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MICROSOMAL ARABINOSYLATION OF POLYSACCHARIDE AND ELICITOR-INDUCED CARBOHYDRATE-BINDING GLYCOPROTEIN IN FRENCH BEAN

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; French bean; suspension cultures; elicitor-induction; arabinosylation; carbohydrate-binding glycoprotein.

Abstract—In contrast to microsomal membranes from suspension cultured cells undergoing primary wall synthesis which incorporated arabinose directly from UDP- β-L-arabinose into arabinan, membranes from cells treated with fungal elicitor catalysed the formation of a lipid oligosaccharide intermediate in the arabinosylation of an inducible M_r 42 500 glycoprotein. These variations in the patterns and mechanism of arabinosylation observed between the cells in response to the differing stimuli were detected in both the kinetics of incorporation and the demonstration that the lipid oligosaccharide after purification on ion-exchange chromatography could act as primary donor for the glycoprotein and not polysaccharide. These results distinguish mechanisms for the transfers of arabinose onto glycoprotein and polysaccharide by enzyme systems known to be immunologically distinct. The fungal elicitor-induced M_r 42 500 glycoprotein binds to thyrogolubulin- and fetuin-Sepharoses in a specific manner and this binding is prevented by chitin oligomers. The glycoprotein thus appears to be a carbohydrate-binding protein and the sugar specificity together with the demonstration of hydroxyproline residues in acid hydrolysates of the glycoprotein purified by affinity chromatography indicates a close similarity to the arabinosylated hydroxyproline-rich lectins of the Solanaceae which can function as bacterial agglutinins. The M_r 42 500 glycoprotein which undergoes rapid transient induction also clearly differs from other extensin-like hydroxyproline-rich glycoproteins, arabinogalactan proteins and the characteristic bean seed-lectin, phytohaemagglutinin, in a number of properties.

INTRODUCTION

L-Arabinose is found in the primary cell wall of higher plants in characteristic polysaccharides and glycoproteins which are synthesized, modified and exported within the endomembrane system. Arabinan, probably mostly $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 5$ linked is a constituent of neutral pectin and at least three types of hydroxyproline-rich glycoproteins are arabinosylated. The range of types of these glycoproteins and their roles in cell structure and function and in disease resistance have now been established [1–3]. It has also become apparent that there is a considerable capacity for the differential deposition and mobilization of wall material in response to a variety of exogenous stimuli. For example, during secondary growth following primary wall formation there is a cessation of pectin deposition accompanied by an increase in hemicellulose and cellulose deposition together with the initiation of lignin synthesis [4] whereas lignin and brown pigment deposition [5], secretion of hydroxyproline-rich bacterial agglutinins [6, 7] and provision of putative endogenous elicitors [8] characterize responses during host–pathogen interactions. Most of these changes have now been observed during plant growth regulator [9–12] or fungal elicitor [13, 14] treatments of bean cell cultures. These changes are also reflected in alterations in the synthetic capacities within the endo-membrane system.

Advantages can be taken of these temporally separated different synthetic capacities to study the synthesis and modifications of particular components where common requirements may interfere with interpretation of data. Thus in previous [13] and the present work mechanisms of arabinosylation have been studied in cell suspension cultures of bean following plant growth regulator treatment and during the synthesis of polysaccharide and some glycoprotein and compared with fungal elicitor application during which arabinose is transferred solely by isolated membranes onto glycoprotein. These differences have been exploited in a reassessment of the possible role of lipid-linked pentosyl-oligosaccharides in intermediate transfers. Previous evidence suggested that UDP-arabinose serves as primary donor for the synthesis of arabinan [15–17]. However, roles for pentosyl lipids in the glycosylation of arabinogalactan proteins at the very least have been elucidated [18, 19]. The results described here are consistent with a direct transfer from UDP-arabinose onto polysaccharide while transfers onto an M_r 42 500 glycoprotein require lipid oligosaccharide intermediates.

Preliminary evidence has identified the M_r 42 500 as an arabinosylated hydroxyproline-rich glycoprotein which is co-ordinately induced with prolyl hydroxylase and protein: arabinosyl transferase by elicitor treatment [13, 14, 20]. This glycoprotein has now been shown to bind to thyroglobulin- and fetuin-Sepharoses in a specific manner and shares a sugar specificity with the arabinosylated hydroxyproline-rich lectins from *Datura* and potato [21–24]. The potato lectin functions as a bacterial

agglutinin in disease resistance [6, 7] and a similar role may be adopted by the M_r 42 500 bean glycoprotein.

RESULTS

Incorporation of L-[1-^{3}H]*arabinose from UDP-β-*L-[1-^{3}H]*arabinose by isolated membranes*

Table 1 shows the distribution of radioactivity in material of differing solubilities extracted from membranes incubated *in vitro* with UDP-β-L-[1-^{3}H] arabinose. Both membranes from growth regulator treated cells synthesizing mainly polysaccharide and from elicitor treated cells synthesizing glycoprotein incorporate arabinose into the same range of products but in differing amounts. Material soluble in chloroform–methanol (3:2) has been previously characterized as has the polysaccharide material soluble in 10% trichloroacetic acid [11, 16]. The material insoluble in 10% trichloroacetic acid has been characterized as mainly an M_r 42 500 glycoprotein [13, 14] although in some preparations material of M_r 300 000 (arabinogalactan proteins) and M_r 50–90 000 (extensin-like) can also be seen faintly on fluorographic analysis. The material soluble in chloroform–methanol–water (10:10:3) was previously thought to be mainly UDP-arabinose or arabinooligosaccharide [11]. However the first unequivocal demonstration using superior extraction method for charged lipid pentoses [18] to that used previously [11] has prompted re-examination of this material using these methods.

Characterization of lipid oligosaccharides

Chromatography of the material soluble in chloroform–methanol–water (10:10:3) on DEAE cellulose gave a distribution of radioactivity (Fig. 1) broadly similar to that observed in pea [18]. Table 2 gives the R_f for these materials on TLC analysis. Whereas the neutral pentolipid showed a similar mobility to steryl glycoside, the charged pentolipid did not migrate on silica gel. Total hydrolysis of the charged material gave radioactive arabinose only; controlled acid or enzymic hydrolysis [11] gave some radioactive arabinose, some arabinose containing disaccharide and an arabinosylated oligosaccharide greater than four residues long (Fig. 2). This material which was synthesized by membranes from both subcultured and elicited cells therefore appeared very similar

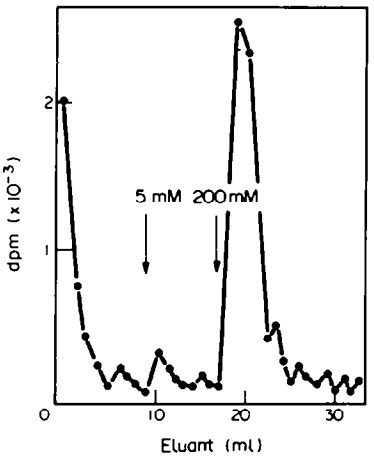

Fig. 1. DEAE-cellulose chromatography of [^{3}H]arabinosyl lipids formed from UDP-β-L-[1-^{3}H]arabinose. Membrane preparations (200 μg protein from elicited cells) were incubated with UDP-β-L-[1-^{3}H]arabinose (10^6 dpm) in a total vol. of 150 μl. Lipids were extracted and analysed on a column (0.5 cm × 2 cm) of DEAE cellulose equilibrated with $CHCl_3$–MeOH–H_2O (10:10:3) and sequentially eluted with $CHCl_3$–MeOH–H_2O (10:10:3) then with the same solvent containing ammonium formate (5 mM) then with 200 mM.

to that observed in pea which has been characterized to a much greater extent [18]. The purified lipid-linked [^{3}H]arabinosyl oligosaccharide was resuspended in 0.5% lysophosphatidylcholine and added back to microsomal membranes as substrate (Table 3). Fractionation of the products clearly showed transfer onto glycoprotein which was shown by fluorographic analysis to be mainly M_r 42 500 glycoprotein. Radioactivity appeared to be transferred more efficiently than from an equivalent amount of radioactive UDP-arabinose. Material extracted into the aqueous fraction appeared to be oligosaccharide possibly released by hydrolysis of the lipid oligosaccharide.

Kinetics of pentose incorporation into various products

Time courses for formation of pentosyl lipids from radioactive UDP-arabinose under different conditions are shown in Fig. 3A–D. In these figures curves have been

Table 1. Distribution of radioactivity from enzyme incubation into aqueous and lipid solvent fractions

	Radioactivity incorporated			
	membranes from elicited cells		membranes from subcultured cells	
Fraction	(dpm)	(% of mean total incorporation	(dpm)	(% of mean total incorporation)
$CHCl_3$–MeOH (3:2)	1634 ± 359	3.2	909 ± 127	2.2
H_2O soluble	889642 ± 63872	—	858283 ± 37965	—
$CHCl_3$–MeOH–H_2O (10:10:3)	2234 ± 582	4.4	2931 ± 451	7.1
10% trichloroacetic acid soluble	1905 ± 364	3.8	32302 ± 7694	78.2
10% trichloroacetic acid insoluble	44440 ± 8978	88.5	5158 ± 1675	12.5

Membranes (120 μg protein/assay) isolated from cells 48 hr after subculture or from cells treated with elicitor were incubated for 10 min with UDP-β-L-[1-^{3}H]arabinose (10^6 dpm approx.) and analysed. Mean values ± s.d. for six incorporations are shown in each case.

Table 2. Chromatographic analysis of lipid arabinosides

Solvent and system	Material soluble in $CHCl_3$–MeOH (3:2) arabinose labelled	Material soluble in $CHCl_3$–MeOH–H_2O (10:10:3) arabinose labelled
$CHCl_3$–MeOH–H_2O (65:24:4)/SILG	R_f 0.89	R_f 0.035
$CHCl_3$–MeOH–HOAc–H_2O (50:30:8:4)/SILG	—	R_f 0.035
$CHCl_3$–MeOH–NH_4OH (75:25:4)/SILG	—	R_f 0.092
$CHCl_3$–MeOH–H_2O (10:10:3)/DEAE plates	—	100% retained
H_2O/paper	—	93% retained

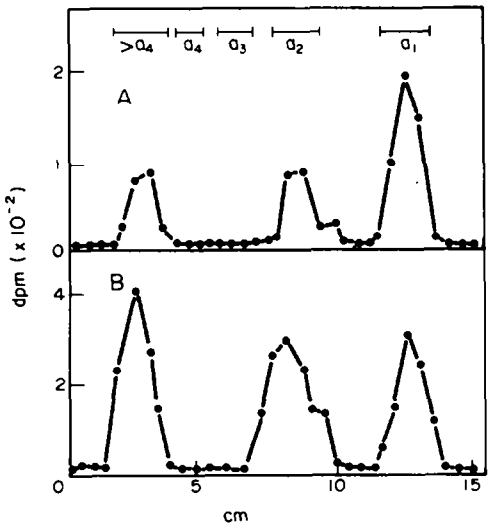

Fig. 2. TLC of partial acid hydrolysis of lipid-linked arabinosyl oligosaccharide. Partial acid hydrolysate of lipid-linked arabinosyl oligosaccharides from (A) subcultured cells; (B) elicited cells. The position of arabino-oligosaccharide markers is shown.

Table 3. Comparison of the distribution of radioactivity in products formed from lipid linked [1-^{3}H]arabinosyl oligosaccharide or UDP-[1-^{3}H]arabinose

Incubations:	Radioactivity incorporated (dpm) 1	2	3	4
Fraction				
Aqueous washed	6062	19778	23295	23370
Lipid	13336	230	279	201
Glycoprotein	2034	1527	1086	285

Membranes (297 μg protein in 110 μl standard incubation buffer) from elicited cells were incubated at 25° for 40 min with 40 μl containing *ca* 2×10^4 dpm of either (1) lipid-linked [^{3}H]oligosaccharide in 0.5% lysophosphatidyl choline or UDP [1-^{3}H]arabinose with (2) or without (3) the presence of 0.5% lysophosphatidyl-choline. A boiled control (4) with UDP-[1-^{3}H]arabinose was included. Incubations were terminated by the addition of 1 ml $CHCl_3$–MeOH (1:1) and analysed.

normalized with respect to the maximum incorporation observed for each fraction to show the relative patterns and levels of incorporation. Figure 3A shows that membranes from subcultured cells do form lipid oligosaccharide but possible transfer is limited by the small amounts of glycoprotein formed. In contrast rapid turnover of the lipid oligosaccharide occurs in tandem with the rapid incorporation into glycoprotein in membranes from elicited cells (Fig. 3B). Further evidence that hydroxyproline residues may be involved in part during transfer mechanisms was obtained from the kinetics of incorporation in the presence of poly-L-proline and poly-L-hydroxyproline. Addition of poly-L-proline proved to inhibit arabinosyl transferase activity while activating prolyl hydroxylase activity (Fig. 3C). In contrast poly-L-hydroxyproline stimulated turnover of lipid oligosaccharide, enhanced incorporation into glycoprotein, although transfer onto the added substrate has not been visualized on fluorographs, and inhibited prolyl hydroxylase (Fig. 3D). These effects have been interpreted in the light of enzyme localization data as suggesting that prolyl hydroxylation is completed in the endoplasmic reticulum before subsequent arabinosylation in the Golgi apparatus [20]. Transfers carried out by membranes from elicited cells were not affected by the inclusion of tunicamycin (100 μg/ml) or dolichol phosphate (2 μg) as has previously been found for membranes from unelicited cells [cf. 16, 18]. However, bacitracin (2 mM), which binds to dolichol pyrophosphates, inhibited total incorporations by about 12%. In some extended incubations the amount of radioactivity incorporated into the M_r 42 500 glycoprotein fell by 20% between 20 and 60 min suggesting some trimming process may follow initial transfer. Also up to 20% of the incorporated arabinose was subject to β-elimination in alkali (pH 9), the remainder, presumably linked to hydroxyproline residues, is stable in alkali.

Carbohydrate building properties and characterization of the M_r 42 500 *protein*

A number of affinity ligands were developed to test the possible carbohydrate binding properties of the M_r 42 500 protein due to its apparent similarity to other lectins such as those from potato or *Datura*. Thyroglobulin- and fetuin-Sepharoses proved to be most efficient in binding the glycoprotein, which did not bind in the presence of

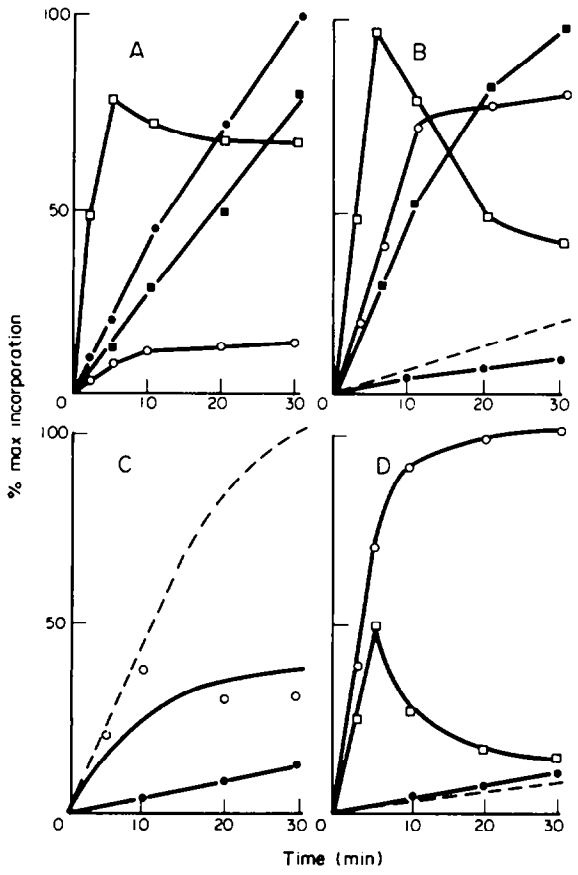

Fig. 3. Time courses for the synthesis of various products by membranes from UDP-[1-^{3}H]arabinose. Membranes (500 μg protein) from (A) subcultured cells, (B–D) elicited cells were incubated with UDP-β-L-[1-^{3}H]arabinose (10^6 dpm) and in a total vol. of 500 μl. 75 μl samples were fractionated into (■) neutral lipid (□) oligosaccharide-lipid, (●) 10% trichloroacetic acid soluble, (○) 10% trichloroacetic acid insoluble material at sequential time points. The graphs depict a direct comparison of the incorporation in the four incubations with incorporations into each fraction expressed as a % of the maximum observed. The effect of the addition of (C) poly-L-proline (400 μg) and (D) poly-L-hydroxyproline (400 μg) is shown. (- - - - -) Time course of prolyl hydroxylase activity. Maximum incorporations (100%) into each fraction were; neutral lipid, 2566 dpm; oligosaccharide-lipid, 3437 dpm; trichloroacetic acid-soluble, 54 916 dpm; trichloroacetic acid-insoluble, 53 142 dpm.

detergent (Table 4). Hypotonically lysing membranes led to up to 50% binding which was prevented in the presence of chitin oligomers. The failure to bind to *p*-aminobenzyl-1-thio-*N*-acetyl-β-glucosaminide-sepharose may indicate a specificity for *N*-acetylglucosamine oligosaccharides.

The glycoprotein could also be eluted from thyroglobulin-Sepharose at pH 9 (Fig. 4) and therefore differs from the characteristic bean lectin phytohaemagglutinin which is eluted at pH 3. The M_r 42 500 glycoprotein is not eluted at this pH. The M_r 42 500 glycoprotein was characterized by alkaline and acid hydrolysis and gave the same results as described for the size fractionated material described previously [13].

The presence of hydroxyproline was checked following hydrolysis of the eluted material. A detailed amino acid

Table 4. Efficiency of binding of M_r 42 500 glycoprotein to affinity ligands

Ligand	% binding		
	Binding buffer/ PBS	PBS/ Tween 20	Tris/ Triton
None unsubstituted (blocked) CNBr Sepharose	100	100	100
Fetuin (15 mg/g) Sepharose	< 1	< 1	< 1
Thyroglobulin (10 mg/g) Sepharose	17–30	3.6	< 1
anti-(PHA) IgG (50 mg/g) Sepharose	29–54	5.3	< 1
p-aminobenzyl-1-thio-*N*-acetyl glucosamide-Sepharose	6	< 3	< 1
	5	< 3	< 1

Radioactive membranes (50 μl) were solubilized in 1 ml detergent or hypotonic buffer with sonication, before end over end incubation with 50 μl of each gel. Each gel was washed with 1 ml of the same buffer three times before preparation for SDS-PAGE on 10% gels. % binding was estimated from scanning densitometry of fluorographs comparing the intensity of each band with that from 50 μl of membranes not exposed to affinity gels.

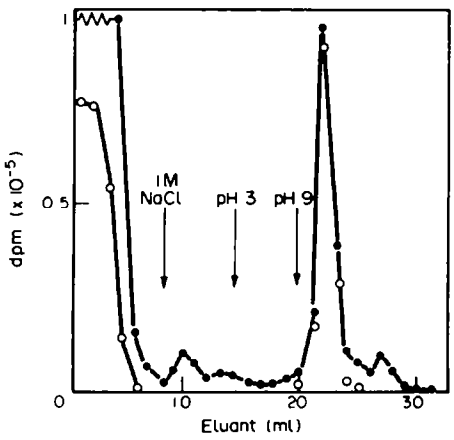

Fig. 4. Elution of M_r 42 500 glycoprotein from thyroglobulin Sepharose. Membranes from elicited cells were incubated with UDP-[1-^{3}H]arabinose, hypotonically lysed and sonicated and added directly to the column (0.5 ml) of thyroglobulin-Sepharose equilibrated with PBS. The column was sequentially eluted with PBS; PBS containing 1 M NaCl, 100 mM glycine–HCl, pH 3.0, containing NaCl and 50 mM Tris–HCl, pH 9.0. (●) Radioactivity. (○) Radioactivity insoluble in 10% trichloroacetic acid.

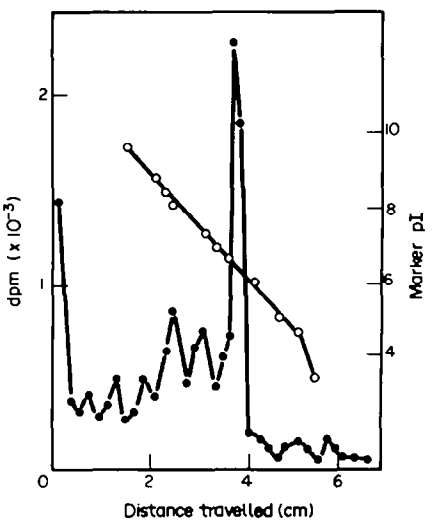

Fig. 5. Isoelectric focussing profile of radioactivity incorporated by membranes *in vitro* after elicitation. Radioactive membranes were prepared for isoelectric focusing by removal of lipid and polysaccharide and digestion with nucleases. The material was denatured, analysed and compared on several micro electrofocussing gels [44] and the distribution of radioactivity (●) in gel slices was determined. Gels containing marker proteins of known pI (Pharmacia, U.K.) were run in parallel to check the gradient (O).

analysis has not been carried out on the eluted fraction because analysis on sodium dodecyl sulphate/polyacrylamide gel electrophoresis showed that the M_r 42 500 glycoprotein, which stained poorly with both Coomassie blue and silver staining procedures but is readily detected overnight by fluorography, co-purified with small amounts of another protein of M_r 65 000. Interestingly, this is the M_r of prolyl hydroxylase [20] which may remain bound to its putative protein substrate during this affinity procedure. 2D-TLC of the acid hydrolysates, nevertheless, demonstrated the presence of large amounts of hydroxyproline together with other major amino acids provisionally identified as lysine, serine, tyrosine, valine, aspartic acid and glycine. Interestingly, some UV fluorescent compound, probably ferulic acid, was also released by hydrolysis of this material. Some of these residues are also extremely abundant in the arabinosylated potato and *Datura* hydroxyproline-rich lectins but not in phytohaemagglutinin [25]. The pI for the M_r 42 500 of 6.4 (Fig. 5) compares with that for the potato lectin of 9.5 and its acidic isolectins of 8.4 and 7.7 [25], of phytohaemagglutin of 5.5 [25] and bean arabinogalactan protein of 2.5–3 [26].

DISCUSSION

With the probable exception of cellulose, polymers located in the wall such as polysaccharides and proteins are synthesized and transported within the endomembrane system, comprising the endoplasmic reticulum and Golgi complex [15, 27, 29]. Changes in wall composition during growth and differentiation of meristematic cells and suspension cultured cells such as those from bean are dependent on differential timing of appearance of the biosynthetic systems within the membrane [11, 12, 30, 31]. These previous studies have established that there is a capacity for rapid quantitative and qualitative changes in membrane associated glycosylation reactions in response to different exogenous stimuli, this being especially true for the effect of the elicitation response on patterns of arabinosylation. Following elicitation arabinose is directed away from polysaccharide biosynthesis and onto specific proteins, in particular an M_r 42 500 hydroxyproline containing glycoprotein [13]. This glycoprotein is co-ordinately induced with a protein: arabinosyl transferase immunologically distinct from an arabinan synthase [13, 31] and with prolyl hydroxylase (proline: 2-oxoglutarate dioxygenase, EC 1.14.11.12) against a background of induction as a consequence of increased gene expression *de novo* of enzymes involved in the synthesis of isoflavonoid phytoalexins and lignin-like material [13, 32, 33].

Comparison of arabinosylation by membranes from cells synthesizing mainly polysaccharide with those from cells synthesizing glycoprotein has enabled elucidation of the mechanisms. Previous evidence that arabinose was transferred directly from UDP-arabinose to arabinan by bean membranes mainly within the Golgi apparatus [16] has been re-examined in the light of the demonstration that intermediate lipid arabinosides serve as donors for glycosylation of arabinogalactan proteins in pea [18]. Other arabinose-containing lipid intermediates are involved in the glycosylation of protein carriers during the synthesis and secretion of maize root slime [34]. The present study has shown that a charged lipid-linked arabinosyl oligosaccharide is formed in small amounts relative to added UDP-arabinose with kinetics that indicate its rapid turnover *in vitro*. Incubation of the purified lipid-linked arabinosyl oligosaccharide with membrane preparations allowed transfer onto the M_r 42 500 glycosylation. Further evidence that this material was concerned in the glycosylation of hydroxyproline residues was provided by the fact that poly-L-proline was a potent inhibitor of the protein: arabinosyl transferase activity whereas poly-L-hydroxyproline enchanced incorporation of arabinose into trichloroacetic acid-insoluble material. Some water-soluble material was formed in incubations with the purified lipid linked arabino-oligosaccharide but this appeared to be mainly low molecular weight oligosaccharide. Similar material has been observed during incubations of suspension cultured spinach cells with [1-^{3}H]arabinose *in vivo* [17]. In contrast the kinetics of incorporation by membranes synthesizing much reduced glycoprotein and considerably increased amounts of arabinan were not consistent with transfer onto polysaccharide from lipid-oligosaccharide. These results, together with previous observations [16], distinguish mechanisms for transfer to arabinan from the lipid intermediate requirement for transfers onto glycoprotein by enzyme systems which have already been demonstrated to be immunologically distinct [13].

The M_r 42 500 glycoprotein has now been shown to bind in a specific manner to thyroglobulin- and fetuin-Sepharoses which is prevented by chitin oligomers. The glycoprotein is clearly a carbohydrate-binding protein which is also distinct from the characteristic bean lectin, phytohaemagglutinin, since it is not bound by anti-(PHA) IgG under the stringent conditions required for the establishment of antigen–antibody binding. Although the two lectin-glycoproteins bind to thyroglobulin, their relative pIs and a preliminary amino acid analysis of the M_r 42 500 show clear differences. Phytohaemagglutin is

an N-linked high mannose-type glycoprotein and the M_r 42 500 appears to be an arabinosylated hydroxyproline-rich glycoprotein similar to those described from *Datura* and potato. The fact that it is induced by fungal elicitor suggests a possible role in disease resistance. The bean lectin clearly differs from other hydroxyproline-rich glycoproteins in both its subunit M_r and timing of appearance after elicitation. The rapid transient appearance [14] contrasts with a much slower induction of mRNA coding for extensin in elicited bean cultures [35] and for the appearance of extensin-like proteins in elicitor-treated melon tissues [36].

EXPERIMENTAL

Derivation of all suspension cultures of French bean (*Phaseolus vulgaris*; cv. Canadian Wonder) was described previously [12] and Poly-L-proline (M_r av. 40 000), poly-L-hyroxyproline (M_r av. 14 000), thyrogobulin, fetuin, *anti*-(phytohaemagglutin) IgG, *p*-aminobenzyl-1-thio-*N*-acetyl-β-glucosaminide, CNBr-Sepharose and CH-Sepharose were all obtained from Sigma, U.K. UDP-β-L-[1-^{3}H]arabinopyranose and non-radioactive UDP-β-L-arabinopyranose were synthesized as described previously [11]. Chitin oligomers were prepared by the method of ref. [24].

Treatment of cultures. Preparation of fungal elicitor was described in ref. [37]. Maintenance, growth and elicitation of cell suspension cultures were as described [13].

Preparation of membranes. Cells were homogenized in 50 mM HEPES pH 6.8 containing 1 mM dithiothreitol, 0.4 M sucrose and 10 mM $MgCl_2$ (1 g tissue/ml of buffer) at 4° in a pestle and mortar. The slurry was filtered through muslin and centrifuged at 1000 *g* for 15 min. The supernatant was then centrifuged at 15 000 *g* for 10 min to sediment most of the larger organelles. The supernatant was then subjected to centrifugation at 200 000 *g* for 1 hr. The final microsomal pellet was resuspended in homogenization medium.

Enzyme assays. Proline hydroxylation coupled to decarboxylation of oxoglutarate was measured by a microscale adaption of the method of ref. [38], described in ref. [20]. The standard assay for arabinan synthase activity using high voltage electrophoresis was as described in ref. [11]. The standard assay for the fractionation of products into lipid-soluble, water-soluble and trichloroacetic acid-insoluble products has now been redesigned using the method of ref. [18]. Membrane preparations (up to 150 μl) were incubated with UDP-β-L-[1-^{3}H]arabinose as described previously [13]. Reactions were terminated by the addition of 1 ml $CHCl_3$–MeOH (1:1). Lipid-linked sugars were extracted and fractionated on DEAE-cellulose as described [18]. The final polymer pellet was then extracted into 10% TCA and soluble and insoluble products obtained [13]. The insoluble glycoprotein products were then analysed by SDS-PAGE and detected by fluorography and densitometer scanning as described previously [14]. Gels were silver stained by the method of ref. [39]. Protein was estimated by the method of ref. [40].

Analysis of lipid. Pentosyl lipids were fractionated into neutral and charged fractions. Analysis by ascending TLC was performed on prewashed polygram SILG silica gel plates (20 cm × 20 cm × 0.25 mm) in $CHCl_3$–MeOH–H_2O (65:25:4), $CHCl_3$–MeOH–HOAc–H_2O (50:30:8:4) and $CHCl_3$–MeOH–17.5 M NH_4OH (75:25:4). Charged fractions were also treated with ascending chromatography on DEAE cellulose plates in $CHCl_3$–MeOH–H_2O (10:10:3) or washed by application to Whatman No. 1 chromatography paper and elution with H_2O.

Hydrolysis of charged lipid was performed enzymatically [11] or in 0.01 TFA in 50% propan-1-ol in a sealed tube at 100° for 10 min [18]. Oligosaccharides were separated by ascending TLC in EtOAc–pyridine–H_2O (10:4:3) using a partial hydrolysate of bean callus arabinan [41] as markers which were detected with the $AgNO_3$ reagent [42]. Radioactive sugars were detected by scintillation counting of strips cut from the chromatogram.

Isolation of glycoprotein. Fetuin, thyroglobulin and rabbit anti-(phytohemagglutinin) IgG were separately coupled to CNBr-activated Sepharose 4B in 0.1 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl for 4 hr. The proportion coupled was determined using the Coomassie blue protein assay [40]. The gel was then treated for 1 hr with 1 M ethanolamine, pH 9.0, prior to extensive washing with 0.1 M NaOAc, pH 4.0, followed by 0.1 M sodium borate, pH 8.0 (both buffers containing 0.5 M NaCl). The gels were stored in 50 mM Tris–HCl buffer, pH 8.0, containing 0.02% (v/v) NaN_3. *p*-Aminobenzyl-1-thio-*N*-acetyl-β-glucosaminide was coupled to CH-activated Sepharose 4B in 0.1 M $NaHCO_3$ buffer, pH 8.0. Unreacted groups were blocked overnight in 1 M ethanolamine, pH 9.0, and the gel washed and prepared as for other gels. CNBr-activated-Sepharose 4B was blocked with ethanolamine and used as an unsubstituted control.

Membrane preparations (up to 200 ml) labelled with UDP-β-L-[1-^{3}H]arabinose were incubated with affinity gels with the following types of treatment, either addition of 800 μl of 50 mM Tris–HCl, pH 7.4, containing 0.75 M NaCl and 1% (v/v) Triton X-100 (standard antibody binding conditions) or 10 mM KPi, pH 7.4, containing 0.15 M NaCl containing 0.5% (v/v) Tween 20. Alternatively membranes were made hypotonic with 800 μl 10 mM NaPi buffer, pH 7.4, and allowed to lyse for 30 min at 4° followed by a brief sonication to release their contents. Following incubation by end over end rotation at 4° overnight the gels were washed × 3 with 1 ml of the requisite incubation buffer before analysis of bound material by one dimensional SDS-PAGE electrophoresis, as described previously [43]. Microgel isoelectric focusing was performed on 5% polyacrylamide gels using 2.4% (v/v) ampholines (pharmalyte, Pharmacia, U.K., pH 3–10) [44]. Following electrophoresis at 500 V for 16 hr, gels were extruded and cut into 2 mm slices when frozen. Radioactivity was determined by scintillation counting after incubation of each slice with 50 μl H_2O_2 (30% w/v) at 60° for 4 hr.

M_r 42 500 was isolated from the hypotonically treated membranes by affinity chromatography on thyroglobulin-Sepharose [45]. The gel was washed extensively with 10 mM KPi buffer, pH 7.4, containing 0.15 M NaCl and then 10 mM KPi buffer, pH 7.4, containing 1 M NaCl. The bound M_r 42 500 glycoprotein was released with 50 mM Tris, pH 9, and detected by scintillation counting; the pooled fraction was dialysed against H_2O for 3 hr at 4° and lyophilized.

Analysis of affinity purified glycoprotein. Following total acid hydrolysis, glycoprotein was analysed for sugar content as described previously [13] and for amino acid content using 2D-TLC in *n*-BuOH–HOAc–H_2O (4:1:1) in the first dimension and PhOH–H_2O (3:1) in the second. Hydroxyproline was detected by its characteristic R_f and colour when stained with ninhydrin.

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