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Characterization of GDP-Fucose

POLYSACCHARIDE FUCOSYL TRANSFERASE IN CORN ROOTS (*ZEA MAYS* L.)[1]

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ABSTRACT

The peripheral root cap cells of corn (cv. SX-17A) secrete a fucose-rich, high molecular weight, polysaccharide slime via the dictyosome pathway. To study the synthesis of this polysaccharide, a technique for isolating and assaying GDP-fucose:polysaccharide fucosyl transferase activity was developed. Corn roots were excised from germinated seeds, incubated 12 hours at 10 C in water, and ground in 100 millimolar Tris or Pipes buffer (pH 7.0) with or without 0.5 molar sucrose. The membrane-bound enzyme was solubilized by sonication in the presence of 2 molar urea and 1.5% (v/v) Triton X-100 and assayed by monitoring the incorporation of GDP-[^{14}C]fucose into endogenous acceptors. Optimum enzyme activity is expressed at pH 7.0 and 30 C in the presence of 0.8% (v/v) Triton X-100. The enzyme does not require divalent cations for activation and is inhibited by concentrations of $MnCl_2$ or $MgCl_2$ greater than 1 millimolar. Corn root cap slime will serve as an exogenous acceptor for the enzyme if it is first hydrolyzed in 5 millimolar trifluoroacetic acid for 60 minutes at 18 pounds per square inch, 121 C. This procedure prepares the acceptor by removing terminal fucose residues from the slime molecule. Kinetics of fucose release during hydrolysis of native slime and *in vitro* synthesized product suggests that the two polymers possess similar linkages to fucose.

The root cap cells of corn synthesize and secrete a polysaccharide slime rich in fucose (11). The pathway of slime secretion has been determined by high resolution autoradiography (14, 16) and by localization of fucose-containing polymers in subcellular fractions of corn roots incubated in [^{14}C]glucose (3–5) or [^{3}H]fucose (17). These studies indicate that the dictyosomes are involved in the secretion and probably the synthesis of corn root slime.

To localize further the site of biosynthesis of the fucose-containing slime, we developed an assay for fucosyl transferase activity for use on organelle fractions derived from homogenized corn roots. This paper describes the isolation and assay of GDP-fucose: polysaccharide fucosyl transferase activity and discusses the characterization of the enzyme and the *in vitro* product.

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays* cv. SX-17A, kindly provided by Cargill, Inc., Minneapolis, Minn.) were rinsed in four changes of tap water to remove partially the Captan and Malathion coating. They were imbibed at 23 C in continuously aerated tap water for 36 h and then germinated for 45 h in the dark at 23 C between two layers of water-saturated Dacron (E. I. du Pont de Nemours and Company) batting in glass trays. At the end of this treatment the emergent roots were 2 to 4 cm long.

Enzyme Preparation. Root tips, 1 cm long, were excised from germinated corn seeds and incubated in distilled H_2O for 12 h at 10 C. The cold incubation optimizes enzyme activity in isolated preparations. The roots were chopped with a motorized razor blade chopper or ground in a Polytron homogenizer (model PT 20, Brinkmann Instruments, Westbury, N. Y.) in medium containing either 100 mM Pipes[4] or Tris (pH 7.0) with or without 0.5 M sucrose. The homogenate was filtered through four layers of bleached cheesecloth and centrifuged at 310g for 10 min to remove cell debris. The supernatant fraction was centrifuged at 20,000g for 60 min and the pellet was resuspended with a syringe in resuspension medium: 0.5 M sucrose, 100 mM Pipes or Tris (pH 7.0), or solubilization medium: 100 mM Tris (pH 7.0), 2 M urea, 1.5% (v/v) Triton X-100 (9). The sucrose-resuspended pellet was assayed directly or frozen until use. The urea, Triton X-100-solubilized pellet was sonicated, incubated on ice for 1 h, and centrifuged at 100,000g for 60 min. The final supernatant fraction was dialyzed against 100 mM Tris (pH 7.0), 1.5% Triton X-100 and then assayed.

Fucosyl Transferase Assay. The sucrose-resuspended pellet containing the enzyme activity and the endogenous acceptor was diluted 1:1 with Triton X-100 to a concentration of 0.5% (v/v) to make a total reaction volume of 30 to 100 μl. The urea, Triton X-100-solubilized enzyme was diluted 1:1 with distilled H_2O or, where specified, hydrolyzed corn root slime. The reactions were initiated by adding 0.02 to 0.1 μCi (0.12–0.6 μmol) GDP-[U-^{14}C]fucose (New England Nuclear, Lot 809-005) to the sample and incubating at 30 C usually for 15 min. The reaction was terminated by adding 1.0 ml distilled H_2O and applying the diluted reaction mix to a Dowex 1 (100–200 mesh) column (0.5 × 4.0 cm) under vacuum. The column was eluted with two 1.0-ml volumes of distilled H_2O. The pooled eluate was made 80% (v/v) ethanol and the product was allowed to precipitate at 4 C overnight. The precipitate was collected by filtration through 2.4-cm Whatman GF/C discs. The discs were placed in plastic scintillation vials to which was added 6.0 ml of scintillation cocktail containing 4.0 g PPO/l toluene. Radioactivity was counted on a Beckman LS-150 liquid scintillation counter. Enzyme activity is expressed as nmol fucose incorporated into neutral, 80% ethanol-insoluble product/min·mg protein.

Preparation and Analysis of Hydrolyzed Slime Acceptor. Corn root slime was collected by pipette suction from root tips or by washing excised roots in a beaker of rapidly stirred distilled H_2O. The slime and washings were combined then filtered *in vacuo* in a Büchner funnel through 20-μm mesh Nitex cloth (Kressilk Products, Inc., New York). The filtrate was brought up to 80% (v/v) ethanol and the slime was allowed to precipitate at 4 C. The

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[4] Abbreviations: Pipes: piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); TFA: trifluoroacetic acid.

ethanol was aspirated off and the residue was dissolved in distilled H_2O and then reprecipitated in 80% ethanol. The precipitate was collected on Whatman No. 1 filter paper and air-dried.

Approximately 30 mg of dried corn slime was dissolved in 8.0 ml of 5 mM TFA, sealed in a 10-ml hydrolysis ampule, and autoclaved 60 min at 121 C. The TFA was evaporated off in a stream of N_2. The gray residue was resuspended in 5.0 ml distilled H_2O and centrifuged 15 min at 1,000*g* to remove the insoluble portion. The final supernatant fraction was frozen until use.

For identification of hydrolysis products hydrolyzed samples were chromatographed on Bio-Gel P-30 (Bio-Rad Labs, Richmond, Calif.). Corn root slime (8.0 mg) and 2.0 ml of 5 mM TFA were placed in hydrolysis ampules and hydrolyzed in an autoclave (18 p.s.i., 121 C) for up to 60 min. After drying in a stream of N_2, the contents of each ampule were resuspended in 1.0 ml of 0.1 N ammonium acetate and transferred to conical centrifuge tubes. The insoluble residue was pelleted by centrifugation for 5 min at 300*g* and 0.8 ml of the supernatant fraction was applied to a Bio-Gel P-30 column (1.5 × 60 cm) eluted with 0.1 N ammonium acetate.

To confirm that the radioactivity incorporated from GDP-[^{14}C]fucose into neutral 80% (v/v) ethanol-insoluble product was in the form of fucose, radioactive product was hydrolyzed with 1 N TFA at 121 C for 2 h. The hydrolysate and standards were chromatographed on Whatman 3MM chromatography paper in solvent containing 95% (v/v) ethanol:1 N ammonium acetate (pH 7.5) (5:2, v/v). For radiocounting the chromatogram was cut into 2-cm strips and placed in cocktail containing 3 g PPO/l solvent, mixed xylenes:Triton X-114 (3:1, v/v) (1). Sugar standards were located on chromatograms using the silver nitrate/NaOH procedure of Trevelyan *et al.* (22).

General Procedures. Protein was determined by the method of Lowry *et al.* (13).

Methyl hexose and hexose were detected by the method of Dische and Shettles (8). H_2SO_4 (4.5 ml of 72% [v/v]) was added to 1.0-ml samples containing less than 50 μg methyl hexose which were then placed in a boiling water bath for 10 min. After cooling, 0.1 ml 3% (w/v) cysteine-HCl was added to each sample. For methyl hexose the *A* at 396 and 427 nm was measured and for hexose at 380 and 413 nm.

Uronic acids were determined by the carbazole method of Dische (7).

RESULTS

Characterization of Fucosyl Transferase Activity. A time course of incorporation of [^{14}C]fucose from the GDP-[^{14}C]fucose donor into the neutral, 80% ethanol-insoluble product is shown in Figure 1. Incorporation was linear with time during the first 15 min and decreased considerably after 30 min. The inclusion of 1.0 M $MnCl_2$ in the reaction mixture proved to be a satisfactory control, since high concentrations of divalent cation dramatically inhibit the enzyme.

In addition to 0.5 M sucrose, 0.5% (v/v) Triton X-100, and buffer, initial reaction mixtures included 0.1% (w/v) BSA, 1 mM DTT, 1 mM EDTA, 1 mM $MnCl_2$, and 1 mM $MgCl_2$. In later experiments these components were omitted with little or no effect on enzyme activity. To verify that neither Mg^{2+} nor Mn^{2+} is required for enzyme activation, the resuspended enzyme pellet was treated with 0.2 M EDTA to chelate endogenous metal cations and then dialyzed exhaustively against buffer. When $MgCl_2$ and $MnCl_2$ were added back to the reaction mixture at various concentrations, there was little difference between water controls and either $MgCl_2$ or $MnCl_2$ at concentrations of 1 to 100 μM (Fig. 2). Concentrations higher than 1 mM of either $MgCl_2$ or $MnCl_2$ strongly inhibited enzyme activity.

The activity of fucosyl transferase at a range of temperatures and pH values was determined. Enzyme activity was optimal at temperatures between 25 and 30 C and over a range of pH from 6.0 to 7.6.

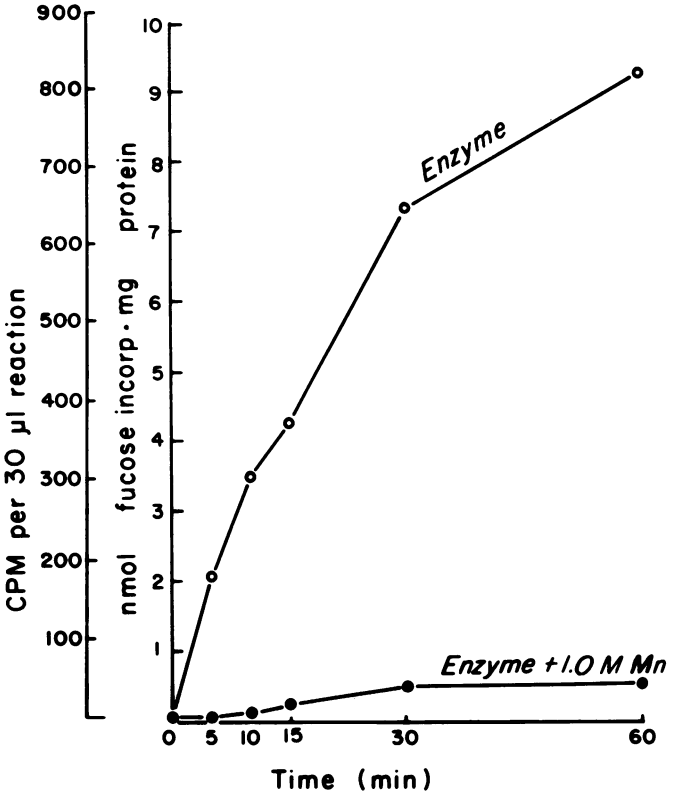

FIG. 1. Kinetics of incorporation of radioactivity from GDP-[^{14}C]fucose into ethanol-insoluble product in the absence of exogenous acceptor and in the presence (●) or absence (○) of 1.0 M $MnCl_2$.

The efficiency of detergent solubilization of transferase activity was assessed by including several concentrations of Triton X-100 in the assay mixture (Fig. 3). Maximum activity was expressed at concentrations of Triton X-100 between 0.5 and 0.8% (v/v) and represents an almost 2-fold increase in activity over non-Triton-treated controls.

Characterization of the Product. To enhance activity and to identify the product, untreated (nonhydrolyzed) and hydrolyzed corn root slime were examined for their ability to serve as exogenous acceptors. When untreated slime was added to sucrose-resuspended enzyme pellet, it increased the rate of [^{14}C]fucose incorporation into 80% ethanol-insoluble material by up to 25%, and when added to solubilized enzyme it had no effect on the rate of [^{14}C]fucose incorporation (Table I). The addition of slime hydrolyzed with 5 mM TFA at 121 C to reaction mixtures, however, enhanced transferase activity in both resuspended and solubilized enzyme preparations (Table II). The rate of incorporation of [^{14}C]fucose into 80% ethanol-insoluble material of sucrose-resuspended enzyme pellets was 38% higher in the presence of 50 μg hydrolyzed slime than in the absence of slime. In preparations of solubilized enzyme 50 μg hydrolyzed slime produced a 53% increase in reaction rate and 5 μg hydrolyzed slime a 12% increase. In another experiment a 3-fold increase in transferase activity was observed with 10 μg of hydrolyzed slime present.

The relationship between TFA concentration and the degree of slime hydrolysis is demonstrated by the Bio-Gel P-30 elution pattern in Figure 4. At 5 mM TFA, only methyl hexose units are hydrolyzed from the polymer, with uronic acids and hexoses eluting in the void volume of the column. With an increase in TFA concentration to 20 mM and 200 mM, there is a concomitant decrease in the hexose and uronic acid components of the void volume peak and an increase in the total volume peak.

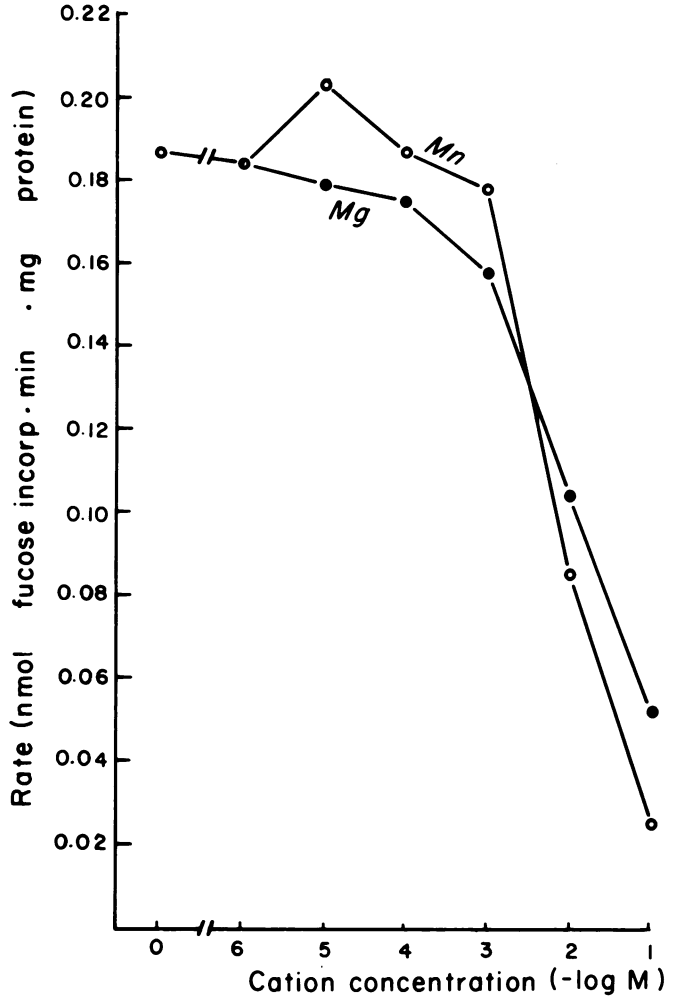

FIG. 2. Effect of Mn^{2+} (O) and Mg^{2+} (●) on fucosyl transferase activity measured in the absence of exogenous acceptor.

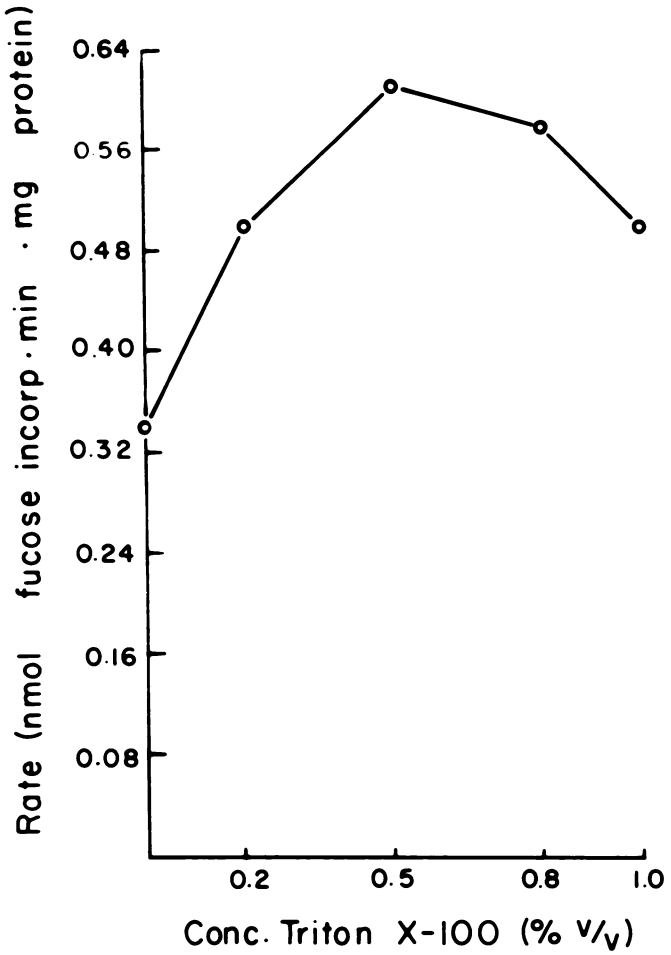

FIG. 3. Effect of Triton X-100 on fucosyl transferase activity measured in the absence of exogenous acceptor.

To verify that the radioactivity incorporated from GDP-[^{14}C]fucose into neutral 80% ethanol-insoluble product was in the form of fucose, the *in vitro* enzyme product was completely hydrolyzed and chromatographed against sugar standards (Fig. 5). More than 90% of all of the radioactivity co-chromatographed with the fucose standard.

The kinetics of hydrolysis of purified slime polysaccharide was also compared with that of the product synthesized *in vitro* to aid further in identification (Fig. 6). The release of fucose during hydrolysis of ethanol-insoluble polymer from both sources was very similar, with a $t_{1/2}$ of hydrolysis of 29 min for the product synthesized *in vitro* and 35 min for the purified slime polysaccharide.

DISCUSSION

In the present study procedures for isolation and assay of GDP-fucose:polysaccharide fucosyl transferase were developed. These efforts were aided initially by the discovery that incubating excised roots for 12 h at 10 C prior to homogenization increased the activity of the final resuspended pellet 3- to 4-fold (12). The assay procedure was greatly simplified by using small Dowex 1 columns to terminate the reactions and at the same time remove unreacted GDP-[^{14}C]fucose donor from the assay mixtures. This procedure reproducibly removed background radioactivity while allowing the neutral product to elute.

GDP-fucose:polysaccharide fucosyl transferase from corn roots is optimal at temperatures between 25 and 30 C and over a pH

Table I. *Effect of Untreated Polysaccharide Slime on Fucosyl Transferase Activity*

Untreated Slime Added	Fucosyl Transferase Activity	
	Resuspended pellet	Solubilized enzyme
μg	*nmol fucose incorporated/min·mg protein*	
0	0.648	0.549
1	0.808	0.496
10	0.785	0.516
100	0.659	0.512

Table II. *Effect of Hydrolyzed Polysaccharide Slime on Fucosyl Transferase Activity*

Hydrolyzed Slime Added	Fucosyl Transferase Activity	
	Resuspended pellet	Solubilized enzyme
μg	*nmol fucose incorporated/min·mg protein*	
Experiment A		
0	0.302	
5	0.351	
50	0.417	
Experiment B		
0		0.234
5		0.261
50		0.359
Experiment C		
0		0.225
10		0.610

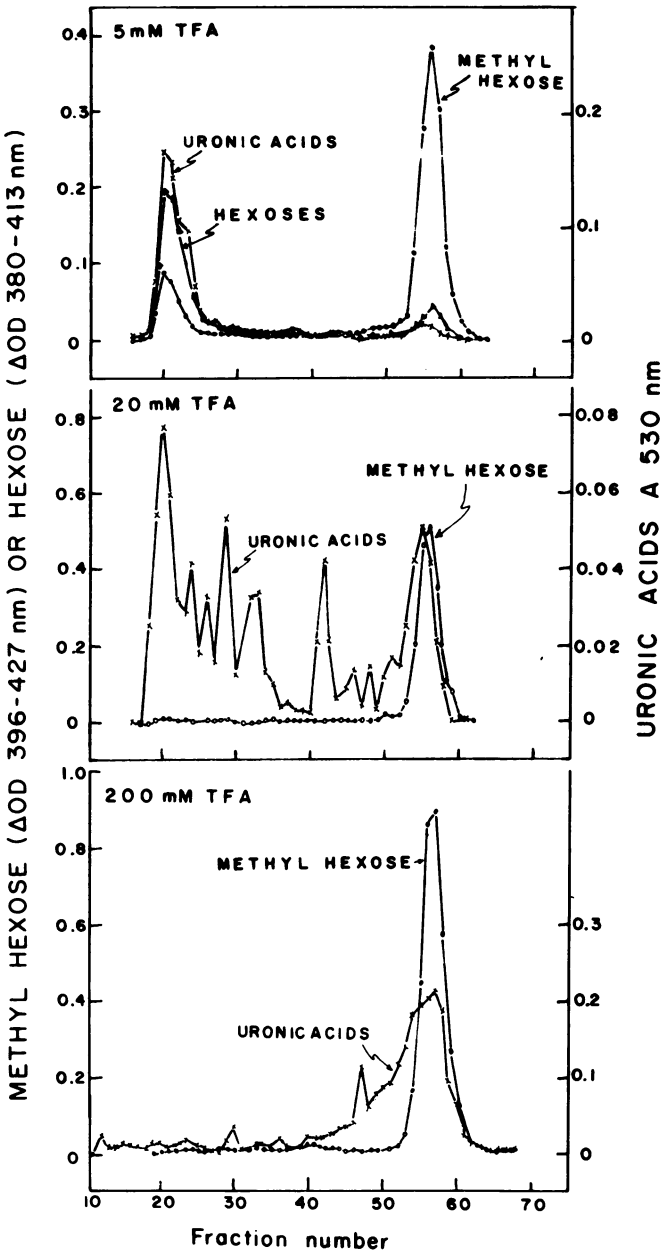

FIG. 4. Bio-Gel P-30 chromatography of hydrolysates after incubation of polysaccharide slime with 5, 20, and 200 mM TFA. Hexose (△), methyl hexose (○), and uronic acid (×).

range from 6.0 to 7.6, with a decrease in activity above pH 7.6. Most fucosyl transferases described from animal (2, 15, 21), plant (6), or bacterial (20) sources possess sharp pH optima. The broad pH optimum of the transferase in this study may indicate more than one enzyme. Since numerous fucose-containing polymers are found within corn roots (10), there should also exist numerous fucosyl transferases.

Fucosyl transferase from corn roots does not appear to require the divalent cations Mg^{2+} or Mn^{2+} for optimum activity. This contrasts with fucosyl transferases from animal, plant, and bacterial systems. Coughlin (6), for example, working with a fucosyl transferase from a brown alga, noted a Mg^{2+} and Mn^{2+} requirement. Powell and Brew (19) reported an activation of galactosyl transferase by Mn^{2+} in bovine colostrum, and in a study of onion stem they noted a Mn^{2+} dependence for galactosyl transferase and Mg^{2+} dependence for glucosyl transferase (18). Bosmann *et al.* (2)

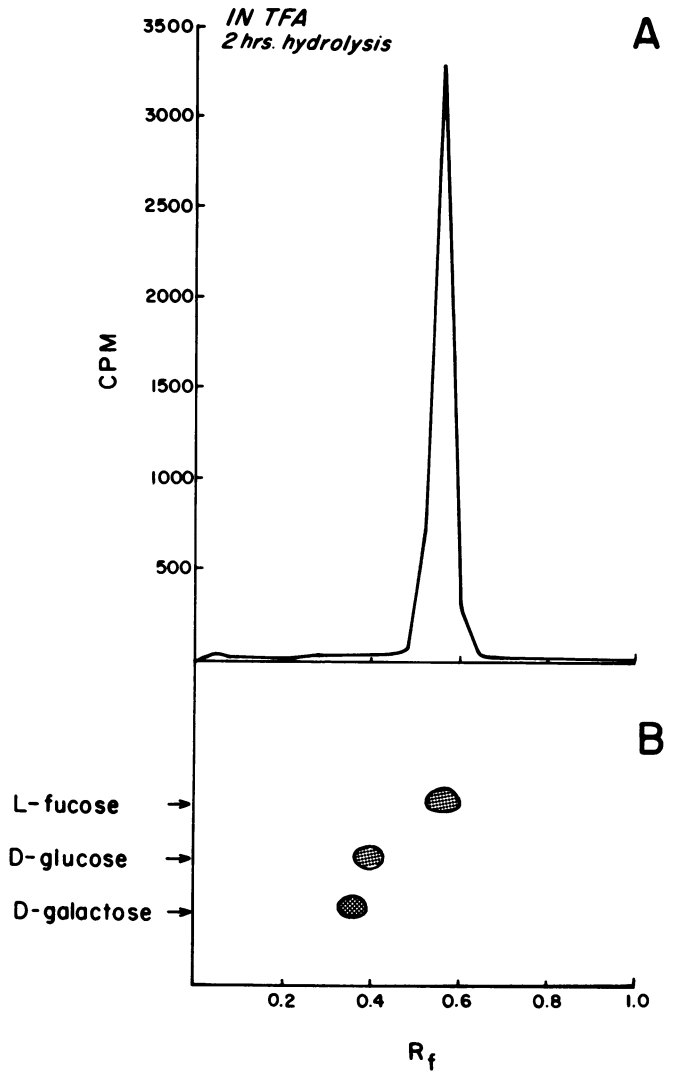

FIG. 5. Paper chromatography after complete hydrolysis of labeled product synthesized *in vitro* using fucosyl transferase and GDP-[^{14}C]-fucose. A: position of radioactivity; B: positions of sugar standards.

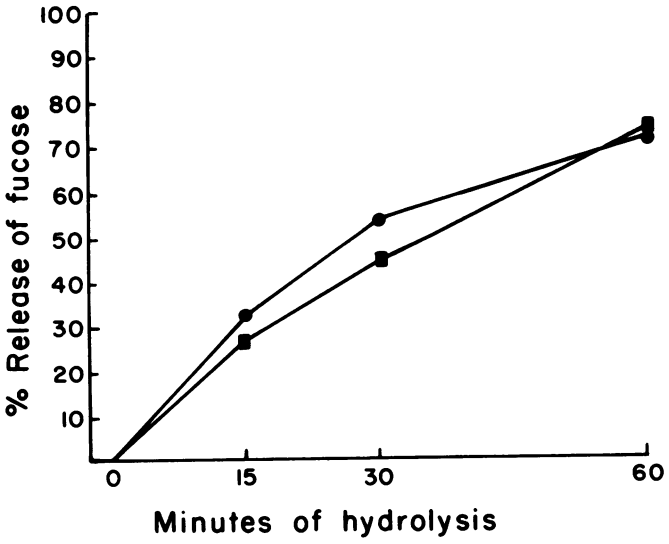

FIG. 6. Kinetics of fucose release during hydrolysis of purified slime polysaccharide (■) and the product synthesized *in vitro* using fucosyl transferase and GDP-[^{14}C]fucose (●).

reported that fucosyl transferase associated with glycoprotein biosynthesis in HeLa cells did not require metal ion activation.

Several lines of evidence suggest that the fucosyl transferase is membrane-bound. Greater than 95% of the enzyme activity is pelleted from root homogenates by a 30 min, 20,000*g* centrifugation (12). The solubilization of the enzyme is facilitated by sonication in the presence of 2 M urea and 1.5% (v/v) Triton X-100 and the addition of Triton X-100 to the reaction mix stimulates enzyme activity, with maximum activity at concentrations between 0.5 and 0.8% (v/v).

Hydrolysis of polysaccharide slime with 5 mM TFA at 121 C results in the removal of methyl hexose from the polymer. When hydrolysis is carried out with higher concentrations of TFA, complete hydrolysis of the slime polymer is observed. This pattern of hydrolysis suggests that the link to fucose is terminal and more labile than other pyranose linkages in the polymer. When the polymer has been hydrolyzed under conditions which cause only the removal of fucose, it will serve as a useful exogenous acceptor in the fucosyl transferase assay. Thus, addition of 5 to 50 μg of partially hydrolyzed slime to the transferase assay mixture stimulates transfer of [^{14}C]fucose from GDP-[^{14}C]fucose by from 50 to 300%.

The product synthesized *in vitro* using fucosyl transferase and GDP-fucose possesses many of the characteristics diagnostic for polysaccharide slime. Both polymers are insoluble in 80% ethanol and soluble in water and both are excluded for a Bio-Gel P-300 molecular sieve column (data not shown). The identification of the product synthesized *in vitro* was further aided by using hydrolysis. When radioactively labeled product from an *in vitro* mixture is hydrolyzed, the only labeled sugar in the hydrolysate co-chromatographs with L-fucose. Furthermore, when the kinetic data of hydrolysis of purified, polysaccharide slime are compared with the kinetics of hydrolysis of the product synthesized *in vitro*, they are very similar. When this evidence is coupled with the observation that partially hydrolyzed slime polysaccharide can act as an exogenous acceptor for fucose *in vitro*, then the conclusion that the product produced by the fucosyl transferase enzyme *in vitro* is similar to polysaccharide slime is warranted.

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