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

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Key Word Index—*Lupinus albus*; Leguminosae; lupin; cellulose; β-(1 → 4)-D-glucan; callose; β-(1 → 3)-D-glucan; uridine diphosphate-D-glucose.

Abstract—The particulate enzyme responsible for the synthesis of β-(1 → 4)-D-glucans from UDP-[^{14}C]-D-glucose has been solubilized and some of its properties have been characterized. Mg^{2+} markedly enhanced synthesis of β-(1 → 4)-D-glucans and inhibited synthesis of β-(1 → 3)-D-glucans. The optimal pH for synthesis of β-(1 → 4)-D-glucans is near pH 8 and the synthesis was enhanced in these preparations by D-glucose, methyl-β-D-glucopyranoside and cellobiose.

INTRODUCTION

AN APPRAISAL of the status of polysaccharide biosynthesis in higher plants including cellulose has recently been made by Nikaido and Hassid[1] and a similar evaluation of cellulose biosynthesis has been made by Shafizadeh and McGinnis.[2] Since these reviews were completed, Tsai and Hassid[3] have reported that they were able to separate the activities responsible for the synthesis of β-(1 → 3)-and β-(1 → 4)-D-glucans from UDP-D-glucose after solubilization. The source of their enzymic activity was *Avena sativa* and solubilization was achieved by treatment with digitonin. More recently Clark and Villemez[4] reported the results of a re-evaluation of β-(1 → 4)-D-glucan formation from UDP-α-D-glucose catalyzed by a particulate enzyme isolated from *Phaseolus aureus*. They made an evaluation of several factors which could be involved in β-(1 → 3) and β-(1 → 4)-D-glucan formation including temperature of growth of the seedlings and enzyme isolation procedures.

Stafford and Brummond[5] have reported that the enzymic activity responsible for β-(1 → 4)-D-glucan synthesis can be solubilized from particulate enzyme preparations obtained from *Lupinus albus* and this report describes some of the properties of the solubilized enzyme.

RESULTS

As shown in Fig. 1, Mg^{2+} enhanced synthesis of β-(1 → 4)-D-glucan from UDP-(^{14}C)-D-glucose as previously reported by Brummond and Gibbons[6] for the particulate enzyme.

[1] NIKAIDO, H. and HASSID, W. Z. (1971) *Advances in Carbohydrate Chemistry and Biochemistry* (TIPSON, R. S., ed.), Vol. 26, pp. 351–483, Academic Press, New York.
[2] SHAFIZADEH, F. and MCGINNIS, G. D. (1971) *Advances in Carbohydrate Chemistry and Biochemistry* (TIPSON, R. S., ed.), Vol. 26, pp. 297–349, Academic Press, New York.
[3] TSAI, C. M. and HASSID, W. Z. (1971) *Plant Physiol.* **47**, 740.
[4] CLARK, A. F. and VILLEMEZ, C. L. (1972) *Plant Physiol.* **50**, 371.
[5] STAFFORD, L. E. and BRUMMOND, D. O. (1970) *Phytochemistry* **9**, 253.
[6] BRUMMOND, D. O. and GIBBONS, A. P. (1965) *Biochem. Z.* **342**, 308.

Under the reaction conditions employed, increasing concentrations of Mg^{2+} up to 10 mM stimulated β-(1 → 4)-D-glucan synthesis while callose synthesis was inhibited about 50%. Higher concentrations of Mg^{2+} inhibited both synthetic reactions. All experiments reported here were run at 20 mM Mg^{2+} unless otherwise indicated, a concentration which showed nearly the optimal ratio of β-(1 → 4)-D-glucan to total glucan synthesis.

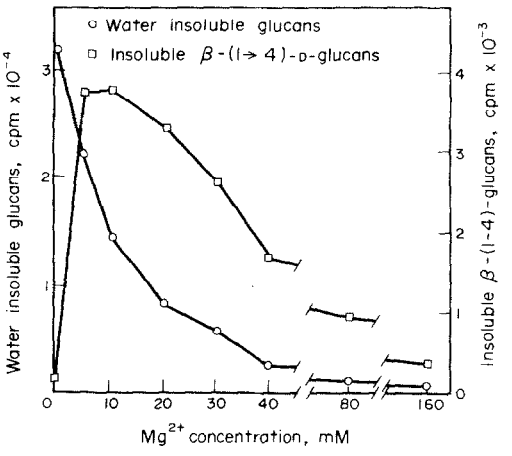

FIG. 1. THE REACTION MIXTURES (0·50 ml) CONTAINED THE FOLLOWING: Tris-HCl pH 8·0, 100 mM, dithiothreitol, 10 mM, D-glucose, 900 mM, UDP-[^{14}C]-D-glucose containing 160 000 CPM, 3 mM, 0·2 ml of soluble enzyme eluted from Sephadex G25 (Exp. I) and $MgCl_2$ concentrations as indicated. The reaction mixtures were incubated for 1 hr at 35°.

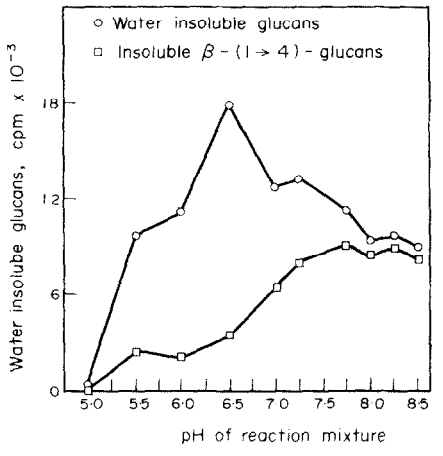

FIG. 2. REACTION MIXTURES (0·50 ml) CONTAINED THE FOLLOWING: Dithiothreitol, 10 mM, $MgCl_2$, 20 mM, D-glucose, 1200 mM, UDP-[^{14}C]-D-glucose containing 160 000 CPM, 3 mM, and 0·2 ml of enzyme eluted from Sephadex G25 (Exp. II) Acetate buffers at pH 5·0 and 5·5, 100 mM, Phosphate buffers at pH 6·0 and 6·5, 100 mM, and the remaining Tris-HCl buffers, 100 mM. Reaction mixtures were incubated for 1 hr at 35°.

The formation of total water insoluble glucan and β-(1 → 4)-D-glucan was proportional to the concentration of enzyme present in reaction mixtures incubated for one hour and proportional to time at a constant enzyme concentration. There was deviation from linearity under conditions in which very small quantities of product were formed and some scatter was observed in the amount of insoluble glucan determined in reaction mixtures incubated for periods of time greater than 1 hr. This scatter is largely due to problems encountered in the isolation of the insoluble glucan fraction.

D-Glucose (*ca* 7-fold at 1 M), methyl-β-D-glucoside (*ca* 11-fold at 1 M) and cellobiose (*ca* 3-fold at 0·2 M) all stimulate β-(1 → 4)-D-glucan formation. This stimulation has been noted at all stages of purification both for the particulate enzyme and the solubilized enzyme.

The optimal pH for the formation of water insoluble glucans in the presence of 20 mM Mg^{2+} is near 6·5 and that the pH optimal for formation of insoluble β-(1 → 4)-D-glucan covers a region from pH 7·2 to 8·5. (Fig. 2).

The radioactivities in various fractions determined on PCs, after partial acid hydrolysis of insoluble glucans after incubation with UDP-^{14}C-glucose is shown in Figs. 3 and 4. The glucans were isolated from reaction mixtures incubated for 1 hr followed by the addition of 10 mg of powdered cellulose. After mixing, the insoluble material was removed by centrifugation and washed several times with water. In these experiments the protein was not denatured. The isolated glucans were subjected to partial acid hydrolysis

and chromatography as previously described.[5] Figure 3 shows the pattern obtained from the insoluble glucan fraction formed in the absence of added Mg^{2+} and is similar to patterns reported by Flowers *et al.*[7] for a β-(1 → 3)-D-glucan originally described by Feingold *et al.*[8] Figure 4 shows the pattern obtained from he insoluble glucan fraction formed in the presence of 20 mM Mg^{2+} and corresponds to the pattern reported by Glaser[9] for cellulose.

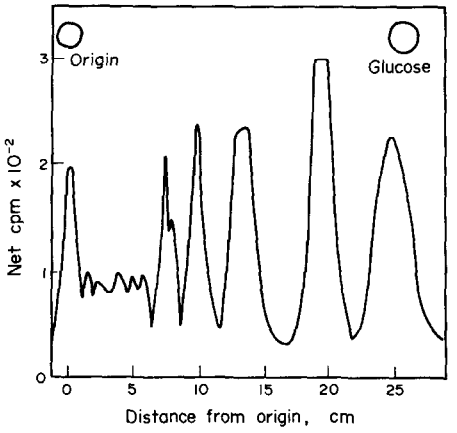

FIG. 3. THE DESCENDING PC WAS RUN ON WHATMAN No. 1 PAPER IN A BuOH–PYRIDINE–H_2O (6:4:2) SOLVENT SYSTEM AND THE RADIOACTIVITY ON THE PAPER WAS DETERMINED WITH A PACKARD 7200 RADIOCHROMATOGRAM SCANNER.
The reaction mixture (0·50 ml) contained the following: Tris-HCl pH 8·0, 100 mM, dithiothreitol, 10 mM, D-glucose 900 mM, UDP-[^{14}C]-D-glucose containing 160 000 CPM, 3 mM and 0·2 ml of enzyme eluted from Sephadex G25 (Exp. III). The reaction mixture was incubated for 1 hr at 35°.

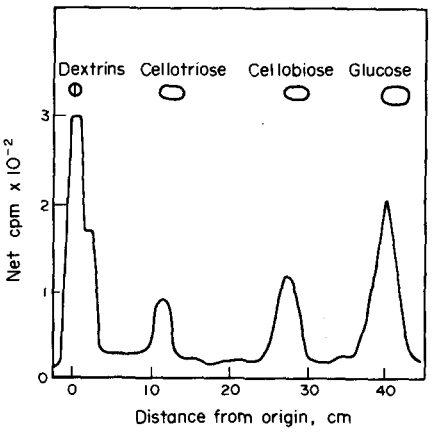

FIG. 4. THE DESCENDING PC WAS RUN ON WHATMAN No. 1 PAPER IN A BuOH–PYRIDINE–H_2O (6:4:2) SOLVENT SYSTEM AND THE RADIOACTIVITY ON THE PAPER WAS DETERMINED WITH A PACKARD 7200 RADIOCHROMATOGRAM SCANNER.
The reaction mixture (0·50 ml) contained the following: Tris-HCl pH 8·0, 100 mM, dithiothreitol, 10 mM, $MgCl_2$, 20 mM, D-glucose, 900 mM, UDP-[^{14}C]-D-glucose containing 1 600 000 CPM and 0·2 ml of enzyme eluted from Sephadex G 25 (Exp. II). The reaction mixture was incubated for 1 hr at 35°.

DISCUSSION

The process of solubilization of the particulate enzyme isolated from lupin hypocotyl tissue reported here was accomplished by treatment of the insoluble fraction with 1% digitonin—0·5 M NaCl–0·5% bovine serum albumin. Other concentrations of digitonin (0·67–1·33%) also produced soluble fractions which had activity. In experiments in which NaCl was not included in the solubilizing medium, only very small quantities of activity were detected in the solubilized fraction. Concentrations of NaCl up to 2·67 M produced active soluble fractions. The presence of albumin significantly increased the amount of enzyme detected in the soluble fraction. The concentration of the particulate fraction most reproducible in providing active soluble preparations was that described in the methods section. More concentrated fractions frequently resulted in soluble preparations containing very small quantities of synthetic activity.

The enzyme preparations solubilized by this procedure and used in these experiments showed some variations in activities. The insoluble glucan fraction produced by some

[7] FLOWERS, H. M., BATRA, K. K., KEMP, JENNIFER and HASSID, W. Z. (1968) *Plant Physiol.* **43,** 1703.
[8] FEINGOLD, D. S., NEUFELD, E. F. and HASSID, W. Z. (1958) *J. Biol. Chem.* **233,** 783.
[9] GLASER, L. (1958) *J. Biol. Chem.* **232,** 627.

preparations under assay conditions was over 95% β-(1 → 4)-D-glucan while other preparations formed less than 50% of this product. The reason for this variation is not known.

The synthesis of D-glucans was proportional to enzyme concentration and to time at a constant enzyme concentration. Deviation was observed under conditions in which only small quantities of glucans were formed, however, and some scatter from linearity was observed in reaction mixtures incubated for longer periods of time. One explanation for the deviation at low enzyme concentrations or in short term incubation experiments is that a small but constant quantity of glucan passed through the filter and was lost. This mechanical loss of some of the glucans formed would give a greater deviation from linearity than a comparable loss in experiments in which greater quantities of product were formed. Although β-D-glucosidase activity was detected in soluble enzyme preparations, this probably would not account for this effect. The scatter of points in reaction mixtures incubated for periods of time greater than 1 hr also appears to be mechanical. It was observed that reaction mixtures increased in turbidity with time of incubation. This turbid material markedly retarded the rate of filtration especially in experiments incubated for these longer time periods. Filtration was accomplished only with one or more changes of filter papers and carried the potential for some mechanical loss. Centrifugation techniques were tried but were no more successful as this material was not readily sedimented. Reaction mixtures incubated for 1 hr or less usually could be processed in a reasonable length of time through a single filter and hence the chance for mechanical loss was decreased.

The amount of glucan formed in the presence of glucose, methyl glucoside and cellobiose was stimulated several-fold. Brummond and Gibbons[6] reported previously that D-glucose and cellobiose stimulated β-(1 → 4)-D-glucan synthesis while maltose inhibited. Unpublished observations of Stafford and Brummond showed that sucrose (0·2 M) and ethylene glycol (3·7 M) did not increase incorporation with particulate enzyme preparations over the rate in their absence. Glycerol (2·7 M) did double the amount incorporated while D-glucose (1·2 M) increased synthesis over 7-fold. In one experiment in which methyl-α-D-glucoside (1·26 M) was used with the soluble enzyme preparation, β-(1 → 4)-D-Glucan synthesis was significantly less than that in the presence of D-glucose or methyl-β-D-glucoside. Phenyl-β-D-glucoside was tested in another experiment with a soluble preparation at 0·2 M concentration and incorporation was about half that observed in the presence of D-glucose or methyl-β-D-glucoside. These latter experiments may not be conclusive but indicate that some specificity may exist as to the nature of this activation rather than just ionic strength or osmotic concentration.

Two probable explanations for these observations are possible, the first is that the added material is acting as an acceptor for D-glucose and that subsequent transfer reactions add D-glucose serially onto existing chains. Feingold *et al.*[8] have reported a similar activation by D-glucose and some of its derivatives on β-(1 → 3)-D-glucan synthesis but concluded that the "activator" was not incorporated into the glucan formed. The second possible explanation would involve an allostearic type of activation with a very high dissociation constant, at least for the compounds tested. The latter hypothesis would partially explain the methyl-β-D-glucoside effect in which the upper portion of the curve would indicate saturation while with cellobiose and D-glucose saturation was not accomplished. One would expect, however, that these effects would be sigmoidal rather than linear if this were the reason. Additional experimentation will obviously be necessary to explain this effect.

To retard denaturation of the enzyme responsible for synthesis of β-(1 → 4)-D-glucans, enzyme preparations were made in the presence of bovine serum albumin. The presence of this added protein makes quantitative expression of the purification difficult consequently these data are not included.

EXPERIMENTAL

Lupin seeds (*Lupinus albus*) were washed 3 × H_2O and allowed to soak overnight in H_2O. The swollen seeds were planted in sand and allowed to grow in the dark at 25–29°. The plants were kept moist with H_2O. After 6–10 days, the elongated hypocotyls were excised, washed (H_2O), blotted dry, weighed, and chilled at 0°. The excised hypocotyls, 15–22 cm long, were placed in a pre-chilled mortar with $KHCO_3$ (1 g/100 g). The mixture was ground with sand and the brei (pH *ca* 7·5) was strained through cheesecloth, and centrifuged at 9000 rpm (Sorvall RC-2B with the SS-34 rotor, maximum centrifugal force at the tip-9770 *g*) for 15 min. The precipitated pellet was suspended in 30–40 ml 100 mM Tris-HCl pH 8·0 buffer and recentrifuged at 9000 rpm for 15 min. The supernatant was combined with the original supernatant and centrifuged at 50 000 rpm (Beckman Spinco Model L with a 50·1 aluminum rotor, maximum centrifugal force at the tip-275 000 *g*) for 70 min. The precipitated pellet was suspended in 100 mM Tris-HCl pH 8·0 buffer and recentrifuged at 50 000 rpm (Beckman Spinco Model L with a Ti-50 rotor, maximum centrifugal force at the tip-226 400 *g*) for 70 min. The pellet resulting from this centrifugation was used as the particulate enzyme.

Solubilization was accomplished by suspending the particulate enzyme in a vol. in ml equivalent to 0·05 the wt in g of the excised lupin hypocotyl tissue originally used. The suspension contained 1% bovine serum albumin (BSA)-100 mM Tris-HCl pH 8·0 buffer. An equal volume of 2·0% digitonin-1 M NaCl solution was added and this suspension was mechanically stirred at 0° for 15 min. The mixture was centrifuged at 50 000 rpm (Ti-50 rotor-226 400 *g*) for 70 min. The supernatant soln was fractionated with a satd $(NH_4)_2SO_4$ soln which had been adjusted to pH 8·0 with NH_4OH containing 10 mM EDTA. Enough satd $(NH_4)_2SO_4$ soln was added to the digitonin supernatant soln to make a 40% $(NH_4)_2SO_4$ satd soln. This was centrifuged at 20 000 rpm (Sorvall SS-34 rotor-48 200 *g*) for 15 min. The precipitate was discarded and the supernatant soln was adjusted to 90% $(NH_4)_2SO_4$ and centrifuged at 20 000 rpm (Sorvall SS-34 rotor) for 15 min. The precipitate was suspended in 100 mM Tris-HCl pH 8·0 buffer-1% BSA and passed over a Sephadex G25 column. The first UV absorbing peak was collected and used as the soluble enzyme in these experiments.

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