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Note

Structural studies of the extracellular β-D-glucans from *Phytophthora parasitica* Dastur

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Abstract

Several extracellular glucans have been isolated from *Phytophthora parasitica* Dastur, a phytopathogenic fungus of the carnation. These polysaccharides consist of a mixture of $(1\rightarrow3)(1\rightarrow6)$-β-D-glucans whose molecular masses varied from 1×10^4 to 5×10^6 Da. All of these polysaccharides have a main chain of β-$(1\rightarrow3)$-linked D-glucose residues substituted with mono-, di- and oligo-saccharidic chains attached through $(1\rightarrow6)$ linkages. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Phytophthora parasitica Dastur; Extracellular β-D-glucans

1. 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 [3–5]. In previous work, we isolated several β-D-glucans from cell walls of *P. capsici*, a phytopathogenic fungus of pepper (*Capsicum annuum*) and of *P. parasitica* Dastur, a phytopathogenic fungus of the carnation [6,7]. They consist of $(1\rightarrow3)$-β-D-glucans with various $(1\rightarrow6)$ branched oligosaccharide chains, the length of these chains being modulated by the culture conditions [6,8]. These $(1\rightarrow3)(1\rightarrow6)$-β-D-glucans exhibited a prominent activity against the allogenic solid sarcoma 180 on CD1 mice [9] and an antiviral activity on tobacco leaves inoculated with tobacco mosaic virus [10]. These biological activities possibly require specific structural features.

In continuation of our work on β-D-glucans of *Phytophthora* genus, we tested possible variations in the structure and specially in the branching of extracellular polysaccharides of *P. parasitica* Dastur.

Glucans were prepared from the culture filtrates of carnation isolate 26 of *P. parasitica* by column chromatography on DEAE-cellulose. The analysis of the first fraction, eluted with 10 mM potassium phosphate buffer pH 7, showed that D-glucose was the unique component. These D-glucans were eluted in a yield of 115 mg/L.

To recognize ordered structures in these fungal glucans the formation of complexes with Congo Red was investigated. The λ_{max} of Congo Red shifted to a higher wavelength in

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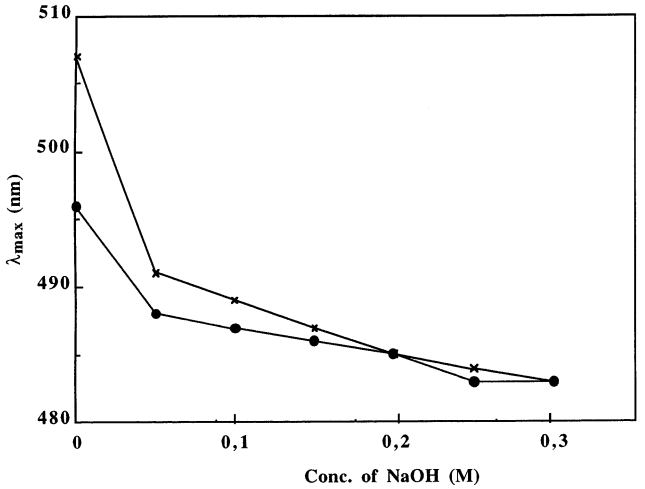
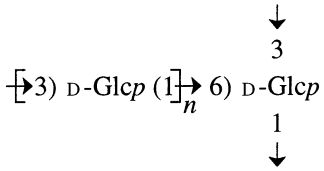


Fig. 1. Plot of maximum absorption of Congo Red complexed with fungal glucans vs. the concentration of sodium hydroxide. Complexation was carried out in the presence of native glucans (×). Control without polysaccharide (●).

the presence of glucans, while the λ_{max} of complexes of glucans with Congo Red decreased with the addition of sodium hydroxide (Fig. 1). This indicates the presence of a low-organized conformation.

The glucans were twice methylated by the Hakomori method [11]. After 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 ratio of 1:4:0.3:1. These results established that these glucans consist of (1→3)-, (1→6)-, and (1→3)(1→6)-linked and non-reducing terminal glucopyranose components with a low proportion of (1→6)-linked residues. The relatively low amount of the 2,4,6-tri-*O*-methyl derivative in comparison with the tetra-*O*-methyl and the di-*O*-methyl derivatives agrees with a highly branched structure and the low value of the 2,3,4-tri-*O*-methyl derivative suggests that the backbone chain of the glucans consists essentially of consecutive (1→3)-linked D-glucose residues.

The glucans were submitted to sequential periodate oxidation, borohydride reduction, and hydrolysis under mild conditions by heating with 0.5 M trifluoroacetic acid at 20 °C for 15 h (Smith degradation) [9]. The Smith-degraded products were separated by column chromatography on Sephadex G-15. The highest molecular weight fraction (S1), composed of only glucose monomers and corresponding to the Smith-degraded glucans, was eluted with the void volume and its structure was analyzed by methylation. The methylated fraction S1 was hydrolyzed and the released glucose residues were reduced with sodium borohydride and acetylated. The products were analyzed by GC–MS which showed the alditol acetates of 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl- and 2,4-di-*O*-methylglucose in the molar ratio: 1:7:1. This result was in agreement with a (1→3)-linked backbone chain. Since 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. These data indicate the presence of the following sequence in the branched oligosaccharide chains of the native glucans:



Detection of D-glucose but not of glycerol in the S1 fraction indicates the presence of 1→6 bonds only in the side chains. On the other hand, the increased content of the 2,4,6-tri-*O*-methylglucose after methylation analysis of the residual Smith-degraded glucans indicates the presence of monosaccharidic side chains and or possibly of short chains of (1→6)-linked glucosyl units in the native glucans. These short chains and the monosaccharide residues have been degraded by the Smith sequence. In addition, amongst the products liberated by the specific Smith degradation, there were free glycerol, originating principally from terminal glucosyl residues, and two fragments S2 and S3 composed of glycerol and glucose. S3 was eluted at a volume (K_{av} = 0.80) identical with that of sucrose on the same column. S2 was eluted at a smaller volume ($K_{av} = 0.50$) than S3, indicating that the S2 molecule was larger than this. This was confirmed by the results of methylation analysis of S2. The methylated S2 gave on acidic

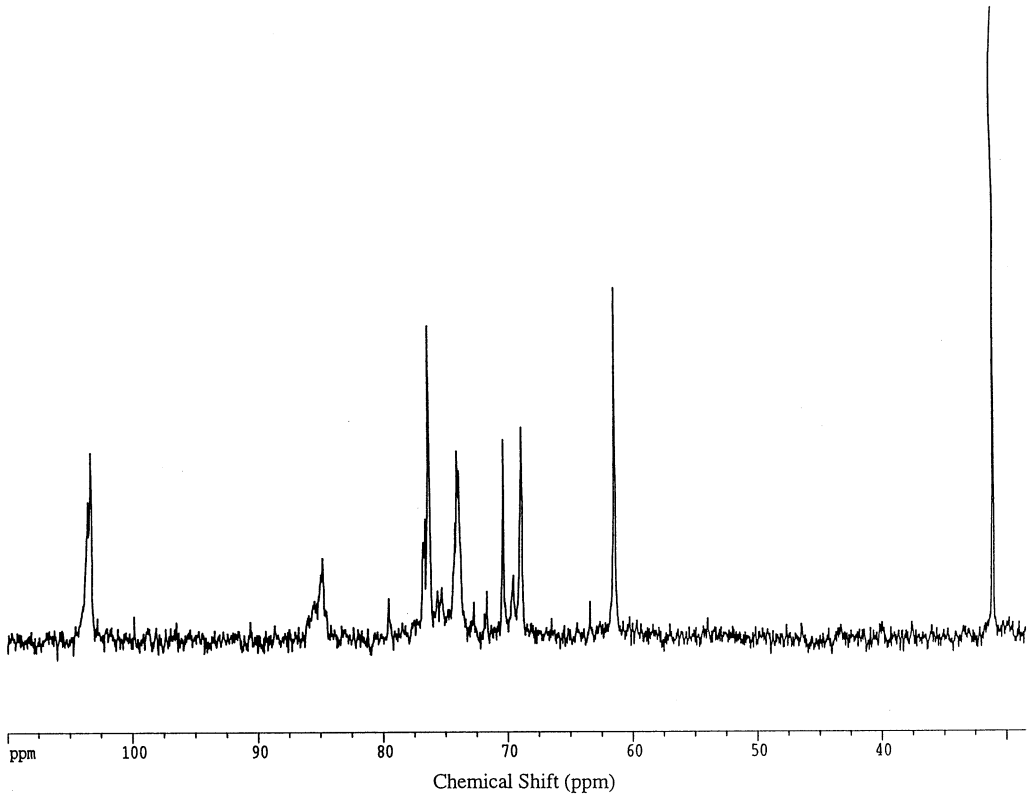


Fig. 2. ^{13}C NMR spectrum at 75.46 MHz for solution in D_2O of native extracellular glucans of *Phytophthora parasitica* Dastur.

hydrolysis, 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methylglucose in the molar ratio 1:1. Thus, S2 had the following structure:

[D-Glc*p*-(1 → 3)D-Glc*p*-(1 → glycerol)]

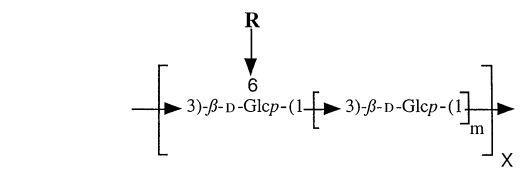
These glycosidic fragments S2 and S3 arose from the cleavage of acetal linkages of the polyhydroxyl groups derived from terminal glucose residues and O-6-glucose units in:

D-Glc*p*-(1→[3)-D-Glc*p*-(1→]$_{n'}$6) D-Glc*p*-(1→

$n' = 0, 1$ or 2

The ^{13}C NMR spectrum of native glucans (Fig. 2) was similar to that of fungal β-D-glucans [9]. This spectrum showed multiple resonances which reveal the structural complexity of the extracellular glucans of *P. parasitica* Dastur. The β configuration of the D-glucosyl residues was evidenced by the presence of two anomeric peaks in the region δ 103.4–103.6 and branchings at C-6 were shown by signals of *O*-substituted C-6 at δ 70.4 and of unsubstituted C-6 at δ 61.5. The multiplicity of the signals and the broad C-3 signal at δ 84.8 could be ascribed to the presence in the glucans of linear β-D-(1 → 3), branched β-D-(1 → 3, 1 → 6), and terminal β-D-glucopyranosyl residues. All these resonances agree with a branched (1 → 3)-β-D-glucan structure.

The results of methylation analysis and Smith degradation suggest the partial structure **1** proposed for the extracellular β-D-glucans of *P. parasitica* Dastur.



R = β-D-Glc*p*-(1 →

R = β-D-Glc*p*-(1 → 6)-β-D-Glc*p*-(1 →

R = β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 6)-β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 →

n' n

m+n+n' = 4 (n' = 0, 1 or 2)

These extracellular β-D-glucans of *P. parasitica* Dastur consist of a mixture of glucans whose molecular weights varied from 5×10^6 to 1×10^4 with a preponderance of high molecular weight material (yield, 67%). They were similar to the D-glucans of some species

of the *Phytophthora* genus [6–8]. Some differences were found in the structure of the branched oligosaccharide chains and in the distribution of the molecular masses.

Our data show that these extracellular β-D-glucans were highly branched and have a complex structure. Previous studies have shown that a high degree of branching and an adequate size of side chains seem correlated to biological activities of fungal β-D-glucans [9,10,12–14]. These extracellular β-D-glucans are thus good models for further studies on these biological activities.

2. Experimental

Organism, growth conditions, and isolation of extracellular polysaccharides.—The isolate 26 of *P. parasitica* from the INRA Culture Collection (Antibes, France) was grown on Huguenin liquid medium [15] for 12 days at 24 °C without shaking. The culture filtrate was prepared as described previously [16]. The culture filtrate was sterilized by membrane filtration (Millipore, 0.22 μm) and evaporated to 25% of the original volume. These concentrates were dialyzed extensively against ultrapure deionised water and freeze-dried. The resulting material was applied to a DEAE-cellulose column (Whatman, 2.5 × 30 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The flow-rate was 12 mL/h. The column was first eluted with the same buffer and then with a linear gradient of NaC1 (0–1 M) in the same buffer. Each fraction (7 mL) was dialyzed and subjected to chromatography on a column of Sephadex G-25 eluted with water.

General analytical methods.—The total glucose content was determined by colorimetric assays according to the procedure of Fischer and Zapf [17] and by gas chromatography (GC) of the glucitol acetate [18]. GC was carried out on a Intersmat apparatus (model 120 FL) fitted with a capillary SP 2380 column (0.25 mm id × 20 m).

The molecular weight was determined by chromatography on a column (1.6 × 80 cm) of Sepharose CL-4B with water eluant. The column was equilibrated with standard dextrans from Pharmacia (France).

Glucosyl-linkage determination.—Glucans or oligosaccharides were methylated twice by the Hakomori method [11] and hydrolyzed with M CF_3CO_2H at 100 °C for 6 h [19]. 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 × 60 m) and a temperature program (120–160 °C at 5 °C/min and 160–280 °C at 2 °C/min). Mass spectra were taken at an ion energy of 70 eV, current intensity of 200 μA and temperature at 180 °C.

Periodate oxidation and Smith degradation.—Glucans were oxidized and degraded as described by Fabre et al. [7] and by Bruneteau et al. [9].

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. [22]. Glucans in NaOH solution (1 mg/mL) and Congo Red in NaOH solution (38 μM) were mixed in equal volumes and the λ_{max} values were measured using an Uvikon 810 spectrophotometer.

NMR spectroscopy.—The ^{13}C NMR spectrum was recorded with a Bruker 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.

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