Partial Characterization of Glycopeptides from Culture Filtrates of *Fulvia fulva* (Cooke) Ciferri (syn. *Cladosporium fulvum*), the Tomato Leaf Mould Pathogen

By J. M. DOW AND J. A. CALLOW

Department of Plant Sciences, Baines Wing, University of Leeds, Leeds LS2 9JT

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Culture filtrates of races 0, 4 and 1.2.3. of *Fulvia fulva* were fractionated by sequential gel chromatography on Bio-Gel P-2, ion-exchange chromatography on DEAE-cellulose and affinity chromatography on concanavalin A-Sepharose 4B. The fractions were characterized for their carbohydrate, phosphate and protein contents and sugar compositions. D-Galactose, D-glucose and D-mannose were the major sugars with much smaller amounts of D-glucuronic acid, D-galactosamine and D-glucosamine. Phosphate was present as a diester. The carbohydrate was present in a family of glycopeptides of disparate molecular weight, charge and sugar composition. The molecular organization of these polymers was further investigated by mild acid and alkali degradation and by acetolysis of the polysaccharide remaining after these treatments. A possible structure for the polymers is suggested.

INTRODUCTION

All fungi synthesize and secrete polysaccharides and glycoproteins into their walls and growth media (Bartnicki-Garcia, 1968; Gander, 1974). Recently attention has been paid to the possible rôle of such polymers as elicitors of phytoalexin accumulation and determinants of specificity in host-phytopathogen interactions (Albersheim & Anderson-Prouty, 1975; Keen, 1975; Ayers *et al.*, 1976*a,b,c*; Callow, 1977; Stekoll & West, 1978). In particular, polymers from culture filtrates of *Fulvia fulva* (syn. *Cladosporium fulvum*) have been suggested to have a rôle in the determination of specificity in the gene-for-gene interaction between this pathogen and the host tomato (Van Dijkman & Kaars Sijpesteijn, 1971, 1973). The active moieties of these polymers are probably carbohydrate in nature (Dow & Callow, 1979). In this paper, we report the characterization of glycopeptides from culture filtrates of three different races of *F. fulva* with particular reference to the carbohydrate moiety. This work is part of a series of investigations into the biochemical nature of varietal specificity in the *F. fulva*-tomato system. The possible rôle of these polymers in the host-pathogen interaction has been discussed elsewhere (Dow & Callow, 1979).

METHODS

Growth of the organism. As a precaution against loss of pathogenicity, *Fulvia fulva* races 0, 4 and 1.2.3. were maintained on potato-dextrose-agar medium at 4°C in the dark. Conidia were harvested from cultures grown at 24°C in the dark on potato-dextrose-agar. For labelling, fungi were grown in 125 ml medium in 1 l Erlenmeyer flasks. Two media were used: medium A contained (per litre) 10 g glycerol, 2 g Casamino acids, 1.5 g MgSO₄·7H₂O, 0.1 g KCl, 2 mg ZnCl₂ and 2 mg MnSO₄·4H₂O in tap water buffered to pH 6.0 with 2.5 mM-phosphate buffer; medium B contained glucose (2 g l⁻¹) instead of glycerol. Medium A was designed to allow the exclusive labelling of carbohydrate from D-[U-¹⁴C]glucose as described

Fig. 1. Protocol for the fractionation of culture filtrates of *Fulvia fulva*.

previously (Dow & Rubery, 1977). Media and flasks were sterilized together by autoclaving at 120°C for 20 min. D-[U-¹⁴C]Glucose was added through a presterilized Millipore filter (0.22 µm diam. pore) before media were inoculated with a washed conidial suspension of the fungus to a final concentration of 10⁵ ml⁻¹. Flasks were shaken in an orbital incubator (200 rev. min⁻¹) at 24°C. Radiochemicals were from The Radiochemical Centre, Amersham.

Fractionation of culture filtrates. After 21 d growth, the fungus was removed from the culture fluid by passage through Whatman GF/A glass-fibre paper discs. Culture filtrates were fractionated sequentially by gel chromatography on Bio-Gel P-2, ion-exchange chromatography on DEAE-cellulose and affinity chromatography on concanavalin A-Sephrose 4B (see Fig. 1).

Hydrolysis conditions, electrophoresis and analytical methods. These were as described previously (Dow & Rubery, 1977) except that acidic hydrolysates were neutralized with BaCO₃.

Acetolysis of fractions. Acetolysis was carried out at 37°C for 16 h by the method of Stewart *et al.* (1968). De-acetylated oligosaccharides were dissolved in water and fractionated by gel filtration on a column of Bio-Gel P-2 (bed dimensions 2 × 96 cm).

Liquid scintillation spectrometry. Samples were prepared as described previously (Dow & Rubery, 1977). Radioactivity was determined on a Unilux III liquid scintillation counter.

Other analytical procedures. Colorimetric estimation of sugars was done by the method of Dubois *et al.* (1956). Total organic phosphorus was estimated by the method of Ames (1966). Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Mild acid hydrolysis was carried out in 0.1 M-formic acid, pH 2.4, for 1 h at 100°C. Mild alkaline hydrolysis was carried out in 0.5 M-NaOH for 24 h at 20°C.

Concanavalin A-Sephrose 4B chromatography. The column (bed dimensions 0.5 × 2.5 cm) was equilibrated with 10 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-MnCl₂, 10 mM-MgCl₂ and 10 mM-CaCl₂. Fractions in this buffer were allowed to equilibrate with the column for 1 h at 20°C. The column was eluted with the equilibration buffer to yield the unbound fraction and then with a gradient of 0 to 50 mM-α-methyl mannoside in the same buffer to yield the bound fraction.

Fig. 2. Sephadex G-150 gel filtration of the high molecular weight fraction of race 0 grown in medium A (carbohydrate labelled exclusively) before (○) and after (●) treatment with 0.5 mg pronase ml⁻¹ (Sigma; 24 h at 24°C, pH 7.0). The column was equilibrated and eluted with 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl. Fractions (5 ml) were counted as described in Methods. The Blue Dextran (BD) and CuSO₄ elution volumes are shown.

RESULTS

The protocol for the fractionation of culture filtrates is shown in Fig. 1. Analyses of the carbohydrate and amino acid compositions of the high molecular weight fraction of filtrates of medium A showed that the carbohydrate moieties were labelled exclusively from D-[U-¹⁴C]glucose and that amino acids were unlabelled. The specific activities of the different neutral sugars were identical under the labelling conditions employed. The high molecular weight fraction from all three races contained D-galactose, D-glucose and D-mannose as major constituents with much smaller amounts of D-glucuronic acid, D-glucosamine and D-galactosamine.

Gel filtration of the high molecular weight fraction on Sephadex G-150 equilibrated and eluted with 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl, showed a disperse peak of carbohydrate at an estimated molecular weight of 70000 (Fig. 2). After treatment of this fraction with pronase, the carbohydrate-containing material was eluted from the column in a larger volume (Fig. 2) giving preliminary evidence that this carbohydrate was covalently bound to protein in a glycoprotein or glycopeptide. Similar results were found for all three races.

Ion-exchange chromatography of the high molecular weight fraction

Although this fraction eluted as a single, though disperse, peak through Sephadex G-150, ion-exchange chromatography on DEAE-cellulose resolved it into several components of different ionic charge (Fig. 3). Three sub-fractions were defined eluting at 10 mM-NH₄HCO₃ (fraction I), 10 to 100 mM-NH₄HCO₃ (fraction II) and 100 to 500 mM-NH₄HCO₃ (fraction III). The distribution of carbohydrate, protein and phosphate and the sugar analyses of these fractions from medium A are shown in Tables 1 and 2. Fractions I and II each contained little protein and approximately one-third of the total carbohydrate. The molar ratio of phosphate to carbohydrate residues increased from fraction I to fraction III in all races. Treatment with pronase did not affect the distribution of carbohydrate between these fractions indicating that the degree of phosphorylation of carbohydrate moieties may have been responsible for the heterogeneity on ion-exchange chromatography.

Fig. 3. Ion-exchange chromatography of the high molecular weight fraction of race 0 on DEAE-cellulose at pH 7.8. The column (1.5×6 cm) was equilibrated with 10 mM- NH_4HCO_3 , then eluted successively with 10 mM- NH_4HCO_3 (30 ml), a gradient of 10 to 100 mM- NH_4HCO_3 (total gradient volume 50 ml) and a gradient of 100 to 500 mM- NH_4HCO_3 (total gradient volume 50 ml). Fractions (5 ml) were counted as described in Methods. $\circ$, Radioactivity; $\bullet$, NH_4HCO_3 concentration (estimated conductimetrically).

Table 1. *Distribution of carbohydrate, protein and phosphate amongst fractions I, II and III (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium A)*

The amounts of protein and carbohydrate in each fraction are given as percentages of the total present in the high molecular weight fraction. The amount of phosphate is given as the molar ratio of phosphate to sugar residues as glucose equivalents.

Fraction		Race of <i>F. fulva</i>		
		0	4	1.2.3.
I	Protein	11.0	10.3	10.0
	Carbohydrate	31.2	31.6	38.1
	Phosphate	1:81	1:80	1:77
II	Protein	11.8	10.9	11.1
	Carbohydrate	30.0	36.7	44.6
	Phosphate	1:34	1:30	1:32
III	Protein	77.2	78.8	78.9
	Carbohydrate	38.8	31.7	17.3
	Phosphate	1:17	1:17	1:18
Total in high mol. wt fraction (μg)	Protein	2233	2102	1940
	Carbohydrate	4251	5043	4413

Concanavalin A-Sepharose 4B chromatography of fraction I

Concanavalin A-Sepharose 4B divided fraction I into a fraction which did not bind to, or had only a low affinity for, the column (fraction IA) and a fraction which bound to the column but which could be eluted with α -methyl mannoside (fraction IB) (Fig. 4). When the unbound fraction was re-applied to the column, none of the radioactivity bound. Fractions IA and IB had very different carbohydrate compositions (Table 3) but both contained small amounts of protein (approximately 10%). Fractions II and III could be similarly divided by this technique (data not shown). The sugar compositions of fractions from culture filtrates of fungus grown in medium B differed qualitatively from those found in glycerol-grown cultures, but equivalent fractions from all three races were similar (Tables 4 and 5).

Table 2. Sugar compositions of fractions I, II and III (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium A)

The amount of each sugar in a fraction is given as a percentage of the total radioactivity (d.p.m.) in the sugars in that fraction.

Sugar	Percentage of total radioactive sugar								
	Fraction I			Fraction II			Fraction III		
	0	4	1.2.3.	0	4	1.2.3.	0	4	1.2.3.
GlcA	1.0	1.0	0.7	2.0	1.1	2.0	0.2	trace	trace
GalN	5.1	0.5	trace	6.8	0.8	trace	5.0	trace	trace
GlcN	trace	0.5	trace	trace	trace	4.6	trace	trace	trace
Gal	7.5	14.0	16.3	17.4	19.3	22.3	15.3	25.2	30.7
Glc	7.0	13.2	13.9	9.2	10.1	3.6	10.5	14.2	14.9
Man	79.4	70.8	69.1	64.6	68.7	67.5	69.0	60.6	54.4
Total radioactivity (d.p.m.)	1667	2043	3102	1328	4132	2205	1396	2990	1994

Fig. 4. Concanavalin A-Sepharose 4B affinity chromatography of fraction I of race 1.2.3. (carbohydrate was labelled exclusively in this fraction from medium A). ○, Radioactivity; ●, α -methyl mannoside concentration, estimated by the method of Dubois *et al.* (1956). The elution position of galactose (unbound) is shown.

These results suggest that *F. fulva* synthesizes a family of polymers which differ in their detailed carbohydrate compositions and in the degree to which they are phosphorylated. All of the derived fractions probably contain a population of molecules of similar, but not identical, characteristics.

Nature of phosphorus in fractions I, II and III

Acid phosphatase failed to release inorganic phosphate from unlabelled or ^{32}P -labelled polymers unless they were previously treated with phosphodiesterase or mild acid, indicating that the phosphorus was probably in a diester form in the polymers. Enzyme treatments were carried out under the conditions of San Blas & Cunningham (1974).

Table 3. *Sugar compositions of fractions IA and IB (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium A)*

The amount of each sugar in a fraction is given as a percentage of the total radioactivity (d.p.m.) in the sugars in that fraction.

Sugar	Percentage of total radioactive sugar					
	Fraction IA			Fraction IB		
	0	4	1.2.3.	0	4	1.2.3.
GlcA	1.0	trace	0.7	1.0	trace	trace
GalN	trace	trace	trace	0.2	trace	trace
GlcN	trace	trace	trace	0.2	trace	trace
Gal	10.0	10.2	8.3	9.4	12.3	9.5
Glc	30.3	32.4	28.8	10.5	4.0	5.3
Man	58.7	57.4	62.2	78.7	83.7	85.2
Total radioactivity (d.p.m.)	4064	8186	6268	5664	5980	2018
Total radioactivity (% radioactivity in fraction I)	40.9	39.9	54.4	59.1	60.1	45.6

Table 4. *Sugar compositions of fractions I, II and III (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium B)*

The amount of each sugar in a fraction is given as a percentage of the total radioactivity (d.p.m.) in the sugars in that fraction.

Sugar	Percentage of total radioactive sugar								
	Fraction I			Fraction II			Fraction III		
	0	4	1.2.3.	0	4	1.2.3.	0	4	1.2.3.
GlcA	1.5	2.1	1.7	2.5	3.3	2.8	2.0	3.2	2.1
GalN	trace	trace	trace	trace	trace	trace	trace	trace	trace
GlcN	0.6	0.4	0.4	0.3	0.2	0.3	0.1	trace	0.1
Gal	21.0	21.1	18.8	32.3	36.2	31.2	26.2	47.5	41.6
Glc	23.3	33.2	28.4	5.0	4.6	6.7	6.0	7.5	7.0
Man	53.6	43.2	50.7	59.9	55.7	59.0	65.7	41.8	49.2
Total radioactivity (d.p.m.)	20242	27143	21003	24130	53977	77130	14177	15408	16903

Mild acid treatment of fractions

Mild acid treatment of fractions failed to release inorganic phosphate but did release monomeric sugars as determined by Bio-Gel P-2 chromatography against standards. All fractions from medium A showed similar behaviour although the degree of degradation varied between 8 and 14% of the total carbohydrate in the fraction. Carbohydrate analysis of the monomeric fraction showed that it was largely (90%) D-galactose. The amount of sugar released is too great to be accounted for by hydrolysis solely of phosphodiester bonds and this acid lability is circumstantial evidence for the presence of galactofuranosyl residues in the polymers in the fractions. This treatment did not remove all of the galactose from the polymer. Although acid treatment led to a shift of the elution profile on Sephadex G-150, the polymer was not broken down into fragments of much smaller size indicating that the galactofuranosyl residues were not internal. Fractions from medium B showed no such

Table 5. Sugar compositions of fractions IA and IB (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium B)

The amount of each sugar in a fraction is given as a percentage of the total radioactivity (d.p.m.) in the sugars of that fraction.

Sugar	Percentage of total radioactive sugar					
	Fraction IA			Fraction IB		
	0	4	1.2.3.	0	4	1.2.3.
GlcA	1.9	2.0	1.3	trace	1.0	1.8
GalN	trace	trace	trace	trace	trace	trace
GlcN	trace	trace	trace	trace	trace	trace
Gal	21.2	20.4	19.4	25.3	25.6	21.5
Glc	45.0	47.1	50.0	8.4	9.0	10.0
Man	31.9	30.5	29.3	66.3	64.4	66.7
Total radioactivity (d.p.m.)	5600	4523	6011	2440	1970	4131

acid labile galactose; the degree of degradation was small (less than 3%) and could probably be accounted for by the hydrolysis of phosphodiester bonds. All races showed similar behaviour.

Mild alkali treatment of fractions

Mild alkali treatment of all fractions from both media caused the release of oligosaccharides with a degree of polymerization of 1 to 4 as determined by Bio-Gel P-2 gel chromatography. These oligomers contained mannose as the sole sugar and accounted for up to 10% of the total carbohydrate in the fraction. Treatment with mild acid did not affect the degree of degradation on subsequent mild alkali treatment or vice versa.

Nature of the protein in fractions I and II

The protein moieties of fractions were labelled exclusively from L-[U-¹⁴C]leucine. Fractionation through the protocol outlined in Fig. 1 confirmed that only a small percentage of the total protein in the high molecular weight fraction was associated with the carbohydrate in fractions I and II. This protein was considered to be covalently bound to the carbohydrate since carbohydrate and radioactivity could not be separated on glass-fibre paper electrophoresis at pH 2.0, 3.5 and 6.5 (data not shown). Mild alkali treatment, however, could separate the protein and carbohydrate. Gel chromatography showed that alkali splits the fraction I polymer into three distinct fragments: a high molecular weight polysaccharide which is eluted from Sephadex G-50 in the blue dextran void volume; a peptide which is eluted in the CuSO₄ void volume of Sephadex G-50 but in the blue dextran void volume of Sephadex G-25; the oligosaccharides described above. The peptide portion must have a molecular weight of between 5000 and 10000. The phosphate and the galactofuranosyl residues are associated with the polysaccharide portion of the molecules. The nature of the covalent linkages between the peptides and both the polysaccharide and oligosaccharide moieties is as yet unknown but may involve linkages to seryl or threonyl residues in the peptide, bonds which are capable of β -elimination in alkali (Neuberger *et al.*, 1966). Similar features were found in all races and in fractions I and II from both media.

Acetolysis of polysaccharide fragments after acid and alkali treatments

The polysaccharide of fractions I, II and III from medium B which remained after mild acid and alkali treatments was subjected to acetolysis. This technique has been used to compare the structures of mannan polymers from different yeast species (Stewart & Ballou,

Fig. 5. Bio-Gel P-2 gel filtration of acetolysis products of the polysaccharide moiety of fraction I of race 1.2.3. remaining after mild acid and alkali treatments. Bed dimensions were 2×96 cm and the column was eluted with distilled water; 5 ml fractions were collected. The elution volumes of blue dextran (BD), stachyose (X_4), raffinose (X_3), sucrose (X_2) and mannose (X) are shown. X_5 denotes the expected elution position of a pentasaccharide.

Table 6. *Distribution of radioactivity between oligosaccharides derived by acetolysis of polysaccharide moieties of fractions I, II and III (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium B)*

Polysaccharides were obtained by successive mild acid and mild alkali treatments of fractions I, II and III. The amount of sugar in a fraction is given as a percentage of the total radioactivity applied to the column (see Fig. 5 for details of chromatography).

Oligo-saccharide	Percentage of total radioactivity								
	Fraction I			Fraction II			Fraction III		
	0	4	1.2.3.	0	4	1.2.3.	0	4	1.2.3.
$X_{\geq 6}$	66.8	67.2	60.7	73.1	63.4	72.2	65.4	60.9	62.2
X_5	18.7	23.5	16.3	26.9	36.6	27.3	33.6	32.7	37.8
X_4	4.0	3.2	11.2	trace	—	0.5	1.0	2.5	trace
X_3	trace	4.4	8.8	trace	—	—	—	—	—
X_2	8.7	1.7	3.0	—	—	—	—	3.9	—
X	1.8	—	—	—	—	—	—	—	—
Total radioactivity (d.p.m.)	2808	2937	4746	4186	9989	6967	2489	8496	4660

1968). When de-acetylated oligosaccharides were chromatographed on Bio-Gel P-2, the fragmentation patterns obtained were similar, showing slight differences between fractions of the same race and between the same fractions of different races (Fig. 5, Table 6). The major identifiable oligomeric product of all races and fractions was eluted in the expected position of a pentasaccharide. Analysis of this fraction (Table 7) showed that the only sugars present were D-glucose, D-mannose and D-galactose. Although there was considerable diversity in the ratio of these sugars between fractions, the same fractions from different races were similar. Electrophoresis on glass-fibre paper in 0.05 M-borate buffer at pH 9.2 showed that all fractions tested were heterogeneous, consisting of several molecular species.

Table 7. Sugar compositions of pentasaccharide fragments derived by acetolysis of polysaccharide moieties of fractions I, II and III (see Fig. 1) of culture filtrates of races 0, 4 and 1.2.3. (medium B)

The amount of each sugar in a fraction is given as a percentage of the total radioactivity (d.p.m.) in the sugars in that fraction.

Sugar	Percentage of total radioactive sugar								
	Fraction I			Fraction II			Fraction III		
	0	4	1.2.3.	0	4	1.2.3.	0	4	1.2.3.
Gal	50.1	50.3	49.5	24.1	26.1	15.3	60.6	65.5	52.5
Glc	19.3	18.3	20.9	15.5	14.9	23.6	3.0	2.6	3.5
Man	30.6	31.4	29.6	60.4	59.0	61.1	36.4	31.9	44.0
Total radioactivity (d.p.m.)	1910	3429	1010	2023	1119	920	1994	2328	896

Fig. 6. Schematic representation of the possible organization of peptide, carbohydrate and phosphate moieties in the glycopeptide of *F. fulva*. The degree of degradation achieved with mild alkali indicates that there is probably more than one oligomannose chain for each polysaccharide in the polymer.

DISCUSSION

The results demonstrate that culture filtrates of several races of *F. fulva* contain a family of glycopeptide polymers of disperse molecular weight and composition. The degradative data are consistent with the generalized structure for these polymers shown schematically in Fig. 6. Polymers from different fractions differ in their detailed carbohydrate composition and degree of phosphorylation (and hence charge). The proposed structure is similar to that suggested for the secreted peptidophosphogalactomannan of *Penicillium charlesii* (Gander *et al.*, 1974; Rick *et al.*, 1974). Small differences between fungal races are found in the fragmentation patterns on acetolysis. The relationship (if any) of these differences to the differences in genotype defined by avirulence on tomato cultivars is not known. Differences in culture conditions lead to differences in the sugar composition of the polymers and also in the presence or absence of galactofuranosyl residues. The glycopeptides may derive from turnover of cell wall polymers as similar molecules may be extracted from the walls of *F. fulva* by treatment with water at 120°C (J. M. Dow, unpublished results).

Fractions I, II, IA and IB from all races caused a rapid leakage of electrolytes from isolated leaf mesophyll cells of tomato. These effects and their possible rôle in the host-phytopathogen interaction have been described elsewhere (Dow & Callow, 1979).

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