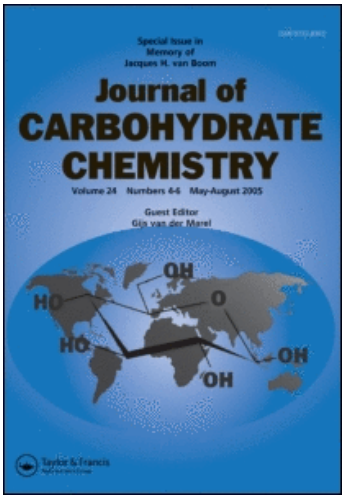

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Structural Analysis on β-d-Glucans from *Phytophthora capsici*

Philippe Rouhier[a]; Maud Bruneteau[a]; Georges Michel[a]
[a] Laboratoire de Biochimie Microbienne, Université Claude Bernard Lyon I, Villeurbanne Cedex, France

To cite this Article Rouhier, Philippe , Bruneteau, Maud and Michel, Georges(1995) 'Structural Analysis on β-d-Glucans from *Phytophthora capsici*', Journal of Carbohydrate Chemistry, 14: 2, 247 — 254
To link to this Article: DOI: 10.1080/07328309508002067
URL: http://dx.doi.org/10.1080/07328309508002067

J. CARBOHYDRATE CHEMISTRY, 14(2), 247-254 (1995)

STRUCTURAL ANALYSIS ON β-D-GLUCANS FROM *PHYTOPHTHORA CAPSICI*

Philippe Rouhier, Maud Bruneteau* and Georges Michel

Laboratoire de Biochimie Microbienne, Université Claude Bernard Lyon I, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

Received May 3, 1994 - Final Form November 10, 1994

ABSTRACT

Several glucans have been isolated from the cell walls of *Phytophthora capsici*, a phytopathogenic fungus of pepper. These polysaccharides consist of a mixture of (1→3)(1→6)-β-D-glucans whose molecular masses varied from 1 x 10^4 to 2 x 10^6 daltons. All these polysaccharides have a main chain of β-(1→3)-linked D-glucose residues. They differ in the presence of 1→6 branched oligosidic chains with 1 or 2 D-glucose residues. The effect of growth conditions on the nature of these glucans has been studied. There are significant differences in the size and in the branching of these fungal glucosaccharides depending on the strain and the growth medium.

INTRODUCTION

Glucans have been found as common polymers of the fungal cell walls in basidiomycetes, ascomycetes[1] and oomycetes.[2] In the *Phytophthora* genus, some species have been studied.[3,4,5] In a previous work, we have isolated β-D-glucans from cell walls of *P. parasitica* Dastur, a phytopathogenic fungus of carnation.[6] They consist of (1→3)-β-D-glucans with various (1→6) branched oligosaccharide chains, the length of these chains being modulated by the culture conditions.[7] These (1→3)(1→6)-β-D-glucans exhibited a prominent activity against the allogenic solid sarcoma 180 on CD1 mice.[8] This biological activity possibly requires specific structural features.

In continuation of our work on β-D-glucans of *Phytophthora* genus, we now describe a comparative structural analysis of the glucans isolated from three strains of *P. capsici*, a phytopathogenic fungus of pepper (*Capsicum annuum*), grown in different media commonly used for fungi cultures.

Table 1. Analysis of D-glucans from *P. capsici*

Glucan sources (strain)	Culture medium	Yield[a] (%)	Molecular mass (kDa) 1500<Mr<2000	100<Mr<500	10<Mr<30
			Percentage		
107	M1	0.5	48	35	17
	M2	0.6	18	19	63
	M3	2.2	90	0	10
197	M1	2.2	36	38	26
375	M1	1.4	40	46	14
	M4	3.1	41	59	0
	M5	1.6	43	57	0

a. The yield is expressed as the glucan percentage of the dry weight of fungal cell walls.

RESULTS AND DISCUSSION

The cell walls from *P. capsici*, strains 107, 197 and 375, prepared as previously reported,[6] were obtained with a yield of 3.5% of the fresh weight mycelium. The glucans were obtained either by hot water extraction[6] (strains 107 and 197) or by successive hot water and sodium hydroxide extraction[9] (strain 375). They were purified by chromatography on DEAE-cellulose. The analysis of the first fraction, eluted with 10 mM potassium phosphate buffer, showed that D-glucose was the unique component of the fraction obtained from strain 375. With the strains 107 and 197, this fraction contained glucose and some trace of mannose. The fraction was further purified by column chromatography on concanavalin-A Sepharose. The unbound fraction consists of pure D-glucans.

The percentages of glucans extracted from the mycelial cell walls are given in Table 1. The differences indicate that the glucan content is somewhat affected by the nature of the growth medium. *P. capsici*, strain 375 is characterized by the highest glucan amount in the M4 medium.

The molecular weights of the glucans were determined by gel permeation chromatography on Sepharose CL-4B. The glucans gave three fractions with molecular masses from 2×10^6 to 1.5×10^6, 5×10^5 to 1×10^5 and 3×10^4 to 1×10^4 daltons, respectively (Table 1).

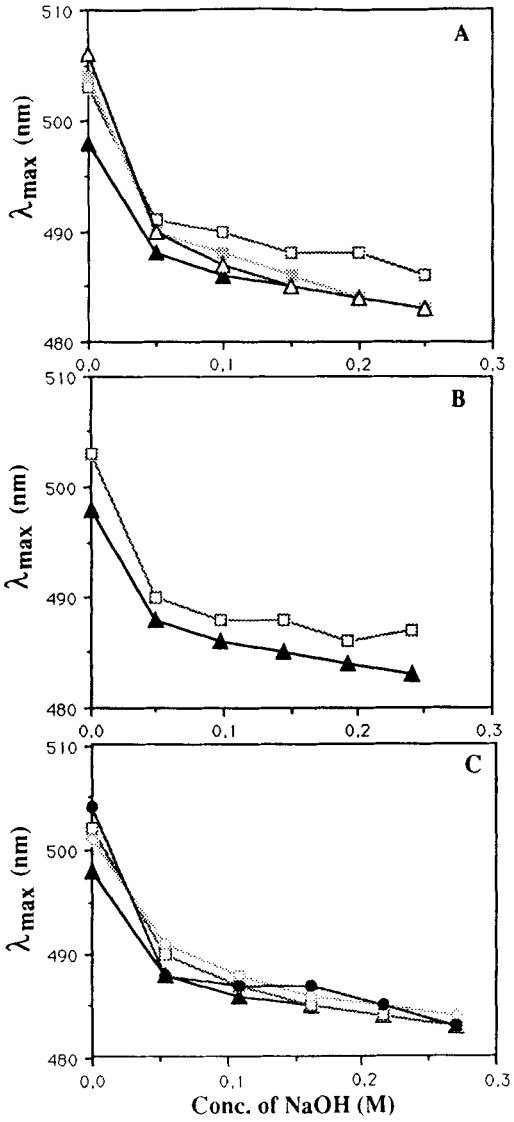

Figure 1. Plot of maximum of Congo Red complexed with glucans (**A**, strain 107 ; **B**, strain 197 ; **C**, strain 375) *vs* the concentration of sodium hydroxide in the presence of glucans grown in —□— M1, —■— M2, —△— M3, —○— M4, —●— M5 media. —▲— in the absence of polysaccharides.

Table 2. Methylation Analysis of the Glucans (Molar ratios)

O-Methyl-D-Glucose	Glucan sources						
	Strain 107			Strain 197	Strain 375		
	M1	M2	M3	M1	M1	M4	M5
2,3,4,6 - Me_4	1	1	1	1	1	1	1
2,4,6 - Me_3	1	1.8	2.5	1	2.2	1.6	1.5
2,4 - Me_2	1	1	1	1	1	1	1

When the three strains were grown in M1 medium three classes of glucans were recovered while in other media completely different distributions between the three classes were found. An increase in the lowest molecular weight fraction was observed with strain 107 grown in M2 medium which contains fosetyl-Al while the amount of the highest molecular weight fraction was higher in a Plich medium (M3).[10] Phosphonate did not affect the percentage of molecular weight fractions of glucans from strain 375 grown in M5 medium. In addition, the glucans from strain 375 grown in M4 (Coffey medium[11]) and M5 media did not contain the molecular masses from 3×10^4 to 1×10^4 daltons.

To recognize ordered structures in the fungal glucans the formation of complexes with Congo Red was investigated. The λ_{max} of Congo Red shifted to higher wavelenght in the presence of glucans, while the λ_{max} of complexes of glucans with Congo Red decreased by the addition of sodium hydroxide (Figure 1). This indicates the presence of a low organized conformation.

The various glucan fractions were methylated according to Hakomori.[12] After acid hydrolysis, borohydride reduction and acetylation, GC of alditol acetates from fully methylated glucans showed three peaks corresponding to 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl- and 2,4-di-*O*-methyl derivatives (Table 2).

These results indicate a (1→3) linked backbone with (1→6) linked branches. The relatively low amount of the 2,4,6-tri-*O*-methyl derivative in comparison with the tetra- and the di-*O*-methyl derivatives agrees with a highly branched structure.

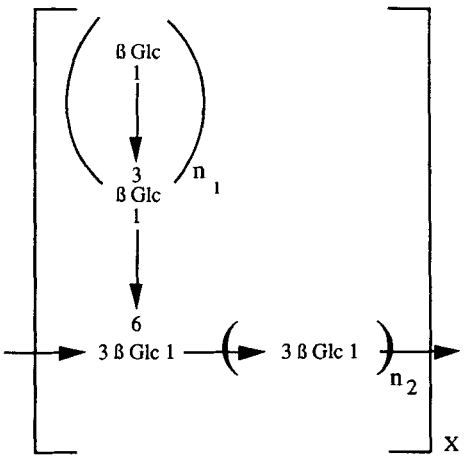

Figure 2. Proposed structures for glucans isolated from *P. capsici* [strain 107, n_1+n_2 = 1 (M1), 1.8 (M2), 2.5 (M3); strain 197, n_1+n_2 = 1 (M1); strain 375, n_1+n_2 = 2.2 (M1), 1.6 (M4), 1.5 (M5)].

The structure proposed for the glucans from *P. capsici* (Figure 2) was further investigated by Smith degradation and acetolysis of the glucans from the three strains grown in M1 medium.

The glucans were submitted to periodate oxidation followed by borohydride reduction. The glucan polyalcohols thus obtained were subjected to mild acid hydrolysis to remove the polyhydroxy groups derived from the D-glucosyl groups. Analysis of the Smith-degradation products showed the presence of glycerol originating from terminal glucose residues, and a Smith-degraded glucan. The latter was produced by cleavage of acetal linkages of the polyhydroxyl groups derived from terminal glucose residues. Methylation analysis of the new glucan yielded 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl- and 2,4-di-*O*-methyl-glucose. Thus branching still occured after one sequence of Smith degradation and these undestroyed glucose residues which substituted the main chain must be a part of 1→3 linked oligosaccharide branching units in the native polysaccharide. These results indicate the presence of monosaccharidic and oligosaccharidic side chains in the native glucans. The acetolysis of the glucans from the three strains grown on M1 medium gave D-glucose, which is compatible with monosaccharidic side chains in the native glucans, and a mixture of two oligoglucosides separated by column chromatography on Sephadex G-15.

The highest molecular weight oligoglucoside was eluted with the void volume. After hydrolysis of the methylated oligoglucoside, only 2,3,4,6-tetra-*O*-methyl- and 2,4,6-

tri-*O*-methyl-glucose but not 2,4-di-*O*-methyl-glucose were detected. The molar ratio of 1:10 between tetra- and tri-*O*-methyl derivatives suggests that acetolysis probably also cleaved some 1→3 bonds of the backbone. The results of methylation analysis of the second oligoglucoside gave a 1:1 molar ratio for 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-glucose corresponding to a disaccharide Glc1→3Glc. This disaccharide substitutes some *O*-6 glucose units of the native glucans backbone. The absence of 2,3,4-tri-*O*-methyl-glucose after methylation analysis of native glucans involves that the length of branching oligosides would not exceed one disaccharide unit.

The β configuration of the D-glucosyl groups was evidenced by the presence of characteristic signals (in the 103-104 ppm region) in the ^{13}C NMR spectrum which was similar to that of fungal β-D-glucans.[8]

P. capsici produce β-D-glucans highly branched of the same type as those of *P. parasitica* Dastur.[6,7] This structure seems characteristic of the genus *Phytophthora.* In *P. capsici,* branches are only mono- and disaccharides as all fungal polysaccharides previously described.[13,14]

Our data show the influence of the composition of culture medium on the size of glucans. Recently, we had observed similar results for glucans of *Aspergillus giganteus.*[15]

EXPERIMENTAL

Microorganism and Culture Conditions. Three strains 107, 197 and 375 of *P. capsici* were grown in various media; strain 107 on M1, M2 and M3 media; strain 197 on M1 medium; and strain 375 on M1, M4 and M5 media.

M1 was a chemically defined medium.[16] The M2 medium had the same composition as M1 but was supplemented with 10 mg L^{-1} fosetyl-Al. M3 was the Plich and Rudnicki medium.[10] M4 was the Ribeiro synthetic medium modified according to Fenn and Coffey.[11] M5 medium was M4 medium supplemented with 5 mM disodium phosphite and 0.8 mM KH_2PO_4. Fungi were grown at 24 °C under 16 h of light on M1, M2, M4 and M5 media. Strain 107 was grown in the dark on M3 medium. Mycelia were collected after three weeks of growth.

Extraction of Glucans and Purification. Glucans were isolated from mycelial cell walls. The cell walls were prepared as previously reported.[6] The glucans from strains 107 and 197 were isolated as described earlier by Fabre et al.[6] and from strain 375 as previously reported by Bruneteau et al.[17] The purification was performed by column chromatography on DE52 DEAE-cellulose (Whatman). The column was equilibrated with 10 mM potassium phosphate buffer pH 7. The column was first eluted

with the same buffer, and then with a linear gradient of NaCl (0-1M) in the same buffer. Each fraction was dialyzed and subjected to chromatography on a column of Sephadex G-25 eluted with water. The first fraction (eluted with 10 mM phosphate buffer) from the strains 107 and 197 was purified further by column chromatography on concanavalin-A Sepharose.

Estimation of Molecular Weight. The molecular weight was determined by chromatography on a column (1.6 x 80 cm) of Sepharose CL4B with water as eluant. The column was calibrated with standard dextrans from Pharmacia (France).

Determination of Glucose. Total glucose content was determined by colorimetric assays according to the procedure of Fischer and Zapf[18] and by gas chromatography (GC) of the glucitol acetate.[19] GC was carried out on an Intersmat apparatus (model 120FL) fitted with a capillary SP 2380 column (0.25 mm id x 20 m).

Methylation Analysis. Glucans were methylated twice by the Hakomori method.[12] The permethylated polysaccharide was hydrolyzed by heating in 85 % formic acid at 100 °C for 6 h. The reaction mixture was concentrated to dryness and the residue was hydrolyzed with M trifluoroacetic acid under the same conditions.[9] The methylated sugars were analyzed as alditol acetates by GC.[20] The identification of methylated sugars was performed by combined gas chromatography mass spectrometry (GC-MS).[21] GC-MS was performed on a VG Micromass 305 apparatus equipped with a capillary column BP1 (0.25 mm id x 60 m) and a temperature program (120 °C to 160 °C, rate 5 °C/min and 160 °C to 280 °C rate 2 °C/min). Mass spectra were taken at an ion energy of 70 eV, current intensity of 200 μA and temperature of 180 °C.

Periodate Oxidation and Smith Degradation. Glucans were oxidized and degraded as described by Fabre et al.[6] and by Bruneteau et al.[8]

Acetolysis. Acetolysis of glucans was performed according to Bayard and Montreuil[22] in a mixture of acetic anhydride-acetic acid-sulfuric acid (10:10:1 v/v/v) as previously described.[6] After acetolysis the sample was fractionated by column chromatography on Sephadex G-15.

Complexes Formation with Congo Red. The change of absorption maximum of Congo Red in the presence of glucans was measured by the procedure of Ogawa et al.[23] Glucans in NaOH solution (1mg mL^{-1}) and Congo Red in NaOH solution (38 μM) were mixed in equal volumes and the λ_{max} values were measured using a Beckman DU-68 spectrophotometer.

^{13}C NMR Spectroscopy. The ^{13}C NMR spectra were recorded with a Brucker AM 300 spectrometer at 75.46 MHz for solutions in D_2O, with acetone as internal reference taken at δ 31.07 relative to the signal of Me_4Si.

ACKNOWLEDGMENTS

The authors thank Mr. P. Mas (Institut National de la Recherche Agronomique, Montfavet, France), Mr. M. Becchi (Service Central d'Analyses du Centre National de la Recherche Scientifique, Solaize, France) and Mrs. O. Miani (Service de RMN de l'Université Claude Bernard, Lyon 1, France) for technical assistance. This work was supported by the Centre National de la Recherche Scientifique (ER.X0018).

REFERENCES

1. S. Bartnicki-Garcia, *Annu. Rev. Microbiol.*, **22**, 87 (1968).
2. S. Bartnicki-Garcia, in *Phytochemical Phylogeny*, J.B. Harborne, Ed.; Academic Press: London, 1970, p 81.
3. M. C. Wang and S. Bartnicki-Garcia, *Carbohydr. Res.*, **37**, 331 (1974).
4. L. P. T. M. Zevenhuizen and S. Bartnicki-Garcia, *J. Gen. Microbiol.*, **61**, 183 (1970).
5. F. A. Wood, R. P. Singh and W. A. Hodgson, *Phytopathology*, **61**, 1006 (1971).
6. I. Fabre, M. Bruneteau, P. Ricci and G. Michel, *Eur. J. Biochem.*, **142**, 99 (1984).
7. J. Perret, M. Bruneteau, G. Michel, M. F. Marais, J. P. Joseleau and P. Ricci, *Carbohydr. Polymers*, **17**, 231 (1992).
8. M. Bruneteau, I. Fabre, J. Perret, G. Michel, P. Ricci, J. P. Joseleau, J. Kraus, M. Schneider, W. Blaschek and G. Frantz, *Carbohydr. Res.*, **175**, 137 (1988).
9. K. Iino, N. Ohno, I. Suzuki, T. Miyazaki, T. Yadomae, S. Oikawa and K. Sato, *Carbohydr. Res.*, **141**, 111 (1985).
10. M. Plich and R. M. Rudnicki, *Phytopath. Z.*, **94**, 270 (1979).
11. M. E. Fenn and M. D. Coffey, *Phytopathology*, **74**, 606 (1984).
12. S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
13. N. Hiura, T. Nakajima and K. Matsuda, *Agr. Biol. Chem.*, **47**(6) 1317 (1983).
14. E. Kishida, Y. Sone and A. Misaki, *Carbohydr. Res.*, **193**, 227 (1989).
15. Ph. Rouhier, M. Bruneteau, G. Michel, J. Fiema and A. Zurzycka, *Carbohydr. Res.*, **262**, 155 (1994).
16. P. Mas and P. M. Molot, *Ann. Phytopathol.*, **6**, 257 (1974).
17. M. Bruneteau, J. Perret, Ph. Rouhier and G. Michel, *Carbohydr. Res.*, **236**, 345 (1992).
18. W. Fischer and J. Zapf, *Hoppe-Seyler's Z. Physiol. Chem.*, **337**, 186 (1964).
19. J. J. Sawardeker, J. H. Sloneker and A. R. Jeanes, *Anal. Biochem.*, **12**, 1602 (1965).
20. H. Björndal, B. Lindberg and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1969); J. H. Sloneker, *Methods Carbohydr. Chem.*, **6**, 20 (1972).
21. P. E. Jansson, L. Kenne, H. Liedgren, B. Lindberg and J. Lönngren, *Chem. Commun., Univ. Stockholm*, **8**, 38, (1976).
22. B. Bayard and J. Montreuil, *Carbohydr. Res.*, **24**, 427, (1972).
23. K. Ogawa, J. Tsurugi and T. Watanabe, *Chem. Lett.*, 689 (1972).