THE OCCURRENCE OF ETHANOLAMINE AND GALACTOFURANOSYL RESIDUES ATTACHED TO PENICILLIUM CHARLESII CELL WALL SACCHARIDES

J. E. Gander and Faye Fang

Department of Biochemistry
College of Biological Sciences
University of Minnesota
St. Paul, Minnesota 55108

Received June 1, 1976

SUMMARY

Penicillium charlesii incorporates ^3H or ^{14}C from ^3H- or ^{14}C-labeled ethanolamine into an alkali soluble, alcohol-insoluble fraction obtained from cell walls. Dansyl ethanolamine was isolated from this alcohol-insoluble fraction following dansylation and hydrolysis. The alcohol-insoluble material was non-dialyzable and contained galactofuranosyl, glucosyl, phosphoryl, amino acyl and variable quantities of uronosyl residues. The lack of detectable quantities of mannosyl residues in this material suggests that the galactofuranosyl-containing cell wall polymer is distinct from the peptidophosphogalactomannan which is obtained from culture filtrates of *P. charlesii* (Gander *et al.*, (1974) J. Biol. Chem. 249, 2063).

INTRODUCTION

The major extracellular peptidophosphogalactomannan, PPGM, produced by *P. charlesii* is composed of a phosphogalactomannan, mannosyl-containing oligosaccharides and mannosyl residues attached to seryl and threonyl residues of a polypeptide (1). The phosphogalactomannan is comprised of a phosphomannan backbone to which approximately 10 galactan chains are attached to the mannan by β(1→3) linkage. The chains contain 2 to 15 5-O-β-D-galactofuranosyl residues. Ethanolamine (2) and N,N-dimethylethanolamine (3) are attached to the mannan.

Ethanolamine has been found in lipopolysaccharide from *Salmonella* species (4) and *E. coli* (5) strains. Choline is a constituent of pneumococcal cell wall teichoic acid (6,7). Pneumococcal cell walls containing ethanolamine are resistant to degradation by autolytic enzymes.

Fungal cell walls contain neither lipopolysaccharide nor teichoic acid, and ethanolamine or its N-methylated derivatives have not been reported as constituents of fungal cell walls previously.

Ascomycetes, the class to which Penicillium species belong, contains chitin-glucan as the major cell wall polysaccharide (8), and the Ascomycetes also contain alkali-soluble, alcohol-insoluble saccharides (9,10,11).

This investigation was undertaken to determine if extracellular PPGM is derived from P. charlesii cell walls.

MATERIALS AND METHODS

a. Penicillium charlesii cell wall isolation and purification. Penicillium charlesii (ATCC 1877) was cultured at 20° on a gyratory shaker (Model 10, Brunswick Scientific Corporation) at a setting of 8 for 3 days in a modified Raulin-Thom medium (1). The cell walls were prepared by a modification of the Mahadevan and Tatum procedure (9). The cells were lyophilized, stirred for 72 hr at 4° in 1% sodium dodecyl sulfate, and then for 24 hr at 25°. The preparation was washed with H_2O and ethanol (9), and extracted 3 times with $CHCl_3$:methanol (1:1 v/v) to remove contaminating lipid. The cell walls were washed with ethanol. Examination of the preparation under phase contrast microscope showed that the cytoplasmic material had been removed and that only cell walls remained.

b. Isolation of cell wall fractions. The cell wall was fractionated into 4 fractions (9). Fraction 1, containing the alkali-soluble substances, was separated into an ethanol-soluble (1b) and ethanol-insoluble-nondialyzable (1a) fractions.

c. Determination of galactofuranosyl residues. Galactofuranosyl residues were determined by exo-β-D-galactofuranosidase catalyzed release of galactose (12,13) followed by quantitation of D-galactose with galactose oxidase reaction (14). Controls contained no galactofuranosidase.

d. Determination of residues carrying free amino groups. Fraction 1a was dansylated (15), the product hydrolyzed in 6 N HCl for 24 hr, and the products chromatographed on polyamide sheets as described previously (2).

e. Analytical procedures. Total carbohydrate was determined by the phenol sulfuric acid method with glucose as a standard (16). Uronic acids were determined by the modified carbazole method (17), following hydrolysis of fraction 1a with 2 N HCl for 2 hr at 100° in a sealed, evacuated tube. Amino sugars were visualized by ninhydrin following hydrolysis in 4 N HCl for 5 hr at 100° in a sealed tube and chromatography on Whatman 1 paper in 1-butanol:pyridine:water (6:4:3, v/v/v). Glucosamine and galactosamine were used as references. Phosphorus was determined by ashing the sample (18) followed by the method of Parvin and Smith (19) for quantitation. Samples containing ^{3}H or ^{14}C were analyzed in a Beckman LS-230 liquid scintillation spectrometer. Radioactive substances were from Amersham/Searle Corp.

f. Amino acid analyses. Samples (10 mg) containing approximately 45 μmoles of anhydrohexosyl residues were treated at 110° with 2 ml of 6 N HCl for 24 hr. The HCl was removed, the residue was dissolved in sodium citrate buffer (0.2 M Na^+), pH 2.2, and the sample analyzed on a Beckman-Spinco amino acid analyzer.

g. Chromatographic separation and identification of sugars. The sugars released by treatment of 5 mg of sample with 2 N HCl in a sealed evaluated tube for 2 hr at 100° were separated by chromatography on Whatman 3 MM with 1-butanol:pyridine:H_2O (6:4:3, v/v/v) as the solvent.

h. Acetolysis of fraction 1a. Fifteen mg of sample was treated for 18 hr at 37° as described by Stewart et al. (20).

i. Smith degradation of fraction 1a. Smith degradation of 10 mg of sample was carried out in 0.05 M $NaIO_4$ (21). Following Smith degradation the products were chromatographed on Whatman 3 MM paper as described for neutral sugars above. Glycerol, threitol, erythritol, glucose and galactose were used as references.

RESULTS AND DISCUSSION

[1-^{3}H]Ethanolamine (200 μCi) was added to 120 ml of P. charlesii culture at 1.5 days after inoculation and the cells harvested on day 3. The cell walls were isolated as described above. Cell walls (1 g) were treated with 2 N NaOH and the distribution of ^{3}H in alkali-soluble (fraction 1) and alkali-insoluble fractions (II-IV) is shown in table 1. Approximately 65% of the ^{3}H is located in fraction 1 and the majority of the remainder of ^{3}H is released by treatment of the residue with 1 N H_2SO_4 (fraction II). The alcohol-insoluble, nondialyzable fraction (1a) contained 77% of the ^{3}H in fraction 1. [2-^{14}C]Ethanolamine provided in the growth medium under similar conditions also resulted in a major fraction of radioactivity in fraction 1a (not shown).

Fractions 1 through IV were treated with galactofuranosidase. Galactose was released from fraction 1a (table 1). Treatment of fractions 1a and 1b with 0.01 N HCl at 100° for 90 min, which hydrolyzes galactofuranosyl but not galactopyranosyl residues, released galactose from fraction 1a but not from fraction 1b.

The composition of fraction 1a was examined by Smith degradation and paper chromatography of the products, by acetolysis, by determining the neutral sugars uronic acids, amino acids and amino sugars following appropriate hydrolysis conditions, and by determining the NH_2-terminal amino acids following dansylation and hydrolysis. Galactose and glucose and from 0 to 5% uronosyl residues were found along with traces of amino sugars. Smith degradation of the cell wall polymer resulted in the destruction of galactose with the formation of threitol and lesser quantities of glycerol. Glucose was not appreciably degraded by this procedure. Acetolysis did not solubilize appreciable quantities of the polymer, and no oligosaccharides were obtained by this treatment. All galactofuranosyl residues were cleaved by this treatment. The polymer(s) contains few if any 1→6 pyranosidic linkages.

Table 1

INCORPORATION OF ^{3}H FROM [1-^{3}H]ETHANOLAMINE INTO A CELL WALL POLYMER CONTAINING GALACTOFURANOSYL RESIDUES

Fraction	cpm	galactofuranosyl residues
		nmole mg^{-1}
I	3.24×10^6	-
Ia	2.3×10^6	108
Ib	0.7×10^6	none
II	1.78×10^6	none
III	3.6×10^3	none
IV	none	none

Fraction Ia was obtained as described in the text after P. charlesii metabolized [1-^{3}H]ethanolamine added to the growth medium. Galactofuranosyl residues were determined by treating 1-5 mg of cell wall fraction with exo-β-D-galactofuranosidase (13) for 24 hr at 37° followed by estimating the galactose released with the galactose oxidase-peroxidase coupled reaction (14).

Table 2

DEGRADATION PRODUCTS OF FRACTION Ia

Treatment	Polymer	Gal	Glc	Man	Threitol	Erythritol	Glycerol
0.01 N HCl, 100°	+++	+++	-	-	-	-	-
2.0 N HCl, 100°[a]	-	+++	+++	-	-	-	-
Smith Degradation	-	-	+++	-	++	-	+
Acetolysis	++++	+++	-	-	-	-	-

Fraction Ia was degraded as described in the "Materials and Methods" section. Samples containing 25 to 75 µmoles of anhydrohexose were used.

[a]Traces of glucuronic or galacturonic acid were found by this treatment.

Table 3

^{14}C FROM [2-^{14}C]ACETATE INCORPORATED INTO FRACTION Ia

Fraction	cpm		
	Total	per μmole sugar	per μmole amino acid
Ia	5.0×10^4	58	-
Bound to Dowex 50	3.9×10^4	-	1200
Unbound to Dowex 50	1.1×10^4	-	-
water soluble	2.0×10^2	-	-
$CHCl_3$:Methanol soluble	2.1×10^3	-	-

Fraction Ia was obtained as described in the text with the following modification. The cells were stirred for an additional 48 hr at 4 in 1% SDS followed by 72 hr at 25°. The cell wall polymer obtained following precipitation with ethanol contained only a trace of uronosyl residues.

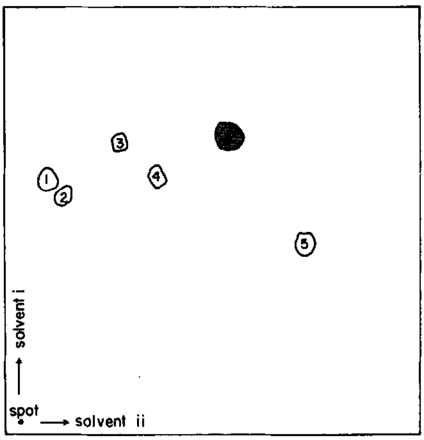

Fig. 1. Schematic representation of a polyamide thin layer chromatogram showing the position of dansyl-ethanolamine (cross-hatched area) and dansyl-amino acids obtained from P. charlesii alkali-soluble, ethanol-insoluble cell wall polymer. 10 mg of fraction Ia was dansylated, the product hydrolyzed and chromatographed as described in "Materials and Methods". Areas labeled 1, 2, 3, 4, and 5 represent dansyl-aspartate, dansyl-glutamate, dansyl-serine, dansyl-glycine, and dansyl-valine, respectively.

Fractions Ia and Ib contain 47.2 and 500 nmoles of amino acid per mg., respectively. The free amino terminal groups in fraction Ia were determined as their dansyl derivatives, and were shown to be aspartate, glutamate, serine, glycine, valine and ethanolamine (Fig. 1). Ten mg of fraction 1a contained no more than 0.1 nmole of each of the dansyl amino acids listed above. Ethanolamine was not released by treatment with 0.01 N HCl for 4 hr at 100° and appears not to be attached to the galactofuranosyl residues.

Phosphate analysis showed that fraction 1a contained 29 nmoles mg^{-1}.

The incorporation of ^{14}C into fraction 1a was determined after P. charlesii was cultured on 250 μCi of [2-^{14}C]acetate for 1.5 days. The cells were harvested at 3 days, fraction 1a obtained and ^{14}C in amino acids and in $CHCl_3$:methanol (1:1, v/v) soluble fraction was obtained. A relatively small quantity of ^{14}C was incorporated into fraction 1a, and 78% of the ^{14}C in that fraction was removed upon passage through a cation-exchange resin. Only about 4% was lipophilic.

DISCUSSION

Penicillium charlesii cell walls treated with 2 N NaOH released a polymer composed primarily of galactofuranosyl and glucosyl residues with minor quantities of uronosyl and amino acyl residues. Radioactivity from ^{3}H or ^{14}C labeled ethanolamine added to the culture was incorporated into the polymer. Dansylation of the polymer followed by hydrolysis resulted in the formation of dansylethanolamine. A hexose to phosphate ratio of 200:1 was found.

The release of glucose by Smith degradation of the cell wall polymer suggests the occurrence of 1→3 glucosidic linkages, and the occurrence of threitol suggests the occurrence of either 1→5 or 1→6 linked galactofuranosyl residues.

The data show that phosphogalactomannan, the major polysaccharide of an exocellular glycopeptide found in the growth medium supporting P. charlesii cultures is not derived from cell walls. If the glycopeptide was derived from the cell wall, treatment of the cell wall with alkali would have released phosphogalactomannan. The other fractions from the cell wall preparation contained no galactofuranosyl residues as determined by the release of galactose by the action of galactofuranosidase. The alkali-soluble galactofuranosyl-containing substance may provide the antigenic determinants that results upon injecting killed whole *P. charlesii* cells into rabbits (23). This antisera reacts with PPGM (23).

ACKNOWLEDGEMENTS

This research was supported by a research grant from the Research Corporation, by Research Grant GB 21261 from the National Science Foundation, by the University of Minnesota Graduate School and the University of Minnesota Agricultural Experiment Station, Scientific Journal Series No. 9511 Agricultural Experiment Station, University of Minnesota, St. Paul, Minnesota 55108.

REFERENCES

1. Gander, J. E., Jentoft, N. H., Drewes, L. R., and Rick, P. D. (1974) J. Biol. Chem. 249, 2063-2072.
2. Rick, P. D., Drewes, L. R., and Gander, J. E. (1974) J. Biol. Chem. 249, 2073-2078.
3. Gander, J. E., and Fang, F. (1974) Biochem. Biophys. Res. Commun. 58, 368-374.
4. Grollman, A. P., and Osborn, M. J. (1964) Biochemistry 3, 1571-1574.
5. Ikawa, M., Koepfli, J. B., Mudd, S. G., and Niemann, C. (1953) J. Amer. Chem. Soc. 75, 3439-3442.
6. Brundish, D. E., and Baddiley, J. (1968) Biochem. J. 110, 573-582.
7. Mosser, J. L., and Tomasz, A. (1970) J. Biol. Chem. 245, 287-298.
8. Mahadevan, P. R., and Tatum, E. L. (1965) J. Bacteriol. 90, 1073-1081.
9. Bartnicki-Garcia, S. (1968) Ann. Rev. Microbiol. 22, 87-108.
10. Mahadevan, P. R., and Tatum, E. L. (1967) J. Cell. Biol. 35, 295-302.
11. Bardalaye, P. C., and Nordin, J. H. (1976) J. Bacteriol. 125, 655-669.
12. Rietschel-Berst, M., and Gander, J. E. (1973) Fed. Proc. 32, 528.
13. Rietschel-Berst, M., Jentoft, N. H., Rick, P. D., Fang, F., and Gander, J. E., J. Biol. Chem. (in press).
14. Fischer, W., and Zapf, J. (1964) Z. Physiol. Chem. 337, 186-195.
15. Gros, C., and Labouesse, B. (1969) Eur. J. Biochem. 7, 463-470.
16. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Anal. Chem. 28, 350-356.
17. Bitter, T., and Muir, H. M. (1962) Anal. Biochem. 4, 330-334.
18. Ames, B. N., and Dubin, O. (1960) J. Biol. Chem. 235, 769-775.
19. Parvin, R., and Smith, R. A. (1969) Anal. Biochem. 27, 65-72.
20. Stewart, T. S., Mendershausen, P. B., and Ballou, C. E. (1968) Biochemistry 7, 1843-1854.
21. Smith, F., and Unrau, A. M. (1959) Chem. Ind. p. 881.
22. Plaisted, P. H. (1958) Boyce Thompson Inst., Contrib. 19, 231-244.
23. Preston, J. F., Lapis, E., and Gander, J. E. (1970) Can. J. Microbiol. 16, 687-694.