A Phytotoxic Glycopeptide from Cultures of Corynebacterium insidiosum

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ABSTRACT

Cultures of Corynebacterium insidiosum produce an extracellular phytotoxic glycopeptide that possesses the ability to wilt plant cuttings. Wilt induced by this glycopeptide is directly dependent upon time and upon concentration with measurable wilt occurring in 40 nM solutions in 1 hour. The organism produces 1.3 grams toxin/liter of culture medium. The toxin was purified, and the physical, chemical, and biological properties were measured. The glycopeptide has an empirical formula of C₈₈H₃₉O₃₅N based on 1 atom of nitrogen. The molecular weight as estimated by light scattering and column gel chromatography indicated values approximating 5 × 10⁶. The toxin does not dissociate into small molecular weight subunits when treated with 8 M urea or 30% pyridine.

The toxin has a specific optical rotation of [α]D⁺³³.⁵ = −166°, an intrinsic viscosity of 0.2307 dl/g, and decomposes at 250°C. It has a blue chromophore due to copper chelation at a concentration of 75 moles copper/mole toxin. Mannose, glucose, galactose and L-fucose, with trace amounts of rhamnose and an unidentified reducing sugar, comprise 83.1% of the toxin. An unknown organic acid appearing chemically similar to a keto-deoxy organic acid comprises 8.8% of the toxin. Lysine, arginine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, leucine, and isoleucine, form a single peptide with glycine as the sole NH-terminal amino acid. The peptide-carbohydrate linkage appears to be of a glycoseidic nature involving the —OH of threonine. This single peptide comprises 2.6% of the toxin, and there are 77 moles peptide/mole of purified glycopeptide.

Corynebacterium insidiosum (McCull.) Jensen causes bacterial wilt of alfalfa (Medicago sativa L.). This disease is characterized by reduction in vigor of plants, yellowing and bleaching of leaves, and finally wilt and death of the plant. The disease is widely distributed in North America and is the most important malady of the crop in the United States. Alfalfa plants are killed so rapidly that fields are unprofitable after 3 or 4 years.

Hodgson et al. (16, 17) and others (9) reported phytotoxic, nonspecific, high molecular weight polysaccharides from bacterial pathogens which cause wilting in plants. Recent studies on Corynebacterium spp. indicate that they also produce phytotoxic polysaccharides in culture media and in the plant. Spencer and Gorin (29) reported that C. insidiosum and C. sepedonicum produced viscous polysaccharide solutions in culture media. The partially purified polysaccharide isolated from C. insidiosum cultures induced wilt in alfalfa cuttings. They detected L-fucose, galactose, and glucose residues in the acid hydrolyzed polysaccharides and offered presumptive evidence that these polysaccharides were present in infected alfalfa and potato plants by demonstration of the rare sugar L-fucose in them. Other plant pathogenic Corynebacteria also produce toxic polysaccharide substances. C. michiganense produces a toxin which is extracellular and contains sugar constituents and six amino acids (24, 25). C. sepedonicum (32, 33) also produces an extracellular glycopeptide in culture as well as in infected plants.

Little attempt has been made to characterize the toxin produced by C. insidiosum, therefore, the purpose of this investigation was to purify the toxic substance produced by C. insidiosum, to measure its physical properties, and to clarify which chemical residues constitute the purified substance.

MATERIALS AND METHODS

Culturing. The culture of C. insidiosum (courtesy of F. I. Froshieier, University of Minnesota) was maintained on a medium containing 1.5% glucose, 2.0% agar, 1.0% yeast extract dialyse, and 0.5% calcium carbonate (33). The organism produced wilt symptoms when inoculated by stem wounding into a 2-year-old alfalfa plant. The toxin was obtained from 250-ml batches of this medium, exclusive of the agar after incubation for 4 days at 20°C on a Potterhometer incubator shaker at 20 rpm.

Preparation and Purification of the Toxin. After incubation the toxin was isolated in a manner similar to that employed by Spencer and Gorin (12, 29), Strobel (33), and Raj and Strobel (24, 25). The culture was centrifuged at 20,000 g for 10 min, and the pellet was discarded. The supernatant liquid was treated with three volumes of acetone (−15°C), and the resulting precipitate was pelleted by centrifugation at 10,000 g for 10 min. The pellet was dissolved in 100 ml of distilled water and passed through a column of Dowex 1 (formate form), 2.5×5.0 cm, 200 to 400 mesh, rinsed with 50 ml of water, and the effluent was passed through a column of Dowex 50 (H⁺ form), 2.5×5.0 cm, 200 to 400 mesh, and rinsed with another 50 ml of water. The effluent was collected and fractionated two
times with ammonium sulfate according to the procedure of Falconer and Taylor (8). Ammonium sulfate (30–40% saturation) precipitated the toxin which was removed by centrifugation at 10,000 g for 5 min and redissolved in 100 ml of distilled water. The toxin was dialyzed against distilled water for 4 days at 4 °C with many changes of distilled water. The solution of purified toxin was stored at −15 °C until used.

**Biological Assay.** The assay procedure used was similar to that employed by Johnson and Strobel (18). Tomato seedlings (Lycopersicon esculentum Mill.) v. Earliana grown in vermiculite under continuous illumination for 10 to 14 days were severed at the crown region and placed in small test tubes containing 0.1 ml of the toxin buffered with 50 mM phosphate to a final pH of 7.0. The test tubes were mounted in a Plexiglas holder surrounded by a transparent Plexiglas box. The top of the box served as a reservoir for water to prevent heating by an artificial light source (100-w bulb and a circular fluorescent bulb) placed above it. After given time intervals the cotyledons were removed with a sharp razor blade, and the extent of wilt in each stem was determined using the wilt-o-meter. This instrument applies a steadily increasing force against a stem until it is no longer able to maintain an erect position. The degree of wilting in any given treatment was the average of three readings obtained from each of five stems. Specific biological activity was determined by the amount of stem strength remaining after a 1-hr treatment in 1.0 mg/ml solution.

**Radioactive Methods.** C. insidiosum was grown on the standard liquid medium containing 25 μC of d-galactose-1-14C (3.0 mc/m mole). The radioactivity in the toxin was determined in a liquid scintillation spectrometer (Nuclear Chicago Model 6804). The toxin solution (10–20 μl) was placed in a vial along with 1.5 ml of absolute methanol and 13.5 ml of scintillation solution containing 4.0 g of PPO and 100 mg of POPP per liter of toluene. The channels ratio method was used to correct for quenching. Autoradiography was performed using Kodak No-screen x-ray film, and electrophoretograms were scanned with a Packard Radiochromatogram Scanner (Model 385). Scanning was performed at 0.5 cm/min with a collimator width of 5.0 mm, a time constant of 10, a linear range of 300, and a gas flow rate of 350 cc/min.

**Molecular Exclusion Chromatography.** 14C-Toxin (5 mg/ml) with a specific radioactivity of 3500 dpm/mg was dried, dissolved in 1 ml of 50 mM tris buffer at pH 7.0 in 40% sucrose, and fractionated with the buffer through columns of Sepharose 2B (1.5 × 45.0 cm). Fractions (1.5 ml) were collected with the aid of a drop counter, and radioactivity was determined in each fraction. This method was also used in attempts to dissociate labeled toxin into subunits with 30% pyridine (26) or 8 m urea followed by fractionation on Sepharose 2B with 50 mM phosphate buffer pH 7.0 containing either 8 m urea or 30% pyridine.

**Electrophoresis.** High voltage paper electrophoresis was conducted in a manner similar to that employed by Strobel (33). One hundred micrograms of toxin dissolved in 20 μl of water was distributed over 25 cm in the center of a 34 × 40 cm piece of Whatman No. 1 filter paper. The paper was pressed between a lower water-cooled plate, and an upper surface was covered by a flexible Plexiglas plate. Pressure was exerted on the upper plate by a water filled rubber bag with a pressure of 6 psi. The temperature of the water in the bag was 4 °C. The molarity and pH of the buffers used were varied in these experiments. All experiments were conducted with a 22.5 v/cm potential for 2 hr. The glycopeptide was detected on the electrophoretograms by the following methods: (a) for reducing ability by the technique of Trevelyan (35), (b) with 0.3% ninhydrin in ethanol, (c) for radioactivity with a chromatogram scanner, (d) visually for the chromophore, and (e) for biological activity after elution of the electrophoretogram with H2O.

Disc gel electrophoresis was performed in 5.0% polyacrylamide gels with glycine-tris buffer, pH 8.8. Fifty micrograms of labeled sample in 40% sucrose was subjected to 2.5 ma in each acrylamide tube (7). After electrophoresis for 30 min, the gels were examined for their reactivity with aniline blue-black for proteins, and Schiff’s base reagent for carbohydrates (23). They were also examined visually for the chromophore and by autoradiography after drying the gel according to the procedure of Herrick and Lawrence (15). Gels were scanned on a Joyce Chromoscan densitometer.

**Physical Properties.** A Cannon-Ubbelohde semi-micro viscometer, Model 75 (Cannon Instrument Co.) was used to determine the specific viscosity at concentrations of 2.6, 1.3, 0.87, 0.65, 0.52, 0.43, and 0.22 mg/ml solutions. The intrinsic viscosity values were calculated by the method of Schachman (27). The intrinsic viscosity was the intercept of the graph of specific viscosity/concentration as a function of concentration. Molecular exclusion chromatography on a column of Sepharose 2B was used to determine molecular size. A second molecular weight estimate was determined with the Brice Phoenix Universal Scattering Photometer (Series 2000) using light with a wavelength of 4358 Å on a series of six solutions ranging from 0.035% to 0.090%. Calculations were based on those of Anacker (2). Double extrapolations to zero angle and zero concentration from the resulting Zimm plot yielded the molecular weight.

**Elemental Analysis.** Five milliliters of 0.1% solution of toxin were treated with 10 ml EDTA at 50 °C for 15 min. The resultant apotoxin was then dialyzed against distilled water for 48 hr after which the ultraviolet and visible spectrum (240–750 nm) were measured on a Cary split-beam spectrophotometer using a 3-ml cuvette with a 1-cm light path.

A 0.1% solution of the toxin was assayed to determine the presence of metal ions which were measured on a Jarrell-Ash atomic absorption spectrophotometer by removing the ion with EDTA and then separating the EDTA-metal-ion complex from the apotoxin by dialysis.

Carbon, hydrogen, and oxygen were determined by Schwarzkopf Microanalytical Laboratory, Woodside, New York; nitrogen was determined by the microKjeldahl technique. From the values of these and the empirical formula was calculated.

**Acid Hydrolysis and Analysis.** Twenty milliliters of 0.5 N sulfuric acid were added to a 50-ml flask containing 50 mg of toxin. The toxin was refluxed for 8 to 12 hr. The mixture was cooled, diluted with 100 ml of distilled water, and neutralized with excess barium carbonate. The precipitate was removed by centrifugation, and the supernatant liquid was passed through a column of Dowex 50 (H+ form), 0.5 × 2.0 cm, 200 to 400 mesh, followed by a 10-ml rinse of water and passage through a column of Dowex 1 (OH− form), 0.5 × 2.0 cm, 200 to 400 mesh. The Dowex 1 column was rinsed with 10 ml of water, and the effluent was dried in a vacuum desiccator over P2O5. This was considered the neutral fraction. Separation and identification of sugar residues in this fraction was done according to the method of Albersheim et al. (1). The sugars were reduced to their alditols with sodium borohydride in 1 N ammonium hydroxide. The reaction was stopped by the addition of a slight excess of glacial acetic acid, and borate was removed by five methanol evaporation. Acetic anhydride (1 ml) was added, and the tubes containing the sugar alcohols were sealed and heated for 3 hr at 121°C. The resultant alditol acetates were identified, and their amounts were estimated by gas-liquid chromatography. The best separation was attained using Gas Chrom P (100 to 120 mesh) coated with 0.2%
poly(ethylene glycol succinate), 0.2% of poly(ethylene glycol adipate), and 0.4% of silicone XF 1150. The F and M gas chromatograph electrometer was set at range 100 and attenuation 4. A 0.4-x 180-cm column was run isothermally at 120 C for 10 min after injection and then raised 1 C per min to 190 C. The detector temperature was 250 C, and the carrier gas flow rate was 30 to 50 cc per minute. The amount of each sugar was determined by a comparison with standard curves established with authentic sugars.

The organic acid fraction was obtained by eluting the Dowex 1 column with 10 ml of 6 N formic acid. The eluant was dried and stored in an evacuated dessicator over P,O, and NaOH pellets. Paper chromatographic analyses of organic acid and sugar residues were done in a one-dimensional descending manner on Whatman No. 1 filter paper in the following solvent systems: (a) 1-butanol-acetic acid-water (4:1:5, v/v), (b) 1-butanol-pyridine-0.1 N hydrochloric acid (5:3:2, v/v), (c) ethyl acetate-acetic acid-water (3:1:3, v/v), (d) 2-butanone-acetic acid-water (8:1:1, v/v), (e) 80% phenol v/v, and (f) 1-propanol-cineole-formic acid (5:5:2, v/v). (g) ethyl acetate-pyridine-water (2:2:1, v/v).

The procedure of Moore and Stein (21) was used for peptide hydrolysis. Toxin (5-10 mg) or peptide (0.25 mg) were placed in 1 ml of constant boiling hydrochloric acid, a crystal of phenol was added, and the tube was sealed under vacuum. The tube was heated for 20 hr at 110 C. Upon cooling, the contents of the tube were dried by flash evaporation at 50 C and stored under vacuum desiccation over NaOH pellets. This preparation was taken up in 5 ml of distilled water and passed through Dowex 50 (H+ form), 0.5 x 1.0 cm, 200 to 400 mesh. A 5-ml distilled water rinse followed, and the amino acids were then eluted with 6 N hydrochloric acid and dried as above.

Identification and quantification of amino acid residues was done on a Beckman automatic amino acid analyzer or by gas-liquid chromatography of the N-trifluoroacetetyl n-butyl esters according to the procedures of Gehrke et al. (10).

**Peptide.** The β-elimination procedure described by Anderson et al. (3) and Tanaka and Pignan (34) to remove peptides in the glycopeptide was used. Toxin (40 mg) was placed in 20 ml of 0.5 N sodium hydroxide and incubated for 216 hr at 4 C in 0.3 M sodium borohydride. The sodium borohydride reaction was stopped by the addition of a slight excess of acetic acid, and borate was removed as methyl borate by five methanol evaporations. The solution was neutralized with sodium hydroxide and passed through a column of Dowex 50 (H+ form), 1.0 x 5.0 cm, 200 to 400 mesh. The retained peptide was then eluted with 6 N hydrochloric acid and dried over sodium hydroxide.

Peptide (1 mg) was spotted on a 34-x 40-cm piece of Whatman No. 1 filter paper and subjected to electrophoresis for 3 hr with 12.5 v/cm potential across the paper in a mixture of 0.54 M formic acid and 1.11 M acetic acid buffer, pH 1.85 (5). This same electrophoretogram was dried and chromatographed in solvent A for 10 hr in the other dimension. The peptide was detected with a modified o-tolidine procedure (6). The electrophoretogram was passed through acetone-ethanol (1:1) solution and while still moist suspended several centimeters over a solution of 1 N hydrochloric acid and 0.5 N potassium permanganate for 5 min on each side. The chromatogram was passed through a solution of 0.5 M potassium iodide mixed 1:1 with saturated o-tolidine in 2 N acetic acid.

**NH Terminal Amino Acids.** The dansyl technique, described by Gray and Hartley (14) was used to determine the NH-terminal amino acid in the peptide. One milligram of the peptide and 25 mg of sodium bicarbonate were dissolved in 0.7 ml of water, and the solution was adjusted to pH 8.2. A solution (0.1 ml) containing 1 mg of dansyl chloride per ml of acetone was added, and the solution was incubated for 12 hr at room temperature. After drying, followed by acid hydrolysis in 1.0 ml of constant boiling hydrochloric acid for 12 hr, the sample was compared against a series of standards by thin layer chromatography on Absorbsil No. 5 in (a) benzenepyridine-acetic acid (16:4:1) v/v, and in (b) toluene-pyridine-ethylene chloride hydrate-0.8 N ammonium hydroxide (10:3:6:6) v/v, upper phase. The dansylated amino acids were detected by ultraviolet light.

**Instrumental Analysis.** Infrared spectra of the organic acid present in the toxin were obtained on a Beckman microspec with 1 to 2 mg of sample in KBr micropellets. Optical rotation measurements were made with a Zeiss circle polarimeter 0.01° with 1 mg/ml H2O in a tube with a 20 cm light path at 34.5° using a Hg lamp and a wavelength of 5460 A. Ultraviolet and visible spectra were made on a Cary Model 14 recording spectrophotometer with quartz cuvettes with a 1.0-cm light path.

**RESULTS**

Figure 1 is a flow chart illustrating the procedure used to isolate the toxin. The toxin, represented by the vertical axis, was extracellular, precipitated with three volumes of cold acetone, and was not retained on Dowex 1 or 50. The effectiveness of each step in removing contaminating materials is shown in Table I. The culture produced 1.3 g of toxin per liter (on a dry weight basis) indicating that approximately 18% of the extracellular material was toxin. The tomato seedling assay developed by Johnson and Strobel (18) was valid for use with this toxin. Tomato seedlings wilted to 100-mg stem strength in a 0.25% solution of buffered toxin in 1 hr when measured on the wilt-o-meter. The stem strength of nontreated tomato stems was 450 mg. Cultivars of alfalfa also wilted when placed in
toxin solutions, but to a lesser extent (200 mg). Tomato seedlings were used in this assay because of their availability, their long hypocotyls, and the ease in applying them to this assay technique. Wilt of test plants was dependent on concentration in a hyperbolic relationship as shown in Figure 2 with measurable wilt occurring in a 0.02% (40 mm) solution of toxin and maximal wilt in 0.15% (0.3 μM) solution of toxin in 1 hr.

**Purity of Toxin Preparations.** Figure 3 demonstrates that when a solution of the purified 14C-toxin with a specific radioactivity of 3500 dpm/mg was fractionated by the addition of 5% of saturation increments of (NH₄)₂SO₄, all of the radioactivity precipitated at the 30 to 40% fraction of saturation. Likewise, all biological activity precipitated in this fraction.

**Table 1. Effectiveness of each Step in the Purification Scheme in the Isolation of the Toxic Glycopeptide**

<table>
<thead>
<tr>
<th>Step in Purification</th>
<th>Dry Weight</th>
<th>Specific Biological Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude culture supernatant</td>
<td>6.8</td>
<td>418</td>
</tr>
<tr>
<td>Acetone precipitate</td>
<td>2.6</td>
<td>285</td>
</tr>
<tr>
<td>Dowex 1 treatment</td>
<td>2.2</td>
<td>238</td>
</tr>
<tr>
<td>Dowex 50 treatment</td>
<td>1.9</td>
<td>219</td>
</tr>
<tr>
<td>1st (NH₄)₂SO₄ ppt.</td>
<td>1.4</td>
<td>127</td>
</tr>
<tr>
<td>2nd (NH₄)₂SO₄ ppt.</td>
<td>3.3</td>
<td>75</td>
</tr>
</tbody>
</table>

1. For each step in the purification procedure, the total dry weight and the specific biological activity were determined. The data are based on 1 liter of crude culture supernatant liquid as the starting material.

2. Specific biological activity is described as the amount of stem strength remaining after a 1-hr treatment in 1.0 mg/ml solution. Stem strength (degree of wilt) is determined with a wilt-o-meter and its units of measure are in mg pressure exerted by the plant stem as it bends beneath the measuring arm of the instrument (18). For instance, a nontreated tomato hypocotyl will give a reading of approximately 450 mg, whereas a wilted stem will give a reading of 75 to 100. The relationship between toxin concentration and degree of wilt of plant cuttings is given in Figure 2.

Fig. 2. Tomato seedlings were placed in 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.50% solutions of toxin in 50 mM phosphate buffer, pH 7.0. Loss of stem response was measured on the wilt-o-meter after 1 hr. The control, excised stems in buffer solution, alone gave a wilt-o-meter value of 380 mg.

Fig. 3. The purified 14C-labeled toxin was fractionated with 5% of saturation increments of ammonium sulfate. After each addition, any resultant precipitate was removed by centrifugation, checked for biological activity, and radioactivity in the supernatant liquid was measured. Radioactivity in dpm/ml was plotted against percentage of saturation with ammonium sulfate.

Fig. 4. A toxin preparation (2 mg/ml) was placed on the column of Sepharose 2B and eluted with 50 mM Tris buffer, pH 7.0. Fractions (1.5 ml) were collected, and 0.2-ml aliquots were counted. Dpm per tube is graphed versus fraction number. The contents of the tubes from both peaks were pooled, dried, and tested for biological activity.

Molecular exclusion column chromatography is frequently used as a criterion of homogeneity and in the estimation of molecular sizes of compounds. A solution (2 mg/ml) of the labeled toxin (7,000 dpm) in 40% sucrose was layered on a Sepharose 2B column (1.5 × 45 cm) which fractionates molecules ranging in size from 2 × 10⁶ to 20 × 10⁶. The toxin was eluted with 50 mM tris buffer at pH 7.0, and 1.5-ml fractions were collected. The presence of two peaks in Figure 4 illustrates the polydispersity of the toxin preparation. Nevertheless, the specific biological activities of both peaks were identical in that they both gave specific biological activity values of 100 when tested in the wilt-o-meter. Furthermore, a determination of total carbon in each tube by techniques previously described (33) yielded a graph that possessed the same pattern as shown in Figure 4.
Disc gel electrophoresis demonstrated that labeled toxin migrated only into the top of the 5% polyacrylamide gel when located by autoradiography after drying the gel. Similarly, the chromophore, the peptide portion, and the carbohydrate moieties remained at the top of the gel. Electrophoresis for longer periods of time with lower gel concentrations also failed to cause migration.

High voltage paper electrophoresis of the toxin showed that at pH 7.0, 0.2 M phosphate buffer, neither the chromophore, carbohydrate, protein, radioactivity, nor biological activity migrated from the origin. Observations of similar experiments conducted at pH 2 and pH 10 revealed no migration of the toxin. The lack of mobility of the toxin during paper electrophoresis is in agreement with gel electrophoresis. Sepharose 2B chromatography indicated a very large compound which may explain the inability of the toxin to migrate through the acrylamide gels. The toxin was not retained on Dowex 50 or Dowex 1, indicating that the compound has no net charge. No other protein, carbohydrate, or radioactive compounds were observed during electrophoresis, indicating only the toxin was present.

Molecular Weight Determinations. One of the properties of Sepharose gels other than to demonstrate the purity of a preparation is their capacity for separating substances according to molecular size. The peak at fraction 28 (Fig. 4) has a $K_v$ value of approximately 0.284. This suggests that the toxin has an average molecular size of $5 \times 10^6$ (28).

A second method used for determining the size of this compound was light scattering. The amount of light scattered by a compound is dependent upon its size. The Zimm plot from light scattering studies is illustrated in Figure 5. Extrapolation to zero concentration (line a) and analysis by the method of least squares to determine the $y$ intercept indicated the toxin had a molecular weight of $5.69 \times 10^6$. The extrapolation to zero angle (line b) and least square analysis to determine the $y$ axis intercept indicated a molecular weight of $4.33 \times 10^6$. These two techniques indicate that the toxin has a molecular weight to approximately $5 \times 10^6$.

Considering the large molecular size of the toxin, dissociation of the compound into lower molecular weight subunits was attempted. Treatments with 8 M urea or with 30% pyridine, or removal of copper with EDTA, failed to reveal subunit formation when fractionated on Sepharose 2B. Toxin preparations receiving such treatments yielded an elution profile similar to untreated toxin. Therefore, the molecule appeared to be one large covalently linked polymer and not a series of loosely bound subunits.

Composition of Toxin. Elemental analysis of the compound indicated 29.52% carbon, 5.14% hydrogen, 48.02% oxygen, and 0.32% nitrogen. Therefore, the empirical formula for the toxic glycopeptide was $C_{68}H_{64}O_{32}N$ based on 1 atom of nitrogen. The toxin had a brilliant blue chromophore. A 0.1% solution of toxin absorbed maximally at 280 nm and 635 nm (Fig. 6). Treatment with EDTA followed by dialysis altered absorbance of the toxin at 280 nm and destroyed absorbance at 635 nm. This would indicate a metal ion was being removed by chelation from the toxin, resulting in a colorless apotoxin. The identity of the metal ion was determined to be copper and was measured on a Jarrell-Ash spectrophotometer (Table II). The EDTA control by itself contained no copper, while the toxin had 13 moles/mole of toxin. After treatment with EDTA, the toxin (apotoxin) contained no copper, while the EDTA-Cu contained 75 moles/mole of toxin. The difference between toxin and EDTA-Cu values was clarified by ashing the toxin in a muffle oven at 650 °C for 20 hr. The ashed toxin contained approximately the same amount of copper as did the EDTA-Cu. Evidently the burning of the carbonaceous toxin masked much of the copper present in it.

The toxin decomposed when heated to 260 °C in a Fisher-Johns melting point apparatus. The intrinsic viscosity was 0.2307 dl/g, and its specific optical rotation was $[\alpha]_d^{25}$ = $-166^\circ$ using a Circle Polarimeter 0.01° with a mercury lamp.

Hydrolysis of the toxin with sulfuric acid followed by formation of the alditol acetates of the sugar residues and gas liquid chromatography indicated that mannose, glucose, galac-

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tose, and L-fucose (1:5:5:10) were the major sugar residues present in the toxin (Table III). Fucose was in the L-form as determined by optical rotation studies made on this sugar isolated by paper chromatography in solvents A and G. Two other reducing compounds were present in trace amounts, rhamnose and an unknown. These carbohydrates accounted for 83.1% of the toxin. The organic acid content of the toxin was quantitatively determined by weighing the dried eluate of the Dowex 1 column on a Cahn electrobalance. This acid gave a positive test with acid-base indicator (4), was weakly reducing (35), and it comprised 8.8% of the toxin (Table III). The anion fraction consisted of one compound which failed to chromatograph with any of the organic acids checked (Table IV). An infrared absorption spectrum of the compound with the Beckman Microspec showed a strong broad absorption band between 2.9 and 3.3 $\mu m$, a smaller band at 3.7 to 4.0 $\mu m$, and other bands at 5.75, 6.0, 6.5, and 7.1 $\mu m$. The absorption spectrum of the compound strongly suggested that it was a carboxylic acid. Analysis of the acid by the thiobarbituric acid method (36) produced an alkali-labile chromophore with an absorption maximum at 549 nm. This reaction is characteristic of $\beta$-formylpyruvate which is formed from 2-keto-3-deoxyaldonic acids.

Acid hydrolysis of the isolated glycopeptide and analysis on the amino acid analyzer indicated its composition to be lysine, arginine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, leucine, and isoleucine, (Table III).

The peptide fraction was removed and separated from the glycopeptide by treatment of the glycopeptide with 0.5 N sodium hydroxide and passage of the preparation through Dowex 1 (formate form), 0.5 x 1 cm, 200 to 400 mesh. The peptide was eluted with 6 N formic acid. After drying, salt was removed by washing the dried peptide residue with 10 ml of acetone containing 1% of concentrated aqueous hydrochloric acid. Undissolved material was removed by centrifugation, and the pellet was washed three times with acetone-HCl, centrifuged, and discarded. The combined supernatant liquids were dried (32) and weighed on a Mettler balance. The peptide represented 2.6% of the toxin on a dry weight basis. Kjeldahl nitrogen analysis indicated 0.32% of the toxin was nitrogen. Since the peptide is 16.6% nitrogen by amino acid analysis and the peptide was 2.6% of the toxin, there was 0.43% nitrogen in the toxin due to the peptide. Therefore, all nitrogen in the toxin was present in the amino acid constituents.

A peptide comprising approximately 2.6% of the toxin and composed of the amino acids shown in Table III would have a molecular weight of 1683. Considering the molecular weight of the toxin (5 x 10$^9$) there would be approximately 77 moles of peptide/mole of toxin. Two dimensional electrophoresis-chromatography indicated a single peptide was present in the toxin (Fig. 7). Electrophoresis of the peptide caused it to migrate 6.9 cm toward the negative pole. Descending paper chromatography in the opposite dimension in solvent A for 10 hr indicated the peptide had an $R_F$ = 0.238. The isolation and demonstration of a single peptide indicates that the amino acids of the toxin are linked together by peptide bonds. Fur-

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### Table III. Chemical Constituents of the Toxic Glycopeptide from C. insidiosum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage of Total Toxin</th>
<th>Ratio Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar residues</td>
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<td></td>
</tr>
<tr>
<td>L-Fucose</td>
<td>38.7</td>
<td>10</td>
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<tr>
<td>Mannose</td>
<td>3.8</td>
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</tr>
<tr>
<td>Glucose</td>
<td>20.0</td>
<td>5</td>
</tr>
<tr>
<td>Galactose</td>
<td>20.6</td>
<td>5</td>
</tr>
<tr>
<td>Rhamnose$^2$</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Organic acid</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
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</tr>
<tr>
<td>Lys, Arg, Asp, Glu, Gly, Ser, Thr, Ala, Val, Leu, Ile</td>
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<td></td>
</tr>
<tr>
<td>Cations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>94.6</td>
<td></td>
</tr>
</tbody>
</table>

1 Refers to the ratio of sugar residues in the total glycopeptide.
2 Rhamnose and the unidentified reducing compound accounted for less than 1% of the total sugars.
3 Refers to the number of residues of each amino acid in the peptide.

### Table IV. $R_F$ Values of Some Sugar Acids Relative to the Carboxylic Acid from the Toxic Glycopeptide of C. insidiosum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent Systems$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>2-Keto-3-deoxygluconic acid</td>
<td>0.21</td>
</tr>
<tr>
<td>5-Keto-glucic acid</td>
<td>0.33</td>
</tr>
<tr>
<td>2-Keto-glucic acid</td>
<td>0.31</td>
</tr>
<tr>
<td>Glucic acid</td>
<td>0.26</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>0.21</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>0.24</td>
</tr>
<tr>
<td>Unknown acid</td>
<td>0.07</td>
</tr>
</tbody>
</table>

1 Chromatography systems given in text.
2 ND = no determination.

---

**Fig. 7.** Two-dimensional electrophoresis-chromatography of the peptide moiety of the toxin. The peptide was subjected to electrophoresis in formic-acetic acid buffer, pH 1.85, at 12.5 v/cm for 3 hr at 10 mamp. After drying, the electrophoretogram was chromatographed in the other dimension in 1-butanol-acetic acid-water (4:1:5) for 10 hr. The electrophoretogram was developed with o-tolidine. The peptide moved 6.9 cm towards the negative pole and had an $R_F$ of 0.238.
ther evidence that the amino acids are covalently bonded together is that dansylation followed by hydrolysis yielded a sole NH2-terminal amino acid, glycine.

Acid hydrolysis of the peptide and analysis of the N-trifluoroacetyl n-butyl ester derivatives by gas-liquid chromatography (10) indicated the apparent peptide-carbohydrate linkage is a glycosidic one through the -OH of threonine as manifested by a 81.7% decrease in threonine with the concomitant appearance of α-amino butyric acid in the β-eliminated and reduced peptide. The molar amounts of the amino acids remaining after β-elimination, reduction, and analysis other than threonine were the same as in the non-β-eliminated toxin. These data are also consistent with the concept that all of the amino acids are covalently linked in one peptide.

Biological Activity and Toxin Stability. The glycopeptide induced wilt in excised plant cuttings of both alfalfa and tomato cultivars. Wilting in plants was dependent on concentration of the toxin and on the length of time the cuttings were in contact with toxin solutions. The toxin was also stable to great fluctuations in temperature and pH. Heating toxin solutions (0.25%) to 121°C for 10 min and allowing them to cool did not affect biological activity in any way. Toxin which had been autoclaved for 2 hr at 121°C demonstrated a lower specific biological activity, but still produced measurable wilt symptoms. The toxin was also stable to fluctuations in pH. Toxin solutions (0.25%) in phosphate buffers at pH 2, 7, and 12 for 24 hr and then adjusted to pH 7.0 still possessed unaltered ability to wilt tomato seedlings. Partial acid hydrolysis of the polysaccharide resulted in a drastic reduction of biological activity with all of the biological activity being lost after 5 min refluxing in 0.5 N sulfuric acid.

DISCUSSION

After purification, a glycopeptide plant toxin (1.3 g) was isolated from a 1-liter culture of C. insidiosum. This yield was in sharp contrast to the 15 mg toxin/liter attained from C. sepedonicum (33) and the 18 mg toxin/liter from C. michiganense (25) and in the same range as that reported by Spencer and Gorin (2 g toxin/liter) (29). The purification attained indicated that most of the material in the culture fluid (18%) produced by the organism was the toxin. The purpose for this massive production of toxin by the organism is not understood, although it may serve as either an energy source and/or a carbon source in later growth, or it may function in aiding pathogenesis of the organism in the plant.

The first (NH4)2SO4 fractionation indicated that contaminating radioactive substances were present after ion-exchange chromatography. The toxin precipitated in the 30 to 40% fractions as did most of the radioactivity. However, about 50% of the label remained in the supernatant liquid after precipitation of the toxin. Based on this information, the toxin isolated by Spencer and Gorin (12, 29) from C. insidiosum was probably a heterogeneous mixture of compounds as their final step in preparation was ion-exchange chromatography. Four experimental tests indicated that the substance after the final step in purification was one compound or one class of compounds. A: A single peptide was found with a single NH2; terminal amino acid, and the number of copper ions present in the molecule were roughly equivalent to the number of peptides; B: all markers of the toxin (radioactivity, chromophore, carbohydrate, protein) migrated into the top of acrylamide gels and did not penetrate further during electrophoresis, and no other compounds were detected migrating through the gels; C: in high voltage paper electrophoresis experiments the toxin did not migrate from the origin when checked in a wide range of buffers; in addition no other radioactive, reducing, or ninhydrin positive compounds migrated; D: Sepharose 2B chromatography of the 14C-labeled toxin obtained after (NH4)2SO4 precipitation demonstrated two peaks in the preparation, both of which had the same specific biological activity and the same specific radioactivity. The presence of two peaks indicated heterogeneity since the elution profile of Blue Dextran on Sepharose 4B demonstrates a similar pattern (28). This peak pattern may be explained by assuming the leading edge of the second peak was being excluded on the column during elution. Although several experiments on the purity of the toxin preparation used in this study demonstrated toxin homogeneity, Sepharose column chromatography, having more resolving capacity for larger sized compounds, showed that the toxin was polydisperse. Thus, the toxin itself appears to be a mixture of high molecular weight compounds, possessing the same biological activity but differing in size probably by degrees of polymerization of sugar residues.

Spencer and Gorin (12, 29) made no estimate of the size of the toxin compound they isolated. Estimation of the molecular size of the toxin preparation by fractionation on Sepharose 2B approached 5 × 106. A molecular weight of approximately 5 × 106 was estimated by light scattering. These weight average molecular weights differ considerably from those reported earlier by Strobel (33) for C. sepedonicum (2 × 106) and Rai and Strobel (24) for the three toxic fractions of C. michiganense after Sephadex G-200 chromatography. The sizes of other phytotoxins isolated from other Corynebacteria in comparison to this (2 × 106 versus 5 × 106) might indicate that this molecule was composed of a series of subunits. Dissociation experiments however failed to reveal subunits.

The specific viscosity of the toxin (0.2307 dl/g) has a similar viscosity to fraction I toxin (0.225 dl/g) of C. michiganense (24) while differing from the toxin of C. sepedonicum (0.125 dl/g) (33). Intrinsic viscosity values for these polysaccharides are frequently proportional to molecular weight, implying that this toxin was larger than fraction I reported by Rai and Strobel (24). The optical activity of the toxin, [α]D = −166°, differed significantly from that reported by Gorin and Spencer (18) for C. insidiosum, [α]D = −98°. The heterogeneity of the toxin prepared by Gorin and Spencer undoubtedly altered their value.

The broad absorption peak at 635 nm obtained by light absorption experiments was the visible chromophore of the toxin. Treatment with EDTA indicated a metal ion chelated to the toxin while quantification of the metal showed copper was present at a concentration of 75 moles copper/mole toxin. These data sharply contrasted with those reported by Kuhn et al. (19, 20), who found and chemically characterized a low molecular weight pigment of C. insidiosum which was also blue. Since this incompatibility of results exist, there may be two pigments present in C. insidiosum cultures, a large molecular weight substance containing copper and a small noncopper-containing compound. Another possible explanation may be that the pigment described by Kuhn et al. was an integral portion of the polysaccharide, and the chromophore of the toxin resulted from this pigment. This, however, is not consistent with the presence of copper in the toxin or the loss of color with EDTA treatment.

Chemical characterization of the toxin suggested this compound was chemically closely related to the phytotoxic glycopeptides from other Corynebacterium spp. Patino-Mendez (22) reported fucose, galactose, glucose, mannose, rhamnose, and an unidentified sugar residue from a toxin isolated from C. michiganense. These same sugars were reported in preparations from C. michiganense (24) and from C. sepedonicum (32, 33). Gorin and Spencer (12) working with C. insidiosum re-
ported the constituent sugars as galactose (32%), glucose (21%), and fucose (46%). In the present study the toxin of *C. insidiosum* contained galactose (21%), glucose (20%), fucose (39%), and mannose (4%), with rhamnose and an unidentified sugar residue present in trace amounts. Another similar characteristic of these toxins was that fucose was present in the L-form, a fact reported by Gorin and Spencer (13) and Strobel (32).

An organic acid from the toxin preparation of *C. insidiosum* described by Gorin and Spencer (13) was 4,6-0-(1-carboxyethylidene)-d-galactose. Conditions of acid hydrolysis on the purified toxin employed in the present study should have hydrolyzed this compound to pyruvic acid and galactose, but pyruvic acid could not be identified as a constituent acid of the purified toxin. This is based on the fact that infrared spectral analysis of pyruvic acid and the unknown organic acid were not similar, since pyruvic acid has strong absorption bands at 6.1 and 7.35 μ, while the unknown organic acid did not. Similarly, the unknown organic acid possessed —CH and —OH stretch bands which pyruvate lacked. Reactions of these organic acids with α-anisidine (31) were different, since pyruvate gave a bright yellow spot and the unknown organic acid gave a dull brown product. Strobel (32) described 2-keto-3-deoxyglucosonic acid from the toxic glycopeptide of *C. sepedonicum* and Ghahambar et al. (11) described 3-deoxy-d-manno-octulosonic acid from cell wall lipopolysaccharides of *Escherichia coli*. The unknown organic acid appeared similar to 2-keto-3-deoxyglucosonic acid and 3-deoxy-d-manno-octulosonic acid because it produced an alkali-labile chromophore by the thiobarbituric acid method and because of its characteristic infrared absorption spectrum, but it did not cochromatograph with 2-keto-3-deoxyglucosonic acid or any of the other organic acids (Table IV). Comparison of the *R*_f values listed for 3-deoxy-d-manno-octulosonic acid (32) and those obtained for the unknown organic acid indicated that they were not the same compound. The presence of an organic acid (8.8%) represented another chemical similarity with the toxins produced by other *Corynebacteria* and it appears to be a keto-deoxy acid.

The presence of amino acids confirms the observations of Strobel (32) and Rai and Strobel (24) with the glycopeptides of *C. sepedonicum* and *C. michiganense*. Gorin and Spencer (12) failed to report that amino acids were present in their preparations but indicated 0.3% of their preparation was nitrogen, virtually the same percentage obtained in this work (0.32%). The isolation and demonstration of a single peptide from the glycopeptide comprising 2.6% of the total toxin is shown in Figure 7. A peptide composed of the amino acids listed in Table III would imply that there would be 77 peptides per molecule of toxin. This is consistent with the finding that there are 75 moles Cu/mole toxin and might indicate that copper chelation is related to the presence and amount of peptide.

The structure of the glycopeptide seems to be quite complex. It appears plausible that the toxin consists of 77 identical peptides linked to the carbohydrate backbone through the three-nine residue of the peptide. Such peptide-carbohydrate linkages are of a glycosidic nature (30). On this basis there must be at least 77 carbohydrate chains which are cross-linked in some unknown manner.

The biological significance of this compound *in vivo* was not investigated, but other glycopeptides reported from other *Corynebacteria* were chemically closely related. They contained essentially the same sugar, amino acid, and organic acid residues and produced similar symptoms *in vivo*. The isolation of 3-fucose from infected alfalfa plants by Spencer and Gorin (29) is presumptive evidence that such a compound is biologically important in the disease process. More substantial evidence concerning the biological importance of these toxins *in vivo* has been offered by Strobel (32). He has isolated the toxin produced by *C. sepedonicum* from both cultures of the organism and from diseased plant parts.

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**LITERATURE CITED**


RIES AND STROBEL


