Carbohydrate Research, 37 (1974) 331–338

MYCOLAMINARANS: STORAGE (1→3)-β-D-GLUCANS FROM THE CYTOPLASM OF THE FUNGUS *Phytophthora palmivora*

M. C. WANG AND S. BARTNICKI-GARCIA

Department of Plant Pathology, University of California, Riverside, California 92502 (U. S. A.)

(Received March 13th, 1974; accepted for publication, April 11th, 1974)

ABSTRACT

Laminaran-like polysaccharides were isolated from zoospores and mycelium of *Phytophthora palmivora*. These glucans, which we have termed mycolaminarans, are readily soluble in cold water, appear highly homogeneous by gel filtration, and contain exclusively D-glucose residues. Methylation analyses and enzymic digestions revealed that mycolaminarans are linear chains of (1→3)-linked β-D-glucose residues, with one or two branches joined by (1→6)-β-D linkages. The zoospore mycolaminaran has one branching residue and $\overline{\text{d.p.}}$ 29. The mycelial mycolaminaran has two branching residues and $\overline{\text{d.p.}}$ 36. The possibility is considered that mycolaminarans may be distinctive storage-polysaccharides of the oomycetous fungi.

INTRODUCTION

In previous studies[1,2], we showed that the cytoplasm of the oomycetous fungus *Phytophthora palmivora* contains substantial proportions of water-soluble β-D-glucans hereinafter referred to as mycolaminarans. In the mycelium stage of *P. palmivora*, only a neutral-type mycolaminaran is present, but in other stages of the life cycle, there are also phosphorylated forms[1] (mycolaminaran phosphates). We now report on the structure of neutral mycolaminaran isolated from mycelium and zoospores of *P. palmivora*.

Glycogen, the usual storage-polysaccharide of fungi, is absent in *Phytophthora* and related organisms. Zevenhuizen and Bartnicki-Garcia[3] found that a soluble (1→3)-β-(1→6)-β-linked D-glucan (mycolaminaran) constituted the storage polysaccharide of *Phytophthora cinnamomi*.

MATERIALS AND METHODS

Microbiological techniques. — A papaya isolate of *Phytophthora palmivora* (P113) was obtained from M. Aragaki of the University of Hawaii. The organism was

formerly designated as *Phytophthora parasitica*[2]. The methods for the maintenance of the culture and the production of mycelium and zoospores were described elsewhere[2]. The mycelium was grown for 7 days in a D-glucose–asparagine liquid medium[4]. The zoospores were harvested from V-8-juice–agar cultures.

Mycolaminaran isolation. — The mycelium or zoospores of *P. palmivora* were consecutively extracted[1] with hot 70% (v/v) ethanol and with hot, distilled water. The combined extracts were dialyzed against water and fractionated by DEAE-Sephadex chromatography[1]. The neutral β-D-glucan fraction was purified by gel filtration using a column (1.9 × 105 cm) of Sephadex G-75 equilibrated with water. This resulted in a single, symmetrical peak of mycolaminaran. Mycolaminaran-containing fractions were combined, concentrated, and freeze-dried.

Methylation analysis. — Mycolaminarans were methylated by the Hakomori[5] method following the procedure employed by Lindberg and his co-workers[6]. The methylsulfinyl sodium reagent was prepared according to the method of Sandford and Conrad[7]. To a solution of mycolaminaran (~20 mg) in methyl sulfoxide (3 ml) in a serum vial (rubber stopper and aluminum cap) sealed under N_2, methylsulfinyl sodium solution (3–5 ml) was introduced, and the sample was incubated overnight. An excess of methyl iodide was then added and the mixture incubated for several hours. The vial contents were dialysed exhaustively against water in a cellophane bag and then evaporated to dryness. The sample was remethylated, as needed, and then hydrolyzed and analyzed by g.l.c. as described below.

Mycolaminaran hydrolysis. — The polysaccharide (methylated or non-methylated) was first soaked with <1.0 ml of 11.25M H_2SO_4 at room temperature for 5 h, the acid was diluted to 425mM, and the container was sealed and heated at 105° for 8–10 h. The acid was neutralized with $BaCO_3$, and the hydrolyzate was deionized through a double-bed column of Dowex-50(H^+) and Dowex-1(AcO^-) resins. The sugars in the hydrolyzate were reduced with $NaBH_4$ overnight. The solution was acidified to decompose excess borohydride and evaporated. Methanol was distilled several times from the residue to remove boric acid. To the dry residue, 1 ml of dry pyridine and 1 ml of acetic anhydride were added, and the sealed container was heated at 105° for 20 min. The solvents were then removed by repeated codistillation with toluene. The final residue, containing the alditol acetate derivatives, was dissolved in a few drops of chloroform and used for g.l.c.

Gas–liquid chromatography and mass spectrometry. — Mycolaminaran hydrolyzates containing alditol acetates of sugars[8], or their methylated derivatives[9], were separated on a glass column (180 × 0.3 cm) packed with 3% (w/w) ECNSS-M on Gas Chrom Q (100–120 mesh). A Perkin–Elmer gas chromatograph, Model 990, with a flame-ionization detector was employed. Retention times (*T*) were calculated by interpolation with the alditol acetates of 2,3,4,6-tetra-*O*-methyl-D-glucose (*T* 1.00) and 2,3,6-tri-*O*-methyl-D-glucose (*T* 2.50) as references. The identity of methylated sugar derivatives was confirmed by mass spectrometry[10] with a Finnigan mass spectrometer, Model 1015, coupled to a gas chromatograph.

Sugar determinations. — Total hexose was determined by the anthrone reagent[11]

with D-glucose as the standard. Following hydrolysis of the polysaccharide, monosaccharide components were separated and determined as alditol acetates by g.l.c.[8] as described above.

Miscellaneous. — The infrared spectra of methylated D-glucans (in chloroform) were obtained with a Perkin–Elmer Infracord spectrophotometer. Optical rotations were measured with a Bendix automatic polarimeter, Model 1100. Molecular weight estimates were made by gel filtration[12] on a Sephadex G-75 column (1.8 × 105 cm) calibrated with *Leuconostoc mesenteroides* dextrans: T40 ($\overline{M}_w = 42{,}400$), T20 ($\overline{M}_w = 22{,}300$), and T10 ($\overline{M}_w = 9{,}300$) purchased from Pharmacia, A.B., Uppsala, Sweden. Zoospore mycolaminaran[1] ($\overline{M}_w$ 4900) was also included as a standard.

Enzymic digestion. — Mycolaminaran (10 mg) was digested with 2.0 units of a purified preparation of exo-(1→3)-β-D-glucanase[13] from a basidiomycete (*Sporotrichum dimorphosporum*) (QM 806) in 4.0 ml of 0.05M sodium acetate buffer (pH 4.8) at 37° for 24 h with a few drops of toluene added as an antiseptic. After heat-inactivation (97° for 5 min), the mixture was directly applied to a Sephadex G-15 column (2.5 × 170 cm), and the products were eluted with water. Fractions of 4 ml were collected. The components of the disaccharide peak were further resolved by descending, paper chromatography, using Whatman 3MM paper. The chromatograms were irrigated with 1-butanol–pyridine–water (6:4:3) or ethyl acetate–acetic acid–water (9:2:2). The sugars were revealed by spraying with aniline hydrogen phthalate. The disaccharides obtained after enzymic hydrolysis were identified[14] by reduction with KBH_4 to the corresponding alditols and permethylation by the Hakomori procedure (as above). The methylated alditols were separated by g.l.c. using a glass column (180 × 0.3 cm) packed with 2.2% SE-30 on Chromosorb W (60/80 mesh). The exo-(1→3)-β-D-glucanase was prepared according to the procedure of Huotari *et al.*[15].

RESULTS

General properties. — Mycolaminarans were isolated from zoospores or mycelium of *Phytophthora palmivora* as white powders in yields of 6 and 34% dry wt., respectively. The u.v. spectra of the two mycolaminarans showed that they were free of protein or nucleic acid. Both samples were readily soluble in cold water, and could also be dissolved in cold 50% ethanol or in hot 70% ethanol. Zoospore mycolaminaran, $[\alpha]_D^{20}$ −15° (*c* 0.34, water), melted in the range 280–287°; mycelial mycolaminaran, $[\alpha]_D^{20}$ −10° (*c* 0.57, water), melted in the range 283–289°. On gel filtration (Sephadex G-75), sharp, symmetric peaks were obtained for both mycelial and zoospore mycolaminarans (Fig. 1 and Ref. 1). From gel filtration values, $\overline{M}_w$ was calculated to be 4900 (zoospore)[1] and 5400 (mycelium). Because of expected differences in shape between mycolaminarans and the Pharmacia (1→6)-α-D-linked dextrans used for calibration, these $\overline{M}_w$ values are only tentative approximations.

On complete, acid hydrolysis, both the zoospore and mycelial mycolaminaran yielded D-glucose as the sole monosaccharide. There was no evidence for mannitol in the gas chromatograms of mycolaminaran hydrolyzates.

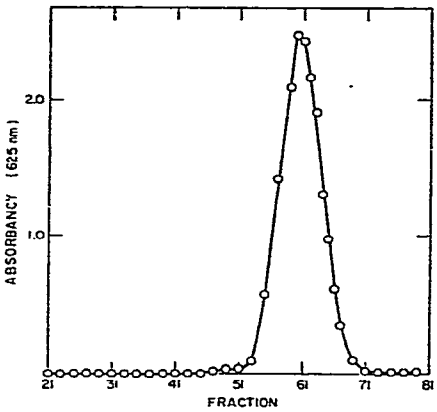


Fig. 1. Gel-filtration profile of mycelial mycolaminaran on a Sephadex G-75 column.

Mycolaminarans were rapidly decomposed by hot alkali. A solution of mycelial mycolaminaran (1.7 mg/ml) in 0.8M KOH, when heated at 97° for 5 min, underwent 85% decomposition, as determined by anthrone assays.

Methylation analysis. — Samples of mycolaminaran were fully methylated after two or three treatments by the Hakomori procedure. After only one methylation, the i.r. spectra showed no hydroxyl absorption (3500 cm^{-1}) and, after hydrolysis, there was no g.l.c. evidence of any unmethylated or monomethylated glucitol derivatives—indicators of incomplete methylation. However, a second or third methylation was necessary to attain ratios of tetra-*O*-methyl and di-*O*-methyl derivatives compatible with a fully methylated polysaccharide. Remethylation also caused the disappearance of two minor peaks having the retention times of alditol acetates of 2,3,6(or 2,3,4)-tri-*O*-methyl- and 2,6-di-*O*-methyl-D-glucose. These were probably undermethylation artifacts.

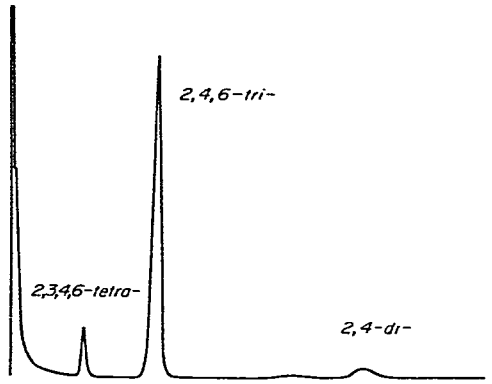
TABLE I

G.L.C. ANALYSIS OF PERMETHYLATED MYCOLAMINARANS FROM ZOOSPORES AND MYCELIUM OF *P. palmivora*

O-Methyl-D-glucitol acetate derivative	*Retention time* (T)	*Molar ratio* (%)	
		Zoospore	*Mycelial*
2,3,4,6-Tetra-	1.00	6.5	8.0
2,4,6-Tri-	1.95	89.9	86.3
2,4-Di-	4.85	3.6	5.8

Three different methylated glucose derivatives were separated in the hydrolyzates of fully methylated mycolaminarans (Fig. 2 and Table I), and their identities confirmed by mass spectrometry. The methylation data show that mycolaminarans are mainly (1→3)-linked D-glucans, with occasional branching at C-6. The same methylation

products were obtained from the zoospore and mycelial mycolaminarans; there were, however, slight differences in relative proportions. From these results, the d.p. values and structural features summarized in Table II were deduced. The zoospore mycolaminaran has only one branching residue per molecule, and is somewhat smaller than the mycelial mycolaminaran, which contains two branches per molecule.



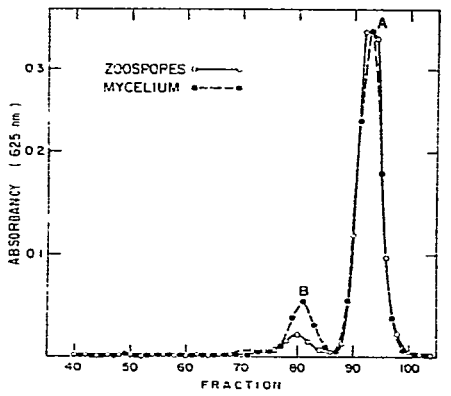


Fig. 2. Separation of methylation products of zoospore mycolaminaran by g.l.c.

Fig. 3. Separation by gel filtration of digestion products of zoospore and mycelial mycolaminarans treated with exo-(1→3)-β-D-glucanase. The entire incubation mixture was applied to a Sephadex G-15 column. No trace of the initial polymer was detected: A, D-glucose; B, gentiobiose.

TABLE II

STRUCTURAL FEATURES OF MYCOLAMINARANS FROM THE MYCELIUM AND ZOOSPORES OF *Phytophthora palmivora*

Type of D-*glucose residue*	*Number of residues per molecule*	
	Zoospore	*Mycelium*
Reducing terminal	1	1
Non-reducing terminal	2	3
Branching residues (C-3 and C-6)	1	2
Linear residues (C-3-linked)	25	30
Sum (d.p.)	29	36

Enzymic digestion. — Treatment of mycelial mycolaminaran with a purified exo-(1→3)-β-D-glucanase for 24 h caused complete digestion of the glucan. Two products, resolved by gel filtration (Fig. 3), accounted for essentially all of the original polymer: D-glucose (89.4%) and gentiobiose (10.6%, identified by paper chromatography). There was also a trace (0.5%) of laminarabiose. The identity of gentiobiose was confirmed by reduction to gentiobiitol with sodium borohydride, permethylation, and gas–liquid cochromatography with an authentic sample of gentiobiitol acetate.

A sample of zoospore mycolaminaran, when similarly digested, gave the same products but with slightly different proportions of D-glucose (93.4%) and gentiobiose (6.6%) (Fig. 3). A minute "peak" of a trimer, probably laminaratriose, was also detected in the gel-filtration profile (Fig. 3).

In both mycelial and zoospore mycolaminarans, the amount of gentiobiose recovered was very close to the value predicted from the methylation data (Table II), *i.e.*, twice the amount of 2,4-di-*O*-methylglucose.

DISCUSSION

Laminaran is a well-known type of storage polysaccharide typically, but not exclusively, found in marine brown-algae (Phaeophyta) (for a review, see Percival and McDowell[16]). The term laminaran (laminarin) does not describe a single, molecular species but a whole range of water-soluble, essentially linear (1→3)-β-D-glucans of relatively low molecular weight, isolated from algae belonging to the Phaeophyta and Chrysophyta. Depending mainly on the source, laminaran samples may differ in their solubility in cold water, presence of D-glucan chains terminated with a mannitol residue, degree of branching at C-6, occurrence of linear (1→6)-linked residues, average branch-length, and overall degree of polymerization (d.p.). Values of 16–33 have been reported[17] for the latter.

Chrysolaminaran[18], a laminaran extracted from fresh-water diatoms (Chrysophyta), differs from the original laminaran[19], prepared from seaweeds of the genus *Laminaria*, in not having mannitol residues.

The soluble D-glucans of the fungus *Phytophthora palmivora* described herein have properties that fall within the above generic description of a laminaran. They are water-soluble polymers of relatively low $\overline{\text{d.p.}}$ (29 and 36). Their levorotatory properties and complete digestion by exo-(1→3)-β-D-glucanase indicated that these polymers consist entirely of β-linked D-glucose residues. Methylation studies showed that mycolaminarans are (1→3)-β-D-glucans with branches at C-6; the mycelial mycolaminaran has 2 branches, whereas the zoospore mycolaminaran contains a single branch per molecule. The position and length of the branches remain to be determined. There was no evidence for linear (1→6)-linked residues as detected in some algal laminarans[20]. Mannitol residues are found in the terminal ends of some types of algal laminaran[16], but there was no evidence of mannitol in the two mycolaminarans studied herein. In fact, the two mycolaminarans behaved as reducing carbohydrates. A reducing end-group determination (Somogyi–Nelson) gave a $\overline{\text{d.p.}}$ of 36 for the zoospore mycolaminaran[1], a value in reasonable accord with other determinations of molecular size by gel filtration, methylation, and enzymic digestion.

There was excellent agreement between methylation-analysis values and enzymic digestion results. Exo-(1→3)-β-D-glucanase depolymerized the mycolaminarans into D-glucose and a small quantity of gentiobiose. The amount of gentiobiose corresponded closely to that expected from the proportion of branching units in each mycolaminaran sample as found by methylation analysis. Seemingly, the enzyme

was able to cleave all glycosidic bonds, bypassing the (1→6)-β-D linkage(s) at the branching point(s). A comparable behavior has been noticed in other glucans (including laminaran), with (1→3)-β-D and (1→6)-β-D linkages, digested by the same enzyme[21–23].

The close agreement between $\overline{M}_w$ estimates of both zoospore and mycelial mycolaminarans (4900 and 5400) and the $\overline{M}_n$ values deduced from the methylation data of Table II (4716 and 5850, respectively), plus the sharply symmetric peaks obtained on gel filtration, indicate a high degree of homogeneity in molecular structure of each mycolaminaran.

(1→3)-β-D-Glucanases capable of attacking mycolaminaran are present in the mycelium and zoospores of *Phytophthora palmivora*[29]. It is unlikely, however, that the mycolaminarans isolated herein were degraded during purification, since these thermolabile enzymes were probably inactivated quickly during the initial, hot extraction (see Materials and Methods).

Soluble D-glucans similar to those described herein, and to which the term mycolaminaran may be applied, were previously isolated from the mycelium of *Phytophthora cinnamomi* (Zevenhuizen and Bartnicki-Garcia[3]) and *Phytophthora infestans* (Wood *et al.*[24]). The glucan from *P. cinnamomi* was reported to be a (1→3)-β-D-glucan having a rather high proportion (12%) of C-6 branching units. However, a re-examination of the structure of this glucan[25] by the superior methylation analysis employed herein (alditol acetates rather than methyl glycosides) revealed that its structure was closely similar to the mycelial mycolaminaran of *P. palmivora* (*i.e.*, only two branches/molecule). The polymer from the mycelium of *P. infestans* was reported to be a (1→3)-β-D-glucan of $\overline{d.p.}$ 23 (formaldehyde determination) with one branch at C-6 (paper chromatography of methylated sugars). Such quantitative differences with the mycelial mycolaminaran of *P. palmivora* could very well be ascribed to the different analytical techniques employed.

The foregoing findings, plus other observations suggesting the occurrence of similar soluble glucans in other species of *Phytophthora*[25] and other genera of Oomycetes (*Pythium*[25,26], *Achlya*[26], *Saprolegnia*[26], *Dictyuchus*[26]), raise the possibility that mycolaminarans may be the characteristic storage polysaccharides of the Oomycetes—a class of fungi where glycogen, the typical reserve polysaccharide of other major classes of fungi (Ascomycetes, Basidiomycetes, Zygomycetes, Chytridiomycetes), is seemingly absent. The accumulation of a laminaran-like polysaccharide may thus be regarded as further indication of the uniqueness of the Oomycetes among the Fungi (see Ref. 27). It may also be indicative of phylogenetic ties between Oomycetes and algae of the Chrysophyta and Phaeophyta (see Ref. 28).

ACKNOWLEDGMENTS

This investigation was supported in part by a research grant (GB 32165) from the National Science Foundation. We thank V. O. Sing for preparing a sample of purified exo-(1→3)-β-D-glucanase from a culture of basidiomycete QM 806 kindly

provided by S. Kirkwood, University of Minnesota. The organism originated from the laboratory of E. T. Reese and M. Mandels, U.S. Army Laboratories, Natick, Mass. We are indebted to E. T. Reese for his valuable suggestions on this work.

REFERENCES

1 M. C. WANG AND S. BARTNICKI-GARCIA, *J. Biol. Chem.*, 248 (1973) 4112.
2 J. TOKUNAGA AND S. BARTNICKI-GARCIA, *Arch. Mikrobiol.*, 79 (1971) 283.
3 L. P. T. M. ZEVENHUIZEN AND S. BARTNICKI-GARCIA, *J. Gen. Microbiol.*, 61 (1970) 183.
4 S. BARTNICKI-GARCIA, *J. Gen. Microbiol.*, 42 (1966) 57.
5 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205.
6 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610.
7 P. A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508.
8 J. S. SAWARDEKER, J. H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 37 (1965) 1602.
9 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801.
10 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Carbohyd. Res.*, 5 (1967) 433.
11 R. DREYWOOD, *Ind. Eng. Chem. Anal. Ed.*, 18 (1946) 499.
12 G. ARTURSON AND K. GRANATH, *Clin. Chim. Acta*, 37 (1972) 309.
13 E. T. REESE AND M. MANDELS, *Can. J. Microbiol.*, 5 (1959) 173.
14 J. KÄRKKÄINEN, *Carbohyd. Res.*, 14 (1970) 27.
15 F. I. HUOTARI, T. E. NELSON, F. SMITH, AND S. KIRKWOOD, *J. Biol. Chem.*, 243 (1968) 952.
16 E. PERCIVAL AND R. H. MCDOWELL, *Chemistry and Enzymology of Marine Algal Polysaccharides*, Academic Press, London and New York, 1967.
17 A. E. CLARKE AND B. A. STONE, *Pure Appl. Chem.*, 13 (1963) 134.
18 A. BEATTIE, E. L. HIRST, AND E. PERCIVAL, *Biochem. J.*, 79 (1961) 531.
19 W. D. ANNAN, E. HIRST, AND D. J. MANNERS, *J. Chem. Soc.*, (1965) 220.
20 A. J. MIAN AND E. PERCIVAL, *Carbohyd. Res.*, 26 (1973) 133.
21 T. E. NELSON, J. V. SCALETTI, F. SMITH, AND S. KIRKWOOD, *Can. J. Chem.*, 41 (1963) 1671.
22 A. S. PERLIN AND W. A. TABER, *Can. J. Chem.*, 41 (1963) 2278.
23 T. E. NELSON, J. JOHNSON, E. JANTZEN, AND S. KIRKWOOD, *J. Biol. Chem.*, 244 (1969) 5972.
24 F. A. WOOD, R. P. SINGH, AND W. A. HODGSON, *Phytopathology*, 61 (1971) 1006.
25 M. C. WANG AND S. BARTNICKI-GARCIA, unpublished data.
26 S. FARO, *J. Gen. Microbiol.*, 72 (1972) 393.
27 S. BARTNICKI-GARCIA, in J. B. HARBORNE (Ed.), *Phytochemical Phylogeny*, Academic Press, London, 1970, p. 81.
28 R. M. KLEIN AND A. CRONQUIST, *Quart. Rev. Biol.*, 42 (1967) 105.
29 V. Z. HOLTEN AND S. BARTNICKI-GARCIA, *Biochim. Biophys. Acta*, 276 (1972) 221–227.