Partial purification of Golgi-bound arabinosyltransferase and two isoforms of xylosyltransferase from French bean (*Phaseolus vulgaris* L.)

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The purification of glycosyltransferases involved in wall matrix polysaccharide synthesis has been attempted. A number of activities readily demonstrated in isolated Golgi membranes are lost following detergent solubilization. However, solubilization releases pyrophosphorylases and phosphatases that hydrolyse the substrate in enzyme assays, whether UDP-glucose, -arabinose or -xylose is used. This hydrolysis, which cannot be completely inhibited, appears to be the major factor in the apparent loss of activity. Separation of this hydrolytic activity during further purification by ion-exchange and gel exclusion leads to recovery of glycosyltransferase activity. Thus two xylosyltransferases and one arabinosyltransferase could be partially purified. These appeared to be differentially expressed. The arabinosyltransferase of apparent M_r 70000 on size-exclusion chromatography was isolated from cells undergoing rapid growth and division. A xylosyltransferase of apparent M_r 38000 on size-exclusion chromatography was associated with cell expansion and primary wall synthesis. A second xylosyltransferase, which was purified to near homogeneity with M_r 40000, showed a peak of activity during the period of maximum secondary wall synthesis.

INTRODUCTION

The plant cell wall represents a major component of the dry mass of plant tissue. It has long been the major source of dietary fibre, and it is recognized that it plays a critical role in determining the rheological properties of plants and food texture. Moreover, it is a dynamic structure and thus has a vital role during plant development and responses to pathogen attack and environmental stress (Bolwell, 1988; Roberts, 1989). However, our understanding of the biosynthesis of plant-cell-wall components is limited, particularly with respect to the polysaccharides. Some progress is being made on the molecular identification of the cellulose-synthesizing complex and the possibly related (1,3)- β -glucan synthase (Amor *et al.*, 1991; Wasserman *et al.*, 1991). The site of synthesis of the matrix polysaccharides has been localized to the Golgi by biochemical studies of fractionated endomembranes (Bolwell, 1988) and organelle subfractions (Brummell *et al.*, 1990; Hobbs *et al.*, 1991) and by elegant immunogold localization of the nascent polysaccharides within the Golgi stack (Moore *et al.*, 1991). However, there are few reports of extensive direct characterization and identification of the glycosyltransferases involved, with the exception of the fucosyltransferase specific for the xyloglucan side chain, a major hemicellulose of the dicotyledonous-plant cell wall (Hanna *et al.*, 1991). Most of the other arabinosyl- and galacturonosyl-transferases in pectin synthesis (Bolwell & Northcote, 1983a; Bolwell *et al.*, 1985), glucosyl- and xylosyl-transferases of primary wall xyloglucan synthesis (Campbell *et al.*, 1988; Gordon & Maclachlan, 1989) and xylosyl- and glucuronosyl-transferases of secondary wall xylan synthesis (Bolwell & Northcote, 1983a; Baydoun *et al.*, 1989; Waldron *et al.*, 1989) are only partially characterized in membrane and solubilized preparations.

The advent of more sophisticated purification technology has allowed the re-examination of the conventional purification of arabinosyl- and xylosyl-transferases from Golgi membranes of

French bean (*Phaseolus vulgaris* L.). This work has allowed the identification of differentially regulated arabinosyltransferase and two isoforms of xylosyltransferase. Partial purification and an estimation of M_r by gel-exclusion chromatography has been achieved for these enzymes.

MATERIALS AND METHODS

Plant material

French bean (*Phaseolus vulgaris* L. var. The Prince) plants were grown under a regime of 16 h light (10000 lux)/8 h dark for the appropriate length of time. Suspension-cultured cells of *P. vulgaris* (var. Immuna) were established and maintained as previously described (Dixon & Lamb, 1979).

Preparation and treatment of plant extracts

For studies of xylosyltransferase activity, bean hypocotyls of the appropriate age were homogenized in 50 mM-Tris/HCl (pH 7.2)/10 mM-MgCl₂/1 mM-dithiothreitol/0.3 M-sucrose/1 % (w/v) polyvinylpyrrolidone at 4 °C. The extract was filtered through three layers of muslin and centrifuged at 10000 g for 10 min to remove cellular debris. The resultant supernatant was spun at 100000 g for 90 min to pellet the microsomes, which were resuspended in 50 mM-Tris, pH 7.2, to give a final protein concentration of between 15 and 30 mg/ml, as determined using the Bradford protein assay (Read & Northcote, 1981).

Studies of arabinosyltransferase activity were carried out using microsomes prepared from bean cell cultures 2–4 days after subculture. After harvesting the cells by vacuum filtration, microsomes were prepared as described above.

Assay procedure

Incubation conditions using radiolabelled substrate were as described previously (Bolwell & Northcote, 1981, 1983a,b). Product analysis was carried out by high-voltage paper electro-

Abbreviation used: RTX, reduced Triton X-100.

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Fig. 1. Distribution of radioactively labelled compounds after incubation of UDP-[1-¹⁴C]glucose, UDP-[1-¹⁴C]xylose or UDP-[1-³H]arabinose with intact or solubilized membranes

Intact or solubilized membranes were incubated with (a) UDP-xylose, (b) UDP-glucose or (c) UDP-arabinose in the standard incubation mix. After 30 min the reaction mix was analysed by high-voltage paper electrophoresis and the amount of radioactivity determined for UDP-sugar, sugar 1-phosphate (sugar 1-P), monosaccharide (Monosacc.) and polymer (origin) in each case. The radioactivity (%) remaining in each component is shown for intact (□) and solubilized (■) microsomes.

phoresis in 8% (w/w) acetic acid/2% (w/w) formic acid also as previously described (Bolwell & Northcote, 1981), or by precipitation and multiple washes of the polymer with cold 70% (v/v) ethanol containing 1 mM-EDTA before (Bolwell & Northcote, 1983a) determination of radioactivity by liquid-scintillation counting.

Protein purification

Microsomes were solubilized by adjusting them to 1% reduced Triton X-100 (RTX) (Aldrich Chemical Co., Gillingham, Dorset, U.K.) and gently stirring on ice for 10 min. The solubilized membranes were then centrifuged at 100 000 *g* for 30 min. Protein content was determined in fractions containing detergent using the BCA protein assay (Pierce, Chester, U.K.) in accordance with the manufacturer's instructions.

Anion-exchange h.p.l.c. was carried out on a 7.5 mm × 75 mm TSK DEAE-5PW column (Anachem, Luton, Beds., U.K.). Samples were loaded in 20 mM-Tris (pH 7.2)/1% RTX, and eluted using a linear gradient of 0–0.75 M-NaCl over 30 min at an overall flow rate of 0.5 ml/min. Fractions were collected and microdialysed against 20 mM-Tris (pH 7.2)/10 mM-MgCl₂/1% RTX/1.4 mM-mercaptoethanol at 4 °C for 1 h before being assayed for glucosyltransferase activity. Active fractions were pooled and subjected to size-exclusion chromatography.

Size-exclusion h.p.l.c. was carried out using a 600 mm × 7.5 mm TSK G2000 SW column (Anachem) equilibrated in 20 mM-Tris (pH 7.2)/0.2 M-NaCl/1 mM-dithiothreitol, at a flow rate of 0.3 ml/min. Fractions were collected and analysed as described above. Western-blot analysis of fractions was carried out as described by Bolwell *et al.* (1986), except that the second antibody was conjugated to alkaline phosphatase.

Polysaccharide analysis

Cell walls and hemicellulose fractions were isolated from hypocotyls as described by Bolwell & Northcote (1983a). Xyloglucan was separated from xylan fractions by chromatography on powdered cellulose (Aspinall *et al.*, 1969). The xylan did not bind, and the xyloglucan was released by urea/NaOH elution. Monosaccharide analysis was carried out after hydrolysis in 3% (v/v) H₂SO₄. After neutralization the sugars were derivatized as

described by Chambers & Clamp (1971) before g.l.c. on a Varian II apparatus (Varian Associates, Palo Alto, CA, U.S.A.).

RESULTS

Effect of solubilization on the assay conditions

When incubated with radiolabelled UDP-sugars, both intact microsomes and membranes, solubilized in various detergents, hydrolysed the substrates, in addition to synthesizing polysaccharides. When the fate of radiolabelled UDP-sugars was monitored *in vitro*, three types of product were seen: polymers, sugar-phosphates and monosaccharides. These products were seen irrespective of the UDP-linked sugar (arabinose, glucose or xylose) used as substrate (Fig. 1). A typical time course of substrate breakdown is shown in Fig. 2. Analysis of the radiochemical composition of the reaction mixture over a period of 30 min suggested a rapid breakdown of the substrate by phosphorylases and phosphatases, the reaction being essentially complete after 6 min (Figs. 1 and 2). Further experiments were carried out on the differential solubilization of these relatively-nonspecific hydrolase activities using UDP-glucose as substrate.

The extent of hydrolysis of UDP glucose during the assay was found to be dependent on the concentration of RTX used to solubilize the microsomes. Whereas the glucosyltransferase was not significantly solubilized until the RTX concentration reached 0.1%, UDP-glucose degradation was already significant at 0.001% RTX. Increasing the ionic strength of the buffer by the inclusion of 0.5 M-NaCl also led to significant degradation of the UDP-glucose, but did not solubilize the glucosyltransferase to any great extent, whereas simply washing the microsomes in homogenization buffer was enough to remove a proportion of the phosphatase activity, but had little effect on the phosphorylase activity. The inclusion of glucose 1-phosphate in the assay mixture at concentrations between 1 and 100 mM reduced the breakdown of UDP-glucose to glucose 1-phosphate, in a concentration-dependent manner; however, it also significantly inhibited the production of the polymer, such that whereas 100 mM-glucose 1-phosphate reduced the degradation of UDP-glucose by 50%, it also inhibited the glucosyltransferase by 66%. A number

Fig. 2. Time course of hydrolysis of UDP-[1-¹⁴C]glucose by solubilized membranes

Solubilized membranes were incubated with UDP-[1-¹⁴C]glucose under standard conditions. Aliquots were taken at the various time points and analysed by high-voltage paper electrophoresis. Radioactivity remaining in UDP-glucose (■), glucose 1-phosphate (□), glucose (●) and polymer (○) is shown.

Table 2. Purification of arabinosyltransferase from suspension-cultured cells of French bean

The purification of arabinosyltransferase from 200 g of suspension-cultured cells of French bean is shown; purification stages were identical with those shown in Table 1.

Step	Vol. (ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Recovery (%)	Purification (fold)
1	0.25	6.0	16.3	2.71	100	1
2	0.25	3.2	3.6	1.13	22	0.42
3	2.0	0.4	9.9	24.9	61	9.2
4	1.8	0.036	7.2	199.0	44	73

of other potential inhibitors, e.g. NaF and sugar nucleotides, were found to be relatively ineffectual.

Fractionation of solubilized microsomes

Solubilized microsomes were subjected to anion-exchange

Table 1. Purification of xylosyltransferases from hypocotyls of French bean

The purification from 80 g of 12-day-old hypocotyls is shown. Purification steps were: 1, microsomal membranes; 2, solubilized membranes; 3, anion-exchange chromatography; and 4, size-exclusion chromatography. Abbreviations: XS1 and XS2, transferases 1 and 2.

Step	Vol. (ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Recovery (%)	Purification (fold)	
1	0.25	7.5	10.4	1.4	100	1	
2	0.25	3.6	1.9	0.53	18	0.4	
3	2.0	0.36	1.14	3.16	11	2.3	XS1
4	1.8	0.031	3.4	109.6	32	78	
3	2.0	0.25	9.33	37.3	90	27	XS2
4	1.8	0.027	7.59	281.3	73	201

Fig. 3. DEAE ion-exchange h.p.l.c. of (a) xylosyltransferase activity in solubilized membranes isolated from 13-day-old hypocotyls of French bean and (b) arabinosyltransferase from suspension-cultured cells 48 h after subculture

Solubilized microsomes (about 15 mg of total protein in either case) was added to a column (TSK DEAE-5PW; 75 mm × 7.5 mm int. diam.) and proteins eluted with a linear gradient of 0–0.75 M-NaCl in 20 mM-Tris/HCl, pH 7.2, containing 1% RTX at a flow rate of 0.5 ml/min for 30 min. Enzyme activity was determined in each fraction (●) by high-voltage paper electrophoresis, which was also used to determine the position of maximum hydrolytic activity (shown by the bar). Pooled fractions for further purification are indicated by stippling. Protein was monitored at 254 nm (—).

Fig. 4. Size-exclusion chromatography of (a) xylosyltransferase eluted early from ion-exchange (XS1), (b) xylosyltransferase eluted late from ion-exchange XS2 and (c) arabinosyltransferase

Pooled fractions for each transferase were separated by size exclusion on a column (TSK G2000SW; 600 mm $\times$ 7.5 mm int. diam) and proteins eluted over a period of 120 min in 20 mM-Tris/HCl (pH 7.0)/200 mM-NaCl/1 mM-dithiothreitol. Enzyme activity was determined in each fraction (●) and protein was monitored at 254 nm (—).

Fig. 5. Differential elution profiles of xylosyltransferases from ion-exchange and isolated from hypocotyls of increasing age

Membranes from hypocotyls of various ages were isolated, solubilized and subjected to anion-exchange chromatography. The activity recovered from (a) 8-day, (b) 12-day and (c) 20-day plants is shown relative to that for the most active fraction (XS2 day 12).

h.p.l.c. Both arabinosyl- and xylosyl-transferase activities could be recovered after fractionation (Tables 1 and 2). In contrast with the effect on the stability of the substrate, solubilization did not lead to any great changes in the properties and products of the three transferases that have been previously determined in microsomes (Bolwell & Northcote, 1981, 1983a). Analyses of column fractions after incubation with the substrate could also be used to determine the positions of hydrolytic activity in the eluate. Fractions containing phosphorylase activity could be identified by the presence of the sugar 1-phosphate upon scanning the paper electrophoretograms of the incubation products; those fractions containing both phosphorylase and phosphatase ac-

Fig. 6. SDS/PAGE of purified xylosyltransferases

After anion-exchange and size exclusion chromatography, fractions corresponding to XS1 and XS2 were subjected to SDS/PAGE on a 10% acrylamide gel and silver-stained. Track 1, M_r markers; track 2, XS2; track 3, XS1. The bands at M_r 65 000/60 000 probably represent the artefact commonly found on SDS/PAGE (Ochs, 1983).

Table 3. Hemicellulose composition of 8-day and 12-day-old hypocotyls of French bean

Xyloglucan and xylan fractions were isolated from whole hypocotyls and analysed for monosaccharide composition. The total amount of each hemicellulose is expressed as a percentage of the dry weight of the tissue. The xylose/glucose ratio is shown for each fraction. Note that young hypocotyls synthesize xylans and that these increase in amount in the older tissue.

Hemicellulose...	Xyloglucan		Xylan	
	Total	Xylose	Total	Xylose
Age (days)	(% dry wt.)	Glucose	(% dry wt.)	Glucose
8	8.8	0.81	1.5	2.44
12	3.6	1.23	5.6	2.68

tivity contained both monosaccharide and sugar 1-phosphate. Analysis of fractions from the column indicated the presence of two peaks of xylosyltransferase activity, designated XS1 and XS2 (Fig. 3a), whereas only one peak of arabinosyltransferase activity was seen (Fig. 3b), depending on the biological source material. Recoveries from these steps were enhanced when compared with the initial solubilized fraction (Tables 1 and 2).

Comparative detailed analysis of reaction mixtures by high-voltage electrophoresis showed that, clearly, in two cases enhanced recovery is due to total separation of the transferase from the phosphatase/pyrophosphorylase activity and this separation is at least partial in the third case (Figs. 3a and 3b). For the xylosyltransferases, the activity of neither peak was enhanced by the addition of xylose oligomers up to xylopentaose in length, xylan or xyloglucan as an acceptor (results not shown).

Characterization of xylosyltransferases

Fractions corresponding to the two peaks of xylosyltransferase were pooled separately and subjected to size-exclusion chromatography (Figs. 4a and 4b). The xylosyltransferase eluted from the ion-exchange column at the lower salt concentrations (XS1) was recovered with a retention time of 74 min, corresponding to an M_r of 38000 relative to the elution of a number of proteins of known M_r . The xylosyltransferase eluted at the higher salt concentration (XS2) had a retention time of 66 min, corresponding to an M_r of 40000. Both fractions were analysed by SDS/PAGE (Fig. 5). The purified XS1 contained a number of polypeptides on silver staining, including what are likely to be well-characterized artefacts between M_r 60000 and 68000 (Ochs, 1983). A major polypeptide of M_r 38000 is clearly visible and may represent the putative XS1. XS2, by contrast, appeared virtually homogeneous, as a single polypeptide of M_r 40000.

The relative recoveries of the two peaks of xylosyltransferase activity was dependent on the age at which the bean hypocotyls were harvested (Fig. 6). At 8 days, the relative activity of the peak we have designated XS1 was greater than that of XS2, whereas between 10 and 12 days the relative activities were reversed. After 20 days a small amount of XS2 activity, only, remained. It is likely that both these forms are associated with xylan synthesis, since xyloglucan synthesis in primary wall hemicellulose biosynthesis requires transfer on to a glucan backbone which is not present in the reaction mix. Both enzymes were still active after the removal of endogenous substrates and were not enhanced in activity under the assay conditions that promote xyloglucan synthesis (Gordon & MacLachlan, 1989). Activities for typical fractions containing XS1 were 0.17 nmol/min without UDP-glucose and 0.19 nmol/min in the presence of 2 mM non-radioactive UDP-glucose. For XS2, the respective activities were 0.38 nmol/min and 0.37 nmol/min. Analysis of hypocotyls showed that xylan was present as about 15% of the total hemicellulose at the peak of XS1 activity, whereas xylans constituted about 60% of the total hemicellulose at 12 days, the peak of XS2 activity (Table 3).

Characterization of arabinosyltransferase

Size-exclusion chromatography of the pooled fractions with arabinosyltransferase activity eluted from ion-exchange chromatography gave one peak of activity with retention time of 62 min, corresponding to an apparent M_r of 70000. In contrast with the two xylosyltransferases, analysis of this fraction by SDS/PAGE showed that it contained numerous bands upon staining with Coomassie Blue or silver. However, use of a monoclonal antibody that inhibits the enzyme and recognizes a multi-antigenic site (Bolwell & Northcote, 1984) recognizes two major bands in this fraction at M_r 70000 and 40000 on Western blotting (Fig. 7). This lower- M_r band is not present in Western blots of membranes

Fig. 7. SDS/PAGE and Western-blot analysis of partially purified arabinosyltransferase

After anion-exchange and size-exclusion chromatography, fractions with the highest arabinosyltransferase activity were subjected to SDS/PAGE on a 10% acrylamide gel and Western blotting. Track 1, M_r markers; track 2, Coomassie Blue-stained arabinosyltransferase fraction; track 3, Western-blot detection with the monoclonal antibody (Bolwell & Northcote, 1984). Note the band at M_r 70000 present in both tracks and in Fig. 8(a) representing the 48 h-old source material and in which the lower- M_r bands are absent.

Fig. 8. Western-blot analysis of arabinosyltransferase in (a) growing and (b) elicited suspension-cultured bean cells

Time courses for the appearance of the M_r -70000 band are shown, which is induced by subculture (Bolwell & Northcote, 1983b, 1984) and disappears on elicitation (Bolwell, 1986). (a) Track 1, 0 h after subculture. 2, 24 h after subculture. 3, 48 h after subculture. 4, 72 h after subculture. (b) Track 1, 0 h after addition of elicitor. 2, 4 h after elicitor addition. 3, 8 h; 4, 12 h; 5, 24 h after elicitor addition. Microsomes were prepared and polypeptides were separated by SDS/PAGE on 7.5% acrylamide gels before blotting. Each track was loaded with 5 μ g protein for Western analysis.

using the monoclonal antibody (Fig. 8) and may represent a proteolytic product. These results probably confirm the M_r -70000 protein as an arabinosyltransferase. The changes in the levels of the M_r -70000 protein after subculture are shown in Fig. 8(a), whereas Fig. 8(b) shows the effect of elicitor treatment on levels of the protein. Levels increased after subculture and rapidly decrease after elicitor treatment, corresponding to the previously reported changes seen in enzyme activities (Bolwell & Northcote, 1984; Bolwell, 1986).

DISCUSSION

Although responsible for the synthesis of much of the bulk of cell-wall matrix materials, Golgi-localized glycosyltransferases involved in the synthesis of pectins and hemicelluloses remain largely uncharacterized. An M_r of 150 000 for only one enzyme, the fucosyltransferase involved in xyloglucan synthesis in pea epicotyl, has so far been obtained and even this enzyme is only partially purified (Hanna *et al.*, 1991). Characterization of enzymes involved in glucan synthesis is advancing (Amor *et al.*, 1991; Wasserman *et al.*, 1991), but for the enzymes that transfer arabinose, galacturonic and glucuronic acids and xylose there has been little progress past the solubilization stage. Indeed previous attempts to solubilize these proteins has led to unacceptable losses of activity. This has been reported to have been due to the release of hydrolytic activities when attempts to isolate the (1-3)- β -glucan synthase of the plasmalemma were made (Saugy *et al.*, 1989). We have also observed this effect upon solubilization of Golgi-bound glycosyltransferases under investigation. Solubilization was also previously shown to inhibit the activity of xylan synthase (Dalessandro & Northcote, 1981) and this may have been due to competition for substrate with phosphorylase and phosphatase. The xylan synthetase of Odzuk & Kauss (1972), however, did not require membrane integrity for activity. In the present study, the activities of xylosyl- and arabinosyl-transferases from French bean were partially recoverable once sufficient further purification away from hydrolytic activity was achieved.

The temporal distribution of the synthases involved in arabinan and xylan synthesis in isolated French-bean membranes has been studied previously (Bolwell & Northcote, 1981, 1983a,b, 1984) as part of a study of the regulation of matrix polysaccharide synthesis during expansion growth and differentiation. The arabinosyltransferase associated with polysaccharide synthesis proved to be induced during the period of cell division in suspension cultures and rapid elongation of hypocotyls. In contrast, following elicitor action, the predominant arabinosylation activity in membranes was found to be concerned with protein glycosylation (Bolwell, 1986). Furthermore, a monoclonal antibody that was inhibitory towards the transferase activity concerned with polysaccharide synthesis, but had no effect on protein glycosylation (thus indicating two distinct enzymes), was shown to cross-react with an M_r -70 000 membrane protein (Bolwell, 1986; Bolwell & Northcote, 1984).

The present work shows that a polypeptide of this M_r is present in the most highly purified fraction, whereas other lower- M_r polypeptides observed are absent from the original membrane material and are possibly partial degradation products. The time course of induction in cells during doubling phase, its disappearance from elicitor-treated cells and the presence in partially purified fractions, support the identity of the M_r -70 000 polypeptide as the arabinosyltransferase responsible for the synthesis of arabinan.

In contrast, the xylosyltransferases from hypocotyls were purified to a much higher level. Two forms of the enzyme were observed which could be separated by anion-exchange chromatography. Relative recoveries of the two forms varied with the age of the hypocotyl. It was previously shown that the peak of xylan formation coinciding with the peak of membrane-bound xylan synthase activity at about 12 days post inhibition in French bean (Bolwell & Northcote 1981) was associated with vascular differentiation. That this enzyme is a marker for this devel-

opmental stage has been confirmed during the differentiation of mesophyll cells of *Zinnia* into tracheids (Ingold *et al.*, 1991; Suzuki *et al.*, 1991). The form of the enzyme we have designated XS2, which shows the maximum abundance at this time, was purified to near homogeneity and, on the basis of size fractionation and SDS/PAGE, has an M_r of 40 000. A second activity, designated XS1, appeared to be catalysed by a protein of M_r 38 000, showed a peak of activity at 8 days post inhibition and was associated with early stages of vascular differentiation. Some xylan is synthesized at this period, and since this activity did not have the characteristics of the xyloglucan synthase when incubated under those assay conditions, it appears to be an early induced form of the xylan synthase, but has not been characterized further. The present work serves to define the conditions by which further progress in the purification, identification and characterization of glycosyltransferases in wall matrix polysaccharides can be made.

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