above (greater antiserum dilutions adsorb even less). Furthermore, the antiserum does not inhibit enzyme activity. Possible explanations for these discrepancies include (i) many of the IgGs in this antiserum might be directed against epitopes that are exposed in the SDS-denatured 55 kDa polypeptide used as antigen, but not in the detergent-solubilized active enzyme, and/or (ii) the 55 kDa band might contain more than one polypeptide, GS-II activity being due to only one of these. That immunoadsorption of the 55 kDa polypeptide was incomplete under the conditions of GS-II activity immunoadsorption (see legend to Fig. 3) favors explanation (i).

This report provides the first direct identification of a specific polypeptide as a higher plant cell wall polysaccharide synthase activity. It agrees with the previous suggestions, based on results from partial purification and ligand labeling, that a 52–57 kDa polypeptide is involved in GS-II activity in various plant systems [8,11,21]. Recently Fink et al. [13] con-

cluded, in contrast, that in soybean cells a 31 kDa polypeptide represents (or contributes to) GS-II activity. However, although an apparently specific anti-31 kDa antiserum was obtained [13], it was not shown to adsorb GS-II activity, as our anti-55 kDa antibody does. It seems unlikely that the GS-II activity of rather closely related plants (pea and soybean) could differ so much in mol wt. Both these polypeptides might be components of a multisubunit enzyme complex, as suggested for cotton GS-II [12], although no indication of this is apparent in the present results.

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