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Effect of growth conditions on the structure of β-D-glucans from *Phytophthora parasitica* Dastur, a phytopathogenic fungus

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This paper is dedicated to Doctor Hubert Mayer on the occasion of his 60th birthday.

Several glucans have been isolated from the cell walls of *Phytophthora parasitica*, a phytopathogenic fungus of carnation. These polysaccharides consist of a mixture of (1 → 3), (1 → 6)-β-D-glucans whose molecular masses varied from 7.5×10^3 to 2×10^6 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, 2 or 4 D-glucose residues.

The effect of various growth conditions on the nature of these glucans have been studied. When asparagine and a high concentration of glucose were present in the culture medium and when the culture was shaken, the production of the lowest molecular weight polysaccharides and their degree of branching were increased.

INTRODUCTION

Glucans have been found as common polymers of the fungal cell wall in Basidiomycetes, Ascomycetes (Bartnicki-Garcia, 1968) and in Oomycetes (Bartnicki-Garcia, 1970). They consist of either linear or branched polyglucoses with branching varying from one branch for two glucose residues of the main chain (Dubourdieu *et al.*, 1981; Yamada *et al.*, 1984) to one branch for three or five or six glucose residues (Rosenfeld & Ballou, 1974; Miyazaki & Oikawa, 1976; Hara *et al.*, 1983; Kato *et al.*, 1983; Iino *et al.*, 1985; Yoshioka *et al.*, 1985; Misaki *et al.*, 1986; Kishida *et al.*, 1989). The distribution of glucosyl side chains along the main chain is sometimes irregular: the glucans of *Dictyophora industriata* (Khishida *et al.*, 1989) and of *Volvariella volvacea* (Kishida *et al.*, 1989) have more glucosyl side chains near the nonreducing end of the main chain.

In the *Phytophthora* genus some species have been studied. Their polysaccharides are poorly branched. They contain per molecule one or two branches in *Phytophthora palmivora* (Wang & Bartnicki-Garcia, 1974), *Phytophthora cinnamomi* (Zevenhuisen & Bartnicki-Garcia, 1970) and *Phytophthora infestans* (Wood *et al.*, 1971). In a previous work we found highly branched glucans in *Phytophthora parasitica* (Fabre *et al.*, 1984). They consist of a straight chain of β-(1 → 3)-linked D-glucose residues and mono-, di- and

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trisaccharide branches having 1, 2 or 3 β-(1 → 6)-linked D-glucose residues (Fabre *et al.*, 1984; Bruneteau *et al.*, 1988). These (1 → 3), (1 → 6)-β-D-glucans exhibited a prominent activity against the allogeneic solid sarcoma 180 on CD1 mice; the antitumor effect was dose-dependent with an optimum activity at 1 mg/kg (Bruneteau *et al.*, 1988).

This antitumor activity was found in the glucans containing both β-(1 → 3) and β-(1 → 6)-linkages from other sources such as yeast, fungi, bacteria and plants (Whistler *et al.*, 1976). Structural features are possibly required for the antitumor activity; a high degree of branching and an adequate molecular size seem correlated to a high biological activity (Adachi *et al.*, 1989; Kishida *et al.*, 1989; Saito *et al.*, 1990).

In the present work we tested possible variations in the structure and specially in the branching of polysaccharides of *Phytophthora parasitica*, with growth conditions. Two media currently used in fungi cultures were used in the experiments: Huguenin medium and Hall medium.

MATERIALS AND METHODS

Organism and culture conditions

The isolate 26 of *Phytophthora parasitica* from the fungal culture collection of INRA Antibes was grown either on Huguenin liquid medium (Huguenin, 1974) for 12 days at 24°C without shaking or on the modified Hall liquid medium (Hall *et al.*, 1969) containing (per liter) 2 g asparagine and 30 g glucose for 3 days at 24°C and 125 rpm in a rotary shaker.

Extraction and purification of glucans from mycelial cell walls

The glucans were isolated from mycelial walls as described earlier by Fabre *et al.* (1984). The purification was performed by column chromatography on DE52 DEAE cellulose (Whatman) and elution with 10 mM potassium phosphate buffer pH 7 then with a linear gradient of NaCl (0–1 M) in the same buffer.

Estimation of molecular weight

A solution of glucans (3 mg) in distilled water (0·5 ml) was applied to a column (1·6 × 80 cm) of Sepharose CL-4B. The column was equilibrated and eluted with distilled water at 10 ml/h and the effluent was collected in 4 ml fractions. The carbohydrate content of each fraction was determined with anthrone reagent (Shields & Burnett, 1960). The column was calibrated with standard dextrans from Pharmacia (France).

Analytical methods

Total glucose content was determined by colorimetric assays according to the procedure of Fischer & Zapf (1964) and by gas chromatography (GC) of the glucitol acetate (Sawardeker *et al.*, 1965). GC was carried out on an Intersmat apparatus (model 120 FL) fitted with a capillary SP 2380 column (20 m × 0·25 mm, i.d.). Combined gas chromatography-mass spectrometry (GC–MS) was performed on a UG Micromass 305 apparatus equipped with a capillary column BP1 (60 m × 0·25 mm, i.d.) 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.

Methylation analysis

Glucans (1 to 5 mg) were methylated twice by the Hakomori method (Hakomori, 1964). The methylated polysaccharide was dissolved in 85% formic acid (2 ml) and the solution was heated for 6 h at 100°C. The reaction mixture was concentrated to dryness and the residue was hydrolyzed with N trifluoracetic acid for 5 h at 100°C (Yamada *et al.*, 1984). After evaporation to dryness the methylated sugars were reduced with $NaBH_4$, acetylated with acetic anhydride and analyzed as alditol acetates by GC (Björndal *et al.*, 1967). The identification of methylated sugars was performed by GC–MS (Björndal *et al.*, 1970).

Periodate oxidation and Smith degradation

Glucans were oxidized and degraded as described by Fabre *et al.* (1984) and by Bruneteau *et al.* (1988).

Acetolysis

Acetolysis of glucans was performed according to Bayard & Montreuil (1972) in a mixture of acetic anhydride–acetic acid–sulfuric acid (10/10/1 = v/v/v) as previously described (Fabre *et al.*, 1984). After acetolysis the sample was fractionated by column chromatography on Sephadex G-15.

NMR spectroscopy

The NMR spectra were recorded in DMSO-d_6 on a Brucker AM 300 spectrometer at 300 MHz for the ^1H and at 75·46 MHz for the ^{13}C spectra.

RESULTS

Isolation and fractionation of glucans

The cell walls from *Phytophthora parasitica* prepared as previously reported (Fabre *et al.*, 1984) were obtained

with a yield of 3–4% of the fresh weight mycelium. The wall-released material by hot water extraction accounted for 7–10% of the walls. This extract was fractionated by column chromatography on DEAE-cellulose, the profiles of elution of the extracts from *P. parasitica* are given in Fig. 1(a) for *P. parasitica* grown in Huguenin medium and in Fig. 1(b) for *P. parasitica* grown in Hall medium. The first fractions Ia and Ib were eluted with the 10 mM potassium phosphate buffer. The analysis of these fractions showed that D-glucose was the unique component. Fractions IIa and IIb have a high protein content.

The molecular weights of glucans Ia and Ib were determined by gel permeation chromatography on a Sepharose CL-4B column. Both products gave three fractions A, B and C with molecular masses around to 2×10^6, 3×10^5 and $7{\cdot}5 \times 10^3$ daltons respectively (Table 1). The repartition was quite different in glucans Ia and Ib: the glucan Ia contained the three molecular weight fractions in roughly similar amounts while the glucan Ib contained essentially the lowest molecular weight fraction.

Structural analysis of glucans

The glucan Ia has already been studied (Fabre *et al.*, 1984). It consists of a β-(1 → 3) linear polyglucose chain with mono-, di- or trisaccharide branchings (Fig. 2). The structure of glucan Ib was determined by methylation analysis, Smith degradation, acetolysis and NMR spectrometry.

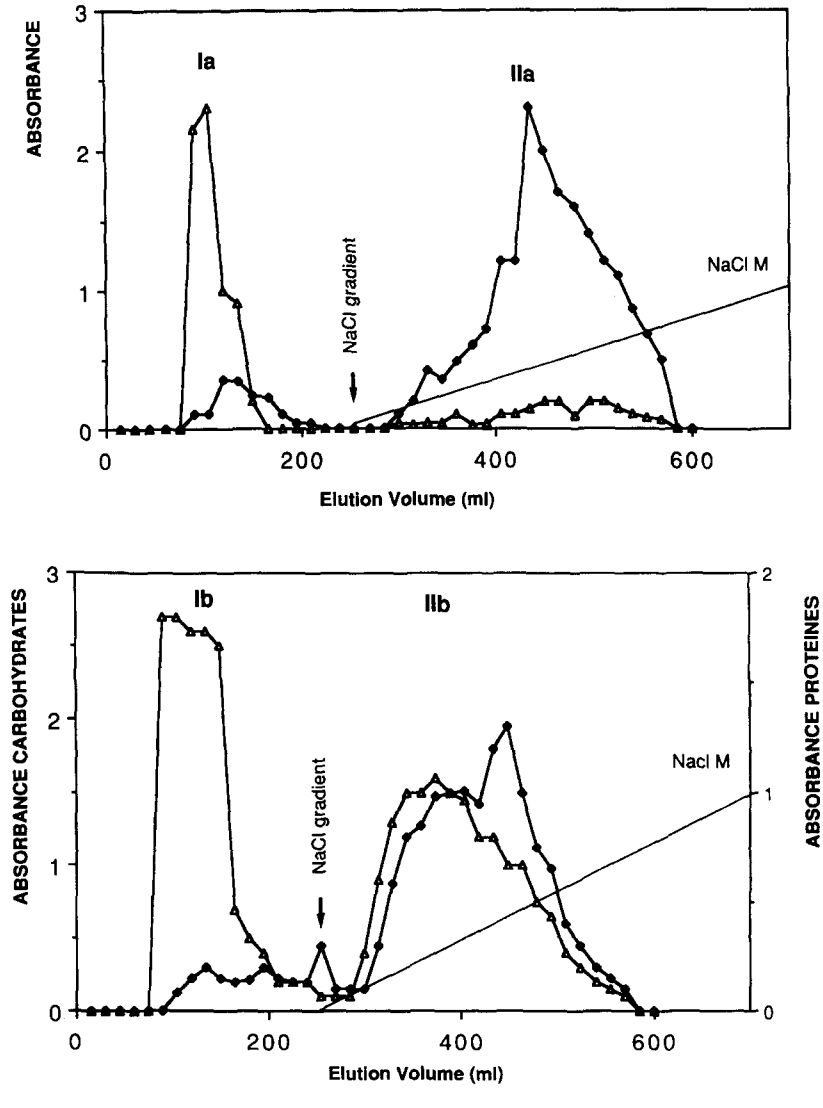

Fig. 1. Ion-exchange chromatography of the extract from cell walls of *Phytophthora parasitica* Dastur grown in Huguenin medium (1(a)) and grown in Hall medium (1(b)). DEAE-cellulose column (25 × 2 cm) was equilibrated with 10 mM potassium phosphate buffer pH 7. The column was run with the same buffer at room temperature with a flow rate of 20 ml/h. Fractions containing 7·5 ml were collected, proteins and carbohydrates were measured spectrophotometrically at 750 nm (—●—) and at 620 nm (—△—) respectively.

Table 1. Repartition on a sepharose CL-4B column of the molecular weights of glucans from *Phytophthora parasitica* grown in Huguenin medium and in Hall medium

	Glucans					
	A		B		C	
Culture medium	Huguenin	Hall	Huguenin	Hall	Huguenin	Hall
Ka_v[a]	0·07	0·07	0·27	0·27	0·87	0·87
Molecular mass (kDa)	2000	2000	300	300	7·5	7·5
Percentage	31	6	26	12	43	82

[a] Ka_v was calculated from the following equation: $Ka_v = \frac{V_e - V_0}{V_g - V_0}$

where V_0 is the void volume, elution of Blue Dextran; V_g is the elution volume of glucose; V_e is the elution volume of fraction A, fraction B or fraction C.

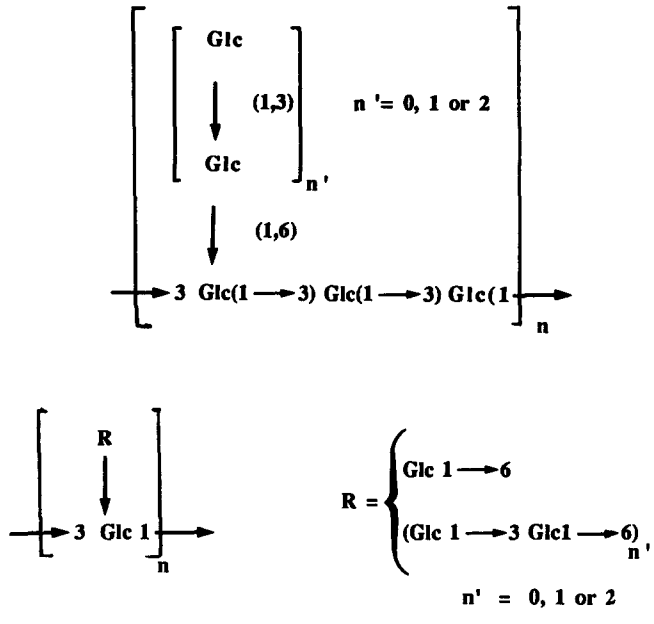

Fig. 2. Proposed structures for glucans Ia (top) and Ib (bottom) isolated from *Phytophthora parasitica* Dastur.

Methylation analysis of glucan Ib

The glucan Ib was methylated according to Hakomori (1964). After acid hydrolysis, reduction and acetylation, analysis of the products by combined GC–MS led to the identification of the following alditol acetates: 2,3,4,6-tetra-*O*-methyl, 2,4,6-tri-*O*-methyl, 2,3,4-tri-*O*-methyl and 2,4-di-*O*-methylglucitol acetates in the molar ratios of 1:1:0·3:1 (Table 2). These results indicate that the glucan Ib contained 1 → 3 and 1 → 6-linkages and the relatively low amount of the 2,4,6-tri-*O*-methyl derivative in comparison with the tetra- and di-*O*-methyl derivatives shows a highly branched structure. This branching is more important in polysaccharide Ib than in polysaccharide Ia.

Smith degradation of glucan Ib

The glucan Ib was submitted to periodate oxidation followed by borohydride reduction and mild acid hydrolysis. The remaining polymeric material was separated from the Smith degradation products by dialysis. Analysis of the dialysable compounds by GC after acetylation showed only the presence of glycerol. When the material was hydrolyzed and then analyzed by GC after reduction and acetylation, glucose appeared in addition to glycerol. This indicated that, amongst the fragments liberated by the specific Smith degradation, there were free glycerol, originating principally from terminal glucosyl residues, and glucosyl-glycerol and/or (1 → 3)-oligoglucosyl-glycerol. The latter arose from side chains containing sequences of one or more (1 → 3)-D-glucosyl residues attached through 1 → 6-linkage to unsubstituted periodate-susceptible glucosyl units: (→ 3-Glc1->)x 6-Glc1 →.

Table 2. Molar ratios of the hydrolysis products of methylated native glucans Ia and Ib and Smith-degraded glucan Ib

O-Methyl hexose derivatives	Molar ratios		
	Ia (native)	Ib (native)	Ib after Smith degradation
2,3,4,6-Tetra-*O*-methyl-D-glucose	1	1	1
2,4,6-Tri-*O*-methyl-D-glucose	3	1	2·6
2,3,4-Tri-*O*-methyl-D-glucose	—	0·3	—
2,4-Di-*O*-methyl-D-glucose	1	1	1

When the dialysable material was examined by HPLC on a NH_2-Lichrosorb column it showed essentially glycerol and no peak corresponding to glucosyl-glycerol. After acid hydrolysis a peak of glucose appeared. It can thus be concluded that glucose arose from short sequences of more than one (1 → 3)-linked glucosyl residues attached to a glycerol.

On the other hand, methylation analysis of the residual Smith degraded polymer yielded 2,3,4,6-tetra-*O*-methyl, 2,4,6-tri-*O*-methyl and 2,4-di-*O*-methyl-glucose in the molar ratios of 1:2·6:1 (Table 2). Thus branching still occurred after one sequence of Smith degradation, these undestroyed glucose residues which substituted the main chain must be a part of 1 → 3-linked oligosaccharide units of the branching in the native polysaccharide. In addition the increased content of the 2,4,6-tri-*O*-methylglucose indicates the presence of monosaccharidic side chains in the native glucan. These monosaccharide residues have been degraded by Smith oxidation.

Acetolysis of glucan Ib

The acetolysis of glucan Ib gave D-glucose and a mixture of two oligoglucosides separated by chromatography on a Sephadex G-15 column. The oligoglucoside which had the highest molecular weight was eluted with the void volume; after methylation and acid hydrolysis 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylglucose were recovered in a molar ratio of 1:11. It can thus be concluded that acetolysis released completely branched oligoside chains and probably also cleaved some 1 → 3-linkages of the straight polysaccharidic chain. The results of methylation analysis of the second oligoglucoside agree with the structure of a disaccharide Glc1 → 3Glc arising from the cleavage of 1 → 6 bonds in poly (Glc1 → 3 Glc1 → 6) residues.

In accordance with the results of methylation analysis, Smith degradation and acetolysis, a structure can be proposed for the glucan Ib (Fig. 2). With the low values of 2,4,6- and 2,3,6-tri-*O*-methylglucose after methylation analysis of glucan Ib, the length of branching oligosides would not exceed, one or two disaccharide units.

NMR spectroscopy of glucan Ib

Due to the low solubility of the glucan in water, the NMR spectra were obtained in DMSO-d_6. The structural complexity of the glucan Ib was immediately revealed in both ^1H and ^{13}C NMR spectra by the multiplicity of the signals in the anomeric regions. In the ^{13}C NMR spectrum more than five resonances can be observed for the glucosyl residues between 102·7 and 103·7 ppm, showing that all the glycosidic linkages had the β configuration and that there was a certain degree of diversity in the relative environment of each D-glucose unit (Table 3). This was confirmed by the ^1H NMR spectrum in which the anomeric protons appeared principally as three doublets or three groups of doublets at 4·0, 4·3 and 4·5 ppm respectively with coupling constant values $J_{1,2}$ of about 8 Hz. The structural complexity is also well illustrated by the multiplicity of the broad complex signals between 86·0 and 87·2 ppm. These resonances correspond to low field chemical shifts of substituted C-3 which is slightly influenced by additional substitution at position 6 and also by the mode of linkage and substitution pattern of the vicinal glucosyl residues. Similarly the characteristic resonances of unsubstituted C-6 at 60·7 and 60·9 ppm and substituted C-6 at 70·1 ppm are also indicative of the complex structure of the glucan Ib. Despite the diversity of the resonances brought by the substitution and branching, the dominant features of the main chain could be seen in the spectrum with the characteristic resonances of this type of β-(1 → 3)-D-glucan chain at ∂ 103·2 (C-1), 86·4 (C-3), 76·1 (C-5), 73·7 (C-2), 88·3 (C-4) and 60·9 ppm (C-6). All these resonances became more prominent in the spectrum of the Smith-degraded glucan Ib (Table 3).

Table 3. ^{13}C NMR chemical shifts of the native glucan Ib and the Smith-degraded glucan Ib

Assignment	Chemical shifts (δ ppm)[a]	
	Native glucan	Smith-degraded glucan
C-1	103·7	
	103·5	103·5
	103·2	
	102·8	102·8
	102·7	
C-2	74·6	74·7
	73·4	
	72·3	
C-3 (substituted)	87·2	
	86·4	
	86·1	86·1
	86·0	86·0
C-3 (unsubstituted)	76·6	76·6
C-4	68·3	68·3
	67·6	
C-5	76·6	
	76·1	76·1
C-6 (substituted)	70·1	70·0
C-6 (unsubstituted)	60·9	60·9
	60·8	

[a]Chemical shifts are in ppm relative to DSS with CH_3 of DMSO at 39·5 ppm.

DISCUSSION

Two lots of purified polysaccharides from *Phytophthora parasitica* were studied: a lot A was purified from organisms grown in a medium without L-asparagine (Huguenin medium), a lot B was obtained from the same strain cultured in a medium with L-asparagine and a high content of glucose (Hall medium). Both polysaccharides Ia from lot A and Ib from lot B consist essentially of branched β-D-glucans whose molecular masses are distributed between $7{\cdot}5 \times 10^3$ and 2×10^6 daltons. However for the polysaccharide Ib the major part had a low molecular mass, about $7{\cdot}5 \times 10^3$ daltons while for the polysaccharide Ia all the sizes were found.

Although all the polysaccharides consist of straight chains having β-(1 → 3)-linked D-glucose residues substituted with glucose or oligoglucoside units, some differences were found in the structure of these branched chains.

The glucan Ia contained (1 → 3)-linked glucose residues which were bound to the main polyglucan chain by β-(1 → 6) bonds (Fig. 2; Fabre *et al.*, 1984). The glucan Ib has a more complex structure (Fig. 2). The branching chains contain either one glucose residue or an oligosaccharide (β-D-Glc 1 → 3 β-D-Glc 1 → 6)$_{1 \text{ or } 2}$. These substituents are linked to the main chain by β-(1 → 6) bonds.

These results show the influence of the composition of culture medium on the structure of the polysaccharides of fungi. The presence of asparagine and a high concentration of glucose (Hall medium) bring modifications in the size and in the branching of glucans. This finding leads to some uncertainty about the structure of some fungal polysaccharides, these structures being modified by the culture conditions. On the other hand the polysaccharides of *P. parasitica* grown on Hall medium present a new type of structure since, in all fungal polysaccharides previously described, branches are only mono- and disaccharides (Hiura *et al.*, 1983; Kishida *et al.*, 1990) while tri- or tetrasaccharides branches were found in *P. parasitica*. Because antitumor activity is dependent on the degree of branching, it would be interesting to produce highly branched polysaccharides, this problem could be solved by the choice of the best culture conditions.

The antitumoral activity of β-(1 → 3)-D-glucans has been demonstrated, specially that of the glucan Ia of *P. parasitica* (Bruneteau *et al.*, 1988). The influence of the size and of the degree of branching of β-(1 → 3)-D-glucans on the antitumoral activity is being studied and the glucan Ib of *P. parasitica* is a good model for these studies.

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