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β-(1 → 4)-D-GLUCAN SYNTHESIS FROM UDP-(^{14}C)-GLUCOSE BY PARTICULATE AND SOLUBILIZED ENZYME PREPARATIONS FROM *LUPINUS ALBUS*

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Abstract—The enzymatic synthesis of β-(1 → 4)-D-glucosyl groups from UDP-(^{14}C)-D-glucose in water-insoluble glucan(s) by a particulate enzyme fraction obtained from *Lupinus albus* is stimulated by high concentrations of D-glucose. At concentrations of 1 M D-glucose or higher, over 10 per cent of the insoluble glucan fraction assayed as β-(1 → 4)-D-glucoside. The enzymatic activity responsible for this synthesis has been preferentially solubilized and partially purified. This partially purified activity produced an insoluble glucan fraction which was assayed to contain up to 80 per cent β-(1 → 4)-D-glucosyl groups. The product, insoluble in water, was partially soluble in hot dilute alkali. After $NaIO_4$ oxidation, (^{14}C)-D-erythrono-1,4-lactone was isolated from the water-insoluble material. Partial acid hydrolysis, followed by chromatographic separation of the products, revealed several components, one of which was identified as cellobiose.

INTRODUCTION

THE SYNTHESIS of glucans from nucleotide sugars catalyzed by particulate enzyme preparations which have the β-(1 → 4)-D-glucosyl type of linkage has been reported to be demonstrable in a variety of tissues including higher plants,[1-3] bacteria[4] and slime molds.[5] Glaser[6] reported that particulate enzyme fractions from bacteria could use UDP-(^{14}C)-D-glucose as substrate for the synthesis of an insoluble glucan which was characterized as cellulose. Feingold *et al.*[7] reported that UDP-D-glucose produced laminarin when incubated with a particulate enzyme fraction isolated from *Phaseolus aureus*. This activity was solubilized and partially purified and the product characterized as a β-(1 → 3)-D-glucan. Elbein *et al.*[1] reported that, in higher plants, GDP-D-glucose could serve as a glucosyl donor for cellulose synthesis. The particulate enzyme preparation used was capable of cellulose synthesis from GDP-D-glucose but other products were produced in the presence of GDP-D-glucose and GDP-D-mannose.[8] Brummond and Gibbons[9] reported β-(1 → 4)-D-glucoside formation from UDP-(^{14}C)-D-glucose catalyzed by a particulate enzyme fraction prepared from *Lupinus albus*. They assayed for β-(1 → 4)-D-glucosyl entities by an acetolysis method sufficiently harsh to facilitate the removal of interfering substances such as laminarin. They also reported that (^{14}C)-D-erythrono-1,4-lactone could be isolated from the water-insoluble glucan(s) formed by the particulate enzyme preparation after $NaIO_4$ oxidation by the

[1] A. D. ELBEIN, G. A. BARBER and W. Z. HASSID, *J. Am. Chem. Soc.* **86**, 309 (1964).
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[8] G. A. BARBER, A. D. ELBEIN and W. Z. HASSID, *J. Biol. Chem.* **239**, 4056 (1964).
[9] D. O. BRUMMOND and A. P. GIBBONS, *Biochem. Z.* **342**, 308 (1965).

method of Wolfrom *et al.*[10] This showed that some of the (^{14}C)-D-glucose incorporated was in β-(1 → 4)-D-glucosyl linkages internal in the chain and, in this case, represented at least 90 per cent of the water-insoluble β-(1 → 4)-D-glucosyl entities assayed by the acetolysis procedure. Partial acid hydrolysis of the insoluble glucan(s) produced soluble components which could be separated chromatographically and from which cellobiose was isolated. The area known to contain cellotriose was examined for β-(1 → 4)-D-glucosyl groups by the acetolysis procedure and the presence of this group was confirmed. Laminarin was shown not to interfere in the acetolysis assay. Based on these observations, β-(1 → 4)-D-glucosyl linkages, demonstrable cellobiose, and presumptive evidence of cellotriose and larger dextrins formed by partial acid degradation, alkali solubility data, partially independent rates of glucan formation, etc., the authors concluded that the product could contain cellulose. Ordin and Hall[3] confirmed the observation of Brummond and Gibbons[9] that UDP-D-glucose could serve as substrate for β-(1 → 4)-D-glucoside formation with *Avena sativa* and reported the presence of a new entity which was described as a trisaccharide with β-(1 → 4) and β-(1 → 3)-D-glucosyl linkages. Ordin and Hall[11] later concluded that three glucans were produced from UDP-D-glucose in their system, laminarin, cellulose and cereal flour type glucan. Villemez *et al.*[12] reported that UDP-D-glucose would serve as substrate for β-(1 → 4)-D-glucoside synthesis as well as β-(1 → 3)-D-glucoside formation. Franz and Meier[13] reported that UDP-D-glucose would serve as substrate for cellulose synthesis in cotton fibrils removed from developing seeds.

Flowers *et al.*[14] reinvestigated the polymer produced by particulate and solubilized enzyme fractions by *P. aureus* from UDP-D-glucose and could find no evidence for any measurable amount of β-(1 → 4)-D-glucose linkages. They were able to demonstrate only trace amounts of β-(1 → 4)-D-glucosyl groups formed from this substrate by *L. albus* particulate fractions and then only when UDP-D-glucose was present in reaction mixtures at 10^{-6} M concentrations. No evidence was obtained for the synthesis of this product by solubilized enzyme preparations from either of these plants. They did mention in their article that preliminary observations made with *A. sativa* confirmed the findings of Ordin and Hall[11] that a β-(1 → 4)-D-glucose linked polymer was produced from UDP-D-glucose *in vitro*. Batra and Hassid[15] reported that over 90 per cent of the glucan produced from UDP-D-glucose by *P. aureus* enzyme preparations was solublized by β-(1 → 3)-D-glucanase in 24 hr.

This report describes a method for the enhancement of β-(1 → 4)-D-glucoside synthesis by particulate enzyme fractions isolated from *L. albus* and for the solubilizationof a portion of that activity. Based on the acetolysis assay method, the portion of β-(1 → 4)-D-glucosyl linkages synthesized by the solubilized enzyme fraction under the assay conditions employed ranged from 35–80 per cent. The solubilized enzyme could still produce non-β-(1 → 4)-D-glucans but whether or not two or three glucans were formed awaits further investigation.

RESULTS

Particulate enzyme fractions prepared from *Lupinus albus* hypocotyls in the manner described in the Experimental section were capable of incorporating the glucosyl entity of

[10] M. L. WOLFROM, J. M. WEBBER and F. SHAFIZADEH, *J. Am. Chem. Soc.* **81**, 1217 (1959).
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[13] G. FRANZ and H. MEIER, *Phytochem.* **8**, 579 (1969).
[14] H. M. FLOWERS, K. K. BATRA, J. KEMP and W. Z. HASSID, *Plant Physiol.* **43**, 1703 (1968).
[15] K. K. BATRA and W. Z. HASSID, *Plant Physiol.* **44**, 755 (1969).

the UDP-(14C)-D-glucose into β-(1 → 4)-D-glucoside linkages of the water-insoluble glucan(s) produced, as illustrated in Fig. 1.

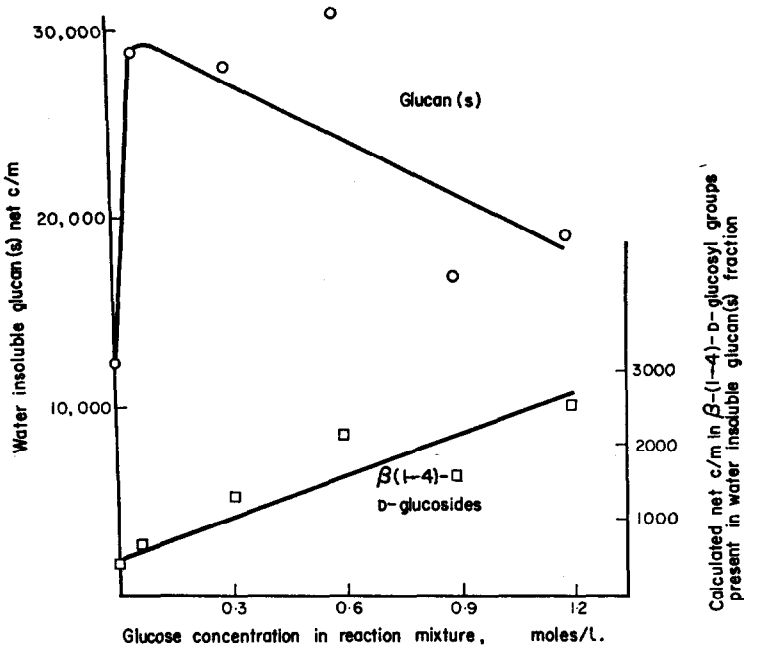

FIG. 1. INCORPORATION OF UDP-(14C)-D-GLUCOSE INTO WATER-INSOLUBLE GLUCAN(S) AND INTO β-(1 → 4)-D-GLUCOSYL LINKAGES IN THIS GLUCAN FRACTION AS A FUNCTION OF GLUCOSE CONCENTRATION IN THE REACTION MIXTURE.

The reaction mixture for this experiment (No. 31) contained in a volume of 0·5 ml the following ingredients: 0·1 M Tris, pH 8·0, 2×10^{-2} M $MgCl_2$, 10^{-2} M cysteine, 3×10^{-3} M UDP-D-glucose, UDP-(14C)-D-glucose 80,000 cpm, 0·2 ml of particulate enzyme, and D-glucose at the concentrations indicated. The reaction was incubated for 1 hr at 35° and terminated by the addition of 10 mg of powdered cellulose, 4·5 mg of insoluble dextrins prepared by the method of Wolfrom and Thompson[16] and 1 ml of N HCl followed by heating on a boiling water bath for 5 min. Insoluble glucans were removed by centrifugation, washed three times with 2-ml quantities of water and twice with 5 ml each of methanol. Samples were counted in a Beckman CPM-100 liquid scintillation spectrometer in heterogeneous suspension. After counting, the dextrins were reisolated, dried and acetolysed.[9] Cellobiose octaacetate was recrystallized at least three times and an average of the cpm/mg used to quantitate β-(1 → 4)-D-glucosyl linkages.

TABLE 1. EFFECT OF STORAGE OF THE SOLUBILIZED ENZYME AT 0° ON THE GLUCAN SYNTHESIZED

Enzyme fraction	Net cpm in water-insoluble glucan fraction	Calculated net cpm in β-(1 → 4)-D-glucosyl groups
Freshly prepared 50–90 enzyme fraction	1674	620
50–90 enzyme fraction after storage at 0° for 14 hr	610	455

The reaction conditions were the same as those presented in the legend for Fig. 1 except that 0·6 M D-glucose was included in each reaction mixture and 0·20 ml of the 50–90 enzyme fraction (experiment No. 44) was used.

[16] M. L. WOLFROM and A. THOMPSON, *Methods in Carbohydrate Chemistry* (Edited by ROY L. WHISTLER), Vol. III, p. 143, Academic Press, New York and London (1963).

The enzymatic activity solubilized from the particulate fraction was also capable of synthesis of the β-(1 → 4)-D-glucoside entities in the presence of D-glucose (Table 1). The effect of storage of the solubilized enzyme at 0° is also included. Feingold *et al.*[7] reported that the solubilized activity responsible for laminarin synthesis usually lost its activity after storage at 0° for several hr.

In another experiment, No. 46, a solubilized enzyme fraction was incubated with UDP-(^{14}C)-D-glucose containing 10^6 cpm and at a concentration of 10^{-3} M. This UDP-(^{14}C)-D-glucose preparation was purchased from International Chemical and Nuclear Corporation and had a specific activity of 130 mc/mmole. The water-insoluble glucans were isolated, counted, reisolated and assayed by different procedures. In experiment 46a, the glucan fraction was acetolyzed. In experiment 46b, the glucan fraction was heated with 0·25 N NaOH in a boiling water bath for 5 min and the residual radioactivity in the insoluble residue was determined. This fraction, after reisolation, was acetolyzed. In experiment 46c, the glucan fraction was treated with $NaIO_4$ for 72 hr by the procedure of Wolfrom *et al.*[10] as previously modified.[9] The results of these assays are presented in Table 2.

TABLE 2. PARTIAL CHARACTERIZATION OF THE GLUCAN(S) PRODUCED BY SOLUBILIZED ENZYME FRACTIONS

Sample No.	Net cpm in water-insoluble glucan(s)	Net cpm insoluble in hot 0·25 N NaOH	Calculated net cpm in β-(1 → 4)-D-glucosyl groups	Calculated net cpm in β-(1 → 4)-D-glucosyl groups in water-insoluble oxy-cellulose fraction
46a	14,800	—	6,800	—
46b	18,600	1,256	990	—
46c	23,300	—	—	690

The reaction mixture contained in a volume of 0·50 ml, 0·1 M Tris-HCl, pH 8·0, 2×10^{-2} M $MgCl_2$, 10^{-2} M cysteine, 0·6 M D-glucose, 10^{-3} M UDP-D-glucose, 10^6 cpm UDP-(^{14}C)-D-glucose and 0·30 ml of the 40–90 solublized enzyme (experiment No. 46). The reaction mixtures were incubated for 1 hr at 35° and terminated as described in the legend for Fig. 1. The calculated net cpm for the β-(1 → 4)-D-glucosyl groups present internal in the glucan(s) was based on two recrystallizations of the D-erythrono-1,4-lactone. The respective radioactivity of these samples was 0·45 and 0·47 cpm/mg and radioactivity measurements were made in 33% ethanol, 67% toluene. The calculated value, $0{\cdot}46 \times \frac{3}{2} \times 1000 = 690$ cpm, represents that fraction of the glucosyl groups internal in the glucan chain which were insoluble in water. Previous experiments[9] showed that about 60% of the powdered cellulose used remained insoluble after such treatment, about 8500 cpm were determined to be present in the soluble phase after $NaIO_4$ oxidation. Any laminarin present in this fraction should not have been solubilized by this procedure.

Several glucan(s) preparations were isolated in which the solubilized enzyme fractions were used. Attempts were made to partially degrade the polymer(s) and chromatographically separate the different dextrin fractions. Initial experiments of this type produced soluble material which remained at the origin of chromatographs or, if rehydrolyzed, produced predominantly glucose. Possible interfering substances present in these glucan fractions were the added albumen and other proteins. In order to minimize their effect, two more glucan fractions were prepared as described in the legend to Table 2, with the enzyme fraction of experiment No. 48. The activity of this enzyme fraction was significantly less than that of No. 46 and 1033 and 682 cpm were obtained in these glucan(s) fractions. The 1033 cpm fraction was hydrolyzed for 30 min at 25° with half conc.–half fuming HCl[14] and the soluble portion chromatographed. The insoluble material remaining was combined with the 682 cpm sample and hydrolyzed in the same manner except for 1 hr. The soluble dextrins

produced were chromatographically separated[6] after removal of the HCl. The results of the chromatographic separation and radioactivity determination are presented in Fig. 2 for the 1 hr hydrolysate. Sample No. 11 from the chromatograph was removed from the counting vial, washed with cyclohexane and the water-soluble materials eluted into a beaker containing 100 mg of cellobiose. Cellobiose octaacetate was prepared, recrystallized and radioactivity determined after each recrystallization. These results are presented in Table 3.

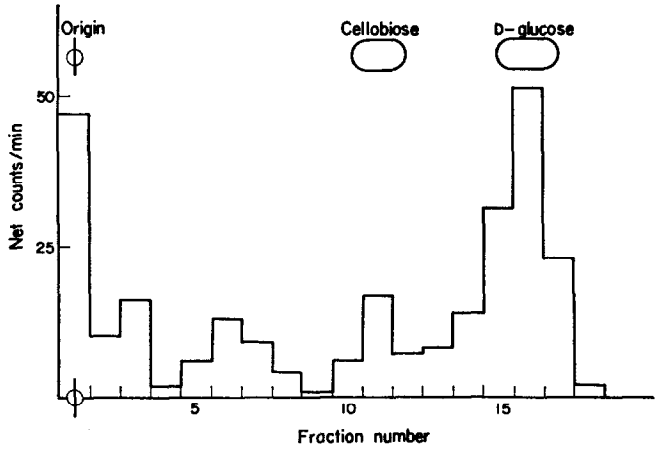

FIG. 2. RADIOACTIVITY DETECTED ON 2-cm SECTIONS OF A CHROMATOGRAPH USED TO SEPARATE SOLUBLE DEXTRINS PRODUCED BY PARTIAL ACID HYDROLYSIS OF GLUCAN FRACTIONS SYNTHESIZED FROM UDP-(^{14}C)-D-GLUCOSE BY A SOLUBILIZED ENZYME FRACTION ISOLATED FROM *Lupinus albus*.

Products of the partial acid hydrolysis (1 hr) of water-insoluble glucan(s) formed from UDP-(^{14}C)-D-glucose after chromatography on Whatman 3 MM paper for 26 hr as described by Glaser.[6] See text for method of glucan preparation, isolation and partial hydrolysis. Radioactivity was determined on 2-cm sections placed on the bottom of counting vials.

TABLE 3. CHARACTERIZATION OF THE RADIOACTIVE MATERIAL PRESENT ON THE CHROMATOGRAPH (FRACTION NO. 11) AS CONTAINING (^{14}C)-CELLOBIOSE

Number of recrystallizations	mg of cellobiose octaacetate	Net cpm
1	141·5	15
2	114·6	13
3	98·8	13

The water-soluble eluate of fraction No 11 was mixed with 100 mg of cellobiose, the sample was dried and cellobiose octaacetate was prepared with 1·5 ml of a mixture of acetic anhydride–H_2SO_4 (1·0–0·09 v/v) at room temp. Cellobiose octaacetate was recrystallized as previously described.[9]

DISCUSSION

These data present additional evidence that β-(1 → 4)-D-glucosides are formed from UDP-(^{14}C)-D-glucose by particulate and solubilized enzyme fractions isolated from *Lupinus albus*. The extent of incorporation was very low in the absence of added D-glucose by particulate fractions but increased to over 10 per cent at concentrations of D-glucose over 1 M. The low incorporation without added D-glucose may explain the failure of Flowers *et al.*[14] to detect β-(1 → 4)-D-glucosyl groups in glucan preparations synthesized from this substrate by *L.*

albus particulate fractions. Solubilized enzyme fractions prepared in the absence of albumin, isolated in a similar manner, did not produce significant quantities of water-insoluble radioactivity. In one experiment with a particulate enzyme fraction, in which 0·2 M sucrose was used in place of D-glucose, β-(1 → 4)-D-glucoside synthesis was inhibited about 50 per cent while total glucan formation was increased.

It is anticipated that the characterization of the radioactive materials which moved at lower R_f's than cellobiose will provide additional information regarding the nature of the glucan(s) produced. Additional purification of the enzymatic activity may also aid in product identification.

EXPERIMENTAL

Lupinus albus seeds were grown as previously described and the acetolysis assay procedure used previously was employed to quantitate β-(1 → 4)-D-glucosyl groups.[9] The particulate enzyme fraction was prepared by a slightly modified procedure. Initial homogenates were prepared at room temperature in a prechilled mortar in 1% $KHCO_3$; all subsequent fractionation was done on an ice bath. Homogenates were centrifuged at 9000 rpm in the Sorvall RCB-2 centrifuge with the S 34 rotor. The insoluble material was suspended in 0·1 M Tris-HCl at pH 8·0 and resedimented. The original supernatant and combined wash was centrifuged at 50,000 rpm in a Spinco 50·1 rotor for 70 min. The pellet was suspended in 0·1 M Tris-HCl at pH 8·0. In experiment No. 31, 96·4 g of hypocotyl tissue was used and the final volume of particulate enzyme was 8·3 ml. In experiments Nos. 44, 46, 48 reported here, bovine serum albumin (1%), Sigma Chemical Company, was added along with the $KHCO_3$ and all 0·1 M Tris-HCl, pH 8·0 buffers employed contained 10 mg/ml albumin. As a result, protein was not determined on these fractions and these values are not reported here.

The procedure used for the solubilization of the UDP-D-glucosyl transferase activity was a modification of the method of Feingold *et al.*[7] in which the final particulate fraction in 4·0 ml containing 0·1 M Tris-HCl at pH 8·0 and 1% BSA was mixed with 8·0 ml of 1% digitonin and slowly homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle in an ice bath for 10 min. The homogenate was centrifuged at 50,000 rpm for 70 min in the Spinco Ti-50 rotor and the soluble portion decanted and fractionated with an $(NH_4)_2SO_4$ solution saturated at pH 7·0. The resultant fractions were collected by centrifugation at 20,000 rpm (Sorvall) and the collected fractions were dissolved in 0·1 M Tris at pH 8·0 and dialyzed for 2 hr against 2 l. of 0·01 M Tris, 0·001 M EDTA and 0·001 M cysteine at pH 8·0

In experiment No. 44, 162 g of hypocotyl tissue was used and the solubilized enzyme used was that fraction which precipitated between 50 and 90% of saturation with $(NH_4)_2SO_4$. The final volume obtained in this fraction was 5·0 ml. In experiment No. 46, 182 g of hypocotyl tissue was used as starting material and the solubilized enzyme fraction used was that which precipitated between 40 and 90% of saturation. The final volume of this fraction was 3·5 ml. In experiment No. 48, 158 g of hypocotyl tissue was used as starting material and the solubilized enzyme fraction used was that which precipitated between 40 and 90% of saturation. The final volume of this fraction was 4·1 ml.

Radioactivity was determined on samples placed in 15 ml. of toluene containing 5·0 g of 2,5-diphenyloxazole and 0·60 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl-)-benzene per liter and counted in heterogenous suspension. Samples of (^{14}C)-cellobiose octaacetate, when counted in this manner gave counts linearly proportional to the mass present for samples up to 1000 mg. Samples of (^{14}C)-D-erythrono-1,4-lactone were counted in a similar manner except that the solvent was 33% ethanol, 67% toluene and the lactone was in solution. Two samples of UDP-(^{14}C)-D-glucose were used, one was that previously described[9] and the other was purchased from the International Chemical and Nuclear Corporation. UDP-D-glucose was purchased from the Sigma Chemical Company and counting vials and phosphors were purchased from the Packard Instrument Company.

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