Abstract:
Phytotoxins produced by Corynebacterium spp. have been reported by several investigators. However, in the case of a toxin produced by C. insidiosum, the complete chemical, physical, and biological characterization has not been established. The present study demonstrates that the toxin of C. insidiosum is a glycopeptide with an empirical formula of C_{108}H_{226}O_{132}N_{1} based on one atom of nitrogen. Chemical analysis shows the toxin is 83.1% polysaccharide composed of mannose (1), glucose (5), galactose (5), and L-fucose (10), with trace amounts of rhamnose and an unidentified reducing sugar. An unknown organic acid comprises 8.8% of the toxin and a single peptide composed of lysine2, arginine1, aspartic acid1, threonine1, serine1, glutamic acid1, glycine2, alanine2, valine2, leucine2, and isoleucine1 accounts for 2.57% of the toxin molecule. The toxin has a blue chromophore due to copper chelation at a concentration of 75 moles copper/mole toxin. Sepharose 2B column chromatography, analytical ultracentrifugation, and light scattering data indicates that the glycopeptide has a molecular weight of 5 X 10^6 daltons. Electron micrographs of the glycopeptide shows it to be an amorphous globule 80.0 - 90.0 μm in diameter. The toxin has a specific optical rotation of -166°, an intrinsic viscosity of 0.2307 dl/g., and decomposes at 260°. The toxin causes wilt in both leaves and stems of test plants and exhibits no specificity between alfalfa or tomato seedlings. Biological activity of the compound is dependent on time and on concentration. The toxin is stable to wide fluctuations in pH and still retains biological activity after heating to 121° for 2 hours. Partial acid hydrolysis results in a drastic reduction of biological activity. The mechanism of action of the toxic glycopeptide is unknown but it may cause plugging of xylem vessels or membrane damage.
CHEMICAL AND BIOLOGICAL PROPERTIES OF A PHYTOTOXIC GLYCOPEPTIDE FROM CORYNEBACTERIUM INSIDIOSUM

by

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

in

Microbiology

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VITA</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>5</td>
</tr>
<tr>
<td>Culturing</td>
<td>5</td>
</tr>
<tr>
<td>Preparation and purification of toxin</td>
<td>5</td>
</tr>
<tr>
<td>Optimum toxin production</td>
<td>6</td>
</tr>
<tr>
<td>Biological assay</td>
<td>6</td>
</tr>
<tr>
<td>Radioactive methods</td>
<td>7</td>
</tr>
<tr>
<td>Molecular exclusion chromatography</td>
<td>8</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>8</td>
</tr>
<tr>
<td>Analytical ultracentrifugation</td>
<td>9</td>
</tr>
<tr>
<td>Light scattering</td>
<td>9</td>
</tr>
<tr>
<td>Specific viscosity</td>
<td>10</td>
</tr>
<tr>
<td>Elemental analysis</td>
<td>10</td>
</tr>
<tr>
<td>Chromophore</td>
<td>10</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>11</td>
</tr>
<tr>
<td>Chromatography</td>
<td>12</td>
</tr>
<tr>
<td>Peptide</td>
<td>13</td>
</tr>
<tr>
<td>NH₂-terminal amino acids</td>
<td>14</td>
</tr>
<tr>
<td>Electron microscopic study</td>
<td>15</td>
</tr>
<tr>
<td>EXPERIMENTAL RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>Purification and biological activity of toxin</td>
<td>16</td>
</tr>
<tr>
<td>Optimum toxin production</td>
<td>26</td>
</tr>
<tr>
<td>Sepharose chromatography</td>
<td>33</td>
</tr>
<tr>
<td>Electrophoresis of toxin</td>
<td>38</td>
</tr>
<tr>
<td>Ultracentrifugation</td>
<td>38</td>
</tr>
<tr>
<td>Light scattering</td>
<td>45</td>
</tr>
<tr>
<td>Elemental analysis</td>
<td>45</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>49</td>
</tr>
<tr>
<td>Dissociation of toxin</td>
<td>49</td>
</tr>
<tr>
<td>Chromophore</td>
<td>49</td>
</tr>
<tr>
<td>Chemical composition</td>
<td>54</td>
</tr>
<tr>
<td>Peptide</td>
<td>57</td>
</tr>
<tr>
<td>Stability of biological activity</td>
<td>64</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>71</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>83</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>85</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table I</td>
<td>Effectiveness of each Step in the Purification Scheme in the Isolation of the Toxic Glycopeptide</td>
<td>19</td>
</tr>
<tr>
<td>Table II</td>
<td>Chemical and Physical Characteristics of the Toxin of <em>C. insidiosum</em></td>
<td>48</td>
</tr>
<tr>
<td>Table III</td>
<td>Metal Ion Quantification</td>
<td>55</td>
</tr>
<tr>
<td>Table IV</td>
<td>Chemical Constituents of the Toxic Glycopeptide from <em>C. insidiosum</em></td>
<td>56</td>
</tr>
<tr>
<td>Table V</td>
<td>R Values of some Sugar Acids Relative to the Carboxylic Acid from the Toxic Glycopeptide of <em>C. insidiosum</em></td>
<td>58</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Purification procedure for the toxin produced by <em>C. insidiosum</em></td>
<td>18</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Effect of toxin solutions on alfalfa and tomato seedlings</td>
<td>21</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Relationship of toxin concentration and the degree of wilt</td>
<td>23</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Effect of glycopeptide concentration as a function of time</td>
<td>25</td>
</tr>
<tr>
<td>Figure 5</td>
<td>First ammonium sulfate fractionation</td>
<td>28</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Second ammonium sulfate fractionation</td>
<td>30</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Toxin production in relationship to growth of <em>C. insidiosum</em></td>
<td>32</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Column chromatography of $^{14}$C-labelled toxin and blue dextran on Sepharose 4B</td>
<td>35</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Column chromatography of $^{14}$C-labelled toxin on Sepharose 2B</td>
<td>37</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Disc gel electrophoresis on the toxin</td>
<td>40</td>
</tr>
<tr>
<td>Figure 11</td>
<td>High voltage paper electrophoresis on the toxin</td>
<td>42</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Ultracentrifugation pattern of the purified glycopeptide</td>
<td>44</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Light-scattering (Zimm) plots for aqueous solutions of toxin</td>
<td>47</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Electron micrograph of freeze-etched replicas of toxin</td>
<td>51</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Absorption spectra of the toxin and apotoxin</td>
<td>53</td>
</tr>
<tr>
<td>Figure 16</td>
<td>The infrared absorption spectrum for the purified organic acid</td>
<td>60</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Two-dimensional electrophoresis-chromatography of the peptide moiety of the toxin</td>
<td>63</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Heat stability of toxin</td>
<td>66</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Stability of toxin to acid hydrolysis</td>
<td>68</td>
</tr>
<tr>
<td>Figure 20</td>
<td>The histogram showing the effect of various size compounds on tomato seedling stem strength</td>
<td>70</td>
</tr>
</tbody>
</table>
Phytotoxins produced by *Corynebacterium* spp. have been reported by several investigators. However, in the case of a toxin produced by *C. insidiosum*, the complete chemical, physical, and biological characterization has not been established. The present study demonstrates that the toxin of *C. insidiosum* is a glycopeptide with an empirical formula of C_{108}H_{226}O_{132}N based on one atom of nitrogen. Chemical analysis shows the toxin is 83.1% polysaccharide composed of mannose (1), glucose (5), galactose (5), and L-fucose (10), with trace amounts of rhamnose and an unidentified reducing sugar. An unknown organic acid comprises 8.8% of the toxin and a single peptide composed of lysine_2, arginine_1, aspartic acid_1, threonine_1, serine_1, glutamic acid_1, glycine_2, alanine_2, valine_2, leucine_2, and isoleucine_1 accounts for 2.57% of the toxin molecule. The toxin has a blue chromophore due to copper chelation at a concentration of 75 moles copper/mole toxin. Sepharose 2B column chromatography, analytical ultracentrifugation, and light scattering data indicates that the glycopeptide has a molecular weight of 5 x 10^6 daltons. Electron micrographs of the glycopeptide shows it to be an amorphous globule 80.0 - 90.0 mp in diameter. The toxin has a specific optical rotation of -166°, an intrinsic viscosity of 0.2307 d1/g, and decomposes at 260°. The toxin causes wilt in both leaves and stems of test plants and exhibits no specificity between alfalfa or tomato seedlings. Biological activity of the compound is dependent on time and on concentration. The toxin is stable to wide fluctuations in pH and still retains biological activity after heating to 121° for 2 hours. Partial acid hydrolysis results in a drastic reduction of biological activity. The mechanism of action of the toxic glycopeptide is unknown but it may cause plugging of xylem vessels or membrane damage.
INTRODUCTION

Bacterial wilt of alfalfa (*Medicago sativa* L.), caused by *Corynebacterium insidiosum* (McCull.) Jensen, is distributed widely in North America and is probably 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. The typical symptoms of this disease are reduction in vigor of the plants, yellowing and bleaching of the leaves, and finally wilting and death of the plants. The taproot shows a pale-brown discoloration of the outer woody tissue.

Phytotoxic polysaccharides from some plant pathogenic bacteria exert their effect on plants by causing wilting. Hodgson, Peterson, and Riker (22, 23) and Feder and Ark (12) reported that certain polysaccharides produced by plant pathogenic bacteria, *Agrobacterium tumefaciens* (Erw. Smith and Town) Conn, *Xanthomonas phaseoli* (Erw. Smith) Dowson, and *Erwinia carotovora* (Jones) Holland, were phytotoxic and possessed wilt-inducing properties. These toxins were non-specific and of high molecular weight.

Further work on toxins produced by *Pseudomonas solanacearum* (Erw. Smith) Erw. Smith (24), the causal agent of southern bacterial wilt of several plants, indicated that the culture filtrate contained pectolytic and cellulolytic enzymes. The heat-treated culture filtrate contained material which also caused wilting in tomato and
tobacco cuttings. This non-enzymatic, heat-stable material was thought to cause plugging in the vessels. Stewart, in 1894 (10), stated that wilting symptoms result from plugging of conductive elements by the pathogen or its products. The mechanical plugging theory has been supported by many investigators working on various bacterial wilt diseases, including wilt of cucumber caused by Erwinia tracheiphila, (Erw. Smith) Holland, wilt of sweet corn caused by Xanthomonas stewarti (Erw. Smith) Dowson, wilt of carnation caused by Pseudomonas caryophilli (Burkholder) Starr and Burkholder (7) and several others. However, in the case of Pseudomonas solanacearum, Hutchinson in 1931 (7) reported that wilting was due to alteration in cellular permeability produced by a toxin.

Recent studies on Corynebacterium spp. indicate that they produce phytotoxic polysaccharides in culture media and in the plant. Spencer and Gorin (39) reported that C. insidiosum and C. sepedonicum (Speckerman and Kotthoff) Skaptason and Burkholder, produced viscous polysaccharide solutions in culture media. Partial purification showed that the polysaccharide isolated from C. insidiosum induced wilt in alfalfa cuttings at a concentration of 0.002%. They detected 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. They postulated that the mechanism of action was plugging of xylem vessels. The acidic nature of these polysaccharides was ascribed to 4,6-O-(1\'-carboxyethylidene)-D-galactose (16). Patino-Mendez (32) isolated a polysaccharide from a culture of *C. michiganense* (Erw. Smith) Jensen, and detected fucose, galactose, glucose, mannose, rhamnose and an unidentified compound from the acid hydrolysate of the polysaccharide. He also found that a 0.01% solution of toxin caused wilting in tomato cuttings and postulated plugging as the mechanism of action. In both cases (32, 39) no attempt was made to characterize the toxins other than through their sugar components. Little attempt was made to study their physical properties, to demonstrate homogeneity, or to study their biological parameters. The complete physical, chemical, and biological characterization of these toxins was needed. Strobel (41, 43) reported that the toxin isolated from *C. sepedonicum* was glycopeptide in nature and possessed 3 sugars (glucose, mannose, L-fucose), 2 unidentified reducing compounds, an unidentified non-reducing compound, 9 amino acids, and 1 organic acid (2-keto-3-deoxygluconic acid). The purified toxin had a molecular weight of 21,400, was highly branched, and had an empirical formula of $C_{48}H_{96}O_{48}N$. He showed that the toxin lost biological activity after acid hydrolysis in 0.5N sulfuric acid, and he demonstrated the presence of the toxin in infected potato
plants by a number of methods including infrared and ultraviolet spectral analysis, X-ray crystallography, and high voltage paper and disc gel electrophoresis experiments. Strobel and Hess (42) proposed an alternate theory to mechanical plugging by demonstrating that membranes were being damaged by the toxin.

Johnson and Strobel (25) established the active site of the glycopeptide as the carboxyl group of the organic acid. Rai and Strobel (34, 35) reported the isolation of physiologically active compounds from C. michiganense. They reported that C. michiganense produces 3 biologically active compounds. These toxins also contained the sugar residues glucose, galactose, mannose, and fucose, and 10 amino acid residues. They postulated that the mechanism of action of these glycopeptides was not plugging but membrane damage. They also demonstrated the presence of the toxins in infected plants by serological methods.

The purpose of this thesis is fourfold: (a) to define the homogeneity of the toxic preparation produced by C. insidiosum, (b) to clarify which chemical residues constitute the purified toxin, (c) to measure the physical properties of the toxin, and (d) to determine and measure various biological parameters of the toxin.
Culturing: A culture of *G. insidiosum* (courtesy of F. I. Frosheiser, University of Minnesota) was maintained on a medium containing 1.5% glucose, 2.0% agar, 1.0% yeast extract dialysate, and 0.5% calcium carbonate (43). The culture was streaked and taxonomically identified as *G. insidiosum* (6). The organism produced wilt symptoms when inoculated by stem wounding into a 2 year old alfalfa plant.

Preparation and purification of toxin: The organism was grown on 250 ml of liquid medium in 1 liter flasks. The culture was aerated on a Psycrotherm Incubator Shaker at 20 rpm at 20° for 4 days. The toxin was isolated in a manner similar to that employed by Spencer and Gorin (15, 39), Strobel (41), and Rai and Strobel (34, 35). The culture was centrifuged at 20,000 xg for 10 minutes and the pellet discarded. The supernatant liquid was treated with 3 volumes of acetone (-15°) and the resulting precipitate pelleted by centrifugation at 10,000 xg for 10 minutes. The pellet was dissolved in 100 ml of distilled water and passed through a column of Dowex 1 (formate form), 2.5 X 5.0 cm, 200-400 mesh, rinsed with 50 ml of water, and the effluent passed through a column of Dowex 50 (H⁺ form), 2.5 X 5.0 cm, 200-400 mesh, and rinsed with another 50 ml of water. The effluent was collected and fractionated 2 times with ammonium sulfate according to the procedure of Falconer and Taylor (11). Ammonium sulfate (20-35%) precipitated the toxin which was removed by
centrifugation at 10,000 x g for 5 minutes 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.

**Optimum toxin production:** Aliquots of a growing culture were removed at various time intervals. The turbidity due to growth was measured with the Klett-Summmerson Photoelectric Colorimeter, and the amount of toxin production determined with the Beckman Laboratory Carbonaceous Analyzer. Toxin solution (20 μl) was injected into the instrument and the amount of carbon determined in ppm from a standard curve after calibration of the instrument with acetic acid standards.

**Biological assay:** The assay procedure used was similar to that employed by Johnson and Strobel (25). Tomato seedlings (Lycopersicon esculentum Mill.) cultivar Earliana and alfalfa seedlings (Medicago sativa L.) cultivars Ladak and Orca grown in vermiculite under continuous illumination for 10-14 days were severed at the crown region and placed in small test tubes containing solutions of the toxin buffered with 0.05M phosphate to a final pH of 7.0. The test tubes were mounted in a plexiglass holder surrounded by a transparent plexiglass box. The top of the box served as a reservoir for water to prevent heating by an artificial light source (100W bulb and a
circular fluorescent bulb) placed above it. After given time intervals the cotyledons were removed with a sharp razor blade and the strength of 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 5 stems.

Radioactive methods: *C. insidiosum* was grown on the standard liquid medium containing 25μC of D-galactose-1-14C (specific activity 3.0 mC/mM). The radioactivity in the toxin was determined in the liquid scintillation spectrometer (Model 6804 Nuclear Chicago). 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 2,5-diphenyl-oxazole and 100 mg of p-bis(5-phenyloxazolyl)-benzene 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: The toxin was taken to dryness and 1-5 mg dissolved in 1 ml of 0.05M Tris buffer at pH 7.0 in 40% sucrose. The sample was then fractionated with the buffer through columns of Sepharose 4B or Sepharose 2B. Fractions (1.5 ml) were collected with the aid of a drop counter, and radioactivity and total carbon determined in each fraction. Attempts to dissociate labelled toxin into subunits with 30% pyridine (36) or 8M urea followed by dialysis against distilled water for 48 hours was also followed by fractionation on Sepharose 2B.

Electrophoresis: High voltage paper electrophoresis was conducted in a manner similar to that employed by Strobel (42). One-hundred μg of toxin dissolved in 20 μl of water was distributed over 25 cm in the center of a 34 X 40 cm piece of Whatman #1 filter paper. The paper was pressed between a lower water-cooled plate and an upper surface covered by a flexible plexiglass plate. Pressure was exerted to the upper plate by a water filled rubber bag with a pressure of 6 pounds per square inch. The temperature of the water in the bag was 4°. 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 hours. The toxin was detected by the methods of Trevelyan (45), with 0.3% ninhydrin in ethanol, for radioactivity with the chromatogram scanner, and visually for the chromophore.
Disc gel electrophoresis was performed in 5.0% polyacrylamide gels with glycine-Tris buffer, pH 8.8. Fifty µg of labelled sample in 40% sucrose was subjected to 2.5 ma in each acrylamide tube (8). After electrophoresis for 30 minutes, the toxin was detected with aniline blue black for proteins, Schiff's base reagent for carbohydrates (33), for the chromophore, and by autoradiography after drying the gel according to the procedure of Herrick and Lawrence (20). Gels developed by each technique were scanned on a Joyce Chromoscan Densitometer.

**Analytical ultracentrifugation:** The sedimentation coefficient of the toxin was determined with a Spinco Model E analytical ultracentrifuge using the schlieren optical system on 1.0, 2.6, and 5.0 mg/ml solutions of toxin. Centrifugation was performed at 64,000 rpm and photographs were taken at 32 min intervals with a bar angle of 50°. The diffusion coefficient was determined by creating a synthetic boundary at 8,000 rpm with solutions of the same concentrations. The partial specific volume of the toxic polysaccharide was assumed to be 0.65 (14). The sedimentation coefficient, diffusion coefficient, and molecular weight calculations were calculated by the method of Schachman (38).

**Light scattering:** The molecular weight of the toxin was determined with the Brice Pheonix Universal Scattering Photometer (Series 2000)
using light with a wavelength of 4358 Å on a series of 6 solutions ranging from 0.035% - 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.

**Specific viscosity:** 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 was the intercept of the graph of specific viscosity/concentration as a function of concentration (38).

**Elemental analysis:** Carbon, hydrogen, and oxygen were determined by Schwarzkopf Microanalytical Laboratory, Woodside, New York; nitrogen was determined by the microkjeldahl technique. From the percentages of these the empirical formula was calculated.

**Chromophore:** Five ml of 0.1% solution of toxin was treated with 0.01M ethylenediamine tetraacetic acid (EDTA) at 50° for 15 minutes. The toxin and apotoxin were then dialyzed against distilled water for 48 hours after which the ultraviolet and visible spectrum (240-750 mp) were measured on a Cary Split-beam spectrophotometer using a 3 ml cuvette with a 1 cm light path.
A 0.1% solution was assayed to determine the presence of metal ions which were measured on a Jarell-Ash atomic absorption flame emission spectrophotometer by removing the ion with EDTA and then separating the EDTA-metal-ion complex from the toxin by dialysis.

**Acid hydrolysis:** Twenty ml of 0.5N sulfuric acid was added to a 50 ml flask containing 50 mg of toxin. The toxin was refluxed for 8-12 hours. 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 passed through a column of Dowex 50 (H⁺ form), 0.5 X 2.0 cm, 200-400 mesh, followed by a 10 ml rinse of water and passage through a column of Dowex 1 (OH⁻ form), 0.5 X 2.0 cm, 200-400 mesh. The Dowex 1 column was rinsed with 10 ml of water and the effluent dried under vacuum desiccation over P₂O₅. This was considered the neutral fraction. The organic acid fraction was obtained by eluting the Dowex 1 column with 10 ml of 6N formic acid. The eluant was dried and stored in an evacuated desiccator over P₂O₅.

The procedure used for amino acid hydrolysis was that of Moore and Stein (30). Toxin (5-10 mg) or peptide (0.25 mg) were placed in constant boiling hydrochloric acid, a crystal of phenol was added, and the tube sealed under vacuum. The tube was heated for 20 hours at 110°. Upon cooling the contents of the tube were dried by flash
evaporation at 50° and stored under vacuum desiccation over sodium hydroxide 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, 200-400 mesh. A 5 ml distilled water rinse followed, and the amino acids were then eluted with 6N hydrochloric acid and dried as above.

Chromatography: Separation and identification of sugar residues was done according to the method of Albersheim, et al. (1). The sugars were reduced to their alditols with sodium borohydride in 1N ammonium hydroxide. The reaction was stopped by the addition of a slight excess of glacial acetic acid and borate removed by 5 methanol evaporation. Acetic anhydride (1 ml) was added, the tubes containing the sugar alcohols sealed, and heated for 3 hours at 121°. The resultant alditol acetates were identified and their amounts estimated by gas-liquid chromatography. The best separation was attained using Gas Chrom P (100-120 mesh) coated with 0.2% poly(ethyleneglycol succinate), 0.2% of poly(ethyleneglycol adipate) and 0.4% of silicone XF 1150. The F and M gas chromatograph electrometer was set at range 100 and attenuation 4. A 6 foot column was used which was heated to 120° for 10 minutes after injection and then raised 1° per minute to 190°. The detector temperature was 250°, and the carrier gas flow rate was 30-50 cc per minute. The amount of each sugar was determined by a comparison with standard curves established with authentic sugars.
Separation of organic acid residues was done by one-dimensional, descending, paper chromatography, on Whatman #1 filter paper in the following solvent systems: (A) 1-butanol:acetic acid:water (4:1:5) v/v, (B) 1-butanol:pyridine:0.1N 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.

Identification and quantification of amino acid residues was done on a Beckman automatic amino acid analyzer.

Peptide: The β-elimination procedure described by Anderson, et al. (3) and Tanaka and Pigman (44) to remove peptides from the glycoprotein was used. Toxin (40 mg) was placed in 20 ml of 0.5N sodium hydroxide and incubated for 216 hours at 4°C in 0.3M sodium borohydride. The sodium borohydride reaction was stopped by the addition of a slight excess of acetic acid, and borate removed as methyl borate by 5 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, 200-400 mesh. The retained peptide was then eluted with 6N hydrochloric acid and dried over sodium hydroxide.

Peptide (1 mg) was spotted on a 34 X 40 cm piece of Whatman #1 filter paper and subjected to electrophoresis for 3 hours with 12.5 v/cm potential across the paper in formic acid (0.54M) acetic acid.
(1.11M) buffer, pH 1.85 (5). This same electrophoretogram was dried and chromatographed in solvent (A) for 10 hours in the other dimension. The peptide was detected with a modified 6-tolidine procedure. The electrophoretogram was passed through acetone:ethanol (1:1) solution and while still moist suspended over a solution of 1N hydrochloric acid and 0.5N potassium permanganate for 5 minutes on each side. The chromatogram was passed through a solution of 0.5M potassium iodide mixed 1:1 with saturated 6-tolidine in 2N acetic acid.

**NH$_2$-Terminal amino acids:** The dansyl technique, described by Gray and Hartley (18) was used to determine the NH$_2$-terminal amino acid in the peptide. One mg of the peptide and 25 mg of sodium bicarbonate were dissolved in 0.7 ml of water and the solution adjusted to pH 8.2. One-tenth ml of a solution containing 1 mg of dansyl chloride per ml of acetone was added and the solution was incubated for 12 hours at room temperature. After drying, followed by acid hydrolysis in 1.0 ml of constant boiling hydrochloric acid for 12 hours, the sample was compared against a series of standards by thin-layer chromatography on Absorbosil #5 in (a) benzene:pyridine:acetic acid (16:4:1) v/v, and in (b) toluene:pyridine:ethylene chlorohydrin:0.8N ammonium hydroxide (10:3:6:6) v/v, upper phase. The dansylated amino acids were detected by ultraviolet light.
Electron microscopic study: A 0.1% solution of toxin was frozen in Freon 22 in the Balzers Apparatus (BA 360M) and freeze-etched as described by Moor (29) and Moor and Muhlethaler (28). The replicas were prepared for examination in the electron microscope as described by Hess and Stocks (21) and examined on a Hitachi HS7 electron microscope.
EXPERIMENTAL RESULTS

**Purification and biological activity of toxin:** Figure 1 is a flow chart illustrating the procedure used to isolate the toxin. The toxin, represented by the vertical axis, was exocellular, precipitated with 3 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. On a specific biological activity basis the purification factor was 5.57X. The culture produced 1.25 g of toxin per liter.

The tomato seedling assay developed by Johnson and Strobel (25) was valid for use with this toxin. Tomato seedlings (Figure 2) wilted to 100 mg stem strength in a 0.25% solution of buffered toxin in 1 hour when measured on the wilt-o-meter, while both cultivars of alfalfa checked wilted to values of 200 mg. The resistant cultivar of alfalfa (Ladak) wilted to the same degree as the susceptible cultivar (Orca) which implied resistance to the disease is apparently not related to the toxin.

Wilt was dependent on concentration (Figure 3) with measurable wilt occurring in a 0.02% (4 X 10^-8 M) solution of toxin and maximal wilt in 0.15% (3 X 10^-7 M) solution of toxin in 1 hour. Wilt of the plant cuttings was a function of time and also concentration up to 0.25% toxin solutions (Figure 4).

The toxin still contained contaminating materials after passage
Figure 1. Purification procedure for the toxin produced by *C. insidiosum*. The toxin was isolated according to the procedure shown. Location of biological activity was determined using the tomato seedling assay. The toxin is exocellular, precipitates with acetone, is not retained on Dowex 1 or 50, and precipitates in the 25-35% fractions with ammonium sulfate.
Growth of Culture

Centrifuge out Cells and CaCO₃

Acetone Precipitation (dissolve ppt. in H₂O)

Dowex 1 (formate form)

Dowex 50 (H⁺ form)

(NH₄)₂SO₄ fractionation

0-25% (discard)

25-35%

35-100% (discard)

(NH₄)₂SO₄ fractionation

0-25% (discard)

25-35%

35-100% (discard)

TOXIN
Table I

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>
<th>Purification Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude culture supt.</td>
<td>6.78 g</td>
<td>418 mg</td>
<td>1.00</td>
</tr>
<tr>
<td>Acetone precipitate</td>
<td>2.62 g</td>
<td>285 mg</td>
<td>1.47</td>
</tr>
<tr>
<td>Dowex 1 treatment</td>
<td>2.17 g</td>
<td>238 mg</td>
<td>1.76</td>
</tr>
<tr>
<td>Dowex 50 treatment</td>
<td>1.93 g</td>
<td>219 mg</td>
<td>1.91</td>
</tr>
<tr>
<td>1st (NH₄)₂SO₄ ppt.</td>
<td>1.39 g</td>
<td>127 mg</td>
<td>3.29</td>
</tr>
<tr>
<td>2nd (NH₄)₂SO₄ ppt.</td>
<td>1.25 g</td>
<td>75 mg</td>
<td>5.57</td>
</tr>
</tbody>
</table>

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

b Defined as the amount of strength of a tomato hypocotyl remaining after 1 hour treatment in a 1 mg/ml solution of each preparation as measured by a wilt-o-meter. Buffer controls had a stem strength of 523 mg.
Figure 2. **Effect of toxin solutions on alfalfa and tomato seedlings.** The toxicity of the compound was measured on two cultivars of alfalfa; Ladak, a resistant cultivar; Orca, a susceptible cultivar, and one cultivar of tomatoes (Earliana). Stem wilt was measured with the wilt-o-meter in mg of pressure and the degree of wilt measured at time intervals of 0, 15, 30, 45, and 60 minutes. Each point represents the average of three readings of five cut stems.

- ● - Orca cont.
- ○ - Orca
- ■ - Tomato cont.
- □ - Tomato
- ▲ - Ladak cont.
- △ - Ladak
Figure 3. Relationship of toxin concentration and the degree of wilt. 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 0.05M phosphate buffer, pH 7.0. Loss of stem response was measured on the wilt-o-meter after 1 hour. Buffer control excised stems gave a wilt-o-meter value of 480 mg.
PLANT RESPONSE (mg Pressure)

BUFFER

TOXIN CONCENTRATION (%)
Figure 4. Effect of glycopeptide concentration as a function of time. Degree of wilt measured with a wilt-o-meter, in mg pressure, of 14 day old tomato seedling stems as a function of time in minutes.

- ▲ - control
- ○ - 0.02%
- △ - 0.05%
- □ - 0.10%
- ● - 0.25%
- ■ - 0.50%
through Dowex 1 and 50 ion exchange columns. Figure 5 demonstrates that when labelled toxin with a specific activity of 3500 dpm/mg was fractionated with ammonium sulfate, a labelled precipitate occurred at the 25-35% fraction. The biological assay established that this was the toxin. Considerable radioactivity, probably labelled contaminants, remained in the supernatant liquid. When the precipitate was redissolved in water, dialyzed, and fractionated again with ammonium sulfate (Figure 6), all radioactivity and biological activity precipitated in the 30-40% fraction.

**Optimum toxin production:** A culture flask (500 ml) was inoculated with *C. insidiosum* and, at time intervals of 0 through 10 days, 5 ml aliquots were removed. After removal of calcium carbonate by settling, bacterial growth was estimated with a Klett-Summerson photoelectric colorimeter (Figure 7). The growth curve is typical of that exhibited for most bacteria with maximal growth occurring at 6 days. From the same culture 2 ml samples were taken at 0, 1, 2, 3, 4½, 6, 7, 8, 9, and 10 days. The toxin was obtained from these samples, dissolved in 1 ml of distilled water, and 20 µl injected into the Beckman Laboratory carbonaceous analyzer. The total amount of toxin fluctuated with culture age (Figure 7) reaching a peak amount at 4½ days. When correlated with the growth curve maximal toxin production occurred at maximal growth. As the growth declined so did the amount of toxin.
Figure 5. First ammonium sulfate fractionation. Labelled toxin after Dowex treatment 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 measured. Radioactivity in dpm/ml was plotted against percent of saturation with ammonium sulfate.
Figure 6. Second ammonium sulfate fractionation. Labelled toxin was treated as described in Figure 2. All radioactivity and biological activity precipitated in the 30-40% of saturation fraction.
Toxin production in relationship to growth of *C. insidiosum*. Turbidity of a culture after removal of calcium carbonate by settling is plotted versus the age of culture in days after inoculation. The amount of purified toxin in μg C/ml produced is also plotted against the same time intervals.

- ▲ - growth curve
- ● - amount of toxin
Sepharose chromatography: Molecular exclusion column chromatography is frequently used as a criterion of homogeneity and in the estimation of molecular sizes of compounds. A 1 mg/ml solution (3500 dpm) of labelled toxin in 40% sucrose was layered on a Sepharose 4B column (1.5 X 45 cm) which fractionated molecules ranging in size from 3 X 10^5 to 3 X 10^6 daltons. The toxin was eluted with 0.05M Tris buffer at pH 7.0 and 1.5 ml fractions collected. It was located by radioactivity determinations. A solution of Pharmacia blue dextran with a molecular weight of 2 X 10^6 daltons was passed through the same column and assayed by measuring absorbance at 625 nm with a spectrophotometer. Figure 8 demonstrates that the toxin was excluded at the void volume, indicating its molecular weight was larger than 3 x 10^6 daltons. The blue dextran, however, exhibited an unexpected elution profile. A broad peak appeared at fraction 35 and a sharp peak at the void volume. This was explained in that the dextran was homogeneous but polydisperse and the tail of the broad peak was being excluded at the void volume.

A 2 mg/ml solution of the labelled toxin (7,000 dpm) was layered on Sepharose 2B which fractionates molecules ranging in size from 2 X 10^6 to 20 X 10^6 daltons. Fractionation conditions were the same as with Sepharose 4B. Figure 9 shows a pattern similar to blue dextran on Sepharose 4B. The two peaks, one at the void volume and the other
Figure 8. Column chromatography of \(^{14}C\)-labelled toxin and blue dextran on Sepharose 4B. One mg of purified toxin (dashed line) was layered on the column and eluted with pH 7.0 buffer. Fractions (1.5 ml) were collected and 0.2 ml aliquots counted by liquid scintillation spectroscopy. The total dpm in each tube is plotted against fraction number. Ten mg of blue dextran (solid line) was fractionated as above and the absorbance at 625 mp plotted against fraction number.
Figure 9. Column chromatography of $^{14}$C-labelled toxin on Sepharose 2B. Toxin preparation (2 mg/ml) was placed on the column and eluted with 0.05M Tris buffer, pH 7.0. Fractions (1.5 ml) were collected and 0.2 ml aliquots counted. Dpm per tube is graphed versus fraction number.
at fraction 28 probably represent the same material with the tail of
the second peak being excluded at the void volume. The molecular
weight of the toxin is somewhere between 2 and 20 million daltons
and it is probably polydispersed as was the blue dextran.

**Electrophoresis of toxin:** Figure 10 demonstrates that the toxin
migrated only into the top of the polyacrylamide gel. All label
remained at the top, as did the chromophore, the peptide portion,
and the carbohydrate moiety. Electrophoresis for longer periods of
time also failed to cause migration.

High voltage paper electrophoresis of the toxin failed to move
the toxin toward either pole. Figure 11 shows that at pH 7.0, 0.2M
phosphate buffer, neither the chromophore, carbohydrate, protein, nor
radioactivity migrated from the origin. Similar experiments at pH 2
and pH 10 failed to cause migration of the toxin.

**Ultracentrifugation:** Figure 12 is the ultracentrifugal pattern of a
2.6 mg/ml solution of the polysaccharide after sedimentation for 3
hours at 64,000 rpm. It indicated that the toxin was heterogeneous,
a spur peak appeared to the right of the major peak. The ultra sharp
characteristic of the slower sedimenting peak does not necessarily
indicate homogeneity but may reflect impurity or polydispersity (14,
38).
Figure 10.  

**Disc gel electrophoresis on toxin.** Labelled toxin (50 µg) was layered on each of four polyacrylamide gels (5%) and subjected to electrophoresis for 30 minutes at 2.5 ma/tube. Components of the toxin were located by autoradiography, and with a densitometer for the chromophore, the peptide (amido schwartz) and the carbohydrate (Schiff base).
AUTORADIOGRAM

Cm

0 1 2 3 4 5

BLUE CHROMOPHORE

AMIDO SCHWARTZ

SCHIFF BASE
Figure 11. High voltage paper electrophoresis on the toxin.

Toxin (100 μg) was subjected to electrophoresis at 22.5 v/cm for 2 hours in 0.2M phosphate buffer at pH 7.0. The electrophoretogram was developed with basic AgNO₃ (A), ethanolic ninhydrin (B), visual observation (chromophore), and radio-chromatoscan tracing (C).
Ultracentrifugation pattern of the purified glycopeptide. A 2.6 mg/ml aqueous solution of the toxin was centrifuged at 64,000 rpm with a bar angle of 50° for 2 hours. Sedimentation is left to right.
Calculations with a series of solutions (1.0, 2.6, and 5.0 mg/ml) showed that the sedimentation coefficient ($S_{20,w}$) was 1.533s. Synthetic boundary experiments at 8,000 rpm determined the diffusion coefficient ($D_{20,w}$) was $11.88 \times 10^{-10} \text{ cm}^2/\text{sec}$. Assuming a partial specific volume of 0.65 (14), calculation of the molecular weight was $4.83 \times 10^6$ daltons.

Light scattering: The Zimm plot from light scattering studies is illustrated in Figure 13. 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 $5.69 \times 10^6$ daltons. The extrapolation to zero angle (line b) and least square analysis to determine the $y$ axis intercept indicated a molecular weight of $4.33 \times 10^6$ daltons.

Elemental analysis: 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 $C_{105}H_{226}O_{132}N$ based on one atom of nitrogen (Table II). The toxin decomposed when heated to 260° in a Fisher-Johns melting point apparatus. The intrinsic viscosity was 0.2307 dl/g and its specific optical rotation was $\left[\alpha\right]_{5460} = -166^\circ$ using a Circle Polarimeter 0.01° with a mercury lamp.
Figure 13. Light scattering (Zimm) plots for aqueous solutions of toxin.

Lines a: results of extrapolation at constant $\theta$ to $c = 0$

Lines b: results of extrapolation at constant $c$ to $\theta = 0$
Table II

Chemical and Physical Characteristics of the Toxin of *C. insidiosum*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>( C_{108}H_{226}O_{132}N )</td>
</tr>
<tr>
<td>Estimated molecular weight</td>
<td>5,000,000 daltons</td>
</tr>
<tr>
<td>Intrinsic viscosity</td>
<td>0.2307 d1/g</td>
</tr>
<tr>
<td>Optical rotation ( [\alpha]_{5460} )</td>
<td>-166°</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>1.533 s</td>
</tr>
<tr>
<td>Melting point</td>
<td>decomposed at 260°</td>
</tr>
</tbody>
</table>
Electron microscopy: Electron microscopic examination after freeze-etching (Figure 14) demonstrates that two different size toxin molecules are present. Particles (P) were amorphous globules and 80.0 - 90.0 μm in diameter. Particles (P') appear as aggregates of the smaller particles.

Dissociation of toxin: The large molecular weight calculated by ultracentrifugation and light scattering data indicated the possibility that the large compound (Figure 14) might be composed of a series of subunits weakly bonded together. This was investigated by treating 1 mg (3500 dpm) quantities of toxin with either 30% pyridine or 8M urea for 1 hour and dialyzing to remove the pyridine or urea. These solutions were concentrated to 1 mg/ml and layered in 40% sucrose on a Sepharose 2B (1.5 X 43 cm) column. Fractions (1.5 ml) were collected using 0.05M Tris buffer, pH 7.0, and radioactivity detected. The toxin did not dissociate into lower molecular weight subunits with either treatment because the elution pattern from Sepharose 2B was the same as that of non-treated toxin.

Chromophore: A 0.1% solution of toxin absorbed maximally at 280 μm and 635 μm (Figure 15). The absorbance in the visible spectrum was attributed to the blue chromophore. Treatment with ethylenediamine tetraacetic acid (EDTA) followed by dialysis altered absorbance of
Electron micrograph of freeze-etched replicas of toxin. A 0.1% solution of glycopeptide was freeze-etched and examined with the electron microscope. Particles (P) observed and measured were 80.0 - 90.0 μ in size. Particles (P') appeared to be aggregates of the smaller particles (P). The field is magnified 84,000 X.
Figure 15. Absorption spectra of the toxin and apotoxin. The absorption spectra of the toxin (solid line) and toxin treated with EDTA (apotoxin) (dashed line) is plotted against wavelength in μm. The bar represents the change from ultraviolet to visible spectrum.
the toxin at 280 mp and destroyed absorbance at 635 mp. 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 III). 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° for 20 hours. 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.

Fractionation of the toxin and apotoxin on Sepharose 2B and assay with the Beckman Laboratory carbonaceous analyzer indicated that the removal of copper did not alter the elution profile.

Chemical composition: 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, galactose, and fucose (1:5:5:10) were the major sugar residues present in the toxin (Table IV). Two other reducing compounds were present in trace amounts, rhamnose, and an unknown. These carbohydrates
Table III

Metal Ion Quantification

<table>
<thead>
<tr>
<th></th>
<th>moles copper/mole toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA Control</td>
<td>0</td>
</tr>
<tr>
<td>Toxin</td>
<td>13</td>
</tr>
<tr>
<td>Apotoxin</td>
<td>0</td>
</tr>
<tr>
<td>EDTA-Cu</td>
<td>75</td>
</tr>
<tr>
<td>Ashed Toxin</td>
<td>89</td>
</tr>
</tbody>
</table>

Quantification of the amount of copper in the toxin, apotoxin, and EDTA after treatment of the toxin with 0.01M EDTA at 50° for 15 minutes followed by removal of EDTA-Cu by dialysis.
### Table IV

**Chemical Constituents of the Toxic Glycopeptide from *C. insidiosum***

<table>
<thead>
<tr>
<th>Compound</th>
<th>% total toxin</th>
<th>Ratio number&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugar residues</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-fucose</td>
<td>38.7</td>
<td>10</td>
</tr>
<tr>
<td>mannose</td>
<td>3.8</td>
<td>1</td>
</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&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Tr.</td>
<td>-</td>
</tr>
<tr>
<td>unknown</td>
<td>Tr.</td>
<td>-</td>
</tr>
<tr>
<td><strong>Organic acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>8.8</td>
<td>-</td>
</tr>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>Lys&lt;sub&gt;c&lt;/sub&gt;, Arg&lt;sub&gt;1&lt;/sub&gt;, Asp&lt;sub&gt;1&lt;/sub&gt;, Glu&lt;sub&gt;1&lt;/sub&gt;, Gly&lt;sub&gt;2&lt;/sub&gt;, Ser&lt;sub&gt;1&lt;/sub&gt;, Thr&lt;sub&gt;1&lt;/sub&gt;, Ala&lt;sub&gt;2&lt;/sub&gt;, Val&lt;sub&gt;2&lt;/sub&gt;, Leu&lt;sub&gt;2&lt;/sub&gt;, Ileu&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cations</strong></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Copper</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total = 94.6

<sup>a</sup> refers to the ratio of sugar residues in the total glycopeptide

<sup>b</sup> rhamnose and the unidentified reducing compound accounted for less than 1% of the total sugars

<sup>c</sup> refers to the number of residues of each amino acid in the peptide
accounted for 83.1% of the toxin.

The organic acid content of the toxin was determined by weighing dried eluate of the Dowex 1 column on a Cahn electrobalance. Table IV indicates the percentage of the organic acid which gave a positive test with acid-base indicator (4) and also was weakly reducing (45). The anion fraction consisted of one compound which failed to co-chromatograph with any of the organic acids checked (Table V). An infrared absorption spectrum of the compound (Figure 16) with the Beckman Microspec showed a strong broad absorption band between 2.9 and 3.3 μ, a smaller band at 3.7 to 4.0 μ, and other bands at 5.75, 6.0, 6.5, and 7.1 μ. Analysis of the acid by the thiobarbituric acid method (46) produced an alkali-labile chromophore with an absorption maximum at 549 mp. This reaction is characteristic of β-formylpyruvate which is formed from 2-keto-3-deoxyaldonic acids.

Amino acid analysis of the toxin with the Beckman automatic amino acid analyzer and integration of the peak areas of each amino acid indicated the composition of the glycopeptide to be Lys$_2$, Arg$_1$, Asp$_1$, Thr$_1$, Ser$_1$, Glu$_1$, Gly$_2$, Ala$_2$, Val$_2$, Leu$_2$, and Ileu$_1$ (Table IV). These residues accounted for 0.4% of the total weight of the toxin.

Peptide: β-elimination effectively removed the peptide from the toxin. Acid hydrolysis and analysis of the peptide indicated the same amino acids as were present in the intact toxin. No significant decrease in
Table V

$R_f$ 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$^a$</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-ketogluconic acid</td>
<td>0.33</td>
</tr>
<tr>
<td>2-ketogluconic acid</td>
<td>0.31</td>
</tr>
<tr>
<td>gluconic 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>

$^a$ chromatography systems given in text

$^b$ no determination
Figure 16. The infrared absorption spectrum for the purified organic acid. Micropellets were prepared by mixing the organic acid in KBr and were scanned at 0.2 inch per minute with 6.8 gain.
the amount of serine or threonine nor increase in valine or \( \alpha \)-amino-
butyric acid occurred after elimination and reduction. Electrophoresis
of the peptide in pH 1.85 formic-acetic acid buffer (5) at 12.5 \( V/cm \)
for 3 hours caused the peptide to migrate 6.9 cm toward the negative
pole. Descending paper chromatography in the opposite dimension in
solvent (A) for 10 hours indicated the peptide had an \( R_f = 0.238 \).
Two-dimensional electrophoresis-chromatography indicated a single
peptide was present in the toxin (Figure 17).

Chromatographic analysis of the dansylated peptide after acid
hydrolysis revealed that glycine occurred as the \( NH_2 \)-terminal amino
acid.

Four hundred mg of toxin treated with 0.5N sodium hydroxide was
passed through Dowex 1 (formate form), 0.5 X 1 cm, 200-400 mesh, and
the peptide eluted with 6N formic acid. After drying, salt was
removed by washing the dried residue with 10 ml of acetone containing
1\% of concentrated aqueous hydrochloric acid. Undissolved material
was removed by centrifugation, and the pellet washed 3 times with
acetone-hydrochloric acid, centrifuged and discarded. The combined
supernatant liquids were dried (40) and weighed on a Metler balance.
The peptide represented 2.57\% 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 and the peptide was 2.57\% of the
Figure 17. 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 hours at 10 mamp. After drying, the electrophoretogram was chromatographed in the other dimension in 1-butanol: acetic acid:water (4:1:5) for 10 hours. 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.
ELECTROPHORESIS

+ POLE

10, 8, 6, 4, 2, 0, 2, 4, 6, 8, 10 cm

CHROMATOGRAPHY

POLE

SOLVENT FRONT
toxin, there was 0.42% nitrogen in the toxin due to the peptide, thus all nitrogen in the toxin was present in amino acids. A peptide with the residues listed in Table IV would have a molecular weight of 1683 daltons. Considering the molecular weight of the toxin there would be 76 peptides per molecule of toxin.

**Stability of biological activity:** The toxin was stable to great fluctuations in temperature and pH. Figure 18 indicates that heating toxin solutions (0.25%) to 121° for 10 minutes and allowing them to cool did not affect biological activity in any way. Toxin which had been autoclaved for 2 hours at 121° 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 hours 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 (Figure 19). All of the biological activity was lost after 5 minutes refluxing in 0.5N sulfuric acid.

Dextran (2.5 X 10^5 daltons), blue dextran (2 X 10^6 daltons), toxin (5 X 10^6 daltons), and soluble starch (70 X 10^6 daltons) were made up to 0.25% solutions. When checked for stem wilt inducing abilities only the toxin significantly possessed this property (Figure 20).
Figure 18. **Heat stability of toxin.** Toxin solutions (0.25%) were heated for 10 minutes at 20°, 30°, 40°, 50°, 60°, 70°, 80°, 90°, and 121°, allowed to cool, and checked for biological activity. A toxin solution was also heated for 2 hours at 121°. Heated buffer solutions failed to induce wilt symptoms.
Stability of toxin to acid hydrolysis. Minutes of exposure of a 0.25% solution of glycopeptide to refluxing in 0.5N sulfuric acid (X axis) is plotted against mg stem strength of tomato stems after 1 hour (Y axis). After each exposure time an aliquot of the acid solution was removed and neutralized with an excess of barium carbonate. After removal of the precipitate the solution was dried and redissolved in a 0.25% solution of the partially hydrolyzed glycopeptide.
The histogram showing the effect of various size compounds on wilt of tomato seedlings. Solutions (0.25%) of dextran ($2.5 \times 10^5$ daltons), blue dextran ($2 \times 10^6$ daltons), toxin ($5 \times 10^6$ daltons), and amylopectin ($70 \times 10^6$ daltons) were checked for ability to induce stem wilt in 1 hour in tomato seedlings.
DISCUSSION

After purification 1.25 g of toxin was isolated from a 1 liter culture of *C. insidiosum*. This yield was in sharp contrast to the 15 mg toxin/l attained from *C. sepedonicum* (42) and the 18 mg toxin/l from *C. michiganense* (34, 35) and in the same range as that reported by Spencer and Gorin (2 g toxin/l) (39). The final step in purification (Figure 5) implied that contaminating radioactive substances were present after ion-exchange chromatography. Based on this information the toxin isolated by Spencer and Gorin (15, 39) from *C. insidiosum* was probably a heterogeneous mixture of compounds. The purification attained (5.57X) indicated that most of the exocellular material (18%) produced by the organism was toxin (Table I).

The optimum incubation time to obtain maximal amount of toxin (4½ days) corresponded to maximal growth of the bacterium (Figure 7). The organism evidently manufactured toxin during exponential growth and then utilized it as either an energy or carbon source during maximal growth. As the toxin decreased the culture entered death phase.

Four experimental tests indicated that the substance after the final step in purification was homogeneous; (1) all labelled material precipitated during the last ammonium sulfate fractionation (Figure 6); (2) all markers of the toxin (radioactivity, chromophore, carbohydrate, protein) migrated into the top of the gel and did not
penetrate further during disc gel electrophoresis (Figure 10), and no other compounds were seen migrating through the gel; (3) in high voltage paper electrophoresis experiments the toxin did not migrate from the origin when checked in a wide range of buffers (Figure 11), no other radioactive, reducing, or ninhydrin positive compounds migrated; and (4) Sepharose 2B chromatography demonstrated 2 peaks in the toxin after elution (Figure 9). This did not necessarily indicate 2 different size compounds. The elution profile of blue dextran on Sepharose 4B demonstrated a similar pattern (Figure 8). This peak pattern may be explained by assuming the later peak was tailing on the column during elution. Thus, this later peak represented the major portion of the compound being fractionated while the peak at the void volume was the tail being grouped and excluded.

One criterion of heterogeneity in the final preparation was shown by ultracentrifugation (Figure 12) in that a minor peak appeared indicating two substances. The slower sedimenting peak represented approximately 10 times more substance than the faster moving peak. These two peaks were never completely resolved even after centrifugation at 64,000 rpm for 7 hours. Further purification was not attempted. The two substances were probably both extremely large, relatively uncharged, and seemed to differ only slightly in densities. Asymmetric or flexible molecules interact hydrodynamically and with
large flexible molecules, extensive physical interaction, i.e., intertwining and entanglement of the threads can result in an exceedingly sharp (hypersharp) boundary which may conceal extensive polydispersity or in these experiments actual heterogeneity (14, 38).

The heterogeneity indicated by ultracentrifugation might be explained by reexamination of the electron micrograph of the freeze-etched toxin (Figure 14). Two particles were observed; \( P \), a particle 80.0 - 90.0 \( \mu \) in size and \( P' \), larger in size than \( P \) but appearing to be an aggregate of the smaller particles. If this was the case the faster sedimenting peak could be the larger particles \( (P') \). The "hypersharp" boundary of the first peak probably reflects polydispersity, common in large polysaccharide substances.

The freeze-etched preparation of the toxin presents an interesting view of a polysaccharide. The structure of each particle observed in Figure 14 closely approximates the shape while in solution. Artifact formation due to fixing and staining is minimal. The shape of the glycopeptide differs considerably from the shape of another protein polysaccharide obtained by Rosenberg, Hellman, and Kleinschmidt (37). Their preparations were dried and stained with uranyl acetate. These protein polysaccharides then appeared as rigid filaments with numerous branching filaments. Freeze-etching employs mild conditions and thus results in an accurate appearance of the polysaccharide.
The toxin appeared to be an extremely high molecular weight compound. Estimation of its molecular weight by fractionation on Sepharose 2B implied it was between $2 \times 10^6$ and $20 \times 10^6$ daltons. Lack of sufficiently large molecular weight standards failed to permit an estimation of molecular weight with the gel sieving technique. A molecular weight of $4.83 \times 10^6$ daltons was estimated by ultracentrifugation studies and a molecular weight of approximately $5 \times 10^6$ daltons by light scattering. These weight average molecular weights differ considerably from those reported earlier by Strobel \cite{41} for \textit{C. sepedonicum} toxin ($2 \times 10^5$ daltons) and Rai and Strobel \cite{34,35} for the three toxic fractions of \textit{C. michiganense} after Sephadex G-200 chromatography (Fraction I $\geq 2 \times 10^5$, Fraction II = $1.29 \times 10^5$, and Fraction III = $3.53 \times 10^5$ daltons). The toxic Fraction I may be a compound related to this toxin. Its molecular weight estimation was made from Sephadex G-200 which excluded molecules larger than 200,000. Thus, Fraction I may be as large as the toxin reported here.

Considering the large molecular size of the toxin, dissociation of the compound into lower molecular weight subunits was attempted. Treatments with 8M urea or with 30% pyridine, or removal of copper, failed to reveal subunit formation when fractionated on Sepharose 2B. The molecule appeared to be one large covalently linked polymer and
not a series of smaller polymers.

The specific viscosity of the toxin (0.2307 dl/g) compared favorably with Fraction I (0.225 dl/g) for *C. michiganense* while differing from the toxin of *C. sepedonicum* (0.125 dl/g). Intrinsic viscosity values for these polysaccharides are frequently proportioned to molecular weight implying that this toxin was larger than Fraction I reported by Rai and Strobel (34, 35). The optical activity of the toxin differed significantly from that reported by Gorin and Spencer (15) for *C. insidiosum* (-98°). The heterogeneity of the toxin prepared by Gorin and Spencer undoubtedly altered their value.

The broad absorption peak at 635 µm obtained by ultraviolet and visible absorption experiments (Figure 15) was the visible chromophore of the toxin. Treatment with EDTA indicated a metal ion chelated into the toxin while quantification (Table III) 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. (26, 27) who found and chemically characterized a low molecular weight pigment of *C. insidiosum* which was also blue. Since this incompatibility of results exists, there may be two pigments present in *C. insidiosum* cultures, a large molecular weight substance containing copper and a small non-copper 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 small pigment. This, however, is not consistent with the presence of copper in the toxin or the loss of color with EDTA treatment (Table III).

Chemical characterization of the toxin suggested this compound was chemically closely related to the phytotoxic glycopeptides from other Corynebacterium spp. Patino-Mendez (32) 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 (34) and from C. sepedonicum (41, 43). Gorin and Spencer (15) working with C. insidiosum reported 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 Spencer and Gorin (39) and Strobel (43).

An organic acid from the toxin preparation of C. insidiosum described by Gorin and Spencer (16) 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 was not 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 as pyruvic acid has strong absorption bands at 6.1 and 7.35 μ while the organic acid did not (Figure 16). Similarly, the unknown organic acid possess -CH and -OH stretch which pyruvate lacked. Reaction of each organic acid with p-anisidine (4) was different since pyruvate gave a bright yellow spot while the unknown organic acid gave a dull brown product. Strobel (43) described 2-keto-3-deoxygluconic acid (KDG) from the toxic glycopeptide of C. sepedonicum and Ghalambor, Levine, and Heath described 3-deoxy-D-manno-octulosonic acid (KDO) from cell wall lipopolysaccharides of Escherichia coli (13). The unknown organic acid appeared similar to KDG and KDO because it produced an alkali-labile chromophore by the thiobarbituric acid method and because of its infrared spectrum, but it did not co-chromatograph with KDG or any of the other organic acids (Table V). Comparison of the $R_f$ values listed for KDO (43) and those obtained for the unknown organic acid (Table V) 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 isolation and demonstration of a single peptide from the glycopeptide is shown in Figure 17. This indicates that the amino acids of the toxin are linked together by peptide bonds. Further evidence that the amino acids are covalently bonded together is that dansylation data indicated a sole \( \text{NH}_2 \)-terminal amino acid, glycine. Acid hydrolysis of the peptide indicated the same amino acid residues were present as those in the untreated toxin. These data are also consistent with the concept that all of the amino acids are covalently bonded to one peptide. After \( \beta \)-elimination and reduction, the amounts of either serine, threonine, aspartic acid, or glutamic acid should have decreased in amount with a concomittant increase in alanine, \( \alpha \)-amino butyric acid, homoserine, or \( \alpha, \beta \)-amino hydroxy-n-valeric acid (3, 31), but in these experiments they did not. A possible explanation for this inconsistency is that sample sizes were small and the peptide represented such a small percentage of the toxin the accuracy of the amino acid analyzer may have suffered. Lysine\(_2\), arginine\(_1\), aspartic acid\(_1\), threonine\(_1\), serine\(_1\), glutamic acid\(_1\), glycine\(_2\), alanine\(_2\), valine\(_2\), leucine\(_2\), and isoleucine\(_1\) were present in the acid hydrolysate of the toxin (Table IV). Integration of peak areas after amino acid analysis of the intact glycopeptide and quantification of the total weight of peptide showed the peptide represented 0.4% of the total glycopeptide. Dry weight determinations of sodium hydroxide
treated toxin suggested the peptide was 2.57% of the toxin. Amino acid hydrolysis in the presence of large amounts of carbohydrate leads to humin formation and a loss of amino acids, a Maillard reaction (17, 40). This is the best explanation for the disparity of the two peptide values. Therefore the estimation utilizing the dry weight is probably the more reasonable value. This value is substantiated when compared with the amount of nitrogen in the toxin. Kjeldahl analysis of the toxin (0.32% N) with a peptide which is 16.6% N indicated that 1.9% of the toxin was peptide. A peptide comprising approximately 1.89 - 2.57% of the toxin and composed of the amino acids listed in Table IV would imply that there would be 56 - 76 peptides per molecule of toxin.

The presence of amino acids confirms the observations of Strobel (43) and Rai and Strobel (34) with the glycopeptides of C. sepedonicum and C. michiganense. Gorin and Spencer (15) 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%).

Biologically the toxin appeared non-specific, affecting tomatoes as well as alfalfa (Figure 12). Other plants such as sanfoin, milk-vetch, and potatoes were also wilted by the toxin. A similar non-specificity was reported by Rai and Strobel (35) with the toxin from
C. michiganense and by Hodgson, Peterson, and Riker (22, 23) using Agrobacterium tumefaciens, Xanthomonas phaseoli, and Erwinia carotovora. The non-specificity of these toxins may show that the effect of the toxins was a mechanical or physical disruption of host tissue. Similarly, the non-specificity of this toxin may suggest that resistance to bacterial wilt of alfalfa is not resistance to the toxin. Disease resistance is probably due to other factors such as hardier roots, which undergo less winter damage and thus deny the bacterium a portal of entry, or that bacterial infection may be walled off.

Wilt induced by the toxin was dependent on concentration and on time. Rai and Strobel (35), Strobel (41), and Johnson and Strobel (25) demonstrated these dependencies with the toxins of other plant pathogenic corynebacteria. The toxins of other corynebacteria were also heat resistant and biological activity was rapidly destroyed by partial acid hydrolysis. The toxin from C. insidiosum demonstrated these parameters while also being stable to wide fluctuations of pH. A covalently-linked structure with little tertiary or quaternary structure necessary for biological activity appeared reasonable.

The mode of action of this toxin is unknown. Figure 20 demonstrates that neither smaller nor larger substances, bracketing the molecular weight of the toxin, caused stem wilt. The indication was that plugging did not occur, as amylopectin (70 X 10^6 daltons) had
no effect on stem wilt. Similar size substances such as blue dextran (2 X 10^6 daltons) did not wilt stems suggesting that the toxin had an active site or sites involved in the wilt process. Electron microscopic studies, electrolyte measurement, and dye transport studies by Rai and Strobel (35) and Strobel and Hess (42) demonstrated with phytotoxins from C. michiganense and C. sepedonicum that membrane damage occurred. The active site was associated with carboxyl groups of 2-keto-3-deoxygluconic acid on the toxic glycopeptide of C. sepedonicum (25). C. insidiosum also contained a significant percentage (8.8%) of an unknown acid which may be related to the toxicity of the glycopeptide in the same manner as 2-keto-3-deoxygluconic acid is to C. sepedonicum. The chemical similarities of this toxin with the other phytotoxins of C. sepedonicum and C. michiganense may indicate a similar mode of action.

The size of the glycopeptide (250 X the molecular weight of C. sepedonicum toxin) may in fact cause plugging of the vascular bundles as has been postulated by several researchers with many examples (7, 10, 19, 24, 32, 39).

The electron micrograph of the freeze-etch preparation indicated the toxin molecules were 80.0 - 90.0 µm in diameter which may plug xylem vessels. From these data (chemical structure and size measurements) it is impossible to ascribe a mechanism of action.
The biological significance of this compound in vivo was not investigated, but other phytotoxins 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 L-fucose from infected alfalfa plants by Spencer and Gorin (39) is presumptive evidence that such a compound is biologically important in the disease process.
SUMMARY

A single exotoxin possessing the capacity to wilt excised stem cuttings was isolated from cultures of Corynebacterium insidiosum and purified, and the physical, chemical, and biological properties measured. The organism produced 1.25 g toxin/1 of culture and approximately 18% of the exocellular material produced was toxin. Optimal toxin production occurred after 4½ days of incubation coinciding with maximal bacterial growth. The toxin wilted alfalfa as well as tomato stems; stem wilt was dependent upon time and upon concentration with measurable wilt occurring in $4 \times 10^{-8}$ M solutions in 1 hour. The toxin appeared homogeneous during Sepharose 2B chromatography, ammonium sulfate fractionation, disc gel and high voltage paper electrophoresis. Heterogeneity was indicated by analytical ultracentrifugation.

The molecular weight as estimated by ultracentrifugation, light scattering, and column gel chromatography was approximately $5 \times 10^6$ daltons. Electron micrographs of freeze-etched replicas of the toxin showed an amorphous globule with a diameter of 80.0 - 90.0 μm. The toxin did not dissociate into small subunits when treated with 8M urea or 30% pyridine. The empirical formula was $C_{108}H_{226}O_{132}N$, intrinsic viscosity 0.2307 dl/g, and the specific rotation $-166^\circ$.

Strong absorbance of the purified toxin at 635 μm gave the toxin a blue chromophore, due to the chelation of copper at a concentration
of 75 moles of copper/mole of toxin. Acid hydrolysis and chromatographic analysis of the purified toxin revealed that mannose, glucose, galactose, rhamnose, fucose (L-form), and an unidentified reducing compound comprised 83% of the toxin. An unknown organic acid which appeared chemically similar to keto-deoxy organic acids comprised 8.8% of the toxin. Lysine, arginine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, leucine, and isoleucine were bonded together forming a single peptide with glycine as the sole NH₂-terminal amino acid. This single peptide composed 2.57% of the toxin indicating there were 76 peptides per molecule of purified glycopeptide.

The toxin was heat resistant, acid labile and water soluble, and in vitro experiments simulated in vivo disease symptomology. An explanation of the mechanism of action was not postulated. Wilt symptoms may be due to plugging of xylem vessels or to membrane damage caused by an active site(s) on the glycopeptide.
LITERATURE CITED


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Chemical and biological properties...