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Characterization of the UDP-glucose: (1,3)-β-glucan (callose) synthase from plasma membranes of celery: polypeptide profiles and photolabeling patterns of enriched fractions suggest callose synthase complexes from various sources share a common structure

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Plasma membranes were purified from celery (*Apium graveolens* L.) by two-phase partitioning and the UDP-glucose (1,3)-β-glucan (callose) synthase was solubilized with the detergent CHAPS. The techniques of moderate pressure anion exchange chromatography (FPLC), gel filtration chromatography, affinity chromatography on UDP-agarose and product-entrapment were then investigated as means for further purification. Enriched fractions were obtained by anion exchange chromatography in 0.1% CHAPS and 50 mM Tris–HCl (pH 7.5, spec. act., 568 nmol min^{-1} (mg prot.)$^{-1}$), by affinity chromatography using UDP-agarose (1405 nmol min^{-1} (mg prot.)$^{-1}$) and by product entrapment (914 nmol min^{-1} (mg prot.)$^{-1}$). Fractions purified by anion exchange chromatography contained numerous polypeptides, however callose synthase purified by the affinity step yielded a silver-stained profile of six prominent polypeptides of 26.8, 30.5, 35, 41, 56 and 72 kDa. In the product-entrapped sample, the polypeptides of 41, 56 and 72 kDa incorporated [^{32}P]5N_3UDP-Glc in a Ca^{2+}-dependent manner, implying that these could play a role in the binding of UDP-Glc to the enzyme. The polypeptides at 26.8, 30.5, 35 and 72 kDa have also been observed in purified preparations from various other sources and could represent other components of the callose synthase complex.

Key words: callose synthase; fast protein liquid chromatography; affinity chromatography; plasma membrane; celery

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Abbreviations: ATPase, adenosine triphosphatase; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(aminoethylether)-*N,N,N′,N′*-tetraacetic acid; FPLC, fast protein liquid chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; IDPase, 'latent' inosine diphosphatase; [^{32}P]5N_3UDP-Glc, 5N_3[^{32}P]UDP-Glc, 5-azidouridine 5′-[^{32}P]diphosphate glucose; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol (av. mol. wt. 3350); P_i, inorganic phosphate; PM, plasma membrane; PMSF, phenyl methyl sulfonyl fluoride; PVP40, polyvinylpyrollidone (av. mol. size 40 000); SDS, sodium dodecyl sulfate; Tris–HCl, tris[hydroxymethyl]aminomethane hydrochloride; 4U fraction, fourth upper-phase after four phase separations; UDP-Glc, uridine diphosphate glucose.

Introduction

Activation of the plasma membrane (PM)-localized callose synthase [1] is one of several mechanisms by which higher plants respond to stress [2]. When plant cells are physically damaged, a disruption in the normal ionic gradient across the PM results leading to an efflux of Cl^- and K^+ ions and an influx of Ca^{2+}. This local increase in Ca^{2+} may lead to synthesis of callose [3].

To further assess the role of callose synthase and its involvement in the protection of damaged plant tissues it is important to understand the polypeptide composition of the callose synthase complex. Although it has proven difficult to enrich the active complex to homogeneity, partially purified

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fractions have been prepared by glycerol gradient centrifugation and product entrapment [4], product entrapment [5], fast protein liquid chromatography (FPLC) [6] and affinity chromatography [7]. From some of these studies, a number of polypeptides have been implicated as components of the complex. For example, Fredrikson, et al. [8] have shown five polypeptides (32, 35, 57, 65 and 66 kDa) were enriched by a combination of FPLC and product entrapment from cauliflower. Reid and Delmer [6] observed that a 42-kDa polypeptide from mung bean bound the affinity label UDP-[^{3}H]pyridoxal. Delmer et al. have more recently reported that a 52-kDa polypeptide was labeled with α-[^{32}P]UDP-glucose in a manner consistent with callose synthase activity and thus may play a catalytic role in this enzyme [9]. Frost et al. [10] identified a 57-kDa polypeptide as a potential UDP-Glc-binding polypeptide using the photoaffinity label [^{32}P]5N$_3$UDP-Glc. Using a monoclonal antibody directed at callose synthase, Meikle et al. were able to enrich four polypeptides of 30, 31, 54 and 58 kDa [11]. Of these the 31-kDa component appeared to be the UDP-glucose binding subunit. A 32-kDa polypeptide was enriched in fractions purified from soy bean cultures [12]. Using glycerol gradients and product entrapment, polypeptides of molecular size, 55 and 64 kDa, were observed to correlate with callose synthase activity [4]. Of these, the 55-kDa polypeptide had been shown to be closely associated with activity [13,14]. Coomassie Blue-stained SDS gels from the product-entrapped fraction used by Wu et al. [5] showed enrichment of closely-migrating polypeptides of 27, 29/31, 35, 43, 57, 70, 83 and 92 kDa.

To elucidate the polypeptide pattern of multicomponent membrane-bound complexes, it is important to be able to compare preparations from different sources. This approach proved to be a valuable tool for constructing a molecular model of the tonoplast ATPase [15] and could provide similar information with regard to pinpointing conserved components of the callose synthase complex. For this reason, it is important to compile a data bank of polypeptide profiles obtained from enriched PM fractions of various plant tissues and to determine which, if any, are common to most systems. During screening experiments with various plant sources, celery was found to contain a highly active and stable callose synthase [16]. Here, techniques for its solubilization and enrichment are described and potential UDP-Glc-binding polypeptides are identified with 5-azido-UDP-Glc. In addition, the polypeptide profile of a fraction obtained by affinity chromatography on UDP-agarose was determined. Similarities between polypeptides found in this fraction and enriched fractions from other sources are discussed.

Materials and Methods

Materials

Celery (*Apium graveolens* L.) was purchased fresh from local markets. PEG 3350 and digitonin were obtained from Sigma and Dextran T-500 from Pharmacia. The detergents Triton X-100, Tween-20, Lubrol PX and Brij 35 (all Surfact-Amps), CHAPS and β-octylglucoside were obtained from Pierce Chemical Co. All steps were performed at 4°C or on ice unless otherwise indicated.

Plasma membrane isolation

Celery stalks (1 kg) were rinsed, drained, cut-up and homogenized in a Waring blender in 1 l of homogenization buffer containing 0.25 M sucrose, 0.5% (w/v) PVP-40, 3 mM EDTA, 3 mM EGTA, 2 mM DTT and 70 mM HEPES (pH 8.0). During homogenization of tissue 1 mM PMSF was included. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 95 000 $\times$ g for 30 min in a Beckman Ti70 rotor. The pellets (microsomal fraction) were combined and resuspended in a solution containing 0.25 M sucrose and 5 mM potassium phosphate (pH 7.8) to a volume of 18 ml. Protein concentrations ranged from 6 to 8 mg ml^{-1}.

A PM-enriched fraction was prepared following the aqueous two-phase method of Larsson [17], with dextran T-500 levels of stock solutions monitored by polarimetry. Microsomes (6 ml) were combined with a solution containing 6.5% (w/w) of both PEG and dextran to give a total weight of 24 g. The tubes were mixed (approx. 50 $\times$, inverted vigorously) and centrifuged at about 500 rev./min for 5 min in a Sorvall GLC-1

benchtop centrifuge to separate phases. The PM-enriched upper phase (PEG) was removed, combined with a new lower phase (dextran) lacking any membranes and adjusted to 24 g with additional upper (membrane free PEG) phase solution. A total of four successive phase separations were done. Membranes from the fourth upper (PEG) phase were recovered by dilution with 2 vol. of a solution containing 0.25 M sucrose and 1 mM HEPES (pH 7.2) [18] followed by centrifugation at 95 000 × *g* for 30 min. This PM-enriched fraction is referred to as '4U' PM. A portion of the final pellet was diluted to 0.5 mg prot. ml^{-1} in a solution containing 0.25 M sucrose, 1 mM DTT, 15% (v/v) glycerol and 1 mM HEPES (pH 7.2) [19] and used immediately for enzyme marker analysis. The remainder was stored at −80°C.

For solubilization experiments, after one '4U' fraction was obtained (as described), membranes remaining in the lower (dextran) phases and interfaces were repartitioned to obtain additional membranes enriched in PM. Since some of the PM goes to the dextran phase and interface during two-phase separations, these can be obtained by repartitioning with new upper-phase solution. This was done by combining the first original lower phase and interface with new phase-separated (dextran-free) PEG solution lacking any membranes. Phases were mixed and separated and the new upper, PEG, phase was removed and repartitioned with the second original lower phase and interface. This was done with the third and fourth lower phases and interfaces until a new 4U fraction was obtained. A total of two complete repartitioning series were done. The three 4U fractions from the original partitioning and the two repartitioning series were pooled, diluted and recovered by centrifugation. Pellets were diluted to 1 mg prot. ml^{-1} prior to solubilization.

Enzyme assays

PM purity was assessed by marker enzyme assays as follows: Vanadate-sensitive ATPase (PM marker) was assayed in the absence or presence of vanadate (0.5 mM) and Triton X-100 (0.0125%) as described [20]. Nitrate-sensitive ATPase (tonoplast marker) was assayed with Triton (as above) and in the absence and presence of 50 mM KNO_3 [21]. Latent IDPase (Golgi marker) was assayed with or without 0.03% Triton X-100 [22]. Activity of these nucleotide phosphatases is expressed as nmol P_i released min^{-1} (mg prot.)$^{-1}$. Cytochrome *c* oxidase (mitochondria) and NADH cytochrome *c* reductase (endoplasmic reticulum) were assayed as described [23]. Specific activities are expressed as μmol of cytochrome *c* oxidized or reduced min^{-1} (mg prot.)$^{-1}$. Callose synthase was assayed as described [18] with units of specific activity expressed as nmol [^{14}C]glucose incorporated into ethanol-insoluble polymer min^{-1} (mg prot.)$^{-1}$.

Protein assay

Protein was assayed by the Bradford microassay with BSA as standard [24]. Membrane-containing samples were assayed by bringing samples to 100 μl in 0.1% (v/v) Triton X-100. In gradient samples containing Tris-acetate, which reacts with assay reagents, average background values were determined for each gradient fraction with no protein introduced and these were subtracted to correct for absorbance due to acetate.

Callose synthase solubilization

PM from the 4U fraction (0.5 ml at 1 mg prot. ml^{-1}) were combined with 0.5 ml of a 1.2% detergent (listed in Table II) solution containing 1 mM each of EDTA and EGTA and 50 mM Tris–HCl (pH 7.5). Suspensions were incubated 30 min on ice and centrifuged at 85 000 × *g* for 30 min. The supernatant fraction was filtered through a 0.22-μm filter and stored on ice for subsequent assay and chromatographic analysis or stored at −80°C. Pellets were resuspended in storage buffer, diluted to 0.5 mg prot. ml^{-1} and assayed for activity and protein.

Samples used for affinity chromatography were solubilized by the two-step method using CHAPS [18]. PM (2 mg ml^{-1}) were combined with an equal volume of a solution containing 0.6% (w/v) CHAPS, 10 mM $MgCl_2$ and 50 mM Tris–HCl (pH 7.5). After incubation for 30 min and centrifugation (as above), the pellet was resuspended in 50 mM HEPES (pH 7.5) containing 15% (w/v) glycerol to 3 mg prot. ml^{-1}. An equal volume of 1.2% (w/v) CHAPS in 50 mM Tris–HCl (pH 7.5)

containing 1 mM EDTA and EGTA was added. Following incubation (30 min), solubilized enzyme was recovered from the supernatant fraction after centrifugation at approx. 100 000 × *g* for 30 min.

Anion exchange chromatography

Ion exchange steps were conducted using a Waters 650 protein purification system equipped with a Waters DEAE-5PW anion exchange column. The system was held at room temperature and buffers were held on ice throughout. Four buffer systems (A–D), each containing 0.1% CHAPS and adjusted to pH 7.5, were compared [25]. Binding buffers systems were as follows: (A) 50 mM Tris–HCl; (B) 50 mM Tris-acetate; (C) 50 mM HEPES-NaOH; (D) 50 mM sodium phosphate. Elution buffers for systems A, C and D consisted of respective binding buffers plus 1.0 M NaCl, and for system B, binding buffer B plus 1.0 M sodium acetate.

Samples (500 μg protein) were injected and allowed to bind for 5 min in binding buffer. A linear gradient of 20–50% elution buffer was introduced for 40 min and this was followed by addition of pure elution buffer for 15 min. Chromatography was conducted at 1 ml min^{-1} at about 300 psi and fractions were immediately placed on ice.

Active fractions from buffer system A (fractions 23–32) were pooled, desalted over a Sephadex G25 column and chromatographed a second time. Approximately 10 ml of the pooled fractions were concentrated to about 4 ml using Centricon 10 (Amicon) ultrafilters.

Affinity chromatography

Callose synthase was solubilized by the two-step procedure using enriched PM from the third upper phase (3U) rather than the fourth upper phase (4U). The 3U fraction contained enriched PM, similar to 4U PM, except one less phase separation had been done. The 3U fraction yielded more PM so the two-step solubilization procedure could provide adequate yields of enzyme. Solubilized enzyme (2 ml) was added to a column containing 1 ml of UDP-agarose (Sigma) and washed with 18 ml of buffer (containing 5 mM $MgCl_2$, 0.01% (w/v) digitonin and 50 mM Tris–HCl (pH 7.5). The enzyme was eluted with 20 mM UDP and fractions of 1 ml were collected and assayed.

Product entrapment and photoaffinity labeling

To determine the specific activity and yields of product-entrapped fractions a reaction mixture of 1.5 ml was prepared by combining 0.24 ml of solubilized enzyme (0.5 mg prot. ml^{-1}) with 1.26 ml of an effector mix to give final concentrations of: 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.02% digitonin, 5 mM cellobiose, 1.5% glycerol and 0.12% CHAPS in 50 mM Tris–HCl (pH 7.5) (EDTA, EGTA, glycerol and CHAPS levels reflect carryover from solubilization). Synthesis of glucan was initiated by addition of 1 mM UDP-Glc. After 40 min at 30°C, the reaction was stopped by cooling to 4°C for 30 min and the mixture was then centrifuged at low speed (7000 × *g* for 40 min at 8°C in a Fisher microfuge). The supernatant fluid was carefully removed and the pellet was resuspended in a solution containing 0.75 ml of 0.1% CHAPS, 3 mM EDTA, 10% glycerol and 50 mM Tris–HCl (pH 7.5).

For photolabeling experiments, reaction mixtures were scaled up to 6 ml and Tris–HCl was replaced with HEPES-NaOH. Following entrapment, pellets were resuspended in 300 μl of a solution containing 10% (v/v) glycerol, 0.05% (w/v) CHAPS, 1 mM EDTA and 5 mM HEPES-NaOH (pH 7.5). Glucan was removed by centrifugation at 80 000 × *g* for 30 min and the supernatant fraction was used for photoaffinity labeling with [^{32}P]5N_3UDP-Glc as described [10].

Electrophoresis

SDS-PAGE was conducted in 7.5–15% gradient gels by the method of Laemmli [26] as modified by Hames [27]. Piperazine diacrylamide (Bio-Rad) was substituted directly for bis-acrylamide to reduce silver staining of background in gels. Samples were diluted 1:1 with a solution containing 20% (v/v) glycerol, 8 M urea, 10% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue and 0.125 M Tris–HCl (pH 6.8) and heated at 90–100°C for 3 min. Samples from very dilute column fractions were concentrated with Centricon-10 ultrafilters. Proteins were visualized by silver staining [28].

Results

Membrane isolation and solubilization

Plasma membrane isolation by two-phase partitioning. Initial screening experiments indicated that PM was purifying in the polymer range of 6.1–6.7% (w/w). PM purification was then optimized. First, the effect of polymer concentration on the phase distribution of PM was determined using Triton X-100 stimulation [29,30] and vanadate inhibition [20] of the PM ATPase after a single phase separation (data not shown). At all polymer concentrations, Triton X-100-stimulated ATPase was found in both phases; however, the magnitude of this stimulation was 2-fold greater in the upper phase. Likewise, vanadate inhibition was greatest in the upper phase. Polymer levels of 6.5% were used for subsequent experiments.

Additional phase separations greatly improved the purity of PM in the upper phase (Fig. 1). The magnitude of Triton X-100 stimulation (Fig. 1A) and vanadate inhibition of the PM ATPase (Fig. 1B) increased until the fourth separation with only a slight further increase after five separations. Therefore, all subsequent isolations were done using four successive separations at a polymer concentration of 6.5% (w/w).

Specific activities of various marker enzymes determined for microsomal and 4U membranes confirmed isolation of a PM-enriched fraction (Table I) since each of the PM markers (ATPase, callose synthase) was significantly enriched. All other markers indicated differing levels of contamination, but their specific activities were greatly reduced in the 4U fraction (Table I) and increased in the first lower phase (not shown). The nitrate-sensitive component of tonoplast membranes was slightly greater in the 4U fraction than with microsomal membranes (125.6 vs. 106.3 units/mg, respectively). However, the actual percentage of total ATPase (ATPase assayed in the presence of Triton X-100) sensitive to nitrate (also assayed in the presence of Triton X-100) was greater in microsomal membranes than in the 4U fraction (25% vs. 11%, respectively).

Callose synthase solubilization. Of the various detergents screened, CHAPS was the most effective (Table II). With CHAPS, 76% of the total activity recovered was in the solubilized form and the specific activity was increased by 2-fold relative to the 4U fraction. Although complete activity was recovered with digitonin, only 30% was solubilized, with the remainder found in the pellet. Each of the other detergents effectively solubilized protein, but inactivated the callose synthase.

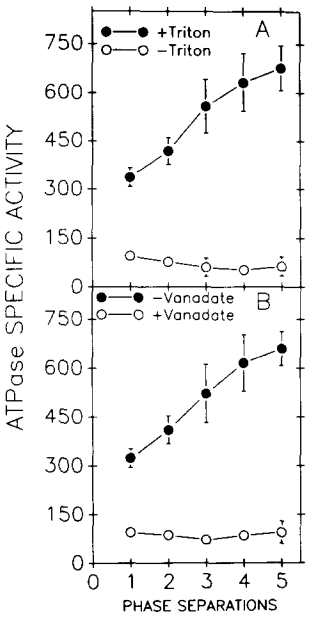


Fig. 1. ATPase activity of membranes from the upper (6.5% PEG) phase following single or multiple phase separations. (A) ATPase activity in the absence and presence of Triton X-100. (B) ATPase activity in the absence and presence of vanadate. ATPase specific activity is expressed as μmoles P_i released min^{-1} (mg prot.)$^{-1}$. The values are the means from three separate isolations ± S.E.

Callose synthase enrichment

To obtain further purification of callose synthase, the techniques of anion exchange chromatography, affinity chromatography with UDP-agarose and product entrapment were each evaluated.

Anion-exchange chromatography. In a previous study, Reid and Delmer [6] explored the use of anion-exchange FPLC to obtain partial purification of digitonin-solubilized callose synthase from mung bean. As a part of this study, its use for

Table I. Membrane enzyme marker analysis of a partially purified PM fraction (4U)

Marker enzyme[a]	Microsomal	4U fraction
PM ATPase		
−Triton X-100	119 ± 15	165 ± 14
+Triton X-100	428 ± 15	1099 ± 34
+Vanadate	164 ± 10	174 ± 32
Vanadate-sensitive component[b]	264 (62%)	924 (84%)
Tonoplast ATPase		
+Nitrate	322	973
Nitrate-sensitive component[c]	106 (25%)	126 (11%)
Callose synthase	115 ± 8	277 ± 13
Inosine diphosphatase		
−Triton X-100	32 ± 7	5 ± 2
+Triton X-100	83 ± 14	19 ± 3
Cytochrome *c* oxidase	0.51 ± 0.02	0.10 ± 0.03
NADH cyt. *c* reductase	0.09 ± 0.02	0.01 ± 0.00

[a]Values are the means of 10 determinations for the PM tonoplast ATPase, 6 for the glucan synthase and cytochrome *c* reductase and 5 for the inosine diphosphatase and cytochrome *c* oxidase ± S.E. Specific activities of nucleotide phosphatases are expressed as nmol P_i released min^{-1} (mg prot.)$^{-1}$. Callose synthase specific activity is expressed as nmol [^{14}C]glucose incorporated into ethanol-insoluble polymer min^{-1} (mg prot.)$^{-1}$. Cytochrome *c* oxidase and reductase specific activities are expressed as μmol cytochrome *c* oxidized or reduced min^{-1} (mg prot.)$^{-1}$.

[b]Vanadate-inhibited activity. Number in parenthesis indicates percent inhibition of total Triton stimulated ATPase activity.

[c]Nitrate-inhibited activity. Number in parenthesis indicates percent inhibition of total Triton stimulated ATPase activity.

Table II. Comparison of various detergents for solubilization of callose synthase activity. Activities and total protein levels of solubilized (sol) and sedimented (pel = pellet) membrane proteins were determined. The control contained all components of the single step solubilization procedure except detergent. Values represent the means from 3 separate isolations ± S.E.

Detergent	Fraction	Total act. (nmol min^{-1})	Total prot. (μg)	Spec. act. (nmol min^{-1} (mg prot.)$^{-1}$)
None	4U	83	519	161 ± 12
Control	sol	2 (4)[a]	74 (14)[a]	20 ± 6
	pel	53 (96)	467 (86)	114 ± 30
CHAPS	sol	87 (76)	284 (66)	308 ± 4
	pel	28 (24)	145 (34)	190 ± 14
Digitonin	sol	29 (30)	179 (42)	163 ± 18
	pel	67 (70)	250 (58)	270 ± 12
Triton X-100	sol	0 (0)	222 (59)	0
	pel	21 (100)	154 (41)	136 ± 13
β-Octyl-glucoside	sol	6 (60)	303 (66)	20 ± 7
	pel	4 (40)	158 (34)	24 ± 10
Tween-20	sol	1 (3)	172 (33)	3 ± 3
	pel	29 (97)	344 (67)	83 ± 16
Lubrol PX	sol	0.3 (3)	403 (75)	1 ± 1
	pel	11 (97)	133 (25)	81 ± 32
Brij 35	sol	2 (8)	254 (45)	8 ± 5
	pel	23 (92)	306 (55)	75 ± 16

[a]Values in parenthesis represent percentages of recoverable activity and protein in each fraction where sol + pel = 100%.

partial purification of CHAPS-solubilized celery glucan synthase was evaluated and factors influencing recovery of enzyme activity were defined.

Buffer choice significantly affected protein profiles and enzyme recovery (Fig. 2). Defined peaks of activity were recovered in the Tris–HCl and Tris-Acetate (Figs. 2A and 2B, respectively) and HEPES-NaOH (Fig. 2C) systems, while phosphate (data not shown) caused complete loss of activity. Activity recoveries were comparable in Tris–HCl (23%) and HEPES-NaOH (26%); however the specific activity of the enzyme chromatographed in Tris–HCl was somewhat higher than the HEPES-NaOH sample (568 vs. 476 nmol min^{-1} (mg prot.)$^{-1}$, respectively). Activity recovery in Tris-acetate was low (9%) and gave a specific activity of 380 nmol min^{-1} (mg prot.)$^{-1}$. An early but consistent peak of callose synthase activity was characteristic of the Tris–HCl system (Fig. 2A). Retention time in HEPES was several minutes longer than in Tris–HCl (Figs. 2A and 2C, respectively). Elution profiles were not significantly affected by CHAPS levels, since similar elution profiles were obtained at 0.1% and 0.6% CHAPS (not shown).

Peak fractions collected from the first chromatographic step were concentrated to 0.4 ml and re-chromatographed in 0.1% CHAPS and 50 mM Tris–HCl using a Waters 300SW gel filtration column. Enzyme specific activity only slightly

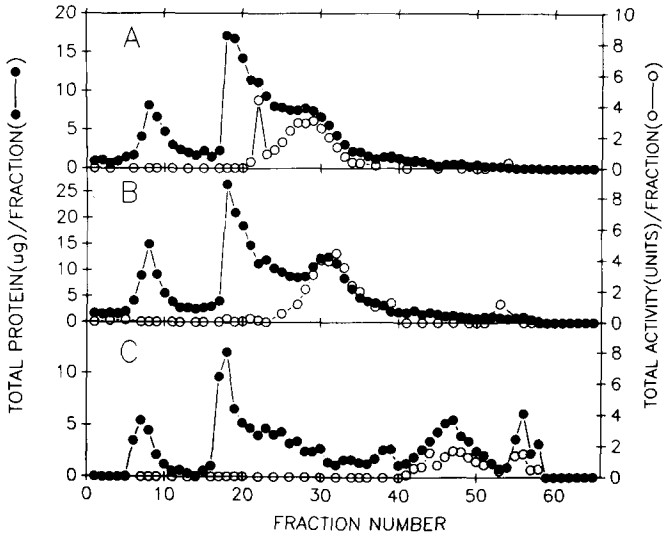
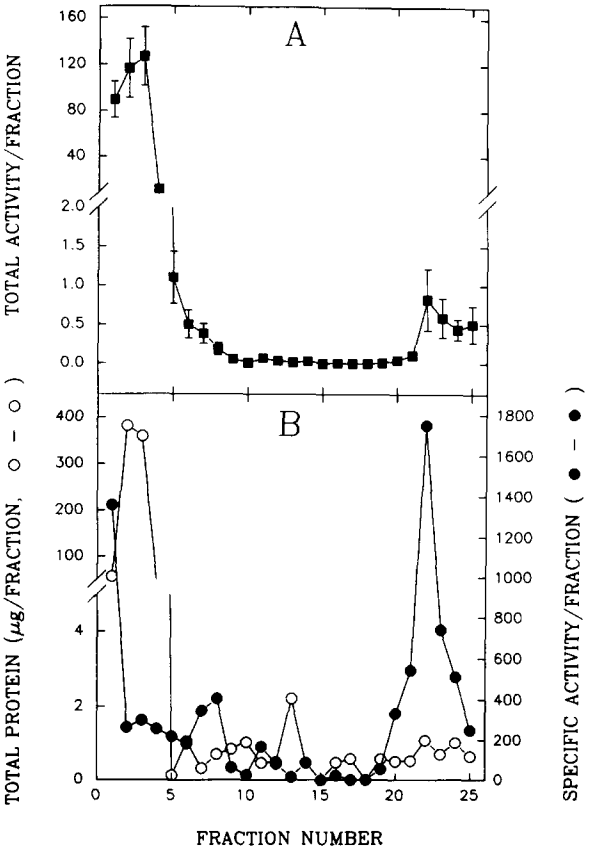


Fig. 2. Anion exchange analysis of CHAPS-solubilized callose synthase. (A) Tris–HCl and CHAPS with an NaCl gradient; (B) HEPES-NaOH and CHAPS with an NaCl gradient; (C) Tris-Acetate and CHAPS with a sodium acetate gradient. See Materials and Methods for concentrations, gradient and flow rate. The total activity is expressed as nmol of [^{14}C]glucose incorporated into an ethanol insoluble polymer min^{-1}.



improved and was present in the void volume thus yielding no further purification (data not shown). Further chromatography using anion exchange led to both lower protein recovery and almost complete loss of activity. It should be noted that inclusion of asolectin following additional anion exchange purification provided some stabilization and recovery of callose synthase but activity levels did not exceed values obtained after one anion exchange step. Recovery of specific activity levels, due to protection by asolectin against inactivation, is consistent with earlier studies where gravity flow columns were used [18].

Affinity chromatography. Figure 3 shows that about 98% of the protein applied to a UDP-agarose column was eluted in the first few fractions. However, upon addition of 20 mM UDP, a

Fig. 3. Affinity chromatography on UDP-agarose of two-step solubilized membrane proteins from an enriched PM fraction. (A) Total activity of callose synthase recovered/fraction. Data points are the means ± S.E. from six separate analyses. (B) Total protein/fraction and specific activity/fraction of a representative chromatogram. The average specific activity of six analyses was 1400 ± 264 (S.E.). Specific activity is expressed as nmol [^{14}C]glucose incorporated into ethanol-insoluble polymer min^{-1} (mg prot.)$^{-1}$.

second peak of activity eluted. The average specific activity of the peak fraction (fraction 22) from six analyses was 1400 nmol min^{-1} (mg prot.)$^{-1}$, a 6.5-fold increase over solubilized enzyme and a 55-fold increase over the homogenate. Since UDP is a potent competitive inhibitor of callose synthase [31] and was not removed before assay, this increase probably underestimates the actual degree of purification. Recoveries of total protein and activity from affinity chromatography were mostly about 96% and 189%, respectively. This near doubling of activity (i.e. 189%), mostly due to an enhancement of the activity of the unretained callose synthase, may reflect either detergent-activation or removal of an inhibitor.

Product entrapment. Preparation of sufficient quantities of active callose synthase for photolabeling experiments requires a rapid enrichment step. Therefore, the product entrapment method was investigated. Using a PM fraction isolated by discontinuous gradient centrifugation [18] and solubilized by the two-step method, product entrapment was conducted incubating standard callose synthase assay mixtures followed by centrifugation (7000 × *g*, 40 min). This procedure increased specific activity from 574 nmol min^{-1} (mg prot.)$^{-1}$ in the solubilized fraction to over 900 nmol min^{-1} (mg prot.)$^{-1}$, with a yield of approx. 35%. Callose synthase was released from the enzyme-glucan matrix by suspension in a buffer containing 3 mM EDTA, 0.1% CHAPS and 10% glycerol, followed by a second centrifugation at 7000 × *g*. Enzyme activity declined by an additional one-third during the resuspension step; however, it was stable when frozen.

Polypeptide components of the affinity-purified enzyme complex

Polypeptide profiles of the enriched fractions were analyzed by SDS-PAGE. UDP-Glc-binding polypeptides from affinity purified fractions and product entrapped fractions labeled with the photoaffinity probe [^{32}P]5N_3UDP-Glc were also resolved.

The fraction obtained by affinity chromatography yielded the cleanest preparation. Figure 4 (lane 6) shows that polypeptides at 26.8, 30.5, 34.8, 41, 56 and 72 kDa were enriched by the UDP-agarose column. No polypeptides of size >72 kDa were seen. It should be noted that although the specific activity of the callose synthase not retained by affinity chromatography (Fig. 3, fraction 1) was always improved (i.e. 1290 nmol min^{-1} (mg prot.)$^{-1}$), it contained numerous polypeptides, including those enriched in fraction 22 (not shown).

Analysis of the fractions prepared by anion-exchange chromatography with either one or two

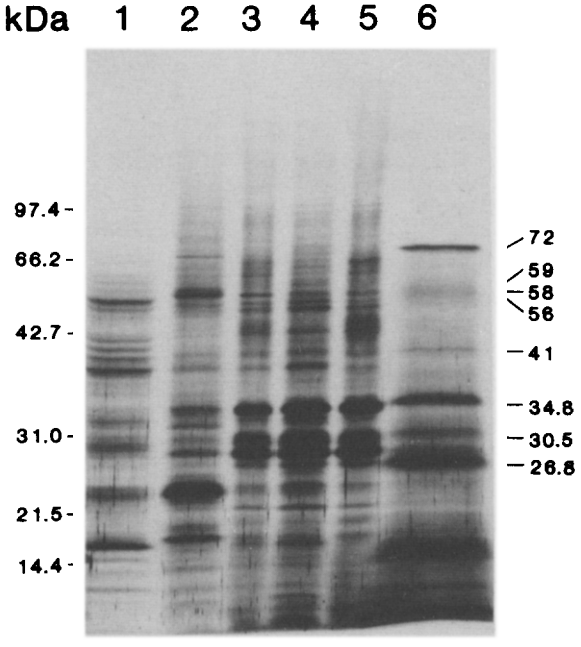


Fig. 4. SDS-PAGE analysis of membrane proteins from successive isolation and solubilization steps and from affinity chromatography purified fractions of callose synthase. Membranes for gel analysis were isolated in the presence of 1 mM PMSF. Lanes: (1) crude celery homogenate; (2) microsomal membranes of celery; (3) membranes from the fourth upper (4U) phase of the two phase system enriched in PM of celery; (4) 4U membrane proteins solubilized after first solubilization step described by Sloan et al. [18] (see also text); (5) membrane proteins solubilized from second solubilization step with 4U membranes; (6) second step solubilized proteins (lane 5) eluted from UDP-agarose affinity column. Approximately 5 μg of protein/lane. kDa relative to the standards: lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 42.7 kDa; serum albumin, 66.2 kDa; phosphorylase B, 97.4 kDa are shown.

chromatographic steps showed that active fractions contained each of the polypeptides observed in Fig. 4 (lane 6); however, many other polypeptides were also present. In accordance with the previous study [6], the instability of the anion exchange fractions precluded further fractionation attempts.

To identify potential UDP-Glc-binding polypeptides, CHAPS solubilized and product-entrapped fractions were photolabeled with [^{32}P]5N$_3$UDP-Glc. Photolabel in the solubilized enzyme was observed in polypeptides of 44, 56, 71 and 80 kDa (Fig. 5, lane 1). The same polypeptides were observed in the product-entrapped sample, however, an apparent shift in the 56-kDa polypeptide to 54 kDa should be noted. The reason for this is not clear, but could be explained by proteolysis during the product entrapment procedure. Figure 5 (lanes 4 and 5) shows that photolabeling of the 44-, 56- and 71-kDa polypeptides required the presence of divalent cations.

Discussion

The callose synthase from celery appears typical of callose synthases with respect to its kinetic characteristics, subcellular localization [32], solubilization properties and behavior upon purification. In addition, when polypeptide patterns of purified fractions derived from various sources are compared, similar polypeptides or groups of closely-migrating polypeptides (clusters) are often seen.

In this study, the fraction of highest specific activity (affinity purified, Fig. 4, lane 6) contained polypeptides of 26.8, 30.5, 34.8, 41, 56 and 72 kDa. Polypeptides that may be equivalent to these have been observed in preparations from other sources, though it must be kept in mind that reported molecular sizes may vary between laboratories, due to differences in molecular weight standards, gel systems and methods used to assign molecular sizes. A number of polypeptides of about 15 kDa or lower appear to copurify with the six other affinity enriched polypeptides. Whether these are additional UDP-binding proteins or solubilized proteins which associate with the other six polypeptides is not known. However, they do not

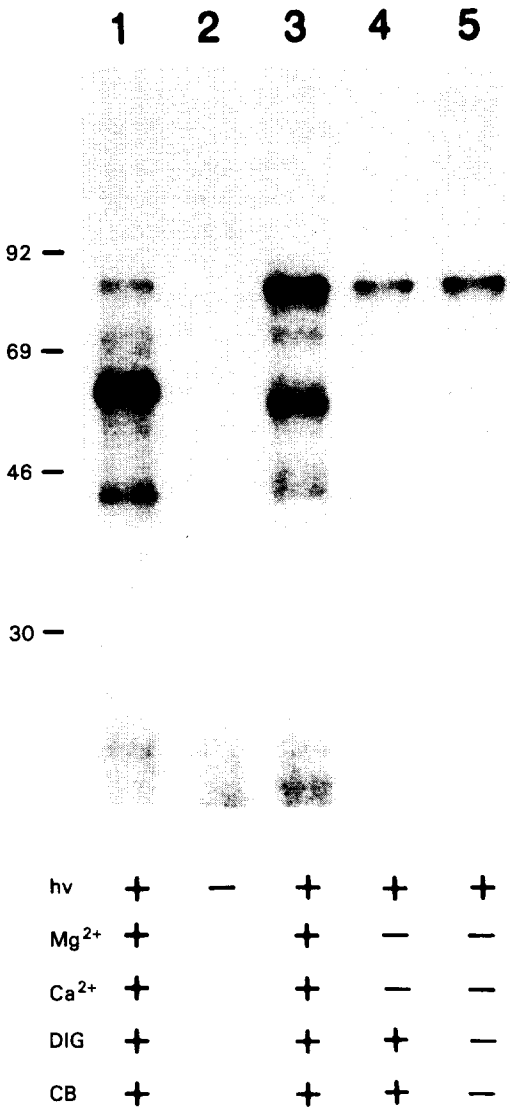


Fig. 5. Photolabeling of solubilized and product-entrapped callose synthase from celery. After photolabeling, samples were separated on SDS-PAGE, the gel was vacuum dried and exposed to X-ray film. Lanes: (1) solubilized enzyme (20 μg, spec. act., 420 units/mg); (2) non-photolyzed product-entrapped control in the presence of all effectors as in lane 1; (3–5) product-entrapped glucan synthase (20 μg, spec. act., 680); (3) with full effector mix; (4) minus divalent cations; (5) minus all activators. Samples were photolabeled with 20 μM probe (16.5 mCi/mol). Molecular weight standards were ^{14}C-methylated (Amersham) and correspond to: carbonic anhydrase, 30 kDa; ovalbumin, 46 kDa; bovine serum albumin, 69 kDa; phosphorylase *b*, 92 kDa.

correspond to any of the polypeptides that have been correlated with callose synthase activity in other studies.

Many parallels can be drawn with the six affinity enriched polypeptides. For example, most significant are the data of Fredrikson et al. [8]. Using anion exchange, gel filtration and product entrapment, five polypeptides were enriched at 32, 35, 57, 65 and 66 kDa. Additional polypeptides associated with the product entrapped enzyme, but which could be 'washed out', were 25, 28, 59, 70 and 73 kDa. Several of the five polypeptides that did not wash out correspond closely with a number of the affinity enriched polypeptides (e.g. the 32-, 35- and 57-kDa and the 30.5-, 34.8- and 56-kDa polypeptides, respectively). Two of the polypeptides which washed out may also correlate with two other affinity enriched polypeptides (e.g. the 28- and 73-kDa and the 26.8- and 72-kDa polypeptides, respectively). Fredrikson et al. [8] suggested that the polypeptides which washed out were nonspecific coprecipitates. It is therefore interesting that two of these polypeptides appear to correspond closely with two polypeptides of affinity-enriched material. Meikle et al. [11] have shown that callose synthase from *Lolium multiflorum* L., immunoprecipitated with a specific monoclonal antibody, was enriched on SDS-PAGE in four polypeptides, e.g. 30, 31, 54 and 58 kDa. Only the 31-kDa polypeptide was able to bind a UDP-Glc photoaffinity label. It should be noted here also that the 30- or 31-kDa and the 54- or 58-kDa polypeptides closely correspond with the 30.5- and 56-kDa polypeptides, respectively, of celery. In addition, Dhugga and Ray [4] have recently shown that two polypeptides of 55 and 64 kDa correlate with activity in glycerol gradients from digitonin or CHAPS-solubilized pea plasma membranes. They had previously reported that only the 55-kDa polypeptide correlated with pea plasma membrane activity [13,14]. In beet storage tissue, a 57-kDa polypeptide photolabels with [^{32}P]5N$_3$UDP-Glc [10]. In the present study a 56-kDa polypeptide of celery binds this probe. In cotton, a 52-kDa polypeptide was shown to bind the photolabel α-[^{32}P]UDP-glucose [9]. Gradient-enriched fractions from *Saprolegnia* [33], cotton [34,35] and pea [4] also contain this polypeptide. A 42-kDa polypeptide that binds UDP-pyridoxal was reported in mung bean [6] and it would be of interest to determine whether this is similar to the 44-kDa polypeptide that photolabels in the present work (Fig. 5). A 34-kDa polypeptide is found in purified fractions of cotton [34], red beet [5] and *Saprolegnia* [33] and in one study [34] it was suggested to be a proteolytic product of a larger polypeptide. As indicated before, several smaller-sized polypeptides at 30.5 and 26.8 kDa have been observed. In beets, a polypeptide of approx. 29 kDa photolabels with [^{32}P]5N$_3$UDP-Glc [10] and in *Lolium* a 31-kDa polypeptide photolabels with 5-[^{125}I]IASA-UDP-Glc [11]. Polypeptides in this size range have been shown to correlate with activity profiles in glycerol gradients of cotton [34] and pea [4]. These may bear a similarity to a 31-kDa polypeptide found in purified preparations from soybean [12]. At this point it cannot be concluded that all of these polypeptides are components of the callose synthase complex, but the fact that similar polypeptides are enriched in callose synthase preparations from diverse sources, using a variety of enrichment methods, strongly suggests that researchers are converging upon a common structural model.

The behavior of the enzyme during purification was largely consistent with expectations, but several observations should be noted. Anion-exchange chromatography increased specific activity by almost 2-fold (Table I and text). Significant activity losses occurred, particularly in Tris-acetate (Fig. 2B) and sodium phosphate (not shown). Reconstitution in asolectin only partially restored enzyme activity. Subsequent gel filtration failed to resolve the callose synthase activity from other proteins and consistently was associated with mixed micelles of >300 kDa that eluted in the void volume.

Affinity chromatography increased purity as judged by specific activity and SDS-PAGE. However, despite the fact that only a small fraction of enzyme bound to the column, this technique consistently yielded protein fractions enriched in relatively few polypeptides, as discussed above. Activity recoveries are probably significantly underestimated, since UDP is a competitive inhibitor with a K_i of 20 μM [31].

Removal of UDP from purified fractions was not attempted.

Though purification of callose synthase to homogeneity in active form remains elusive, this study demonstrates a convergence of polypeptide profiles when data from a range of systems is compared. Any of the 6 groups of polypeptides visualized here could represent components of the complex. At present polypeptides common to two or more systems and those that specifically bind active site probes, are the strongest candidates. Development of a model is further complicated by the occurence of closely-migrating clusters of polypeptides within some of the groups (e.g. the 56-, 58- and 59-kDa polypeptides and several between 26.8 and 34.8 kDa, Fig. 4). It remains to be determined whether polypeptides within each cluster are structurally related. This primitive model of the callose synthase complex will undoubtedly be subject to extensive fine-tuning as additional information is obtained.

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