ISOLATION AND PARTIAL CHARACTERIZATION OF A PHYTOTOXIC GLYCOPEPTIDE FROM A PROTEIN–LIPOPOLYSACCHARIDE COMPLEX PRODUCED BY A POTATO ISOLATE OF *VERTICILLIUM DAHLIAE*

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1. Introduction

Phytotoxins are microbial metabolities which, at low concentrations, are harmful to plants [1]. *Verticillium* spp., a causal agent of fungal wilt in numerous plants, elaborates in culture phytotoxic protein–lipopolysaccharide (PLP) complexes of high M_r ($\sim 3 \times 10^6$) [2–4]. Until now, no clear definition of the active principle has emerged. The PLP produced by cotton isolates of *V. albo-atrum* [2] acted at least in part as a toxin (low M_r) [5] on excised cotton leaves, rather than as a vessel plugging agent (high M_r) [5]. The PLP tended to form lower M_r 'degradation products' in vitro [2].

Phytotoxic PLP, produced by an isolate of *V. dahliae* which is pathogenic for potato, can break down to low M_r peptidic material under non-dissociating conditions [4]. We now report the isolation of a 3000-M_r glycopeptide that retains essentially all of the toxic activity of the PLP from which it derived. A different peptide was isolated from a non-phytotoxic PLP produced by a non-pathogenic mutant strain of the fungus.

2. Materials and methods

2.1. *Cultivation of* Verticillium dahliae

An isolate of *V. dahliae* (Gl), pathogenic for potato plants, was maintained on potato dextrose agar (Difco). A non-pathogenic mutant (V 297), arising from a single spore isolate of Gl, was selected on the same medium. For production of the PLP complex, the fungi were grown in 100 ml medium containing 2 g glucose, 0.2 g asparagine, 0.15 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7\,H_2O$, 1 mg $FeSO_4 \cdot 5\,H_2O$, 0.6 mg $CaSO_4$, 1 mg thiamine HCl and 0.5 mg pyridoxine; pH was adjusted to 6.7 with KOH. The cultures were incubated for 21 days at 26°C in the dark.

2.2. *Isolation of the glycopeptide*

Acetone precipitation of the proteins and gel filtration were performed as in [4]. The mycelium was removed from 21-day cultures by filtration and the filtrate was concentrated under vacuum at 50°C. The concentrate was mixed with 4 vol. cold (–18°C) acetone and allowed to stand at 4°C overnight. The precipitate was loaded onto an Agarose A-5m column (Bio-Rad Labs, Richmond CA), 3 × 70 cm, and eluted with distilled water at room temperature. Fractions (2 ml) were collected at 7 ml/h, monitored for proteins, carbohydrates and lipids and tested for phytotoxic activity. Peak I (pI), eluting between 45–60 ml, was characterized as a 3×10^6 M_r protein–lipopolysaccharide complex (PLP) which contained the majority of the phytotoxic activity [4]. pI was concentrated to ~1 ml, as above, and dialysed against distilled water. The activity was recovered from the dialysate which was concentrated to ~4 ml as above, then to 0.5 ml in a Speed Vac Rotary Concentrator (Savant). PLP and dialysates obtained from cultures of the mutant, V 297, showed no detectable activity in the bioassay [4]. The dialysates were tested for proteins, carbohydrates and lipids.

2.3. *High-performance liquid chromatography (HPLC)*

The reversed-phase HPLC system (RPLC) consisted of a stainless steel column, 4 × 250 mm (Alltech

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Assoc., Deerfield IL), packed with Lichrosorb RP-18, 10 μm (Merck) and a high-pressure mini-pump (Milton Roy, Riviera Beach FL). Samples were loaded onto the column via a high-pressure sample injection valve (Rheodyne, Berkeley CA). The column was eluted under isocratic conditions using 0.3 M pyridine formate (pH 4.0) (buffer A) or 50% 1-propanol in the same buffer (buffer B). Flow rates of 0.4 or 0.8 ml/min were employed and fractions were collected every 2.5 min from the time of injection. Column eluates were monitored with a fluorescamine stream-sampling detection system [6] which diverted 3% of the effluent for detection, while the remainder was directed to a fraction collector. The fractions were assayed for bioactivity.

2.4. *Bioassay*

Fractions were tested for wilt symptom-producing activity by a detached potato leaf bioassay [4]. The first true leaves of 3–6-week-old potato plant seedlings were excised under water and placed in vials containing 20 ml water. Test solution (0.2 ml) was injected into the intercellular spaces of the leaves. The cuttings were placed under continuous illumination. Symptoms were observed after 24 h and the degree of necrosis was scored after 72 h. The concentration of the test solution to be used was determined at each step by dilution end-point on leaves. The highest dilution showing a significant difference between susceptible and resistant plants was employed.

2.5. *Analytical methods*

Protein was determined by absorbance measurement at 280 nm and by the method in [7]. Polysaccharide was determined by an anthrone method [8], using D-glucose as standard. Lipid was determined gravimetrically following hydrolysis of the complex in 1 N HCl for 60 min at 100°C and extraction into diethyl ether. Identification of carbohydrate components of the glycopeptide was carried out by gas–liquid chromatography (GLC) [9]. For amino acid analysis, samples were hydrolysed in 6 N HCl at 110°C for 22 h under vacuum and analysed with a Durrum D-500 automatic amino acid analyser.

2.6. *Oxidation with periodate*

Mixtures of 0.9 ml dialysate and 0.1 ml 5 mM sodium metaperiodate were incubated at room temperature for 3 h. Oxidation was stopped by adding an equimolar amount of mannitol in 0.2 ml. A sample of

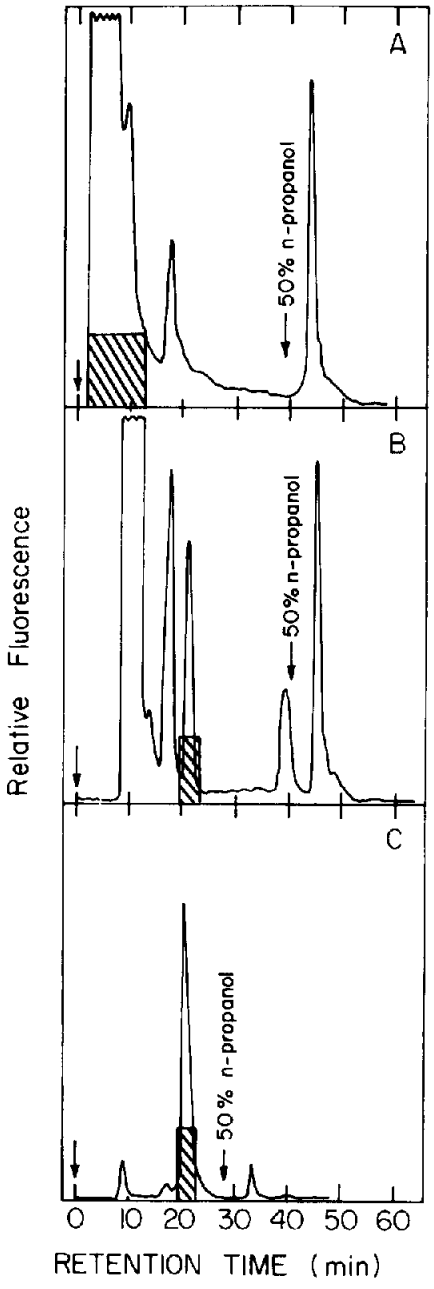

Fig.1. Isolation of the glycopeptide from PLP by RPLC. RPLC of pI_0 on an RP-18 column (4 × 250 mm). The sample was injected via a 500 μl sample injection loop and eluted with 0.3 M pyridine formate (pH 4.0). Fractions of 2.5 min were collected: (A) flow rate 0.8 ml/min; (B) flow rate 0.4 ml/min; (C) rechromatography of fraction 9 of (B) under the same conditions.

Table 1
Purification of the glycopeptide from PLP of *Verticillium dahliae*

Fraction	Total protein (mg/ml)	Protein injected for bioassay (mg)	Purification (-fold)
Filtrate	6	1.2	1
Acetone ppt.	3	0.6	2
Peak I (pI)	0.5	0.1	12
Peak I out (pI_o)	0.2	0.4	30
HPLC fraction 9	0.003	0.0006	2000

the dialysate to which sodium metaperiodate and mannitol were added at the same time served as a control. All reaction mixtures were tested for biological activity; an untreated sample was used as control for sensitivity of the test plant.

3. Results and discussion

When peak I from the agarose column (see section 2.2) of the phytotoxic PLP was exhaustively dialysed against distilled water, essentially all of the activity was recovered in the dialysate, which will be referred to as 'peak I out' (pI_o). pI_o contained protein and carbohydrate, but no lipids were detected. The results indicate that the PLP complex had dissociated and the active principle was of low M_r, supporting the hypothesis [4] that phytotoxic activity is not dependent upon the presence of an intact complex. Phytotoxicity was abolished by incubation with protease (pronase P, Sigma type VI), but was not decreased by treatment with sodium metaperiodate, suggesting that peptidic material is important for biological activity.

Fractionation of pI_o by RPLC, using buffer A at 0.8 ml/min, resulted in elution of a broad zone of toxic activity (fig.1A). Reduction of the flow rate to 0.4 ml/min, a mobile phase velocity much lower than that generally employed in HPLC, resulted in improved resolution of the fractions, with toxic activity eluting in a single, sharp peak (fig.1B). This agrees with the results in [9], using C-8 columns, on the effect of flow rate on HPLC of proteins. Typically, separation of the phytotoxic material was achieved by isocratic chromatography at the reduced flow rate in <25 min. The biologically active fraction [9] was further purified by rechromatography under the same conditions (fig.1C). The procedure resulted in a 2000-fold purification of the active material (table 1).

The amino acid composition of fraction 9 is shown in table 2; it appears that the active principle of the phytotoxic PLP is an acidic peptide with an estimated M_r of ~3000. The process yielded 1 μg peptidic material from 100 ml original culture fluid. GLC analysis of fraction 9 for sugars detected glucose as the major carbohydrate component.

RPLC fractionation of pI_o obtained from non-phytotoxic PLP produced by the non-pathogenic strain, V 297, resulted in a similar elution pattern of fluorescamine-positive material. Amino acid analysis of fraction 9 of both strains (table 2) indicated that a

Table 2
Amino acid composition of glycopeptides isolated from phytotoxic and non-phytotoxic PLP from *Verticillium dahliae*

Amino acid	Gl		V 297	
	nmol	Residue[a]	nmol	Residue[a]
Asp	1.330	3	1.313	3
Thr	0.684	2	0.768	2
Ser	0.958	2	0.518	1
Glu	1.318	3	0.707	2
Pro	0.399	1	0.402	1
Gly	2.308	5	1.136	3
Ala	0.921	3	0.425	1
½Cys	–	0	–	0
Val	0.918	2	1.898	5
Met	–	0	0.027	0
Ile	0.362	1	0.710	2
Leu	0.432	1	0.395	1
Tyr	0.832	2	–	0
Phe	–	0	–	0
Lys	0.271	1	–	0
His	0.139	0	–	0
Arg	0.126	0	–	0

[a] Leu = 1

different peptide was produced by each strain, supporting the conclusion [4] that the toxic moiety produced by the pathogenic isolate of *V. dahliae* may be altered or even absent in the mutant.

These data suggest that the active moiety of the PLP (3×10^6 M_r) is a 3000-dalton glycopeptide and that only the peptidic portion is important for biological activity. The results confirm the contention [2] that the PLP acts as a low M_r toxin on excised cotton leaves. A variety of high M_r and low M_r toxic substances have been reported for this fungus [2], thus a question remains whether the high M_r material represents a precursor of, or an aggregate of, the actual toxic molecule. Further biosynthetic studies are necessary to resolve this problem.

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