ENZYMATIC GLUCOSYLATION OF DITHIOTHREITOL BY URIDINE DIPHOSPHATE D-GLUCOSE AND PARTICULATE PREPARATIONS FROM GERMINATING SEEDS OF PHASEOLUS AUREUS AND PISUM SATIVUM[1]

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SUMMARY

A glucoside has been synthesized in vitro from UDP-D-glucose and dithiothreitol in the presence of particulate preparations from mung bean and pea seeds. This glucoside is produced only when dithiothreitol is included in the incubation mixtures. Acid and enzymatic hydrolysis of the purified glucoside produces D-glucose and dithiothreitol. The glucoside is completely hydrolysed by β-glucosidase but not by mercuric chloride. Therefore, the glucosidic linkage appears to occur through a hydroxyl group of dithiothreitol rather than through a sulfhydryl group. The data suggests that the product is dithiothreityl-2-O-β-D-glucoside.

It is now well established that most of the complex saccharides in nature are synthesized from nucleoside diphosphate sugars (1). However, it has been recently shown that sugar nucleotides do not exclusively donate the glycosyl moiety directly to another sugar for the formation of a complex saccharide. The acceptor may be polyprenol monophosphates, including dolichol monophosphate (2,3) or other non-carbohydrate compounds (4,5,6).

It has been shown in this report that dithiothreitol (DTT) can serve as an acceptor for the glucosyl moiety of UDP-D-glucose in the presence of mung bean or pea seed preparations. Dithiothreitol is being used widely in enzymatic preparations as an antioxidant and enzyme stabilizer (7,8). In the event that this compound is utilized in enzymic incubations involving sugar nucleotide reactions, a considerable error

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may occur in the results.

MATERIALS AND METHODS

UDP-D-glucose-^{14}C, 233 mmoles/mCurie, uniformly labeled in the glucosyl moiety was purchased from Amersham/Searle. Dithiothreitol (Cleland's Reagent), A grade, was purchased from Calbiochem.

Analytical Methods. Paper chromatography (descending) was conducted on Whatman No. 1 chromatography paper with the following solvents: 12:3:5 1-butanol-acetic acid-water (solvent 1), 3:4:4:2.5 ethyl acetate-1-butanol-water-acetic acid (solvent 2), and 7:1:2 1-propanol-water-ethyl acetate (solvent 3). Radioactive chromatograms were analyzed by cutting the paper into one cm strips and counting each in 10 ml of Liquifluor (New England Nuclear) toluene scintillation fluid on a Mark I Nuclear Chicago liquid scintillation counter.

Thin layer chromatograms were conducted on precoated silica gel F-254 plates, 0.5 mM thick, (E. Merck) with the following solvents: 65:25:4 chloroform-methanol-water (solvent A) and 85:15:0.7 chloroform-methanol-water (Solvent B). The plates were activated by heating to 110° C for 30 min immediately prior to use. Radioactive chromatograms were analyzed by scraping off 0.5 cm portions of the silica gel and counting them in liquid scintillation fluid. Nonradioactive materials were located by charring the plates at 100° after spraying with 95% sulfuric acid.

Preparation of Particulate Enzymes. Mung beans (Phaseolus aureus) and peas (Pisum sativum L. var. Alaska) were germinated in the dark at 20° for three or four days. The shoots were removed and the seeds (40 g to 50 g) were decoated and homogenized in a cold mortar with sand in the presence of 50 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 1% bovine serum albumin. The crude homogenates were strained through cheesecloth, and the filtrates were centrifuged at 1000 g for 10 min. The residues were discarded, and the supernatant solutions were centrifuged at 30,000 g for one hour. The precipitates were resuspended in 5 ml of the same buffer.

Enzyme Assay. The incubation mixture consisted of 50 µl of particulate enzyme, 5 µl of UDP-D-glucose-^{14}C (0.3 nmole, 90,000 cpm) in water, and various amounts of DTT in 10 µl of water. After 30 min at 27°, the reaction was stopped by adding 50 µl of glacial acetic acid. The whole mixture was then applied in a 7 cm long streak on Whatman No. 1 paper and irrigated with solvent 1 for 15 hours. Radioactive materials were located as described above.

Preparation of the Glucoside. Particulate enzyme preparation from peas, 50 ml, was incubated with 300 mg (500 µmoles) of unlabeled UDP-D-glucose and 39 mg (250 µmoles) of DTT for three hours at 26°. The reaction was terminated by addition of 40 ml of 2:1 chloroform-methanol followed by mixing and centrifugation. The lower layer was discarded. The aqueous layer containing particulate material was shaken with 40 ml of methanol. After centrifugation the supernatant solution was removed and saved. The pellet was extracted with 60 ml of warm methanol, and this extract was combined with the aqueous extracts. The solution was evaporated in vacuo and mixed with radioactive glucoside, 5 x 10^5 cpm, previously prepared from particulate enzyme and isolated by paper chromatography. The crude glucoside was subjected twice to silica gel column chromatography and eluted with 4:1 chloroform-methanol to give 3.6 x 10^5 cpm, 30 mg of material. The product contained a considerable amount of nonradioactive contaminates, absorbing at 280 mµ, which were removed by repeated (four times) gel filtration on a 1.4 x 89 cm bed of Sephadex LH-20 in ethanol. Pure glucoside, 1.8 x 10^5 cpm, 2.2 mg, was obtained.

RESULTS

Product-Substrate Relation. Particulate enzymes from peas and mung bean seeds were incubated in the presence of DTT and analyzed; the results were presented in a paper chromatogram, as shown in Figure 1. An unidentified radioactive glucoside was also observed with an R_f of 0.68 with

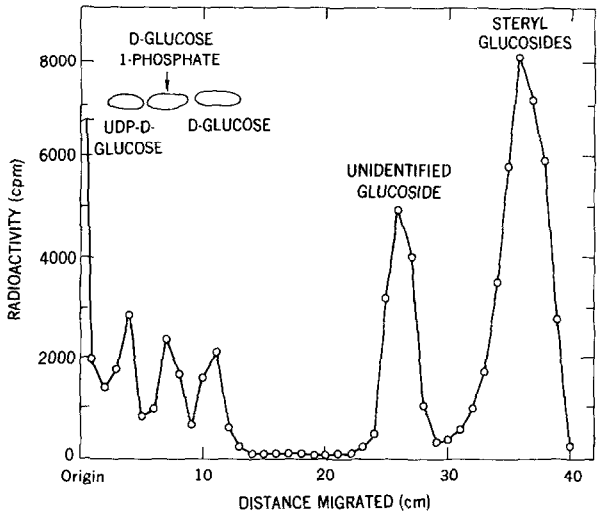

Fig. 1. Paper chromatogram of the incubation of UDP-D-glucose with mung bean particles in the presence of 15.4 mM DTT.

solvent 1. This material could not be extracted from the incubation mixtures with 2:1 chloroform-methanol, but remained in the aqueous phase. Only the extracts from the seeds of the plants contained appreciable glucoside synthetase activity. The activity in shoot extracts from peas was lower, and that from mung bean shoots was negligible.

The glucoside was produced only when DTT was included in the incubation mixtures, and the yield increased with increasing DTT concentrations (Table I). When L-cysteine was substituted for DTT, the glucoside was not formed.

Characterization of the Glucoside. The purified glucoside showed a single spot on all paper chromatograms with silver nitrate/acetone spray (9). Likewise, thin layer chromatograms showed only one spot with sulfuric acid charring. In both cases radioactivity was present in the chemical stains. Formation of stains with silver spray suggested that the unknown compound contained a reducing group. The products from both mung beans

Table I

Effect of Dithiothreitol on the Yield of Glucoside from the Incubation of Uridine Diphosphate Glucose with Particulate Enzymes of Pea and Mung Bean Seeds

Enzyme Source	DTT Concentration	Glucoside Yield
	mmoles/liter	cpm
Pea	0	0
	15.4	1180
	154	4301
Control[1]	154	0
Mung bean	0	0
	7.7	11640
	15.4	15042
	154	20399
Control[1]	154	0

[1]Enzyme particles were boiled two minutes.

and peas were chromatographically identical on paper when treated with solvents 1, 2, and 3 and on silica gel with solvent A, suggesting that the materials were the same compound.

The glucoside was soluble in water, alcohols, and chloroform-methanol mixtures, but was insoluble in chloroform. The material was electrophoretically immobile at pH 7.0. It was neither degraded by alkali under a variety of conditions nor by mercuric chloride. It was completely hydrolyzed by treatment with 2 N HCl at 100° or with β-glucosidase at 37°. Paper chromatographic analysis of the hydrolysate with solvent 1 showed two silver-reducing spots (Figure 2). The first spot corresponded to D-glucose and contained all the radioactivity. Paper chromatographic analysis of the hydrolysate with solvent 3 with samples of D-glucose, D-galactose, and D-mannose showed that the silver-reducing spot and the radioactive spot had the same mobility as D-glucose. The second spot from the hydrolysate corresponded chromatographically to DTT when treated with solvents 1 and 2 (Figure 2) and silica gel with solvent B. DTT reduced silver ion

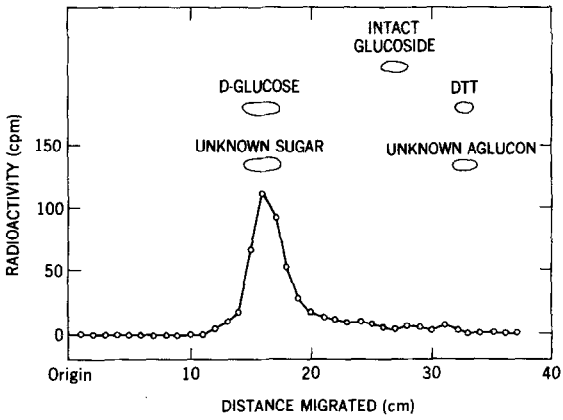

Fig. 2. Paper chromatographic analysis (solvent 2) of the acid hydroslyate from pea glucoside. Part of the chromatogram was analyzed for radioactive components, and the remainder was stained with silver ion along with samples of the intact glucoside, glucose, and DTT.

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as did the unknown aglucon.

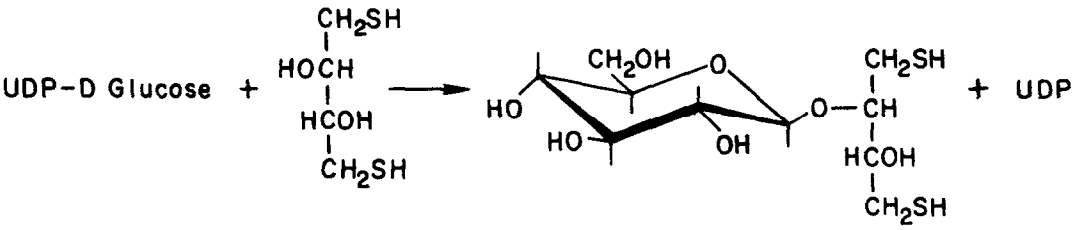
Glucose could be measured in the glucoside by either the phenol sulfuric acid method (10) or with D-glucose oxidase (Glucostat, Worthington Co.). The latter method required hydrolysis and separation of DTT from D-glucose by paper chromatography as it interferred with the analysis. The glucoside contained approximately 50% D-glucose by weight, analytically determined (10), indicating a 1:1 dimer of D-glucose and DTT (theoretical D-glucose content is 57%). In addition, Smith degradation (11) of the intact glucoside gave glycerol as the only non-volatile radioactive product, suggesting a monoglucosyl moiety bonded to DTT.

Attempts to analyze for free sulfhydryl groups by Ellman's Method (12) in the DTT from the hydrolysate were not successful. This was apparently due to partial or complete oxidation of DTT to its cyclic form (7) during isolation by paper chromatography.

DISCUSSION

It has been shown that particulate enzymes from the germinating seeds of mung beans and peas catalyze the formation of a glycoside of DTT. The only sugar found to be in the product was D-glucose. The glucoside was completely hydrolyzed by β-glucosidase but not by mercuric chloride. Since S-glucosides are not affected by β-glucosidase, but are degraded by mercuric chloride (13), the D-glucose is apparently linked to a hydroxyl group of DTT rather than to a sulfhydryl group. The product, thus, is an O-β-D-glucoside of DTT.

The data indicate that the enzymatic formation of DTT-2-O-β-D-glucoside is as follows:



Because the two hydroxyl groups on dithiothreitol are stereochemically equivalent, glucosylation of either hydroxyl forms the same product. The actual formation of UDP in this reaction was not established, but its formation has been demonstrated in the glucosylation of phenolic compounds (5).

The question arises regarding the identity of the natural acceptor for D-glucose in these enzymatic reactions. The enzymes studied here are apparently not UDP-D-glucose:sterol glucosyltransferases shown by Kauss (14) to be active in mung bean shoots. No UDP-D-glucose:DTT glucosyltransferase activity was observed in mung bean shoots in the present study.

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