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HEAT SHOCK TREATMENT RELEASES *PENICILLIUM* PHOSPHOGLYCOPEPTIDE

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Key Word Index—*Penicillium fellutanum*; peptidophosphogalactomannan; ^{13}C and ^{31}P NMR spectroscopy; cell walls.

Abstract—Filtrates of heat (54°) treated day-5 *Penicillium fellutanum* cultures contained 70 mg of peptidophosphogalactomannan-II; an unheated control contained 30 mg. The polymer contained up to 60 phosphodiesters, and 5-*O*-β-D-galactofuranosyl, mannopyranosyl, amino acyl and 2-aminoethanol residues. Its ^{13}C NMR spectrum was nearly identical with that of the control polymer. The major ^{31}P NMR signal was phosphocholine phosphodiester at δ 0.22 ppm; significant phosphodiester signals occurred at δ 1.15, 1.33 and 1.47. Dilute mineral acid released galactofuranosyl residues from the mannan. Signals at δ 1.15–1.47 ppm were associated with molecules of mass less than 3500 and contained galactose, 2-aminoethanol and peptide(s). After this acid treatment, signals at δ 1.0–0.22 remained associated with the mannan. Heat released up to 4-fold more of peptidophosphogalactomannan-III compared with the untreated control; carbohydrate and phosphate content, per mg polymer, were reduced by 4- and 2-fold, respectively. A galactofuranosyl-, phosphoryl- and amino acyl-containing polymer of M_r greater than 14000 was solubilized by alkali treatment of *P. fellutanum* cell walls.

INTRODUCTION

Penicillium fellutanum (formerly, *P. charlesii*), releases extracellular phosphoglycopeptides upon depletion of ammonium in the growth medium [1–3]. The percentage and composition of peptidophosphogalactomannans, $pPGM^{II}$ (**1**) and $pPGM^{III}$ (**2**) in culture filtrates, depend on culture medium composition and age of culture [4, 5]. The location of **1** and **2** within the organism is unknown.

Numerous reports on the composition of *Penicillium* cell walls suggest that the walls contain significant quantities of polymers containing glucosyl, galactosyl and mannosyl residues [6–17]. For instance, *Penicillium notatum* hyphal walls contain glucose, galactose and mannose; phosphate and lipid content are each around 2% [6]. The galactose content of cell walls decreases with age [7]. The cell wall mass of *Penicillium rubrum* is approximately 17% lipid; 10% of this is bound [8]. The phosphate and protein accounted for 0.5% and 10%, respectively. Alkali-soluble and alkali-insoluble fractions of *Penicillium chrysogenum* were shown to contain glucosyl and galactosyl residues [9]. All of the mannosyl as well as galactosyl residues detected in hyphal walls appeared in this fraction together with glucosaminyl residues. However, an intracellular galactomannan was obtained from *P. chrysogenum* [10]; the polymer contained galactofuranosyl residues in (1 → 5) linkage. The quantity of glucosyl, galactosyl and mannosyl residues in cell walls of several other species of *Penicillium* is comparable [6, 11, 12]. Leal and colleagues examined wall polysaccharides of *Penicillium*, *Eupenicillium*, *Aspergillus* and *Talaromyces* species. They found that 1 M Sodium hydroxide solubilized α-(1 → 3)-glucan and β-glucogalactan polymers in walls from *Penicillium* species [13]. *Eupenicillium* walls contain an α-glucan [14] and *Talaromyces* [15] walls contain a β-glucogalactan which is soluble in alkali. The α-glucan-containing material is water insoluble. *Gliocladium viride* contains an alkai-soluble and water-soluble fraction that was identified as a galactomannoglucan [16]. Leal *et al.* [17], have characterized, using ^{13}C NMR and IR spectroscopies, the alkali- and water-soluble fraction from *Eupenicillium crustaceum*, *Penicillium brevicompactum*, *Penicillium decumbens*, *Aspergillus flavipes*, and *Aspergillus ochraceus*. The acid hydrolysate of this fraction from these species contained from 40 to 80% galactose, 5 to 8% of both mannose and glucose with the exception of *P. bre-*

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vicompactum which contained 26% galactose, 22% glucose and 7% mannose. The only significant ^{13}C NMR signals were those of the six carbons of β-(1 → 5)-galactofuranosyl residues; signals at 108 and 77 ppm represent those of galactofuranosyl ^{13}C-1 and ^{13}C-5 atoms, respectively. The lack of signals of the glucosyl or mannosyl residues makes it difficult to determine whether the β-(1 → 5)-galactan is an independent polymer or is part of a galactomannoglucan, or some combination of those hexosyls.

In preliminary studies [18], *Penicillium fellutanum* cell walls were shown to contain an alkali-soluble, ethanol-insoluble fraction composed primarily of galactofuranosyl and glucosyl residues. No mannose was detected in the acid hydrolysate. Treatment of this alkali-soluble fraction with 1-dimethylamino-naphthalene-5-sulphonyl chloride followed by 6 N HCl resulted in the release of fluorescent derivatives of ethanolamine, aspartate, glutamate, serine, glycine and valine. This cell wall fraction contained at least 10-fold less phosphorus compared with that in **1** or **2** from this organism. Taken collectively, the data above do not convincingly show that **1** and **2** are derived from *Penicillium* cell walls.

An alternate source of polymers **1** and **2** exists. Galactofuranosyl-containing polymer(s) isolated from membranes as well as cell walls of *P. fellutanum* are putative precursors of polymers **1** and **2**. Fractionation of *P. fellutanum* membranes by isopycnic ultracentrifugation on a 5–55% sucrose gradient yielded 6 bands [19]. Galactofuranosyl residues were only detected in the membrane band of density 1.21 g ml^{-1}. A polymer, lipo-pPGM, (**3**), from day-3 *P. fellutanum* was purified from this band; **3** contained a similar percentage of galactofuranosyl, mannopyranosyl, phosphoryl, and 2-aminoethanol residues as found in extracellular **1**. Following treatment of **3** with 6 N HCl, amino acid analyses indicated that serine, threonine and alanine represented 50% of the total quantity of amino acid residues and were present in proportions to those in **1**. Sulphur-containing and aromatic amino acids were either absent or present only in trace quantities. None of the other five membrane fractions of *P. fellutanum* contained detectable quantities of galactofuranosyl residues, the major species of carbohydrate found in **3** as well as in **1**.

Membranes from lysed spheroplasts and solubilized cell wall products of *P. fellutanum* contained 1.33 and 0.12 mg, respectively, of galactofuranosyl residues per mg of protein [20]. No detectable galactofuranosyl-containing substances were found in the soluble fractions from intact protoplasts.

Penicillium fellutanum secretes hydrolases that catalyse the release of galactofuranosyl and phosphodiester residues [5, 21]. Cultures on low phosphate media release a non-specific phosphocholine diester:phosphocholine hydrolase activity into the culture filtrates. Phosphocholine phosphodiester residues of **1** are decreased from an average of greater than 10 to less than 5 residues per molecule resulting in **2** that

-[-Man$_1$ α

^{6}Man$_1$ α $_2$Man$_1$ α $_2$Man$_1$ α-]-

Fig. 1. A fragment of the mannan region of peptidophosphogalactomannan-II. The mannan region contains approximately 80 mannopyranosyl residues and is composed of 20 2-*O*-α-D-mannopyranotetraosyl attached by α-(1 → 6)-linkage [24]. A unit of the mannan is depicted above. Phosphocholine diester residues are attached to C-6 position of mannopyranosyl residues of the mannan. 5-*O*-β-D-galactofuranosyl residues branch by 1,3-linkage or 1,3- and 1,2-linkages from the mannan [2, 21].

binds more tightly to DEAE cellulose borate [5]. Exo-β-D-galactofuranosidase-catalyzed hydrolysis of **1** decreases with increased number of phosphodiester groups on the substrate [21, 22]. These enzymic activities assist in recycling of phosphate, choline and galactose, as needed, from **1** back into the mycelia where they are reutilized [23].

A unit of the mannan region of **1** characterized by ^{13}C NMR spectroscopy is shown in Fig. 1 [24]; wet chemistry [3] has provided evidence for attachment of the mannan through an *O*-glycosidic linkage to a seryl or threonyl residue of 3000 Da peptides. The mannan is composed of up to twenty 2-*O*-α-D-mannopyranosyl tetrasaccharide units attached together by α-(1 → 6) linkages; the mannotriose and mannobiose generated during acetolysis of the phosphomannan may result from the non-specific cleavage of α-(1 → 2) linkages [2]. An average of approximately 12 manno-oligosaccharides are also attached through *O*-glycosidic linkages to seryl and threonyl residues of the peptides; mannotriose is the largest of these saccharides.

Treatment of **1** with anhydrous HF removes the saccharides and phosphoesters from the peptides resulting in a preparation that appears homogenous with regard to mass; however, the peptides were separated into four fractions each with a different N-terminal amino acid [19]. Amino acid sequence analysis suggested that each partially purified preparation was composed of numerous peptide species. These peptides may be derived from many proteins.

^{13}C NMR spectroscopy [24, 25] has shown that the galactan region is composed of 5-*O*-β-D-galactofuranosyl residues, wet chemistry [2, 21] has shown that there are approximately 10 of these galactan chains attached to the mannan. Removal of the galactan chains by dilute acid hydrolysis resulted in the loss of about 10 periodate insensitive mannopyranosyl residues; this value is equivalent to the number of formaldehyde residues generated from C_6 by the oxidative cleavage of nonreducing terminal galactofuranosyl residues [2, 21]. This suggests that the galactan chains are attached to the mannan through a 1,3-linkage, or possibly through 1,2- and 1,3-linkages, to a mannopyranosyl residue.

Day-9 cultures of *P. fellutanum* contain approximately 10 phosphodiesters in **1**; negligible peptides or phosphodiesters are released by 10 mM HCl [19, 25, 26]. The major signal at 0.22 ppm results from phos-

phocholine phosphodiesters attached to C_6 of mannopyranosyl residues. In contrast, highly phosphorylated **1** contains an average of up to 60 phosphodiester residues per molecule when isolated from day-5 culture filtrates. The phosphodiesters providing significant signals at 1.15, 1.33 and 1.47 ppm are associated primarily with components of the galactan chains rather than the mannan backbone [27]. A major signal 0.22 ppm and minor signals at 0.9, 0.8 and 0.7 ppm represent phosphocholine, 1-*O*-phospho-2-aminoethanol and *N*-methyl phosphodiester derivatives, respectively, attached to the mannan. Treatment of **1** with boiling, dilute mineral acid results in the formation of galactose and low M_r phosphorylated substances that contain galactose, 2-aminoethanol and small peptides; the chemical shift of the phosphodiester signals remains unchanged suggesting that 10 mM HCl did not cleave the phosphodiesters; furthermore, the chemical shift is independent of pH. Some of the peptide-containing substances passed through 1000 M_r cutoff membranes; the remainder were up to 3500 Da in size. Both fractions were rich in seryl, threonyl and alanyl residues and aromatic and sulfur-containing amino acids were deficient or absent. The data suggest the presence of *N*-peptidyl-2-aminoethanol phosphodiesters of galactofuranosyl residues in this fraction. However, Bonetti's laboratory reported that at least one of the phosphodiesters, released by similar dilute mineral acid treatment of peptidophosphogalactomannan from *Penicillium spinulosum*, is attached to C_6 of a mannopyranosyl residue [28].

This paper shows that culture filtrates from short term heat treatment of fungal cultures have double the quantity of phosphorylated polysaccharide when compared with untreated controls. Based on ^{13}C and ^{31}P NMR spectra, the composition of **1** from treated filtrates were almost identical with **1** present in unheated control filtrates. A portion, if not all, of **1** could have been derived from cell walls.

RESULTS AND DISCUSSION

Influence of heat treatment on quantity and composition of phosphoglycopeptide released

Heat treatment (54°) of day-5 cultures of *Penicillium fellutanum* resulted in a 2-fold increase in extracellular glycopeptide fraction (**4**) obtained from DEAE-cellulose-borate by elution with 0.01 N HCl–0.06 M LiCl. The polymer, **4**, from heat-treated cultures eluted from DEAE in a similar manner as polymer **1** from control cultures. Control (unheated) culture filtrates contained 30 mg of **1**/flask, *vs* an average of 65 mg of **4**/flask for cultures subjected to heat treatment for 30 or 60 min (Table 1). The quantity of the glycopeptide fraction (**5**) eluting in 0.01 N HCl–0.4 M LiCl increased from 8 mg/flask in unheated cultures to 35 mg/flask in heat treated cultures. The polymer, **5**, from heat treated cultures eluted similarly to the control phosphoglycopeptide **2**. Polymers in **4** are composed of **1** released before heat treatment and any polymer(s) released during treatment that fractionate with **1**. In a similar manner, polymers in **5** are composed of **2** and any polymer(s) released during heat treatment that fractionate with **2**.

The carbohydrate and phosphate content of **4** and **5** from heat-treated cultures were compared with their corresponding controls **1** and **2**, respectively (Table 1). The relative quantities of carbohydrate and phosphate in **4**, in cultures heated from 30 to 120 min, were comparable to that in **1**. This suggests that **1** and **4**

Table 1. Influence of time and temperature of heat treatment on quantity and composition of phosphoglycopeptide in culture filtrates

Heat treatment	Time, min at 54° 0*	15	30	60	120†
Fraction	mg of phosphoglycopeptide per flask				
F_4 (**4**)	30	64	59	70	28
F_5 (**5**)	8.5	28	37	34	14
	μmol of carbohydrate mg^{-1} of phosphoglycopeptide‡				
F_4 (**4**)	5.0	3.7	5.0	5.1	6.4
F_5 (**5**)	4.0	0.72	1.4	1.2	1.6
	μmol of phosphate mg^{-1} of phosphoglycopeptide‡				
F_4 (**4**)	0.49	0.42	0.54	0.49	0.52
F_5 (**5**)	0.53	0.11	0.23	0.25	0.22

* Control, fractions **4** and **5** were isolated from culture filtrates of day-5 *fellutanum* cultures in the Standard Growth medium at 23° by described procedures [4]. Cultures F_4 and F_5 not heat treated (column 1), contain only polymer **1** and **2**; all other columns potentially contain polymers **1** and **4**, and **2** and **5**, respectively, in the first and second row indicating mg flasks^{-1}, μmol carbohydrate mg^{-1} of polymer, and μmol phosphate mg^{-1} of polymer.

† Culture was held at 23° for 24 hr following heat treatment before filtration and isolation of polymers.

‡ Carbohydrate and phosphate analysis were determined by phenol–sulphuric acid [29] and the Ames [30, 31] procedures, respectively.

have the same general composition. However, our results suggests that **5** from heat-treated cultures contains significant quantity of polymers different from **2** as well as any **2** released prior to treatment (Table 1). The μmol mg^{-1} sample of carbohydrate and phosphate in **5** at 15 min heat treatment exhibited a 5-fold decrease as compared with the values for control **2**. Increasing the heating time to 30, 60 and 120 min doubled the μmol mg^{-1} of carbohydrate and phosphate in **5** as compared with the values at the 15 min interval. Polymers in **5**, isolated from cultures heated for 30 or 60 min, had approximately one-third of the quantity of carbohydrate and one-half the quantity of phosphate as that in **2**.

The source from which the additional **1**-like polymer in heated cultures is derived is unknown. The soluble cytoplasmic fraction of *P. fellutanum* contains little if any **1** or **2** [20]. However, **2** may be derived from either cell walls [20] or membrane-bound **3** with a composition similar to extracellular **1** [19]. Incubation of 2 hr heated cultures for 24 hr at 23° reduced the quantity of both **4** and **5** by about one-half compared with that heated 60 min.

^{13}C NMR spectra of phosphoglycopeptides

Polymer **4** was analysed by ^{13}C NMR spectroscopy to determine if significant quantities of saccharides and phosphoryls exist other than those found in **1**. Proton-decoupled, ^{13}C NMR spectra of **1** isolated from (a) day-5 culture filtrate, and (b) **4** isolated from filtrate of a day-5 culture heated at 54° for 120 min were obtained. The two spectra are nearly identical; major signals at δ 109.8, 84.2, 79.2, 78.3, and 63.9 are those of the anomeric, C_2 and C_4, C_3, C_5, and C_6 carbons, respectively, of internal galactofuranosyl residues (Fig. 2). The signal at δ 110.5 is the same as that of the nonreducing terminal galactofuranosyl residues of **1**. The small signals at δ 105.1–101.3 are ascribed to the anomeric carbons of mannosyl residues of the mannan [24]. The small signal at δ 107.8 may be that of the anomeric carbon of galactofuranosyl residues attached to the mannan. The quaternary methyl groups of phosphorylcholine phosphodiester are observed at δ 56.9 and the C_2 methylene of phosphoryl-2-aminoethanol is observed at δ 42.9 [25]. Signals at 65 to 75 ppm are likely C-6 residues of mannosyl and galactofuranosyl residues substituted with phosphoesters.

Polymer **4** released from the 120 min heat treated culture [Fig. 2(B)] had essentially identical ^{13}C NMR spectra as that of **1** [Fig. 2 (A)]. This suggests that **4** and **1** have the same major carbohydrate composition. There is no apparent evidence for *N*-acyl-amino-2-deoxyglycosyl sugars in **4** (Fig. 2). The ^{13}C spectra of **2** and **5** were comparable; these spectra differ from those of **1** and **4** by having significantly larger signals for anomeric carbons of mannosyl (δ 101–105 ppm), and a smaller signal at 56.9 ppm (data not shown).

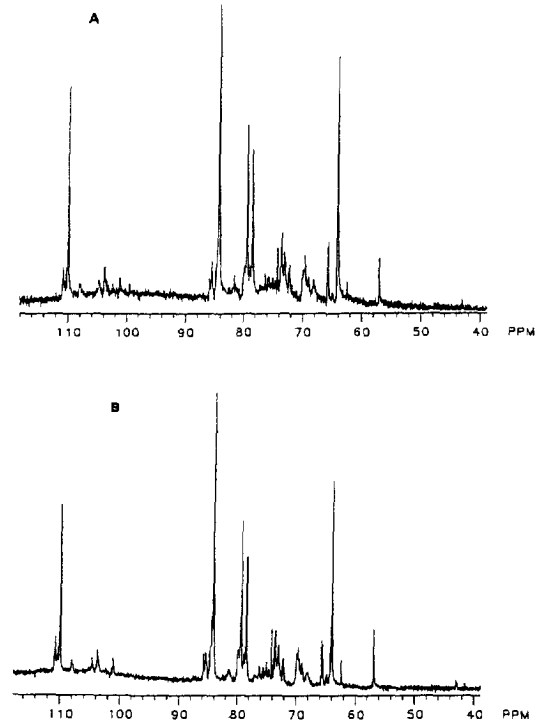

Fig. 2. ^{13}C NMR spectra of phosphoglycopeptide from filtrates of control culture and from heated cultures. Phosphoglycopeptides were isolated from day-5 culture filtrates and fractionated on DEAE-cellulose-borate [4]. The fraction eluted in 0.01 N HCl–0.06 M LiCl was analysed by proton decoupled ^{13}C NMR spectroscopy using a spectrometer with a 70 kG field tuned to 75.46 MHz. The samples were dissolved in approximately 4 ml of ^2H_2O and the spectrometer was locked onto the ^2H signal. The samples were passed through Chelex-100 to remove paramagnetic ions. Chemical shifts for carbon spectra are reported in ppm downfield from internal TSP. The spectrum shown in Fig. 2(A) is that of control phosphoglycopeptide **1** obtained from day-5 culture filtrates; that, **4**, shown in Fig. 2(B), is a spectrum of culture filtrate obtained after heating the culture for 120 min at 54° followed by holding it at 23° for 24 hr. The quantity of each polymer analysed was 30 and 28 mg as shown in Fig. 2(A) and 2(B), respectively. The signal at 56.9 ppm is that of the methyl carbon of mannopyranosyl-6-*O*-phosphocholine phosphodiester; those at δ of 63.9, 78.3, 79.2, 84.2, 109.8 and 110.5 are from galactofuranosyl residues and all others are from mannosyl carbons.

This shows that the percentage galactose and phosphocholine diester are reduced in 2 and 5.

^{31}P NMR spectroscopy of phosphoglycopeptides

The ^{31}P NMR signal of phosphocholine phosphodiester at 0.22 ppm is significantly larger in **1** than in **2** from the same control culture filtrates. This is the major difference in ^{31}P NMR spectra between **1** and **2** and is believed to be the property responsible for differential binding to DEAE cellulose-borate.

Comparison of the ^{31}P NMR spectrum of **1** [Fig. 3(A)] with that of **4** ([Fig. 3(B)] 60 min heat treated

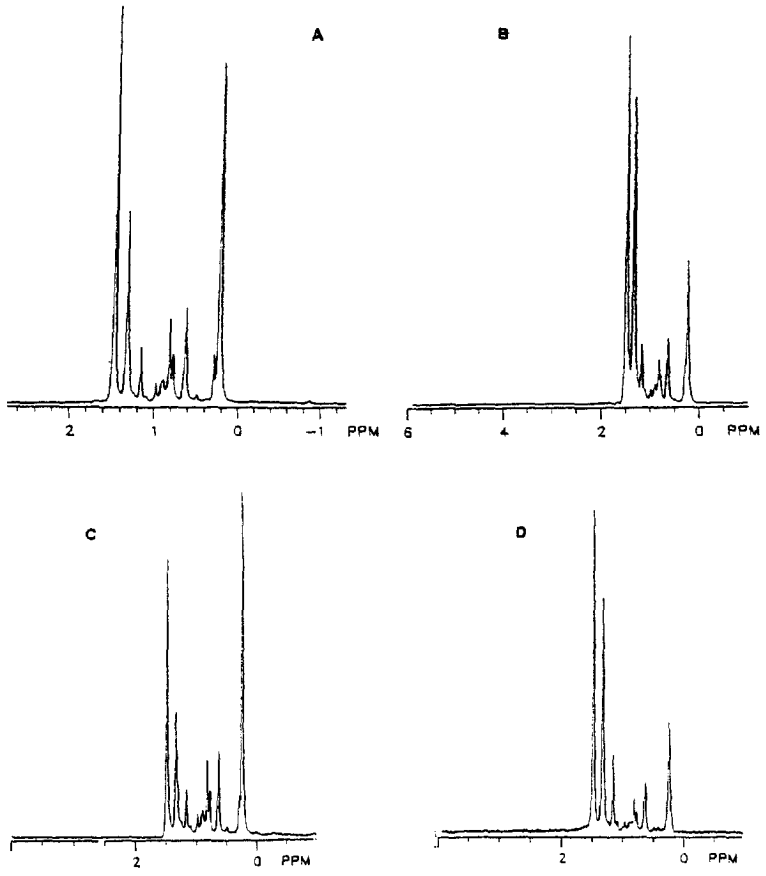

Fig. 3. ^{31}P NMR spectra of phosphoglycopeptides from filtrates of control cultures and from cultures heated for 60 min. Phosphoglycopeptides were isolated from day-5 culture filtrates and were fractionated on DEAE-cellulose-borate [4]. The fractions that eluted in 0.01 N HCl–0.06 M LiCl and in 0.01 N HCl–0.4 M LiCl were analysed by proton decoupled ^{31}P NMR spectroscopy in a spectrometer with a 70 kG field tuned to 121.47 MHz. The proton decoupled ^{31}P NMR spectrum of control phosphoglycopeptide **1** was obtained and is shown in Fig. 3(A). Phosphoglycopeptide from control glycopeptide **2** that eluted in 0.01 N HCl–0.4 M LiCl is shown in Fig. 3(B). Figures 3(C) and 3(D) show spectra of phosphoglycopeptide preparations **4** and **5** from 60 min heat treated cultures fractionated in 0.01 N HCl containing 0.06 M LiCl or 0.4 M LiCl, respectively. The quantity of polymer analysed and shown in Figs 3(C) and 3(D) was 70 and 34 mg, respectively.

shown as representative), isolated after heat treatment of cultures for 15, 30, 60 or 120 min, showed that **1** and **4**, in each preparation were essentially identical with respect to detectable phosphorylated residues. Furthermore, signals at 1.47, 1.33, 1.15 and 0.22 are unchanged as the pH is changed from 2–12 [26, 27]. This property suggests a species with a very low pK_a.

A ^{31}P proton-decoupled NMR spectrum of **5** obtained from 60 min heat treated cultures is shown as representative of this treatment. No significant difference exists between the signals found in the spectrum of polymer **2** from control cultures, and those of **5** obtained from any of the heat treatments. Although very few changes in the types of signals were observed, heat treatment of mycelia did result in changes in the ratio of intensities of phosphodiester species. For instance, as the duration of heat treatment increased to 60 min the signal intensities of **4** of δ 1.32 and that at δ 1.47 declined concurrently, relative to δ 0.22.

In contrast, the prolongation of heat treatment caused an increase in the phosphodiester signal at δ 1.32 of **5** and a simultaneous decline in the signal at δ 1.47. The heat treatment also resulted in release of phosphomonoesterase and non-specific phosphocholinediester:phosphocholine hydrolase activities; the latter may have been responsible for some of the decrease in signal at δ 0.22 during the prolonged incubation at 23° (data not shown). The data (Table 1) show a significant decrease in the mass of fractions **4** and **5** in samples held for 24 hr at 23° following 120 min incubation at 54° when compared with shorter heat treatments. This decrease in mass may reflect the activity of exo-β-D-galactofuranosidase and phosphodiesterases released into the culture filtrates.

The above data, considered collectively, are completely consistent with the conclusion that heating *P. fellutanum* cultures at 54° for up to 60 min results in an increase in quantity of **1** in culture filtrates from 30 to 60 mg or more per 150 ml of culture. The co-release of **4** and **5** by heat treatment, coupled with the fact

Table 2. Carbohydrate, galactose and phosphate solubilized from cell walls with water, mineral, acid or alkali

	M_r range of solubilized cell wall components					
	(M_r < 1000)		(M_r 1000–14000)		(M_r > 14000)	
	μmol g^{-1} cell wall					
Treatments	*CHO/Gal	PO_4	CHO/Gal	PO_4	CHO/Gal	PO_4
Water†	87.5/11.4	0.1	15.4/3.9	1.1	12.6/4.9	4.1
0.01 N HCl‡	1220/102	49	23.9/0	3.5	27.4/0	1.9
2.0 N HCl‡	1580/111	96	17.1/0	0.5	2.1/0	0.7
0.4 N KOH‡	86.4/5.2	14	60.5/51.4	1.9	48.1/37.6	8.1

* Abbreviations: CHO/Gal, carbohydrate/galactose ratio; PO_4, phosphate.

† Cell walls weighing 360 mg were suspended in water at 4° in azide and stirred for 24 hr. Solubilized components were separated from insoluble substances by filtration through a 0.2 μm filter. Fractionation of the soluble components into their respective M_r ranges was achieved by dialysis through appropriate membranes.

‡ Cell walls weighing 36, 49, or 226 mg, were treated with 0.01 N HCl for 90 min at 100°; with 2.0 N HCl at 100° for 3 hr, or with 0.4 N KOH for 24 hr at 4°, respectively. The soluble components were separated from insoluble and the soluble components were fractionated into three range of sizes as described in †, above. Carbohydrate, and phosphate content of the fractions were determined by methods described in the Experimental section [29–31].

that **5** does not resemble **2** from control cultures with respect to percentage of carbohydrate or phosphate, suggests that **5** is derived from **4**.

Extraction of cell wall carbohydrate, phosphate and peptides with dilute mineral acid or alkali

Investigations were performed to determine if galactofuranosyl-containing polymers are present in *P. fellutanum* cell walls. The results of these structural investigations are presented in Table 2. Purified cell wall fractions from *P. fellutanum* were treated with (a) H_2O, (b) 0.01 N HCl, (c) 2 N HCl, or (d) 0.4 N KOH to solubilize cell wall components. Solubilized components were separated by dialysis into approximate M_r of (a) less than 1000, (b) between 1000 and 14000, and (c) greater than 14000. Extraction of cell walls with H_2O solubilized less than 10% of the total carbohydrate, phosphate or peptide that was released by extraction with 2 N HCl. In contrast, treatment of cell walls with 0.01 N HCl resulted in extraction of 80% of the total carbohydrate compared with that released by 2 N HCl; most of this passed into the low M_r fraction. Ninety percent of the galactose was released by this treatment. Galactose, glucose and mannose were present in the dilute acid hydrolysate. Treatment of this hydrolysate with 2 N HCl released glucosamine.

Approximately one half of the phosphate and the peptide was solubilized by dilute mineral acid (as compared to that released by 2 N HCl) and appeared in the low M_r fraction. Amino acids, small peptides and amines in the low M_r fraction were reacted with 1-dimethylamino-napthalene-5-sulphonyl chloride and were fractionated on polyamide sheets. Dansyl derivatives of serine, threonine, alanine and ethanolamine were the primary products (data now shown); lesser quantities of dansyl derivatives of bis-lysine, tryptophan, arginine, lysine and histidine were noted. Further treatment of this dilute acid hydrolysate with 6 N HCl followed by dansylation and chromatography resulted in fluorescent areas indicative of dansyl derivatives of glycine, proline, valine, isoleucine, leucine, and phenylalanine as well as of the amino acids cited above. Considering that most proteins and peptides are stable to short term treatment with boiling, dilute mineral acid under non oxidizing conditions, coupled with evidence for derivatives of 1-dimethylamino-napthalene-5-sulphonyl 2-aminoethanol and amino acids, the data suggest that dilute acid treatment of cell walls releases the carbohydrate, phosphate, amino acids/peptides and 2-aminoethanol as occurs when **1** is treated in similar manner [3]. The ratio of carbohydrate/phosphate is 25 in the dilute acid hydrolysate. This ratio is in the range that has been observed in **1** [1–3].

Treatment of cell walls with 0.4 N potassium hydroxide extracted approximately 12% of the carbohydrate released by 2 N HCl; however, galactose comprised 82% of the carbohydrate in the intermediate and high M_r fractions. The galactose extracted with alkali represents 87% of that solubilized by dilute acid treatment of cell walls. Dilute acid treatment of the neutralized alkaline extract released galactose; this suggests the presence of galactofuranosyl residues in polymers in the extract. Galactose, glucose and mannose were the major monosaccharides found in this fraction following treatment with 2 N HCl. These data suggest that *P. fellutanum* cell walls contain water-soluble polymers of the F1S class reported by Leal *et al.* [17].

The ratio of carbohydrate/phosphate was approximately 30 and 6 in the intermediate and high M_r alkali-extracted fractions, respectively. Although alkali treatment of cell walls was effective in extracting the galactosyl-containing polymers, it extracted only about 30% of the phosphate found in the low M_r fraction solubilized with dilute acid. A carbo-

hydrate/PO_4 ratio of 6 in the fraction of M_r > 14 000 suggests that polymers in this fraction are highly phosphorylated like those found in **1** in day-5 culture filtrates.

Extraction of cell walls with dilute mineral acid also solubilized peptides; half were found in the M_r 1000 fraction. Serine, threonine, alanine and 2-aminoethanol were the major amino acids in the dilute acid-labile fraction. These three amino acids represent approximately 50% of the total amino acyl mass in **1**.

The experiments presented indicate that treatment *P. fellutanum* at 54° results in the release of additional **1** and that incubation of the culture at 23° for 24 hr after the heat treatment decreases the quantity of phosphoglycopeptide in the culture filtrate by at least 50%. This decrease in quantity of phosphoglycopeptide may result from the activity of exo-β-D-galactofuranosidase and phosphodiesterase(s). The μmol of carbohydrate and of phosphate per mg of polymers in **5** decreased several fold as a result of the heat treatment. In contrast, the quantity of phosphate and carbohydrate per mg of polymer **4** did not significantly decrease. It has been shown that exo-β-D-galactofuranosidase has little activity on highly phosphorylated species of **1** [22].

The ^{13}C and ^{31}P NMR spectra provide evidence that the carbohydrate and phosphodiesters in **4** are not significantly different than that in **1**. Changes in the apparent ratios of signal intensities at 1.32 and 1.47 ppm, relative to that at 0.22 ppm occurred. This alteration in relative quantities of phosphodiesters likely resulted from phosphomonoesterase and phosphodiesterase activities released by the heat treatment. There was no evidence in **4** for the release of significant quantity of polysaccharide(s) that are composed of glycosyl residues and linkages not observed in **1**.

The additional phosphoglycopeptide released by heat treatment could have originated from either membranes or from cell walls. It seems likely that heat treatment would have released **3** from the cytoplasmic membrane. This polymer should fractionate with **1** or **2** only if the heat treatment also released the lipid portion from **3**. The relative solubility of a fraction rich in phosphate and carbohydrate, and its insolubility in acid, suggests that protonation of anionic groups serves to decrease solubility of cell wall polymers that are extracted with 0.4 N potassium hydroxide. This conclusion is consistent with a polymer containing many phosphodiester residues.

Characterization of phosphodiesters in **1** *by Smith Degradation*

The structural characterization of phosphodiesters in **1** was performed using Smith degradation [34] and chromatography to isolate phosphodiesters derived from galactan chains separated from those derived from the mannan. Periodate oxidation of **1** followed by reduction with sodium borohydride and treatment with dilute acid yielded threitol phosphodiesters from the galactan chains and glycerol phosphodiesters from the mannan. Anionic threitol phosphodiesters can only be derived from phosphodiesters attached to C6 of 5-*O*-β-D-galactofuranosyl residues in galactan chains; these residues were separated from other products by DEAE-cellulose chromatography and exhibited signals at δ 1.47–1.1 ppm in the ^{31}P NMR spectrum. Threitol was identified by paper chromatography after treatment of the threitol phosphodiesters with 2 N HCl for 3 hr at 100°. These preliminary experiments suggest that the phosphodiesters found at δ 1.47–1.1 are *N*-peptidyl-2-aminoethanol phosphodiesters of galactofuranosyl residues and attached at C-6 of the sugar.

EXPERIMENTAL

Maintenance of Penicillium cultures. Penicillium fellutanum was maintained on Czapek–Dox-agar solid medium with glucose as the primary carbon source as described [4]. Approximately 10^6 conidia were transferred to 500 ml wide mouth Erlenmeyer flasks containing sterile standard growth medium [4] at 23° and aerated on a platform shaker for 5 days.

Heat treatment. Day-5 liquid shake cultures of *P. fellutanum* grown in standard growth media at 23° were subjected to 54° for 15, 30, 60 or 120 min. All cultures were harvested immediately after heat treatment with the exception of those heat-treated for 120 min. The 120 min cultures were returned to a shaker at 23° for 24 hr; these cultures were harvested after this interval.

Isolation and purification of phosphoglycopeptides, pPGM. On day 5 cultures were filtered, and the filtrate was dialysed in 14 000 M_r cut-off tubing in ddH_2O to remove small molecules. Phosphoglycopeptides were isolated and purified by a routine procedure [2, 4]. Polymers were sepd into two classes, **1** and **2**, on DEAE-cellulose-borate; these were the only significant carbohydrate-containing frs isolated from DEAE-cellulose. The salts were removed by dialysis and the solvent was removed by freeze-drying. The mass was determined and the samples were stored over a dessicant.

Isolation of Penicillium fellutanum cell walls. Day-3 mycelia of *P. fellutanum* were obtained by filtration through Whatman no. 4 paper, and were thoroughly rinsed, and frozen. This suspension was freeze-dried and cells were broken with a mortar and pestle at 4° and ground to a fine powder in 50 mM Tris pH 7.2 containing 0.1 M phenylmethylsulphonyl fluoride. Aluminum oxide, washed with EDTA, was added to the paste and grinding was continued until a high percentage of the mycelia were broken. The soluble cytoplasmic fr. was removed by low speed centrifugation at 4° and the cell walls and unbroken hyphae were resuspended in Tris–PMSF buffer and grinding followed by centrifugation. This procedure was continued until the prepn was free of cytoplasmic and membranous components. Aluminum oxide was

removed by centrifugation through a 65% sucrose soln. Sucrose was removed from the cell wall prepn by repeated centrifugation of the prepn suspended in H_2O at 4°. The washed (sucrose-free), prepn was frozen and freeze-dried.

Chemical treatments and partial solubilization of Penicillium cell walls

Treatment with 0.01 N and 2 N HCl. Cell walls, c.w., weighing 360 mg were suspended in 200 ml of 0.3 mM sodium azide at 4° with constant stirring for 24 hr. The suspension was passed through a sterile 0.2 μm filter with several washings with H_2O to remove insoluble c.w. material. Soluble solutes were sepd into mol. wt ranges by dialysis. Dilute acid treatment of c.w. followed a similar procedure with the following modifications. The cell wall prepn (36 mg) in 5 ml of 0.01 N HCl was transferred to a reaction vial, the suspension purged with dinitrogen and heated at 100° for 90 min. The reaction mixt. was neutralized with alkai and the insoluble c.w. material removed. The solubilized c.w. components in 100 ml soln were sepd by dialysis.

Treatment of c.w. (49 mg), with 2.0 N HCl followed the same general procedure except the prepn was held for 3 hr at 100°. Following this treatment, the prepn was neutralized with KOH and the insoluble residue was sepd from the soluble substances. Components in the aq. fr. were further fractionated by dialysis.

Treatment of cell walls with 0.4 N KOH. The procedure for extraction of solutes from c.w. (226 mg), with 0.4 N KOH was similar to that in which H_2O was the extractant except the solubilized material was neutralized with HCl and the insoluble residue was sepd by filtration. Components in the aq. fr. were fractionated by dialysis.

Analytical determinations

Determination of carbohydrates and phosphate. The μmol of carbohydrate per mg of polymer was determined by the PhOH–H_2SO_4 method [29] with glucose as a reference. The μmol of phosphate per mg of polymer was determined by the method of Ames [30] after samples were reduced to ash [31]; KH_2PO_4 was used as a reference.

Determination of protein. Protein was determined by the bicinchoninic acid protocol of Pierce Biochemicals. Bovine serum albumin (10–250 μg ml^{-1}) was used as the reference.

Galactose determination. The galactose content was determined after treatment of samples in 2 N HCl, 100°, for 3 hr, or treatment of samples in 0.01 N HCl, 100°, for 90 min. The galactose determination was performed using the coupled galactose oxidase procedure using *O*-cresol and horseradish peroxidase procedure [32].

Formation of and separation of DNS-amino acids and DANSYL-peptides. Soluble components of sized freeze-dried samples (16 μg protein) were reacted with DANSYL.Cl according to the procedure of Gros and Labouesse [33]. Peptides and amino acids were reacted with DANSYL.Cl. The DNS-derivatives were extracted into organic solvent, freeze-dried, dissolved in Me_2CO and 2-dimensional chromatography performed on polyamide plates with 1.5% HCO_2H for solvent I and C_6H_6–HOAc (9:1) as solvent II [3]. Samples containing peptides and those in which peptides had been converted to amino acids with 6 N HCl were both examined for DNS amino acids.

Smith degradation procedures and product analyses. Polymer **1**, 200 mg, was dissolved in 20 ml of a soln of 50 mM sodium periodate/0.3 M NaOAc, pH 4.5 and allowed to react for 24 hr. The reaction mixt. was neutralized and subsequently treated with 50-fold excess of $NaBH_4$ in 0.3 M $Na_2B_4O_7$, pH 8 for 8 hr at room temp. The reaction mixt. was neutralized and was dialysed *vs* ddH_2O in 1000 MWCO membranes and the retentate containing the polyols was treated with 0.1 N HCl, at 25°, for 16 hr [34]. The hydrolysates were neutralized and subjected to chromatography on DEAE-cellulose to separate phosphocholine phosphodiesters from *N*-peptidyl-2-aminoethanol phosphodiester; the former elute quickly in H_2O wash with the later eluting at higher NaCl concs. Paper chromatography of phosphodiesters was performed following hydrolysis (2 N HCl, 100°, 3 hr) and removal of acid by rotary evapn. The silver staining procedure was used to visualize the Smith degradation products. The ^{31}P NMR spectroscopy of threitol phosphodiester was performed as described previously.

Sizing of solutes by dialysis. Molecular size sepn of components in culture filtrates and solubilized cell wall frns was accomplished by dialysis at 4°. Membranes of M_r cut off of 1000 and 14000 were used to separate components into frs of M_r less than 1000, 1000 to 14000 and greater than 14000.

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REFERENCES

1. Preston, J. F., Lapis, E., Westerhouse, S. and Gander, J. E., *Journal of Biological Chemistry*, 1969, **259**, 324.
2. Gander, J. E., Jentoft, N. H., Drewes, L. R. and Rick, P. D., *Journal of Biological Chemistry*, 1974, **249**, 2063.
3. Rick, P. D., Drewes, L. R. and Gander, J. E., *Journal of Biological Chemistry*, 1974, **249**, 2073.
4. Salt, S. D. and Gander, J. E., *Experimental Mycology*, 1985, **9**, 9.

5. Salt, S. D. and Gander, J. E., *Experimental Mycology*, 1988, **12**, 243.
6. Applegarth, D. A. and Bozoian, G., *Canadian Journal of Microbiology*, 1968, **14**, 489.
7. Martin, J. R., Nicolas, G. and Villanueva, J. R., *Canadian Journal of Microbiology*, 1973, **19**, 789.
8. Unger, P. D. and Hayes, A. W., *Journal of Genetics and Microbiology*, 1975, **91**, 201.
9. Rizza, V. and Kornfeld, J. M., *Journal of Genetics and Microbiology*, 1969, **58**, 307.
10. Miyazaki, T. and Yadomae, T., *Chemical and Pharmaceutical Bulletin*, 1969, **17**, 361.
11. Grisaro, V., Sharon, N. and Barkai-Golan, R., *Journal of Genetics and Microbiology*, 1968, **4**, 145.
12. Applegarth, D. A., *Canadian Journal of Microbiology*, 1967, **120**, 471.
13. Leal, J. A., Moya, A., Gomez-Miranda, B., Ruperez, P. and Guerrero, C. in *Cell Wall Synthesis and Autolysis*, ed. C. Nombela. Elsevier Science, Oxford, 1984, p. 149.
14. Gomez-Miranda, B., Moya, A. and Leal, J. A., *Experimental Mycology*, 1986, **10**, 184.
15. Ruperez, P., Moya, A. and Leal, J. A., *Archives of Microbiology*, 1986, **146**, 250.
16. Gomez-Miranda, B., Prieto, A. and Leal, J. A., *FEMS Microbiology Letters*, 1990, **70**, 331.
17. Leal, J. A., Guerrero, C., Gomez-Miranda, B., Prieto, A. and Bernabe, M., *FEMS Microbiology Letters*, 1992, **90**, 165.
18. Gander, J. E. and Fang, F., *Biochemistry and Biophysics Research Communications*, 1976, **71**, 719.
19. Gander, J. E., Beachy, J. C., Unkefer, C. J. and Tonn, S. J., in *ACS Symposium Series*, Vol. 126, *Fungal Polysaccharides*, ed. P. A. Sanford and K. Matsuda. American Chemical Society, Washington, D.C., 1980, p. 49.
20. Gander, J. E. and Laybourn, C. J. in *Recent Advances in Phytochemistry*, Vol. 15, ed. F. A. Loewus and C. A. Ryan. Plenum Press, New York, 1981, p. 59.
21. Rietschel-Berst, M., Jentoft, N. H., Rick, P. D., Pletcher, C. H., Fang, F. and Gander, J. E., *Journal of Biological Chemistry*, 1977, **252**, 3219.
22. Tuekam, B. A. and Gander, J. E. (unpublished).
23. Park, Y.-I., Buszko, M. L. and Gander, J. E., *Journal of Bacteriology*, 1997, **179**, 1186.
24. Unkefer, C. J. and Gander, J. E., *Journal of Biological Chemistry*, 1990, **265**, 685.
25. Unkefer, C. J. and Gander, J. E., *Journal of Biological Chemistry*, 1979, **254**, 12 131.
26. Unkefer, C. J., Jackson, C. L. and Gander, J. E., *Journal of Biological Chemistry*, 1982, **257**, 2491.
27. Bonetti, S. J. and Gander, J. E., *FASEB*, 1988, **2**, A4291.
28. Cosyleon, A., Bayha, T., Sanchez, D. and Bonetti, S., *FASEB Journal*, 1994, **8**, A1425 abst. 9657.
29. Dubois, M., Giles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., *Analytical Chemistry*, 1956, **28**, 350.
30. Ames, B. N., in *Methods in Enzymology*, Vol. 8, ed. E. F. Neufeld and V. Ginsburg. Academic Press, New York, p. 115.
31. Ames, B. and Dubin, D. T., *Journal of Biological Chemistry*, 1960, **235**, 769.
32. Fischer, W. and Zapf, J., *Zeitschrift für Physiologica und Chemisch*, 1964, **337**, 186.
33. Gros, C. and Labouesse, B., *European Journal of Biochemistry*, 1969, **7**, 463.
34. Smith, F. and Unrau, A. M., *Chemistry and Industry*, 1959, 881.