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Guanosine Diphosphate-L-Fucose Glycopeptide Fucosyltransferase Activity in *Corynebacterium insidiosum*[1]

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The biosynthesis of a phytotoxic glycopeptide of *Corynebacterium insidiosum* involves guanosine diphosphate-L-fucosyltransferase activity. This enzyme activity is most consistently associated with the cellular membranes fraction. The optimal pH for the transfer reaction is 7.5. The partially hydrolyzed toxin serves as an acceptor (primer) of L-fucose.

Corynebacterium insidiosum is the causal agent of wilt of alfalfa. A phytotoxic glycopeptide produced by this organism has been implicated in the disease process (10). This same toxic glycopeptide is one of the major products of *C. insidiosum* in liquid shake culture (9). Ries and Strobel (9) demonstrated that the toxin has a molecular weight of about 5×10^6, binds 75 atoms of copper per mol, and consists of approximately 40% L-fucose, 4% mannose, 20% glucose, 20% galactose, 9% organic acid, and 2.6% peptide.

In beginning a study of toxin biosynthesis, it seemed reasonable to examine the cells for the presence, properties, and location of guanosine diphosphate L-fucose glycopeptide fucosyltransferase activity, since L-fucose constitutes such a large percentage of the total weight of the toxin.

MATERIALS AND METHODS

Culturing. The culture of *C. insidiosum* (McCull) Jensen used in this study was kindly supplied by F. I. Frosheiser, Department of Plant Pathology, University of Minnesota. It was maintained and cultured under the conditions described by Ries and Strobel (9), except that sucrose was substituted for glucose as the carbon source.

Preparation of cell-free extracts. After a 50- to 60-h incubation period at 22 C with shaking at 20 rpm, the cells were collected by centrifugation at 22,000 × *g* for 10 min at 4 C. All subsequent treatment was also at 4 C. The cells were washed 3 to 5 times with a buffer consisting of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.05 M, pH 7.5) and 0.4 M sucrose. The cells were disrupted in an Apparatebau Melsungen (referred to as M.S.K.) by using glass beads (Glasperlen, 0.11–0.12 mm) previously washed in 0.05 M Tris-hydrochloride buffer (pH 7.5). The cells were disrupted in 20-s bursts to a total of 2 min to keep the solution near 4 to 10 C. The disrupted cells were then centrifuged at 5,900 × *g* for 30 min. The supernatant liquid obtained from this centrifugation was centrifugated at 48,500 × *g* for 120 min, and the pellet was resuspended in a solution consisting of 0.05 M Tris-hydrochloride and 0.4 M sucrose (pH 7.5) by using a 15-ml glass homogenizer. The pellet containing cellular membranes was used as a source of enzyme throughout this study.

Protein determination. Protein was determined by the method of Lowry et al. by using bovine serum albumin as a standard (7).

Preparation of primer. The phytotoxic glycopeptide from *C. insidiosum* was obtained as described by Ries and Strobel (9). The toxin was stored in solution at −20 C until used. The primer was routinely prepared by dissolving 100-mg lots of the glycopeptide in 3 to 5 ml of 2 N HCl in a sealed test tube and heating the solution in an oven at 100 C for 30 min. The product was dialyzed against frequent changes of distilled deionized water at 4 C for a minimum of 72 h. The insoluble product which came out of the solution was removed by centrifugation and discarded. The resulting supernatant liquid was dried under a stream of air. When primer was needed, the dried product was resuspended in distilled deionized water. The glycosidase-treated α_1-acid glycoproteins were a gift from H. Schachter, Department of Biochemistry, University of Toronto.

Substrate. Guanosine diphosphate (L-fucose-*U*-^{14}C), (GDP-^{14}C-fucose) ammonium salt, 195 Ci/mol, was obtained from Amersham/Searle and stored at −20 C until used.

Fucosyltransferase assay. Unless otherwise indicated, the standard enzyme assay reaction mixture

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contained 3.7 μg of glycopeptide primer, 128 pmol of GDP-L-fucose, 1.25 μmol of Tris-hydrochloride buffer (pH 7.5), 10 μmol of sucrose, and 20 μg of enzyme protein in a total volume of 0.035 ml. These components were mixed on a strip of Parafilm and then incubated for 30 min in a small-diameter glass tube at 20 C. The reaction was terminated by spotting the reaction mixture on strip of Whatman no. 1 paper. The unreacted substrate was removed by high-voltage paper electrophoresis at 24 V/cm in 0.05 M sodium tetraborate (pH 9.1) for 3 h. Under these conditions, the substrate (and breakdown products of the substrate) migrated, but polymeric products remained at the origin. The amount of fucose incorporated into glycoprotein was determined by cutting out the origin and measuring the radioactivity in a Packard liquid scintillation spectrometer (model 3320). The paper strip containing the origin was immersed in a liquid scintillation cocktail consisting of 1.5 ml of absolute methanol and 13.5 ml of scintillation solution containing 5.0 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-2(5-phenyloxazolyl)-benzene per liter of toluene. Counting was conducted on a Packard liquid scintillation spectrometer by using the channels ratio method to correct for quenching, and counts were converted to disintegrations per minute (dpm). A heat-inactivated control was run in each experiment. Any radioactively remaining at the origin after electrophoresis of the control was subtracted from the value obtained in the active preparation. These control values normally varied from 30 to 50 dpm above background.

Verification of fucose incorporation. The reaction products of 13 standard reaction mixtures were eluted from the origin of the electrophoretograms. The eluate was collected and dried under a stream of warm air. The radioactive product (3,640 dpm) was dissolved in 100 μliters of distilled water. A 30-μliter portion was dried in a test tube and redissolved in 2.0 ml of 1 N trifluoroacetic acid; the tube was sealed and placed at 120 C for 2 h.

Sugars were identified by descending paper chromatography in ethyl acetate-acetic acid-formic acid-water (18:3:1:4; vol/vol/vol/vol). The sugar standards were located by using the methods of Trevelyan (11). The radioactive primer and monosaccharides were located by using a Packard radiochromatogram scanner (model 385).

Identity of product being labeled. Approximately 1 mg of the primer was incubated in 1.0 ml of water at 60 C for 20 min with 6 μCi of NaB^3H_4 having a specific activity of 502 Ci/mol. After stopping the reaction by the addition of an excess of acetic acid, the mixture was evaporated to dryness with successive additions of absolute methanol. The residue was taken up in 2.0 ml of water and chromatographed on a column (98 by 1.5 cm) of Biogel P-100 and eluted in 2-ml volumes with 0.01 Tris buffer (pH 7.2). Radioactivity was determined by mixing the contents of each tube with 12 ml of Aquasol (New England Nuclear Corp.).

After being counted in the liquid scintillation cocktail, the products of 10 standard reactions contained on paper strips were removed by placing the strips in 20 ml of boiling water for 1 min. The aqueous solution was evaporated to dryness and resuspended in 2.0 ml of water. This solution, containing about 1,000 dpm, was applied to the Biogel P-100 column described above, eluted, and counted in an identical manner.

RESULTS AND DISCUSSION

The subcellular location of GDP-L-fucosyltransferase activity is shown in Table 1. Although the high-speed supernatant liquid demonstrated the highest specific enzyme activity, this was not consistently observed in all preparations, possibly indicating that the soluble enzyme was a result of the cell breaking and fractionating procedure. On the other hand, considerable activity was always observed in the cellular membrane preparation. That the membrane fraction did indeed contain membranes was demonstrated by embedding the pellet obtained by high-speed centrifugation into plastic and observing sections by electron microscopy. The extreme heterogeneous nature of the crude preparation may have accounted for the enzyme yield being lower than that obtained after the first centrifugation.

The optimal pH for fucosyltransferase activity was 7.5 (Fig. 1). The time course of the transferase reaction appeared to be linear up to 45 min under standard assay conditions. The concentration of primer in the reaction mixture did affect the incorporation of fucose, the optimum being about 0.15 μg of primer per μliter of reaction mixture with 20 μg of protein. Interestingly enough, however, the reaction proceeded without the addition of any exogeneous primer (Table 2). This may be explained on the basis that the enzyme preparation itself contained some residual glycopeptide which in turn

TABLE 1. *Location of GDP-fucosyltransferase activity in the various subcellular fractions of C. insidiosum*

Subcellular fraction	Volume (ml)	Total activity[a]	Specific activity[b]
Cells after fractionation (crude preparation)	245	4,900	8.0
Cell debris (supernatant liquid) after 5,900 × *g*	150	8,550	47.5
Cell debris (precipitate)	45	4,320	16.0
High-speed supernatant liquid after 48,500 × *g*	115	2,783	22.0
Cell membranes	8	270	13.0

[a] Number of picomoles of fucose incorporated per milliliter of subcellular fraction times the total number of milliliters present.

[b] Number of picomoles of fucose incorporated per milligrams of protein.

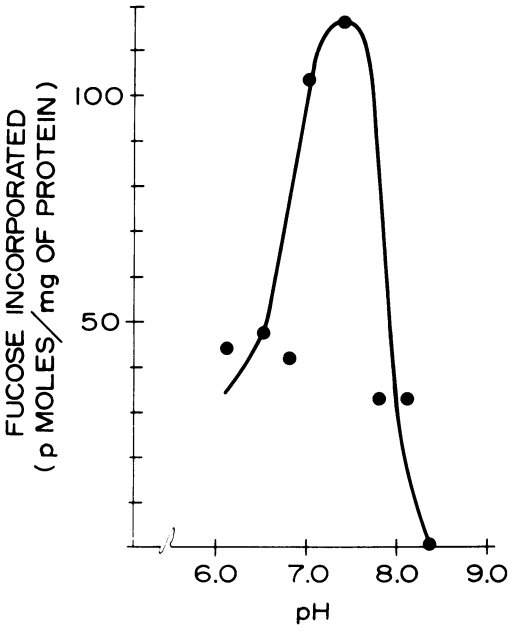

FIG. 1. *Optimal pH of fucosyltransferase activity in membrane preparations of C. insidiosum. The three reactions having an acidic pH contained sodium cacodylate buffer, and the remaining five contained Tris-hydrochloride, otherwise standard reaction conditions were used.*

TABLE 2. *Relative incorporation of ^{14}C-fucose achieved with various primers*

Fucosyl acceptor[a]	Fucose incorporated (pmol)	Relative fucosyl transferase activity (%)
Without primer	0.156	23
Phytotoxic glycopeptide		
15-min hydrolysis (3.2 μg)	0.102	15
30-min hydrolysis (3.2 μg)	0.251	36
60-min hydrolysis (3.2 μg)	0.171	24
α_1 Acid glycoprotein treated with sialidase (2 μg)	0.703	100
α_1 Acid glycoprotein treated with sialidase and β-galactosidase (4 μg)	0.427	61

[a] Standard reaction conditions were used except 13 μg of protein was placed in each reaction mixture.

served as acceptor. In fact, the addition of the 15- or 60-min hydrolysis products of the glycopeptide did not enhance activity. Of the glycopeptide primers used, the 30-min product was the best acceptor of L-fucose. However, the best overall acceptor of L-fucose was the α_1-acid glycoprotein that had been treated with sialidase possibly because of the gentleness and selectiveness of the enzymic hydrolysis used in its preparation. Jabbal and Schachter (6) indicated that α_1-acid glycoprotein, not only treated with sialidase, but also with β-galactosidase, served as acceptors in the porcine fucosyltransferase reaction. Ultimately, more enzyme binding sites and places for glycosidic linkages to form may have been available on these acceptors to likewise be effective primers in the corynebacterium fucosyltransferase reaction.

Jabbal and Schachter (6) showed that the presence of the chelating agent ethylenediaminetetraacetic acid (EDTA) caused a 65% decrease in porcine fucosyltransferase activity. However, Hudgin and Schachter (5) found only a 10% decrease in the activity of sialytransferase with the addition of EDTA. The addition EDTA to the reaction mixture, or the distruption of the cells in the presence of the chelating agent was found to be deleterious to the activity of the *C. insidiosum* transferase (Table 3). Jabbal and Schachter (6) also showed that Triton X-100 was desirable in the reaction mixture of fucosyltransferase and that addition of the detergent beyond optimal concentration did not adversely affect the activity. The detergent apparently acts on the lipid constituents of the membrane, causing it to unfold with the result that more enzyme is exposed. Nevertheless, Triton X-100 was deleterious to *C. insidiosum* fucosyltransferase activity.

Electrophoresis of any reaction mixture containing primer and active enzyme yielded two radioactive peaks. One was at the origin, and the other was a broad peak extending 10 to 16 cm off the origin relative to the movement of a picric acid marker at 20 cm, the broad peak probably being unreacted substrate or breakdown products thereof. The components re-

TABLE 3. *Effect of various agents on fucosyltransferase activity*

Changes in the normal incubation mixture	Relative fucosyltransferase activity (%)
None	100
1 μliter of Triton X-100, 1.0%	6
0.1%	67
EDTA, 1.0 mM	22
0.1 mM	67
GTP, 0.025 μmol	33
None, enzyme preparation from cells fractionated in presence of 1 mM EDTA	50
None, enzyme preparation from cells fractionated in presence of 0.1% Triton	0

maining at the origin were eluted and subjected to acid hydrolysis. Chromatography and detection of the radioactivity by a radiochromatogram scanner showed that ^{14}C-fucose was a product of the acid-hydrolyzed material (Fig. 2). On the other hand, chromatography of the unhydrolyzed product showed all of the radioactivity remaining at the origin.

Evidence that the primer obtained from the toxic glycopeptide was accepting fucose in the transferase reaction is shown in Fig. 3. Treating the 30-min primer with NaB^3H_4 allowed for the localization of primer products as they eluted from the Biogel column. The elution pattern from the column of the primer obtained after the transferase reaction was comparable to that of the tritiated primer. The primer product that seemed to best serve for the acceptance of fucose eluted at tube 19 from the column. This corresponds to a product with a molecular weight of approximately 50,000 as judged by the elution of a set for proteins of known molecular weight from the same column. Furthermore, the radioactivity in this peak eluting from the column represented about 20% of the total radioactivity originally applied to the column from the transferase reaction.

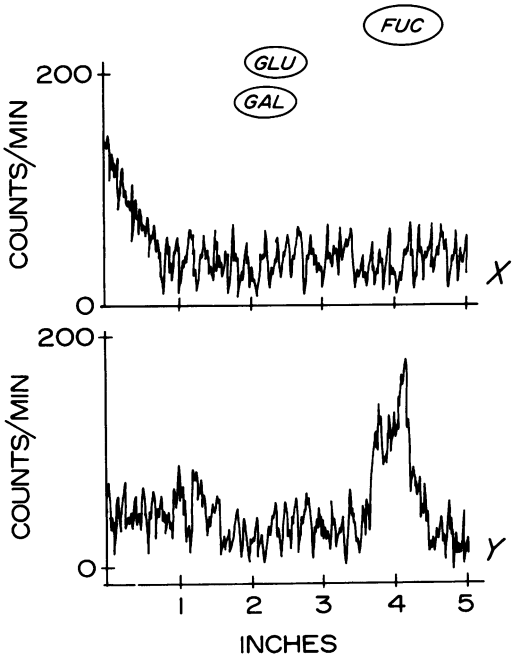

FIG. 2. *Radiolabeling on chromatograms of hydrolyzed (y) and unhydrolyzed (X) products of fucosyltransferase activity. This figure illustrates only 0.5 of the radioactivity remaining at the origin. The products hydrolyzed were eluted from the origin of electrophoretograms of 13 standard reaction mixtures.*

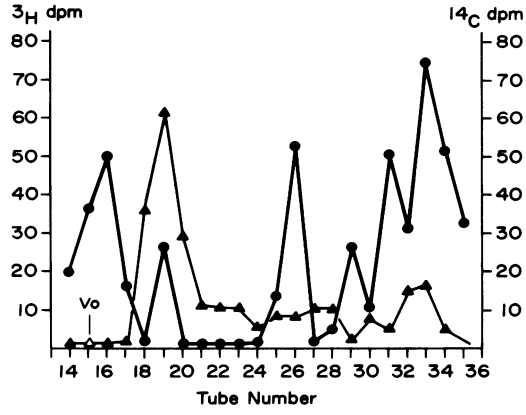

FIG. 3. *Elution of the tritiated primer and the ^{14}C primer recovered from 10 enzyme reaction mixtures with GDP-L-^{14}C-fucose from a 1.5 by 98-cm column of Biogel P-100. Symbols: tritiated primer, ●; ^{14}C primer, ▲.*

Although GDP-L-fucosyltransferase activity has been previously demonstrated in various mammalian systems (1-4, 6, 12), its presence in bacterial systems has remained unknown until this report. It is likely that such transferase activity plays an important role in the production of other fucose-containing toxic glycopeptides (8).

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