Cyclitol Glucosides and their Role in the Synthesis of a Glucan from Uridine Diphosphate Glucose in *Phaseolus aureus*

CHARACTERIZATION OF SOME CYCLITOL GLUCOSIDES AND THEIR SYNTHESIS

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Short-chained sugar compounds, thought to be involved in the synthesis of callose, were formed in small amounts from UDP-glucose by soluble extracts from hypocotyls of seedlings of *Phaseolus aureus*. The properties of the glucosides were investigated by treatment with various chemicals and analysis by paper chromatography, g.l.c. and mass spectrometry. The data obtained support the characterization of these compounds as myoinositol-β-glucoside and diglucosylmyoinositol. The cyclitol moiety was provided by the enzyme extract. Free myoinositol was not the immediate substrate but a compound containing myoinositol, isolated from the enzyme extract, may be involved. The method of synthesis of these glucosides is compared with that of other cyclitol glucosides.

Several naturally occurring glucosides of myoinositol have been found in higher plants. The first report of the synthesis of a compound of this type was by Frydman & Neufeld (1963), who described the transfer of galactose from UDP-galactose to myoinositol to form galactinol in the presence of an enzyme extracted from peas. Senser & Kandler (1967) underlined the importance of galactinol as a galactose donor in the synthesis of the raffinose series of oligosaccharides.

In the present work a connexion has also been established between the formation of glycosidic cyclitols and the synthesis of sugar polymers. A study has been made of the initiation of chains of callose, a β-(1→3)-linked glucan. The subsequent synthesis of this glucan has been described by Feingold *et al.* (1958), Flowers *et al.* (1968) and Elbein & Chambers (1970).

Since callose could be synthesized from UDP-glucose in the presence of a soluble enzyme any substance needed to initiate a polysaccharide chain must be present in solution in the enzyme extract and the present paper describes the results of the search for these compounds and their characterization.

Experimental

Materials

UDP-[1-4C]glucose was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-glucose and anilinediphenylamine spray were obtained from Sigma, Norbiton Station Yard,

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Kingston-upon-Thames, U.K. Cab-O-Sil (colloidal silica), laminaribiose (Glcβ1→3Glc) and laminaritriose (Glcβ1→3Glcβ1→3Glc) were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Glucosylmannitol was kindly provided by Mr. John A. Farrar, Department of Agricultural Science, University of Oxford, Oxford, U.K.

Methods

Growth of etiolated seedlings of *Phaseolus aureus* (*mung bean*). Seeds were obtained from Palms, Oxford, U.K., and before being planted they were soaked in distilled water for several hours. A mixture containing 1.5 litres of sterilized sand and 0.5 litre of distilled water was prepared and the seeds were shallowly planted in this mixture in enamel trays. They were kept in the dark at 25°C and after the first 2½ days were watered daily with about 100ml of distilled water. Seedlings between 3 and 7 days old were used to prepare the enzyme.

Preparation of enzyme. Mung-bean shoots were separated from their roots, cotyledons and young leaves, and were chilled. The enzyme was prepared essentially as described by Flowers *et al.* (1968). Chilled shoots were ground in a pestle and mortar with an equal volume of 0.1M-Tris–HCl buffer, pH 7.5. The suspension was filtered through two layers of cheese cloth and the filtrate centrifuged at 1000g for 5min. The supernatant was centrifuged at 15000g for 30min. All centrifugation was carried out in the cold. The pellet was resuspended in 0.1M-Tris–HCl buffer, pH 7.5, by using 20μl of the buffer for every gram of fresh bean shoots, the resulting suspension containing about 20mg of protein/ml.
This particulate fraction was treated with an equal volume of a 1.6% (w/v) solution of digitonin. In a few early experiments the digitonin was dissolved in 0.05 M-sucrose. The mixture was kept on ice for 15 min with occasional shaking and centrifuged at 15000g for 20 min. The supernatant thus prepared catalysed the formation of glucan and oligosaccharides from UDP-glucose and contained about 2 mg of protein/ml.

Incubation conditions. Incubations (50 μl) were carried out in a low concentration (often 1 μM) of UDP-glucose containing about 20000 d.p.m. of UDP-[14C]glucose and 2 mM-MgCl₂ in 80 mM-Tris-HCl buffer, pH 7.5. Usually 10 μl of enzyme was used and incubations were carried out for different periods at room temperature (18°C). The enzyme reactions were stopped by boiling.

Thin-layer and paper chromatography. Double development of cellulose t.l.c. plates in propan-1-ol—ethyl acetate—water (7:1:2, by vol.) was found to be a fairly quick and satisfactory way of separating oligosaccharides of different sizes. Paper chromatography was found to be superior for separating similar oligosaccharides, especially when large amounts of material were involved. Separation of oligosaccharides on paper was carried out by descending chromatography on Whatman no. 1 paper. Usually the solvent was propan-1-ol—ethyl acetate—water (7:1:2, by vol.). Development was carried out either for 48 h at room temperature or for 36 h at 30°C.

Radioactive paper chromatograms were cut up into half-centimetre strips and counted in a scintillation spectrometer. T.l.c. plates were divided into half-centimetre strips and the material was scraped from the glass base with a razor blade before being counted in the scintillation spectrometer. The cohesive properties of the cellulose enabled accurate recovery of the plate material. Standard sugars were detected with either aniline—diphenylamine spray from Sigma or alkaline silver nitrate spray (Block et al., 1958). Nucleotides were located by their u.v. absorption.

Large-scale preparation of glucosides. Glucosides were prepared from incubations of low concentrations of UDP-glucose with digitonin-solubilized enzyme by using the normal concentrations of Tris—HCl buffer and MgCl₂ but scaling up to several millilitres according to the yield required. For preparations for mass spectrometry and gas chromatography the concentration of UDP-glucose was usually 50 μM and the radioactivity was decreased to the minimum amount required for detection of the product on a paper chromatogram.

After incubation for 10 min the preparation was boiled to kill the enzyme and then freeze-dried before being spotted on to paper and chromatographed in propan-1-ol—ethyl acetate—water (7:1:2, by vol.) for about 36 h at 30°C. The radioactive peak near the laminariobiose (Glcβ1→3Glc) marker was eluted with water and the eluate was evaporated to dryness in a vacuum desiccator over P₂O₅.

Determination of radioactivity. Radioactive samples were counted in a Beckman liquid-scintillation spectrometer, in scintillant containing 7 g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/1 of toluene. Where cellulose-plate material was to be counted for radioactivity 25 g of Cab-O-Sil (colloidal silica)/1 was added to the scintillant and each sample was shaken to disperse and suspend the plate material.

In experiments using compounds labelled only with 14C the counting efficiency was of the order of 70%.

Enzymic hydrolysis. A preparation of β-(1→3)-glucanase, described by Manners & Patterson (1966), was kindly supplied by Professor D. Manners, Herriot-Watt University, Edinburgh, U.K. Approximately 0.75 mg of this enzyme was incubated with about 1 μg of the glucoside in 0.05 M-dithiothreitol in a volume of 200 μl for 1 h or more.

Mild acid hydrolysis. Mild acid hydrolysis was carried out in 1 M-HCl for 15 min at 100°C. The acid was evaporated in a vacuum desiccator over KOH and the products were redissolved in water and then evaporated to dryness twice more.

Borohydride reduction. The reaction mixture consisted of 20 μl of sugar (15000 c.p.m.; 70 pmol), 10 μl of fresh NaBH₄ solution (1 mg/ml) and 10 μl of boric acid (2 mg/ml). Incubation was carried out for 30 min at room temperature and the reaction was stopped by the addition of 20 μl of 10% (v/v) acetic acid. The mixture was then passed through a small Dowex 50 column (H⁺ form) to remove Na⁺ ions. A very high proportion of the radioactive material could be recovered from the eluate, which was evaporated to dryness in a vacuum desiccator over P₂O₅ and then treated with methanol and evaporated to dryness three times. This removed the boric acid as the volatile methyl ester. If boric acid was omitted from the initial incubation the product was glucose, presumably formed by hydrolysis. Reduction of laminariobiose by this method gave a high yield of glucosylglucitol.

Oxidation with lead tetra-acetate. A dried preparation of the unknown disaccharide (approx. 7000 d.p.m.; 0.15 μg) was treated with 20 μl of lead tetra-acetate (0.1 mg/ml), 75 μl of acetic acid and 5 μl of water. The mixture was stirred and left for 2 h. Oxalic acid (1 μl of a solution containing 0.6 mg/ml) was added and after 30 min the mixture was centrifuged at 20000 g for 20 min to separate the precipitate of lead oxalate from the radioactive solution, which was then examined by paper chromatography.

Silylation of sugar compounds. Sugars were converted into the trimethylsilyl derivatives before gas chromatography or mass spectrometry. The samples were completely dried and then treated with bis(trimethylsilyl)acetamide; 1 μl of this reagent was added for each microgram of sugar present. The
reaction vessel was then tightly sealed and left overnight at room temperature. Complete silylation was effected under these conditions.

Gas chromatography. The apparatus used was a Pye model with a flame ionization detector. The column (1.5 m × 0.6 cm) was stainless steel. The support was 1–120 diatomite C and the stationary phase was 3% SE52. The gas-flow rate was 1 ml/s. Operation was carried out with a single column and a constant temperature.

Trimethylsilyl derivatives of monosaccharides were separated at 150°C and those of disaccharides were separated at 180°C. Each injection consisted of about 1 µg of sample in 1 µl. Standards for comparison were prepared under identical conditions and were injected at the same time as the experimental samples. Retention times and half-height peak widths were measured where necessary.

Mass spectrometry. Mass spectrometry of the trimethylsilyl derivatives of the unknown biose and some standards were carried out by Dr. Aplin in the Dyson Perrins Laboratory, Oxford, U.K. About 5 µg of the sugar was used in each run.

Results

An experiment was designed to isolate compounds which might be the products of early stages in the synthesis of the glucan. If some component of the soluble enzyme extract was required for chain initiation then soluble short-chain oligosaccharides might be formed under conditions where the proportion of that component to UDP-glucose was high. To investigate this possibility 1 µm-UDP-glucose was incubated with enzyme which had been extracted from seedlings aged 4–5 days. After incubation for 5 min the mixture was boiled, analysed by t.l.c. and the chromatogram sectioned and counted. Fig. 1 shows that about 80% of the UDP-glucose had reacted to form a compound which appeared in the position of laminaribiose (Glcβ1→3Glc). In some experiments using seedlings of different ages a trisaccharide and a tetrasaccharide were also formed.

Chemical analyses

The fastest-moving compound, which we now designate biose, co-chromatographed with laminaribiose in several solvents. However, the radioactive sample was separated from authentic laminaribiose by developing a paper chromatogram in propan-1-ol-ethyl acetate-water (7:1:2, by vol.) for 72 h at room temperature. The radioactive trisaccharide (designated triose) could be separated from laminaritriose by using the same solvent and developing the chromatogram for 100 h. Under these conditions the unknown biose could be distinguished from some of the commonly occurring disaccharides, namely, celllobiose (Glcβ1→4Glc), maltose (Glcα1→4Glc), lactose (Galβ1→4Glc) and trehalose (Glcα1→α1→Glc).

A straight line was obtained by plotting the mobilities of the biose, triose and tetrasaccharide against the number of sugar units they contained; this, according to French & Wild (1952) is a test of whether the compounds have the same linkage.

When the unknown product was hydrolysed with a β-(1→3)-glucanase about 30% of the radioactivity was converted into a compound with the same chromatographic mobility as glucose. Acid hydrolysis of the biose also gave a radioactive product which had the same chromatographic mobility as glucose. It was probable, therefore, that the unknown biose was the β-glucoside of a non-radioactive moiety.

The unknown biose was treated with lead tetracetate under conditions which would oxidize both

Vol. 142
laminaribiose and cellobiose. The unknown compound was also treated with NaBH₄ under conditions which would reduce both laminaribiose and cellobiose. Neither oxidation products nor reduction products could be isolated, suggesting that the biose itself was non-reducing. Acid hydrolysis of the radioactive compounds left after both these reactions gave a product which had the same mobility as glucose. Clearly there was no free reducing group in the glucose moiety, its C-1 carbon atom being involved in the linkage.

Further chemical identification depended on the isolation of microgram quantities of the biose so that the techniques of g.l.c. and mass spectrometry could be used and thus the identification would no longer depend on the presence of ¹⁴C.

The trimethylsilyl derivative of the unknown compound gave a single g.l.c. peak. This was additional evidence for it being a non-reducing compound. The biose derivative was clearly separated by g.l.c. from the derivatives of most of the common disaccharides and could be distinguished from glucosylβ1→3glucitol, prepared by reduction of laminaribiose and also from glucosylβ1→3mannitol, with this method. However, the unknown compound could not be separated from sucrose.

Assuming that the detector-response factor for the unknown compound was similar to those for known disaccharides, a calculation of the peak height gave an estimate of the amount of biose present. The amount of glucose incorporated into the biose could be calculated from the specific radioactivity of the UDP-[¹⁴C]glucose and the radioactivity in the compound. Comparison of these two values suggested that one sugar moiety was not radioactive, i.e. that the UDP-glucose was the source of only one of the sugar units.

Mass spectroscopy of the trimethylsilyl derivatives of the unknown biose, sucrose and laminaribiose was carried out and the patterns of disintegration were similar but each compound gave peaks that were not present in the other.

The largest fragment produced by the unknown compound has a mass of 903. This was unlikely to be a mass ion but could be a mass ion minus a methyl group. In that case the unknown could have the molecular weight of a dihexose.

Kochetkov et al. (1968, 1973) claim that dihexoses break to give certain fragments which are diagnostic of the linkages in those disaccharides. The spectrum from the unknown biose did not contain any of these specific fragments, suggesting that it was not a dihexose but a glycoside with a C₆ aglycone.

Batches of radioactive biose and triose were then isolated and subjected to mild acid hydrolysis. After removal of the acid, trimethylsilyl derivatives were made and the components separated by g.l.c. Each preparation gave three peaks and appeared to contain the same sugar moieties, which were characterized more fully in the triose. A mixture of the trimethylsilylated hydrolysate of the triose with trimethylsilyl glucose showed three peaks, two of which could thus be identified as trimethylsilyl α-glucose and trimethylsilyl β-glucose. A mixture of the trimethylsilyl hydrolysate with trimethylsilyl fructose, however, gave four peaks, showing that fructose was not one of the hydrolysis products.

The third peak in the hydrolysate had a longer retention time than the trimethylsilyl hexoses and was similar to that of trimethylsilyl myo-inositol. When a myoinositol standard was mixed with the hydrolysate, no fourth peak appeared and the increase in height of the slow peak did not appear to be accompanied by an increase in peak width. Other cyclitols can be clearly separated from myo-inositol under similar conditions (Lee & Ballou, 1965) and although it has not been possible to check the retention times of other cyclitols under the precise conditions used in the present work it can be fairly confidently claimed that the third peak was trimethylsilyl myo-inositol. These results are shown in Fig. 2. It can also be seen that the peaks from glucose and inositol are in the proportions expected if the triose contained 1 molecule of inositol and 2 of glucose. It appeared therefore that the biose was the β-glucoside of myo-inositol and that the triose was diglucosylmyo-inositol. It has already been suggested that the biose, triose and tetraose were possibly from the same family of oligosaccharides. The tetraose might therefore be triglucosylinositol.

**Biosynthesis of the glucosides**

The particulate enzyme fraction obtained before digitonin treatment also catalysed the formation of a disaccharide which had the same mobility as that shown in Fig. 1. It seemed therefore that the treatment with digitonin was not necessary for the formation of the compound and there was no evidence that its synthesis was unphysiological.

Experiments were designed to investigate the biosynthesis of the disaccharide glucosylinositol. Dual-labelling experiments were carried out to see whether myo-inositol and glucose were used as a substrate for biose production. Fig. 3 shows a chromatogram obtained after incubation of [³H]inositol and UDP-[¹⁴C]glucose with the enzyme. A single biose peak was formed which contained both ¹⁴C and ³H, providing good evidence for the incorporation of ³H into the biose together with further confirmatory evidence for the chemical identification of the biose as glucosylinositol.

However, only a very small proportion of the [³H]inositol reacted to form biose and the specific radioactivity of the ³H in the aglycone was clearly much decreased. Table 1 shows the results of an
Fig. 2. Separation and analysis by g.l.c. of the trimethylsilylated products after hydrolysis of the unknown trisaccharide

The unknown trisaccharide was hydrolysed with HCl, treated with bistrimethylsilylacetamide and the products were separated by g.l.c. Details of these methods are given in the text. (a) Hydrolysate + myoinositol; (b) hydrolysate + fructose; (c) hydrolysate + glucose; (d) hydrolysate.

experiment to test the effect of the concentrations of UDP-[14C]glucose and [3H]inositol on their incorporation into biose. It is clear that more [3H] is incorporated at higher concentrations but that the added cyclitol is the source of only a minor part of the inositol in the biose. It seemed unlikely therefore that free inositol was the immediate substrate required to make biose.

Moreover, in all these experiments a small proportion of the [3H] accumulated at the origin of the chromatogram. No way has yet been found of increasing the amount of this product so accurate measurements cannot yet be made to assess whether this unknown product was the source of the inositol in the synthesis of the biose.

Plants aged 4½–5 days were used to show the effect of the concentration of UDP-glucose on the synthesis of the biose. The enzyme preparation was used as the source of the inositol, which may have been limiting the reaction at higher concentrations of UDP-glucose. The results are shown in Fig. 4. Under the conditions used in the experiment the glucoside was neither converted into higher oligosaccharides nor broken down to glucose and the biose was the major product, although about 5% of the UDP-glucose was incorporated into an unidentified product.

It is clear from this experiment that the cyclitol moiety must be present in the extract at a concentration of at least 11 µM. This corresponds to a concentration of about 600 pmol/g of fresh bean tissue. The kinetic data were also used to obtain rough estimates of the apparent $K_m$ (1.8 x 10^{-5} M) and $V_{max}$ (2.4 nmol/min per mg) of the reaction forming the biose with respect to the UDP-glucose.

The addition of 1 mM-UDP or 1 mM-UMP to the incubation mixture completely inhibited the synthesis of the glucoside and under similar conditions 1 mM-laminaribiose, -cellobiose or -glucose decreased
the yield of cyclitol glucoside to less than half. In all these experiments the inhibition was accompanied by the accumulation of two unknown compounds containing glucose but there were no callose oligosaccharides or polymer, although 40% or more of the UDP-glucose remained unchanged.

Discussion

Elbein & Chambers (1970) and Clark & Villemez (1972) have noted the production of a disaccharide by a preparation from mung beans which catalysed the formation of β-(1→3)-linkages in glucans. In both cases this compound was presumed to be laminaribiose from its mobility on a paper chromatogram. Since glucosylmyoinositol and laminaribiose have very similar mobilities it is possible that the product noted by these two groups of workers is identical with the disaccharide described here.

Table 1. Effect of concentration of UDP-[14C]glucose and [3H]myoinositol on the proportion of each incorporated into glucosylmyoinositol

<table>
<thead>
<tr>
<th>Substrate concn. (μM)</th>
<th>UDP-[14C]glucose</th>
<th>[3H]myoinositol</th>
<th>14C incorporated into glucoside (pmol)</th>
<th>3H at origin of chromatogram (pmol)</th>
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<tbody>
<tr>
<td>1</td>
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<td>100</td>
<td>1000</td>
<td></td>
<td>2460</td>
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</table>

Fig. 3. Incorporation of 3H from myoinositol into glucosylmyoinositol

A single chromatogram showing the radiation from UDP-[14C]glucose (1 μM) (a) and from [3H]myoinositol (0.1 μM) (b) after incubation for 10 min with enzyme (10 μl) in 90 mM-Tris-HCl, pH 7.5, and 2 mM-MgCl2 in a total volume of 50 μl. The separation by paper chromatography is described in the text. The counting efficiencies were not estimated in this experiment but they differ markedly from all experiments where a single isotope was used.

Fig. 4. Effect of the concentration of UDP-glucose on the yield of glucosylmyoinositol with the enzyme extract as the sole source of the cyclitol

Enzyme was extracted from 4-day-old beans. Incubations were carried out by the method described in the text, with 20 μl of enzyme. Portions of the resulting mixtures were separated either by paper chromatography for 24h by the method described in the text (○) or by chromatography in 0.3 M-sodium formate, pH 3, for 40 min on paper impregnated with polyethyleneimine (●).
A major problem was that of obtaining sufficient product for analysis. This partly arose from the fact that one substrate was being supplied by the enzyme system and appeared to limit the synthesis at higher concentrations of UDP-glucose. Difficulty was also caused in some cases by the enzymic removal of the synthesized disaccharide for the formation of glucan. This reaction varied with the age of the seedling and increased with higher concentrations of UDP-glucose (Kemp & Loughman, 1973). However, considerable progress with the chemical identification has been achieved. All the evidence supports the conclusion that the disaccharide is myo-inositol-β-glucoside and the trisaccharide is diglucosylmyo-inositol.

The occurrence of glucopyranosylmyo-inositol in potatoes has been shown by Urbas (1968) and the synthesis of two forms of glucosylmyo-inositol by Sporobolomyces has been described by Gorin et al. (1965). It is believed that this is the first description of diglucosylmyo-inositol, although the occurrence of digalactosylmyo-inositol (Petek et al., 1966) and dimannosylmyo-inositol (Tanner, 1967) has been noted.

It is clear that the synthesis of glucosylinositol in mung beans differs markedly from that in Sporobolomyces (Gorin et al., 1965) since in the latter the glucose moiety is supplied from either cellobiose or lactose to free inositol. It also appears that the synthesis of glucosylinositol in the mung bean differs from that of galactosylinositol (galactitol) in the pea (Frydman & Neufeld, 1963) where UDP-galactose reacts with free inositol.

It is fairly clear that free myo-inositol does not play an important role in glucosylinositol synthesis since it is not incorporated with equimolar amounts of glucose. If free inositol cannot act as the direct substrate then the source of the cyclitol, available in the enzyme extract, must be formed in small amounts from free myo-inositol. The substance isolated at the origin of the paper chromatograms in the experiments using [3H]inositol appears to be the only compound which conforms to these requirements and it is possible that the compound is an intermediate in the synthesis of the disaccharide rather than in competition with it.

Recent work by Tanner (1967) has shown the formation of mannosylinositol in yeast and in this system the inositol donor has been shown to be a lipid component. It cannot be ruled out that the system in mung beans parallels that in yeast, since the inositol donor in the former, originally isolated in a particulate enzyme fraction, has been brought into solution by treatment with a detergent, digitonin. It has been found that the enzyme extracted in this way does contain lipids (Flowers et al., 1968).

Most of the experiments described in this paper were carried out under conditions where the disaccharide was the final product of the reaction. However, there is evidence that these cyclitol glucosides are involved in the synthesis of a glucan which is probably callose (Kemp & Loughman, 1973).

We are grateful to Professor D. J. Manners for supplying the β-(1→3)-glucanase, to Dr. R. Aplin for help with the mass spectrometry and to Mr. J. A. Farrar for help with g.l.c. We thank Mr. M. G. Beesley and Mr. K. Mutch for their able technical assistance. We gratefully acknowledge financial support from the Agricultural Research Council.

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