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Presence in a plant of a compound similar to the dolichyl diphosphate oligosaccharide of animal tissue

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A compound with properties identical with the glucose-containing dolichyl diphosphate oligosaccharide present in animal tissues was detected in alfalfa roots incubated with [^{14}C]glucose. The products of mild acid hydrolysis behaved the same on paper chromatography, on treatment with specific glucosidases and on *N*-deacetylation.

A key compound for the glycosylation of asparagine residues in proteins has been found to be a dolichyl diphosphate (Dol-P_2) oligosaccharide which contains three glucoses, nine mannoses and two *N*-acetylglucosamine residues (Kornfeld & Tabas, 1978). This oligosaccharide is transferred to protein faster than similar compounds with less than three glucoses (Staneloni *et al.*, 1980). After transfer the G-oligosaccharide is processed to yield the high mannose or the complex type of asparagine-linked oligosaccharide (see for a review Staneloni & Leloir, 1979). Most of the studies have been carried out in animal cells and some in yeast (Parodi & Leloir, 1979). In plants the lipid-bound G-oligosaccharide has not been detected although the formation of some related compounds has been reported such as lipid phosphate glucose, mannose, *N*-acetylglucosamine and mannose-containing oligosaccharides (Pont-Lezica *et al.*, 1975; Brett & Leloir, 1977; Forsee & Elbein, 1975). Furthermore evidence has been presented showing that in plants the lipid moiety of the intermediate is a derivative of dolichol and not of undecaprenol as occurs in bacteria (Brett & Leloir, 1977; Elbein, 1979).

Materials and methods

Materials

Dolichyl diphosphate G-oligosaccharide labelled in glucose was obtained by incubation of rat liver microsomes with UDP-[^{3}H]Glc or UDP-[^{14}C]Glc (Behrens & Tábora, 1978). Dol-P_2 G-oligosaccharide labelled in both mannose and glucose residues was obtained by incubation of hen oviduct slices with [^{14}C]mannose (Staneloni & Leloir, 1979). Mild acid hydrolysis was carried out as described previously (Parodi *et al.*, 1973).

The following solvents were used for paper chromatography: solvent A, propan-1-ol/nitromethane/water (5:2:4) for oligosaccharides and solvent B, butanol/pyridine/water (6:4:3) for monosaccharides.

Alkaline treatment

The oligosaccharides (about 3000 c.p.m.) were mixed with 8 mg of sodium borohydride and 40 μl of 10 M-NaOH and heated for 2 h at 100°C. The mixture was neutralized with acetic acid and desalted by passage through a gel permeation column (Bio-Gel P-2; 1.3 cm × 17 cm).

Preparation of crude radioactive glycolipid

Medicago sativa (alfalfa) seeds were germinated for 5 days under sterile conditions in the medium as described by Gibson (1963). The root tips were finely chopped with a knife and 3 g were incubated in 7 ml of modified White's medium without sucrose (Street & Henshaw, 1966) containing 300 μCi of [U-^{14}C]glucose, sp. activity 268 Ci/mol. After 3 h at 25°C with gentle shaking the suspension was centrifuged, the liquid was poured off and the solids were washed three times with sugar-free White's medium. The precipitate was homogenized (tissue grinder K-885000; Kontes Glass Co.) in water (5 ml per g of solids). 2 vol. of methanol, 3 vol. of chloroform and 100 mg of

Abbreviations used: Dol-P_2, dolichyl diphosphate; G-oligosaccharide is the oligosaccharide containing: glucose$_{1-3}$, mannose$_9$ and *N*-acetylglucosamine$_2$, which occurs combined with dolichyl diphosphate. The subscripts G_1-, G_2- and G_3- refer to the number of glucose residues.

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microsomal protein as carrier were added. The mixture was centrifuged and the interphase was washed twice with chloroform/methanol/water mixtures in the above mentioned proportions. The interphase was washed three times with 10ml of chloroform/methanol/4mM-$MgCl_2$ (3:48:47). The solid was then extracted three times with 4ml of chloroform/methanol/water (1:1:0.3). The last extraction was allowed to proceed overnight (1:1:0.3 extract). The radioactivity of the fraction varied in different preparations (5–70) $\times 10^4$ c.p.m.

Results

Properties of the glycolipid

Chromatography on DEAE-cellulose resulted in the separation of several peaks, one of which appeared in the position where Dol-P_2 G-oligosaccharide was known to be eluted. The tubes corresponding to that peak were pooled. The yield in six different experiments varied around (1–5) $\times 10^4$ c.p.m. The water-soluble substances were removed by a chloroform/water partition. (Under these conditions Dol-P_2 G-oligosaccharide remains at the interphase.) Rechromatography on DEAE-cellulose of a portion with an internal standard of tritium-labelled Dol-P_2 oligosaccharide, prepared with liver microsomes, showed that they were eluted in a similar manner.

Paper chromatography of the compound with butanol/pyridine/water (4:3:4) as solvent gave an R_F value of 0.60 similar to Dol-P_2 oligosaccharide.

Properties of the mild acid hydrolysis product

Mild acid hydrolysis of the plant glycolipid yielded about 50% of a radioactive water-soluble substance. No information was obtained on the identity of the substances corresponding to the other 50%. As shown in Fig. 1(*a*) after chromatography of the water-soluble substances in solvent A only one peak was detectable and its mobility was identical with that of the G-oligosaccharide sample (Fig. 1*b*). It may be pointed out that chromatography under these conditions leads to the separation of G-oligosaccharide differing in one, two or three glucose residues.

The oligosaccharide eluted from a paper chromatogram like that of Fig. 1(*a*) and a control of G_3-oligosaccharide were treated with a mixture of purified liver microsomal glucosidases which specifically release glucose from the G_1-, G_2- and G_3-oligosaccharides (Ugalde *et al.*, 1979). The products of glucosidase treatment were then chromatographed on paper with solvent B. A peak which hardly moved from the origin and another moving like glucose were observed. No mannose was detectable. The peak at the origin corresponding to the oligosaccharides was then eluted and re-run in solvent A. As shown in Fig. 1(*c*) three peaks appeared with the same mobility as those formed from G-oligosaccharide treated with glucosidase (Fig. 1*d*).

In another experiment the plant oligosaccharide obtained as in Fig. 1(*a*) was heated in 1M-HCl for 4h at 100°C and chromatographed with solvent B.

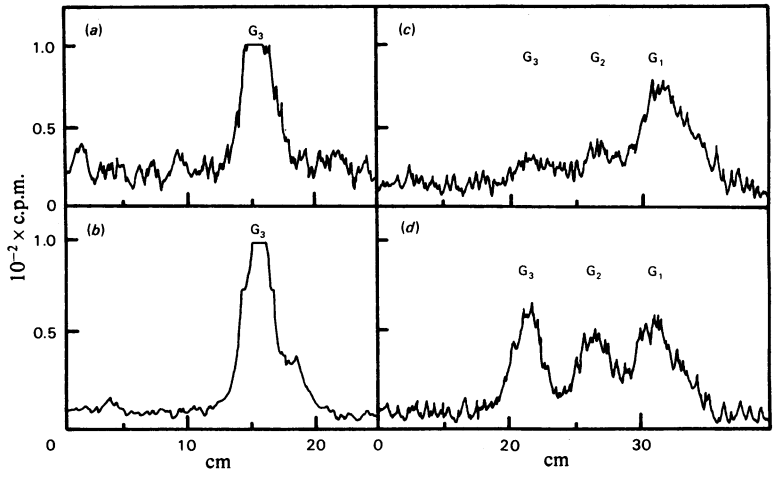

Fig. 1. *The action of liver microsomal glucosidases on the oligosaccharides*
(*a*) Product of mild acid hydrolysis of the pooled fractions of DEAE-cellulose chromatography of the plant glycolipid. (*b*) G-oligosaccharide labelled in glucose and mannose residues. (*c*, *d*) The radioactive products of (*a*) and (*b*) respectively were eluted from the papers and treated with liver microsomal glucosidases as described previously (Ugalde *et al.*, 1979). The hexoses released were identified by paper chromatography with solvent B and the oligosaccharides were rechromatographed with solvent A for 5 days.

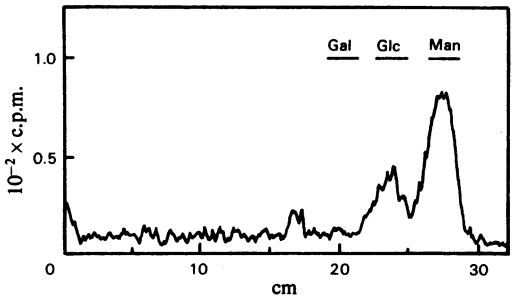

Fig. 2. *Total hydrolysis of the oligosaccharides*
A sample of oligosaccharide from the plant glycolipid, purified by paper chromatography as in Fig. 1(*a*) was hydrolysed in 1 M-HCl for 4 h at 100°C and chromatographed on paper with solvent B.

Radioactivity was detectable in the zones corresponding to mannose and glucose in the ratio 2.7 (Fig. 2). A control G-oligosaccharide treated in the same way gave a ratio of 2.8. Equilibration of glucose and mannose in various tissues is usually very rapid but is slower between hexoses and hexosamines. Nevertheless in the experiment of Fig. 2 a small peak of radioactivity appeared in the position corresponding to glucosamine (R_{Glc} = 0.71).

Alkaline treatment

Previous work showed that treatment of the G-oligosaccharide with 2 M-KOH at 100°C resulted in the formation of positively charged substances which corresponded to the removal of one or two *N*-acetyl residues (Parodi *et al.*, 1973). Protection of the reducing group was effected by preparing the methyl derivative or by reduction with sodium borohydride. Treatment of the plant oligosaccharide under these conditions led to the formation of two positively charged substances having the same electrophoretic mobility as those obtained from the G-oligosaccharide. These results are shown in Fig. 3. The charged compounds were eluted from the paper and re-acetylated as described previously (Staneloni & Leloir, 1979) and it was found that the products were neutral in formic acid electrophoresis.

Discussion

Previous attempts to detect Dol-P_2 G-oligosaccharide in plant material were negative. The procedure used was that for animal tissues which consists of incubating microsomes with labelled UDP-Glc in order to obtain the transfer of radioactive glucose to an endogenous acceptor. In the experiments reported here a compound formed by incubation of alfalfa root segments soluble in

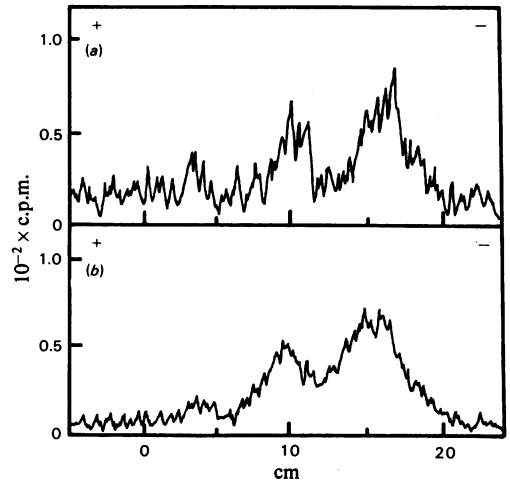

Fig. 3. *Alkaline treatment of the oligosaccharide*
Electrophoresis in 5% formic acid, at 50 V/cm for 3 h of samples heated in sodium borohydride and 10 M-NaOH as described in the Materials and methods section. (*a*) Plant oligosaccharide and (*b*) G-oligosaccharide.

chloroform/methanol/water (1:1:0.3) was compared with a known sample of Dol-P_2 G-oligosaccharide. Both compounds behaved in the same manner on paper and DEAE-cellulose chromatography. The water-soluble products obtained by mild acid hydrolysis behaved identically during paper chromatography, in their degradation by specific glucosidases, on *N*-deacetylation and in yielding mannose and glucose by complete hydrolysis.

The finding of the glucose-containing oligosaccharide in plant material is of some interest because it is a further indication that *N*-glycosylation in plants occurs as in animal tissues.

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