

Partial purification of a GTP-insensitive (1 → 3)-β-glucan synthase from *Phytophthora sojae*

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Abstract A (1 → 3)-β-glucan synthase activity was identified in cell membrane preparations from the oomycete *Phytophthora sojae*, a soybean pathogen. The activity could be solubilized using the zwitterionic detergent CHAPS at relatively low concentrations (3 mg/ml). High salt concentrations were not effective in removing the activity from the membranes. Detergent solubilization of the enzyme resulted in a six-fold increase of calculated $V_{\max}$ values (2.5 vs. 0.4 nkat/mg protein) but only minor alteration of the K_m (10.6 vs. 10.7 mM). Analysis of the reaction product of the solubilized enzyme by enzymatic degradation and by 2D NMR spectroscopy confirmed its identity as a linear high molecular weight (1 → 3)-β-glucan. Glucan synthase activity in both membrane and solubilized preparations was not activated by GTP or divalent cations as reported for other fungal or plant glucan synthases. The activity was inhibited, as expected, in a competitive manner by UDP with a K_i of 2.9 mM. Partial purification of the enzyme was achieved by anion exchange chromatography followed by product entrapment. This procedure resulted in the selective enrichment of a protein band with apparent M_r 108 000 in SDS-PAGE which was not visible in any of the steps preceding product entrapment. The glucan pellets from product entrapment contained up to 3% of the initial enzyme activity present in the fraction used for the procedure.

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Key words: Cell wall synthesis; (1 → 3)-β-Glucan synthase; Product entrapment; *Phytophthora sojae*

1. Introduction

The mycelial walls of oomycetes are composed primarily of (1 → 3)-β-linked glucans with lesser amounts of (1 → 6)-β linkages [1–3]. The additional presence of up to 10% (1 → 4)-β linkages and the absence of chitin are among the distinctive features of this taxon which is now part of the kingdom Chromista [4]. The wall and soluble extracellular branched (1 → 3), (1 → 6)-β-glucans serve not only structural and storage functions but are also involved in chemical signaling in plant-pathogen interactions. The specific induction of phytoalexins by β-glucans of the oomycete phytopathogen *Phytophthora sojae* in soybean and other legumes has been well documented [5–8]. A cell surface protein has been characterized in soybean which binds a (1 → 3), (1 → 6)-β-heptagluco-side derived from

P. sojae mycelial walls and which may function as a receptor mediating plant defense responses [9,10]. In view of this evidence, and the fact that many oomycetes are plant pathogens of significant impact to agriculture, we considered it of interest to proceed with the study and biochemical characterization of *Phytophthora* cell wall synthesizing enzymes. The enzymatic synthesis of (1 → 3)-β-glucans in this genus was among the first to be reported [11,12].

A number of genes have been identified which are involved in the biosynthesis of (1 → 3)-β-glucans in yeast and *Neurospora crassa*. Genes *GS-1* and *KNR4/SM11* appear to encode transcriptional regulatory proteins [13] while *ETG1/FKS11* *CWH53/GSC1* and *GSC2/FKS2* encode similar 200 kDa proteins which have (1 → 3)-β-glucan synthase activity but are subject to differential regulation during the cell cycle [14–17]. Similar efforts have been made for medically more relevant genera such as *Aspergillus* where a *FKS1* homolog, *fkxA*, has been isolated which encodes a protein of 200 kDa, subsequently identified and partially purified [18], or *Candida* where a 187 kDa protein has been enriched [19] and a *FKS1/GSC1* homolog has been cloned [20]. On the other hand, no β-(1 → 6) linkage forming activity has been identified to date although at least the products of two genes, *KRE-6* and *SKN1*, appear to be involved in (1 → 6)-β-glucan biosynthesis in yeast [21]. The obvious importance of the wall glucans to fungal cell survival and the fact that mammals lack a wall have made glucan synthases a desirable target for antifungal therapy in medicine. This might not appear to be the case for fungal plant pathogens where both organisms involved in the interaction possess a cell wall. However, the initial characterization of a number of oomycete, fungal and plant (1 → 3)-β-glucan synthases suggests differences between these proteins regarding the effect of putative regulatory activators such as GTP [22,23] or other reported stimulators and stabilizers such as sucrose, glycerol, cellobiose, bovine serum albumin and divalent cations [2]. A better biochemical understanding of these differences would be useful to ascertain if the fungal or oomycete glucan synthases might be suitable targets for biotechnological or chemical approaches for pathogen control in crop plants. We report here on a (1 → 3)-β-glucan synthesizing activity in *P. sojae* cell membranes which is stable, can be solubilized using the zwitterionic detergent CHAPS, is amenable to partial purification by anion exchange chromatography and product entrapment techniques and displays no activation by GTP or divalent cations in vitro.

2. Materials and methods

2.1. Fungal cultures and microsomal fractions

P. sojae race 1 was obtained from our laboratory collection. Mate-

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; HPTLC, high performance thin layer chromatography; Zwittergent 3-12, N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate

rial for enzyme preparation was obtained from the mycelium of 3-week-old cultures grown in liquid medium. Inoculum for the liquid cultures was prepared fresh each time by growing the fungus on solid Lima bean agar (Difco, Detroit, MI USA) at 26°C for 1 week. Three or four 1 cm² agar pieces were cut and inoculated into each of 1.8 l Fernbach flasks containing 400 ml of modified Erwin synthetic liquid growth medium [24]. The flasks were grown for 3 weeks at 26°C in the dark. The mycelium was harvested by collection on a 300 µm nylon mesh and washed extensively with cold tap water and then distilled water to remove excess calcium carbonate. The washed mycelium was either frozen and stored at –20°C or used immediately to prepare a microsomal fraction.

The microsomal fraction used as starting material for the solubilization was prepared by homogenizing the mycelium in a Waring blender with 5 volumes of a buffer containing 50 mM Tris-HCl, pH 7.5, 1 M sucrose, 10 mM EGTA, 5 mM MgCl₂ and 10 mM NaF (buffer A). The homogenate was filtered through a 300 µm nylon mesh and centrifuged at 3500×g for 20 min. The supernatant was collected and centrifuged at 40 000×g for 45 min. The supernatant was discarded and the pellets collected, frozen in liquid nitrogen and stored at –80°C until used.

2.2. Solubilization and partial purification of the enzyme

Membrane pellets were washed by resuspension in 50 mM Tris-HCl, pH 7.5, 1 M sucrose, 25 mM NaF (buffer B) and centrifuged at 40 000×g for 45 min. The pellets were then resuspended in buffer B containing 3 mg/ml CHAPS (Calbiochem, Bad Soden, Germany) at a final protein concentration of 2 mg/ml and shaken slowly on an ice tray for 1 h. The suspension was then centrifuged at 180 000×g for 50 min. The supernatant was collected and the pellet reextracted under the same conditions in half the initial volume of buffer B containing CHAPS. Solubilized material was filtered through a 0.45 µm pore membrane, frozen in liquid N₂, and stored at –80°C until used.

Anion exchange chromatography of solubilized proteins was carried out on a prepacked 10×150 mm Fractogel EMD-DEAE Superformance cartridge (Merck, Darmstadt, Germany). Detergent-solubilized sample (20–30 mg protein) was diluted 1:1 with distilled water and loaded onto a column equilibrated with 25 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 10 mM NaF and 2 mg/ml CHAPS (buffer C). The column was then washed with 30 ml of buffer C containing 0.2 M NaCl. Elution of the activity was carried out using a 50 ml gradient from 0.2 M to 0.6 M NaCl in buffer C at a flow rate of 1 ml/min. One ml fractions were collected and those eluting between 28 and 38 min were pooled and used for characterization and further purification of the enzyme activity.

Product entrapment was carried out as described by Inoue et al. [17] with minor modifications. The DEAE pool or crude solubilized protein was incubated with 10 mM UDP-glucose for 1 h at 25°C. The glucan containing the entrapped protein was collected by centrifugation at 4000×g for 10 min and the pellet was washed twice in buffer C containing 5 mM UDP-glucose and centrifuged as before. The pellet was then resuspended in buffer C and centrifuged at 200 000×g for 10 min for a final wash. The resulting pellet was homogenized in buffer C using a Potter-Elvehjem homogenizer with a teflon pestle and centrifuged at 200 000×g for 10 min. The pellet was again resuspended in buffer C, homogenized and the resulting suspension was used directly for all assays.

Aliquots from all purification steps were analyzed by SDS-PAGE using 7×10 cm, 1 mm thick gels containing 8% (w/v) total acrylamide. Proteins were visualized by silver staining according to the method of Wray et al. [25]. Protein concentration was determined according to Bradford [26] using bovine serum albumin as standard.

2.3. Enzyme assays

Glucan synthase activity was measured as previously described [27]. The assay mixture consisted of 25 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 10 mM NaF, 5 mg/ml bovine serum albumin, 5 mM UDP-[¹⁴C]glucose (Amersham Buchler, Braunschweig, Germany) diluted to a final specific radioactivity of 7.4 MBq/mol with unlabeled UDP-glucose, in a final volume of 40 µl. The reaction was carried out for 1 h at 25°C and terminated by addition of 0.5 ml of cold 20% (w/v) trichloroacetic acid and filtration through 2.5 cm Whatman GF/B filters. The filters were washed once with 10 ml water and radioactivity was measured by scintillation counting. Calculation of catalytic constants (K_m , K_i , V_{max}) was performed by non-linear regression

of the data using the program SigmaPlot 4.0 (SPSS, Erkrath, Germany).

2.4. Product analysis

For enzymatic degradation experiments, a detergent-solubilized fraction (0.1 mg protein) was incubated for 2 h with 5 mM UDP-[¹⁴C]glucose (7.4 MBq/mmol) as described in Section 2.3 in a total reaction volume of 250 µl. The reaction was terminated by heating at 100°C for 10 min. The sample was then centrifuged at 15 000×g for 20 min. The supernatant was discarded and the pellet was washed 3 times by resuspension in 1 ml of distilled H₂O and centrifugation at 15 000×g for 10 min. The final pellet was resuspended with 250 µl of H₂O. Fifty microliter aliquots of the suspension were incubated with either additional 50 µl of 2 mg/ml laminarinase solution in 10 mM sodium acetate buffer, pH 5, or of 10 mg/ml α-amylase solution in 25 mM Tris-HCl, pH 7.5, or only buffer for controls. After incubation at 37°C for 2 h, the tubes were centrifuged at 15 000×g for 10 min and 10 µl aliquots of the supernatants were spotted onto 20×20 cm Merck silica gel 60 aluminum-backed HPTLC plates. The plates were run in 4:5:2 (v/v/v) butanol/acetone/water [28]. [¹⁴C]Glucose (148 MBq/mmol; Amersham Buchler, Braunschweig, Germany) was applied both separately and as an internal standard in aliquots of the samples. The plates were air-dried and scanned using a Fujifilm BAS-1500 phosphorescence image analyzer. Non-radioactive standards of glucose, laminaribiose and gentiobiose (Sigma, Deisenhofen, Germany) were applied onto separate lanes and visualized using a 95:5 (v/v) ethanol/sulfuric acid spray and heating to 120°C for 5 min.

For NMR analysis, non-radioactive glucan collected by centrifugation was washed extensively with 2 M NaCl followed by distilled water and was then freeze-dried. NMR spectra were recorded with a Bruker DMX 500 spectrometer (proton frequency: 500.13 MHz) using an inverse geometry 5 mm probehead (90°: 10.8 µs ¹H; 10.0 µs ¹³C) in DMSO-d₆ at 30°C (¹H/¹³C: 2.49/39.50 ppm). The ¹³C-NMR spectrum was recorded with broad band decoupling and an acquisition time of 825 ms (relaxation delay d1: 1 s). Gradient enhanced absolute value HH-COSY and phase sensitive (TPPI) HMQC spectra were acquired using Bruker standard software (gradient pulse: 1 ms, gradient recovery: 450 µs; HMQC: aq: 102 ms, sw: 5040 Hz, d1: 2 s, ¹J (CH): 145 Hz, ¹³C GARP decoupling: 70 µs, number of increments in F1: 257; HH-COSY: aq: 117 ms, sw: 4370 Hz; 512 increments in F1). The FID of the HMQC spectrum was multiplied by a $\pi/2$ shifted sine bell in F2 and by an unshifted sine bell in F1 dimension; the HH-COSY spectrum was processed with unshifted [square (F2)] sine bells; both spectra were zero filled providing a 1k×1k data matrix.

3. Results and discussion

3.1. Product characterization

Glucan synthase activity was easily detectable and quantifiable in microsomal preparations of actively growing *P. sojae*

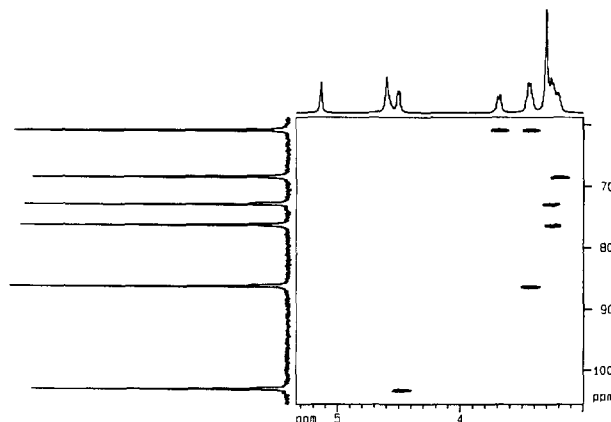


Fig. 1. ¹H (horizontal axis), ¹³C (vertical axis), and HMQC NMR spectra of the product of the enzymatic reaction with UDP-glucose in the presence of glucan synthase from *P. sojae*.

cultures. Initial tests suggested the need to include some previously reported supplements in the assay such as sucrose at 0.5 to 1 M, NaF at 10 mM and bovine serum albumin at 5 mg/ml which activated and stabilized the enzymatic activity. The kinetics of the *in vitro* reaction at 25°C showed linear glucose incorporation into insoluble glucan for up to 1 h and non-linear but significant product formation for up to at least 4 h after addition of substrate (not shown). The activity in microsomal preparations was fairly stable with no more than a 25% loss being observed after 2 days at 4°C (not shown).

The ^{13}C -labeled insoluble glucan product could be fully degraded using commercially available laminarinase. The sole product of this reaction was glucose as shown by thin layer chromatography. The presence of (1→6)- β -linked oligosaccharides or of the disaccharide gentiobiose could not be detected (not shown). The glucan was not degraded by α -amylase, and was insoluble in water but soluble in 0.1 M NaOH or DMSO. NMR spectra of the unlabeled glucan, dissolved in DMSO- d_6 , showed resonances typical of carbohydrates. The ^{13}C -NMR spectrum displayed only six resonances while the ^1H spectrum showed broadened signals indicating a short transverse relaxation time corresponding to a high molecular weight polymer. Two of the low field proton resonances did not produce cross peaks in the HMQC-NMR spectrum, therefore representing non- (or slowly) exchanging hydroxyl groups (Fig. 1). These could be assigned by their vicinal couplings in an HH-COSY spectrum. The assignment was confirmed by HH-COSY and ^1H , ^{13}C -HMQC-NMR spectra which gave chemical shift values closely resembling literature values for (1→3)- β -D-glucans: ^1H -NMR δ 5.15 (1H, s (br), 2-OH), 4.60 (1H, s (br), 4-OH), 4.58 (1H, s (br), 6-OH), 4.50 (1H, d, $J_{12(\text{obs})}$ = 6.9 Hz, H1), 3.69 (1H, d, $J_{6a6b(\text{obs})}$ = 9.3 Hz, H6a), 3.44 (2H, m, H6b, H3), 3.30 (1H, m, H2), 3.25 (1H, m, H5), 3.20 (1H, m, H4); ^{13}C NMR δ 103.02 (C1), 86.22 (C3), 76.32 (C5), 72.82 (C2), 68.39 (C4), 60.85 (C6) [29–32]. The Lorentzian tails associated with the rather high linewidth of the ^1H -NMR resonances (approximately 7 Hz) combined with the low dispersion of the chemical shift precluded an accurate estimate of impurities from the proton and HH-COSY NMR spectra alone. However, in both spectra no resonances were visible other than from a (1→3)- β -glucan. The purity of the sample was deduced from the signal to noise ratio in the ^{13}C -NMR spectrum and from the absence of additional cross peaks in the ^1H , ^{13}C -HMQC-NMR spectrum. Both spectra showed a single

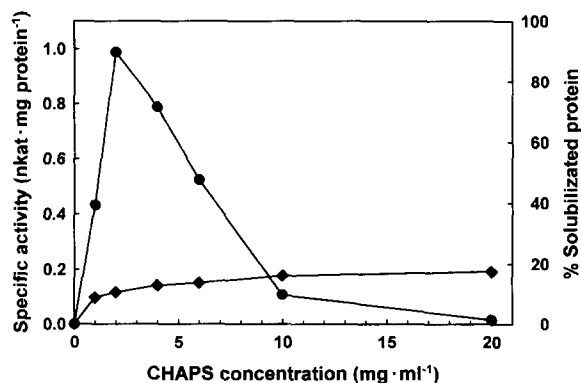


Fig. 2. Solubilization of (1→3)- β -glucan synthase activity from a *P. sojae* membrane fraction using CHAPS. Specific enzyme activity of the solubilize (●) and total solubilized protein (◆).

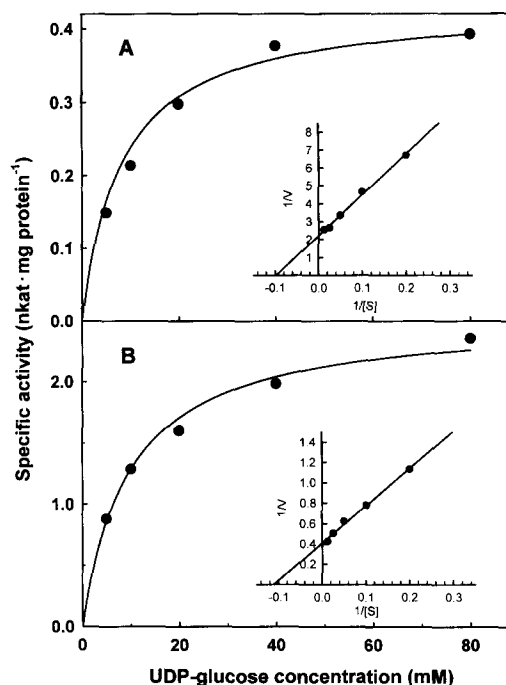


Fig. 3. Dependence of (1→3)- β -glucan formation on UDP-glucose concentration in the presence of glucan synthase from *P. sojae*. Panel A shows product formation in the presence of the microsomal fraction and panel B in the presence of CHAPS-solubilized protein. The inserts represent the Lineweaver-Burk plots of the data. The catalytic constants were calculated directly from v versus $[S]$ diagrams by non-linear regression.

uniform glucan with impurities and branching other than (1→3)- β -D-glucan not exceeding 3–4%.

3.2. Solubilization of glucan synthase activity

A number of detergents were tested, including Zwittergent 3-12, octyl glucoside and CHAPS. Both of the detergents containing alkyl chains, Zwittergent 3-12 and octyl glucoside, proved highly inhibitory. Only CHAPS was effective in solubilizing the activity with an optimal concentration for solubilization of 3 mg/ml (Fig. 2). This concentration resulted in both highest total and specific activities in the 180 000 $\times$ g supernatant. For routine preparative solubilization of the activity, a detergent/protein (w/w) ratio of 1.5 to 1 was employed. Moreover, it was crucial for increasing solubilization efficiency to add EGTA to the buffer for preparing the cell-free extract, likely because of removal of an excess of Ca^{2+} from the growth medium that might have been trapped by the mycelium. Detergent-solubilized activities displayed a degree of stability similar to that of the membrane-bound enzyme.

The use of CHAPS resulted in a significant increase in glucan synthase activity similar to the reported effect of detergents on enzymes in other cases [2]. Analysis of the substrate saturation curves for both membrane bound and solubilized preparations showed a six-fold increase in V_{max} values in the solubilized fraction, from 0.4 to 2.5 nkat/mg protein, while the apparent K_m value remained almost unchanged (10.6 and 10.7 mM for membrane and solubilized fractions, respectively) (Fig. 3). This effect has also been observed with the *A. nidulans* glucan synthase [18]. The apparent K_m values are in the high range of what has been found for other oomycete enzyme sources. Values reported in the literature range

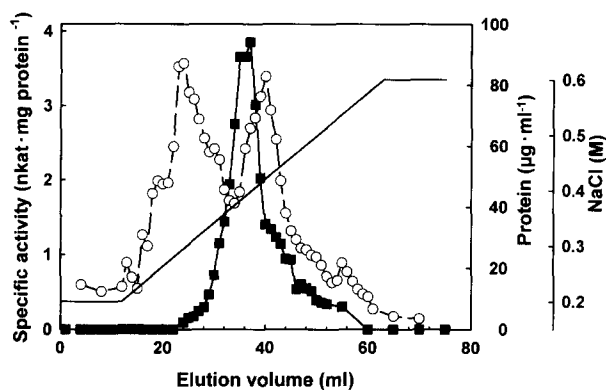


Fig. 4. Anion exchange chromatography of detergent-solubilized glucan synthase preparation from *P. sojae* cell membranes. Chromatography was carried out on a 10×150 mm Merck DEAE-Superformance column equilibrated with 25 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 10 mM NaF. The sample consisted of 20 mg of CHAPS-solubilized protein. Enzyme activity (■) and protein concentration (○) were determined in selected fractions.

between 0.7 and 7.1 mM [2,27]. In contrast with other reports, however, both membrane and solubilized preparations displayed no activation of enzyme activity by GTP in the range between 4 μ M and 4 mM. The divalent cations, Mg^{2+} and Ca^{2+} , did not affect enzyme activity at concentrations of up to 10 mM, whereas both Mn^{2+} and Fe^{2+} inhibited enzyme activity to about 70% at 10 mM. Neither EDTA nor EGTA showed any effect on enzyme activity (results not shown). GTP regulation of glucan synthase activity in yeast, *C. albicans*, and *A. nidulans* has been attributed to the presence of the Rho1 subunit which copurifies with the complex [22,23]. It is unclear whether the *P. sojae* enzyme is susceptible to this type of regulation in vivo, however, it is noteworthy that the enzyme from another oomycete, *Achlya ambisexualis*, also displays no activation by GTP and has a high apparent K_m for UDP-glucose [27]. UDP, as expected, was a competitive inhibitor of the reaction with an average K_i between 2 and 3 mM (not shown). Unlike other reports for *Aspergillus* [33], UDP proved a significantly more efficient inhibitor of the activity than pyrophosphate. Maximal inhibition by pyrophosphate was about 50% and was achieved at a concentration of 10 mM.

3.3. Partial purification of glucan synthase

Unlike other glucan synthases reported in the literature, the activity solubilized from *P. sojae* membranes could be enriched by standard chromatography procedures. An increase in the specific activity by a factor between 3 and 6 could be obtained by chromatographing the crude solubilized fraction on DEAE or quaternary amine anion exchangers. At pH 7.5, the activity was retained and eluted in NaCl gradients between 0.35 and 0.45 M. As shown in Fig. 4, the activity eluted between the two major protein peaks observed in the elution profile and a recovery of about 50% was obtained. The fraction obtained from this step had the added advantage of being one-tenth of the volume of the original crude solubilized fraction. This significantly reduced the total amount of UDP-glucose to be used in a subsequent product entrapment step.

Product entrapment has proven the most effective affinity procedure to date for the purification of glucan synthases. For the *P. sojae* enzyme, product entrapment was tested initially

using 5–10 mg amounts of the crude detergent-solubilized protein fraction. A concentration of UDP-glucose near the apparent K_m value (10 mM) was used for the initial incubation. The incubation at 25°C for 1 h was followed by two intermediate wash steps with low speed sedimentation and resuspension of the pellet in buffer containing 5 mM UDP-glucose. The final wash was performed with buffer without UDP-glucose and the pellet was sedimented at 200 000×g. The resulting compact pellet was homogenized in buffer without UDP-glucose and was assayed for activity and analyzed by SDS-PAGE. The initial experiments showed that the activity was difficult to separate from the glucan material in the pellet, even after thorough resuspension with a Potter-Elvehjem homogenizer. Activity could be assayed using the suspended pellet directly, but not in supernatants obtained after homogenization and centrifugation at 200 000×g. Recoveries of activity for all the fractions obtained during the procedure, including wash supernatants and glucan pellets (3%), averaged 90%.

SDS-PAGE analysis was carried out by treatment with denaturing buffer of the final homogenized glucan pellet suspension, the supernatant obtained after 200 000×g centrifugation of this fraction and the resulting 200 000×g pellet. Of these three fractions, only the suspended pellet and the denatured pellet showed in SDS gels the enrichment of a band with an apparent M_r of 108 000 (Fig. 5, lane B) while other visible protein bands present in previous fractions had become clearly weaker in the product entrapment step. The presence of this band correlated with that of glucan synthase activity in

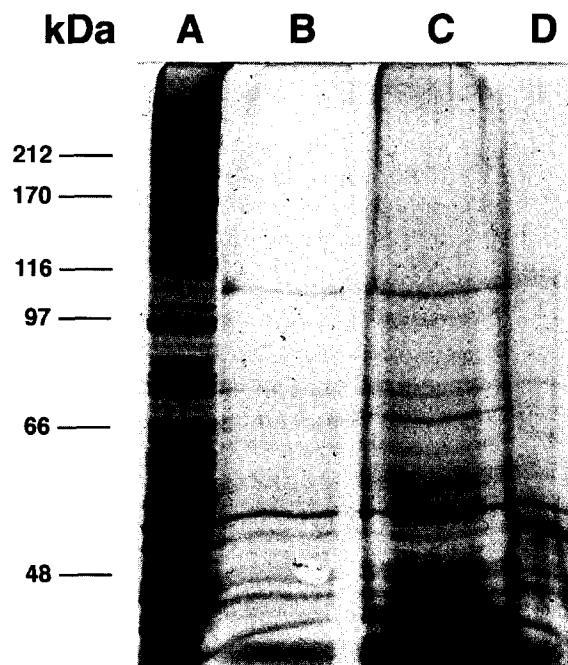


Fig. 5. SDS-PAGE of crude detergent-solubilized proteins from *P. sojae* membranes and fractions after affinity purification using product entrapment. Lane A contains CHAPS-solubilized proteins used for product entrapment, lane B contains proteins extracted from the pellet after product entrapment with crude solubilize, lane C contains proteins extracted from the product entrapment pellet that was obtained with an enzyme fraction partially purified by anion exchange chromatography, lane D contains the pellet remaining after denaturation and extraction with SDS buffer (see B) that was directly applied to the gel.

the fractions. A similar result was obtained when the protein pool from anion exchange chromatography was used for product entrapment (Fig. 5, lane C).

The extreme difficulty observed in removing the activity from the glucan pellet is illustrated by the slow removal of the 108 kDa protein under denaturing conditions. Treatment of the 200 000×g pellet obtained from product entrapment directly with denaturing buffer followed by heating and centrifugation released most of the 108 kDa band into the supernatant as visualized in SDS gels. However, when the pellets remaining after denaturation and centrifugation were directly applied onto a sample well, the resulting electroelution of residual protein left in the pellet showed that there was enough of the 108 kDa band to be clearly visualized by silver staining (Fig. 5, lane D).

For both the CHAPS-solubilized protein fraction and the DEAE pool, the stability of the enzyme activity during the entrapment procedure was remarkable. Thus, it was possible to continue with product entrapment after the first glucan pellet was collected. The second pellet, obtained from the supernatant of the first product entrapment by incubation for an additional hour at 25°C also displayed the 108 kDa band in SDS-PAGE, although with lower intensity (not shown).

3.4. Conclusions

This is the first report on the partial purification of a (1→3)- β -glucan synthase from an oomycete with characteristics that appear to be different from those of yeasts, filamentous fungi or plants. The properties of the enzyme confirm those reported for an enzyme of another oomycete, *Achlya ambisexualis*, which also does not display activation by GTP, has a relatively high K_m for UDP-glucose and does not appear to require divalent cations for activity [27]. The fact that the oomycetes have been included in the kingdom Chromista appears to be reflected in these properties that might be called intermediate between plants and fungi. If the 108 kDa protein band that has been primarily enriched by product entrapment represents the glucan synthase for this species, it would also represent a distinctive difference in molecular mass from that reported for yeast and that deduced from homology cloning in a filamentous fungus, such as *Aspergillus* [18]. Although the inability to dissociate the entrapped protein adequately from the glucan pellet has precluded a measurement of the purification factor, semiquantitative densitometric analysis suggests an enrichment of this band of over two orders of magnitude with this procedure. Ongoing efforts are directed towards sequencing of this protein and cloning of its cDNA in order to better understand the β -glucan biosynthesis in oomycetes. This will also allow a comparison with (1→3)- β -glucan synthases from plants and fungi.

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References

- [1] Bartnicki-Garcia, S. and Wang, M.C. (1983) in: *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology* (Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H., Eds.), American Phytopathological Society, St. Paul, MN.
- [2] Stone, B.A. and Clarke, A.E. (1992) *Chemistry and Biology of (1→3)- β -Glucans*, La Trobe University Press, La Trobe.
- [3] Fontaine, T., Mouyna, I., Hartland, R.P., Paris, S. and Latge, J.P. (1997) *Biochem. Soc. Trans.* 25, 194–199.
- [4] Erwin, D.C. and Ribeiro, O.K. (1996) *Phytophthora Diseases Worldwide*, American Phytopathological Society, St. Paul, MN.
- [5] Sharp, J.K., Valent, B. and Albersheim, P. (1984) *J. Biol. Chem.* 259, 11312–11320.
- [6] Cheong, J.-J., Birberg, W., Fügedi, P., Pilotti, A., Garegg, P.J., Hong, N., Ogawa, T. and Hahn, M.G. (1991) *Plant Cell* 3, 127–136.
- [7] Waldmüller, T., Cosio, E.G., Grisebach, H. and Ebel, J. (1992) *Planta* 188, 498–505.
- [8] Cosio, E.G., Feger, M., Miller, C., Antelo, L. and Ebel, J. (1996) *Planta* 200, 92–95.
- [9] Cosio, E.G., Frey, T. and Ebel, J. (1992) *Eur. J. Biochem.* 204, 1115–1123.
- [10] Mithöfer, A., Lottspeich, F. and Ebel, J. (1996) *FEBS Lett.* 381, 203–207.
- [11] Wang, M.C. and Bartnicki-Garcia, S. (1966) *Biochem. Biophys. Res. Commun.* 24, 832–837.
- [12] Wang, M.C. and Bartnicki-Garcia, S. (1982) *Exp. Mycol.* 6, 125–135.
- [13] Enderlin, C.S. and Selitrennikoff, C.P. (1994) *Proc. Natl. Acad. Sci. USA* 91, 9500–9504.
- [14] Douglas, C.M., Foor, F., Marrinan, J.A., Morin, N., Nielsen, J.B., Dahl, A.M., Mazur, P., Baginsky, W., Li, W., El Sherbeini, M., Clemas, J.A., Mandala, S.M., Frommer, B.R. and Kurtz, M.B. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12907–12911.
- [15] Eng, W., Faucette, L., McLaughlin, M.M., Cafferkey, R., Koltin, Y., Morris, R.A., Young, P.R., Johnson, R.K. and Livi, G.P. (1994) *Gene* 151, 61–71.
- [16] Ram, A.F.J., Brekelmans, S.S.C., Oehlen, L.J.W.M. and Klis, F.M. (1995) *FEBS Lett.* 358, 165–170.
- [17] Inoue, S.B., Takewaki, N., Takasuka, T., Mio, T., Adachi, M., Fujii, Y., Miyamoto, C., Arisawa, M., Furuichi, Y. and Watanabe, T. (1995) *Eur. J. Biochem.* 231, 845–854.
- [18] Kelly, R., Register, E., Hsu, M.-J., Kurtz, M. and Nielsen, J. (1996) *J. Bacteriol.* 178, 4381–4391.
- [19] Frost, D., Brandt, K., Estill, C. and Goldman, R. (1997) *FEMS Microbiol. Lett.* 146, 255–261.
- [20] Mio, T., Adachi-Shimizu, M., Tachibana, Y., Tabuchi, H., Inoue, S.B., Yabe, T., Yamada-Okabe, T., Arisawa, M., Watanabe, T. and Yamada-Okabe, H. (1997) *J. Bacteriol.* 179, 4096–4105.
- [21] Roemer, T., Paravicini, G., Payton, M.A. and Bussey, H. (1994) *J. Cell Biol.* 127, 567–579.
- [22] Dgrnova, J., Drgon, T., Tanaka, K., Kollar, R., Chen, G.-C., Ford, R.A., Chan, C.S.M., Takai, Y. and Cabib, E. (1996) *Science* 272, 277–279.
- [23] Qadota, H., Python, C., Inoue, S., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. and Ohya, Y. (1996) *Science* 272, 279–281.
- [24] Keen, N.T. (1975) *Science* 187, 74–75.
- [25] Wray, W., Boulukas, T., Wray, V.P. and Hancock, R. (1981) *Anal. Biochem.* 118, 197–203.
- [26] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [27] Cabib, E. and Kang, M. (1987) *Methods Enzymol.* 138, 637–642.
- [28] Monschau, N., Stahmann, K.P., Pielken, P. and Sahn, H. (1997) *Mycol. Res.* 101, 97–101.
- [29] Yu, L., Goldman, R., Sullivan, P., Walker, G.F. and Fesik, S.W. (1993) *J. Biomol. NMR* 3, 429–441.
- [30] Müller, A., Ensley, H., Pretus, H., McNamee, R., Jones, E., McLaughlin, E., Chandley, W., Browder, W., Lowman, D. and Williams, D. (1996) *Carbohydrate Res.* 299, 203–208.
- [31] Gutierrez, A., Prieto, A. and Martinez, A.T. (1996) *Carbohydrate Res.* 281, 143–154.
- [32] Kottutz, E. and Rapp, P. (1990) *J. Gen. Microbiol.* 136, 1517–1523.
- [33] Beauvais, A., Drake, R., Ng, K., Diaquin, M. and Latge, J.P. (1993) *J. Gen. Microbiol.* 139, 3071–3078.

[1] Bartnicki-Garcia, S. and Wang, M.C. (1983) in: *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology* (Erwin, D.C.,