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Pythium aphanidermatum: culture, cell-wall composition, and isolation and structure of antitumour storage and solubilised cell-wall (1 → 3),(1 → 6)-β-D-glucans

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ABSTRACT

Under optimal conditions for the culture of the fungus *Phytium aphanidermatum*, no polysaccharides were excreted into the medium. The mycelium contained up to 38% of a slightly branched, storage (1 → 3),(1 → 6)-β-D-glucan with a MW of 20000. The cell-wall polysaccharides of the mycelium comprised 18% of cellulose and 82% of (1 → 3),(1 → 6)-β-D-glucans. Of the non-cellulosic glucans, ~ 33% could be solubilised by extraction with water at 121°, and they had a MW of 10000, were highly branched, and contained 6% of (1 → 6) linkages. Treatment of the cell wall with 0.1 M trifluoroacetic acid released ~ 50% of the non-cellulosic glucans. The acid-soluble cell-wall (1 → 3),-(1 → 6)-β-D-glucans of lower MW (6000) were still highly branched and contained 14% of (1 → 6) and 8% of (1 → 4) linkages. The storage glucan and the hot-water-soluble cell-wall glucan exhibited strong activity against the Sarcoma 180 in CD-1 mice, whereas the acid-soluble cell-wall glucans were inactive. The hot-water-soluble cell-wall glucan was also active against the DBA/2-MC.SC-1 fibrosarcoma in DBA/2 mice.

INTRODUCTION

The fungus *Pythium aphanidermatum* belongs to the Oomycetes, which are exceptional in that their cell walls contain cellulose instead of chitin, and (1 → 3),-(1 → 6)-β-D-glucans are major cell-wall components[1,2]. (1 → 3),(1 → 6)-β-D-Glucans (mycolaminarans) also act as storage polysaccharides instead of glycogen[3,4]. Some (1 → 3),(1 → 6)-β-D-glucans display antitumour activity by stimulation of the immune system of the host[5–7]. Thus, schizophyllan and lentinan, isolated from Basidiomycetes, are in clinical use in Japan[8–11]. These antitumour glucans are of particular interest because of their low toxicity[12,13]. The search for new structural

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variants with antitumour potency is of great importance. Since these high molecular weight glucans cannot be synthesised chemically, the search for adequate biological sources is necessary. In previous studies[14,15], the antitumour activity of some solubilised cell-wall (1 → 3),(1 → 6)-β-D-glucans from *Phytophthora* species also belonging to the Oomycetes was demonstrated.

The aims of the present investigation were to *(a)* optimise the conditions for the culture of *P. aphanidermatum*, *(b)* isolate and characterise the water-soluble (1 → 3),(1 → 6)-β-D-glucans, *(c)* test the glucans for antitumour activity, and *(d)* gain an insight into the chemical composition of the cell wall.

EXPERIMENTAL

Culture of the fungus.—Pythium aphanidermatum [(Edson) Fitzp., CBS 287.79 ex soil under Fragaria sp.] was obtained from the Centraalbureau voor Schimmelcultures (AG Baarn, The Netherlands). The fungus was grown in a glucose–asparagine medium[16] (based on a Hendrix medium[17]) that contained 21.6 g of D-glucose, 5.8 g of asparagine, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 4.8 mg of $FeCl_3$, 4.4 mg of $ZnSO_4$, 0.4 mg of $CuSO_4$, 0.03 mg of $MnSO_4$, 0.13 mg of $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 10 mg of EDTA, 100 mg of $CaCl_2$, 20 mg of β-sitosterol, and 2 mg of thiamine HCl per L of distilled water. Stock cultures were kept on agar plates (1.5% of agar)[16]. For comparison of growth in various media, cultures were grown in 250-mL Erlenmeyer flasks each containing 200 mL of medium at 28° on a rotary shaker (100 rpm)[16]. For large-scale batch cultures, the fungus was grown at 28° in a 5-L Bioflo-II fermenter (New Brunswick Scientific, Edison, NJ, USA) with two eight-bladed flat-disc impellers (100 rpm), aeration through a ring sparger at 2.1 L/min, and automatic control of pH sensed by a glass electrode and maintained at 6.0 by a controller which operated two peristaltic pumps connected to acid (1.5 M HCl) and base (1.5 M NaOH).

During culture, samples of the medium were collected for enzymic/photometric determination of the concentrations of D-glucose, sucrose, and asparagine. D-Glucose and sucrose were determined at 340 nm with test kits from Boehringer (Mannheim) which contained ATP : D-hexose 6-phosphotransferase/D-glucose 6-phosphate : $NADP^+$ 1-oxidoreductase/ATP/NADP and without and with β-fructosidase, respectively. Asparagine was determined[18] using L-asparagine amidohydrolase/L-aspartate : 2-oxoglutarate aminotransferase/L-malate : NAD^+ oxidoreductase/α-ketoglutarate/NADH at 340 nm (all enzymes were obtained from Boehringer, Mannheim).

After the times indicated, the mycelium was harvested, washed with distilled water, freeze-dried, and ground to a particle size of 0.25 mm as described[16].

Isolation of the cell-wall material and glucans.—The culture medium (1 L) was concentrated (to 50 mL) under reduced pressure and dialysed (MW cut off, 10000). The glucans were precipitated by the addition of EtOH (to 80%, 5°, 12 h),

collected by centrifugation (20000*g*, 15 min), dried, and purified[16] by anion-exchange chromatography on DEAE-Sephacel (Pharmacia, Freiburg).

Cytoplasmic storage glucans were isolated from the mycelium by double extraction (1 g of mycelium/100 mL) with 0.1 M sodium phosphate buffer (pH 7.25) at 4° for 2 and 1 h, respectively, and centrifugation at 10000*g* for 10 min. The combined supernatant solutions were concentrated under reduced pressure, dialysed (MW cut off, 10000), and freeze-dried, and the residue was purified by chromatography on DEAE-Sephacel[16]. The residue was washed (1 g of residue/100 mL) with distilled water, 1:1 $CHCl_3$–MeOH, and acetone, then dried to yield the cell-wall material. Each washing step was repeated twice by stirring for 30 min at 20° and centrifugation at 10000*g* for 10 min[16].

The cell-wall material was extracted with distilled water (1 g of cell-wall material/100 mL) at 121° (1.2 bar, 30 min). Insoluble material was removed by a glass-fibre filter, and the filtrate was treated[16] with pronase E (Serva, Heidelberg), dialysed, and freeze-dried, and the residue was purified by chromatography on DEAE-Sephacel[16] to yield the hot-water-soluble cell-wall glucans.

Acid-soluble cell-wall glucans were released from cell-wall material by treatment at 100° for 1 h each with 0.05, 0.1, or 0.2 M trifluoroacetic acid. After evaporation of the acid under reduced pressure, the cell-wall material was extracted with distilled water (30 min, 121°, 1.2 bar). The acid-soluble glucans were obtained after centrifugation, dialysis of the supernatant solution (MW cut off, 3500), and freeze-drying.

Analysis of the cell-wall material and the glucans.—The sugar composition of each glucan preparation was determined after hydrolysis (2 M trifluoroacetic acid, 121°, 1 h), conversion of the resulting monosaccharides into alditol acetates, and GLC[16,19].

By sequential hydrolysis of the cell-wall material with 2 M trifluoroacetic acid and 72%/4% H_2SO_4, according to Saeman et al.[20], combined with GLC of the alditol acetates derived from the products, the proportions and compositions of non-cellulosic and cellulosic cell-wall polysaccharides were determined[19]. The cellulose content of the cell walls was determined also by the Updegraff method[21] on isolated cell-wall material and the insoluble material left after treatment of the cell walls with 0.1 or 2 M trifluoroacetic acid.

The MW of the glucans were determined by gel-permeation chromatography on Superose^{TM}12 (Pharmacia, Freiburg) with 0.1 M NaCl, using pullulans (Macherey Nagel, Düren) as standards[16].

Linkage analysis of each glucan preparation was effected by methylation analysis and GLC–MS of the derived partially methylated alditol acetates[16,22].

^{13}C-NMR spectroscopy.—The spectra (external Me_4Si) were obtained at 62.89 MHz with a Bruker WM spectrometer for a solution of each glucan (50 mg) in D_2O (2 mL) at 33°.

Binding of Congo Red.—The assay was performed as described by Gomaa et al.[23]. The shift of λ_{max} of 0.38 μM Congo Red (Sigma) was recorded with a Shimadzu double beam UV 210-A spectrophotometer.

Removal of lipopolysaccharide from the glucan preparations.—Prior to the antitumour tests, contaminating LPS was removed from the glucan preparations, if necessary, either by chromatography on agarose-bound Polymyxin-B (Boehringer, Mannheim) or by treatment with alkali (0.1 M NaOH, 10 h, 30°) followed by neutralisation with acetic acid, and dialysis. The degree of contamination by LPS was tested by the limulus–amoebocyte–lysate assay with the Pyrogent^{R} gel-clotting test kit (Byk-Sangtec, Dietzenbach). The tests were performed according to the instructions of the supplier under aseptic conditions, with sterilised (220°, 4 h) glassware and pyrogen-free water.

Antitumour tests.—CD1, BDF1, and DBA/2 mice, 7–9 weeks old, purchased from Charles River Wiga (Sulzfeld), were kept under standard conditions, and received standard diet (Altromin) and tap water *ad libidum*.

Sarcoma 180, kindly provided by Dr. G. Bogden (Mason Research Institute, Worcester, MA, USA), was maintained by routine passages (ip) of ascites fluid (5×10^6 tumour cells) inoculated into female BDF1 mice every week. Testing was performed by sc inoculation of 0.1 mL of ascites fluid (5×10^6 tumour cells) into the right groin of female CD1 mice (10 mice/group). A solution of the test sample in saline was injected ip daily from days 1 to 10, starting 24 h after inoculation of the tumour. Tumour growth was monitored by measuring the tumour area (length $\times$ width) with a caliper every 10 days. The mice were sacrificed after 30 days, and the tumours were excised and weighed. The rate of inhibition was calculated by comparing the weights of the tumours of the treated group with those of the controls[14,15]. DBA/2-MC.SC-1 fibrosarcoma was induced in female DBA/2 mice by sc application of 3-methylcholanthrene (0.5 mg/mouse). The tumour was maintained by sc transplantation of tumour pieces (1 mm^3) into the right groin of female DBA/2 mice every 4–5 weeks with a trocar. The same procedure was used for testing (10 mice/group). Treatment involved ip application of each test sample three times per week, starting 24 h after transplantation of the tumour. Monitoring of the tumour growth and determination of the tumour weight (day 40) was done as described above[15,24].

Statistical analysis.—Statistical evaluation was performed using Student's *t*-test (double-sided) at a level of significance of $p < 0.05$.

RESULTS AND DISCUSSION

Culture of the fungus.—*(a) Conditions. Pythium aphanidermatum* exhibited excellent growth in the culture medium[16] used for *Phytophthora parasitica*. This asparagine–glucose medium contains macro- and micro-amounts of various salts, thiamine, and β-sistosterol. Exogenous thiamine is needed for the growth of many *Peronosporales (Phytiaceae)*[25], and was also required by *P. aphanidermatum*. Optimum growth occurred at 28°. For *P. acanthicum*, growth in the range 2–38° was described[26] with optimum growth at 30°. The optimum pH of the culture medium was 6.0, as was the end value when the starting pH was in the range 5.0–7.5.

TABLE I

Influence of calcium, β-sitosterol, and the nitrogen source on the growth [a] of *P. aphanidermatum*

Ca^{2+} (0.1 g/L)	β-Sitosterol (20 mg/L)	N-Source (g/L)		Yield of mycelia (%)
−	−	Asp	(5.8)	29
−	+	Asp	(5.8)	65
+	−	Asp	(5.8)	71
+	+	Asp	(5.8)	100
+	+	KNO_3	(7.8)	65
+	+	NH_4NO_3	(3.09)	60
+	+	NH_4Cl	(4.13)	19

[a] In the asparagine–glucose medium (see Experimental), calcium or β-sitosterol were present (+) or omitted (−), or asparagine (Asp) was replaced by equimolar amounts of inorganic sources of nitrogen.

(b) Influence of calcium and β-sitosterol. Calcium stimulates growth of several fungi[27–31] and had a two-fold effect on that of *P. aphanidermatum* (Table I). Sterols can improve the growth of Oomycetes and induce spore production and sporangium formation[28,32,33]. For *P. aphanidermatum*, β-sitosterol had strong growth-promoting properties comparable to that of calcium (Table I). However, in submerged culture of *Pythium*, growth stimulation was not associated with induction of reproduction, and microscopy showed that the mycelium consisted only of branched, unsepted hyphae. By the combined action of calcium and β-sitosterol, growth could be stimulated more than three-fold (Table I).

(c) Effects of various nitrogen sources. Reduced growth was observed when the organic nitrogen source was replaced by equimolar amounts of inorganic nitrogen salts (Table I). Growth on KNO_3 or NH_4NO_3 was still satisfactory, whereas, on NH_4Cl, growth nearly stopped (Table I). Although inorganic nitrogen may be utilised by some members of *Phytiaceae*, organic nitrogen usually seems to stimulate growth[25–27].

(d) Time course of growth and consumption of carbon and nitrogen sources. The time-course of fermentation of *Pythium* is shown in Fig. 1. The fungus grew at least as well on sucrose as on glucose. After a lag phase of ~ 1 day, growth proceeded rapidly up to day 3, when the biomass was almost maximal. After 4 days, when the sucrose and asparagine had been almost completely consumed, 7–8 g of freeze-dried mycelium per L of culture medium could be harvested. At least part of the sucrose was hydrolysed by the action of an extracellular invertase. The concentrations of glucose and fructose increased up to day 3 and then declined. Glucose and fructose probably share a common carrier system that has a higher affinity for glucose, since the glucose concentration was always lower than that of fructose. Thus, the sugar transport system resembles that described[34] for *Phytophthora palmivora*, in which one high-affinity carrier transports glucose and fructose, and a low-affinity carrier is responsible for the uptake of sucrose.

Characterisation of the cell wall and isolation of glucans.—(a) Extracellular glucans. Only negligible amounts of polysaccharides (20–30 mg glucan/L) could be

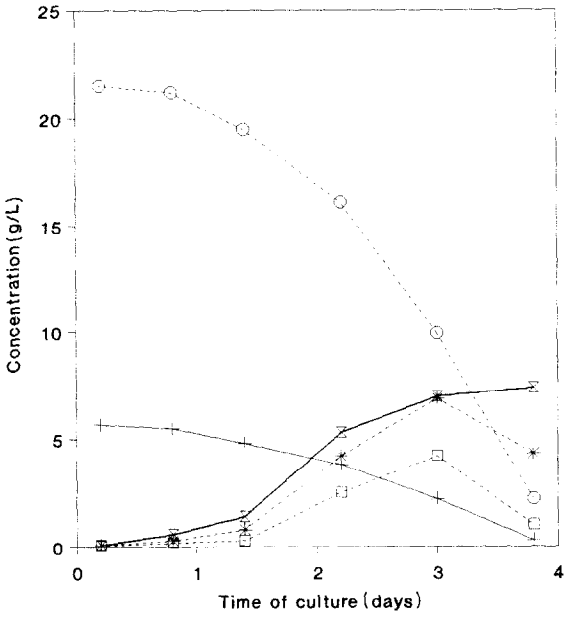

Fig. 1. Time course of growth of *P. aphanidermatum* in a 5-L fermenter in an asparagine–sucrose medium starting with a sucrose concentration of 21.6 g/L (see Experimental): ○, sucrose; +, asparagine; *, fructose; □, glucose; , biomass.

isolated from the culture medium. These glucans were subjected to structure analysis (Tables II and III), but there was not sufficient material for antitumour experiments. The low level of glucans in the medium indicates that the fungus does not excrete polysaccharides, and the small amounts of glucans present probably arise by autolysis of cell walls of dying mycelium.

(b) Yield of storage glucans. Large amounts of freshly harvested mycelium could not be homogenised satisfactorily by conventional methods. Therefore, the mycelium was freeze-dried and milled before isolation of the cytoplasmic storage glucans and cell-wall glucans[16]. Protein and other contaminants in the glucan

TABLE II

MW of storage and solubilised cell-wall glucans of *P. aphanidermatum* determined by gel-permeation chromatography

	Storage glucans	Cell-wall glucans solubilised with			
		hot water	0.05 M CF_3CO_2H	0.1 M CF_3CO_2H	0.2 M CF_3CO_2H
Range of MW	8000–40000	5000–30000	5000–10000	3000–8000	2000–7000
MW at the elution maximum	20000	10000	7000	6000	4000

TABLE III

Linkages (mol%) in the medium, storage, and solubilised cell-wall glucans of *P. aphanidermatum* determined by methylation analysis

Sugar residue	Medium glucans	Storage glucans	Cell-wall glucans			
			Hot-water-soluble	Soluble by CF_3CO_2H treatment		
				0.05 M	0.1 M	0.2 M
Glc	20	6	13	16	16	16
(1 → 3)-Glc	45	85	65	41	42	39
(1 → 3,6)-Glc	20	8	16	20	20	19
(1 → 6)-Glc	12	1	6	15	14	16
(1 → 4)-Glc	3			8	8	10

preparations were removed by treatment with pronase and ion-exchange chromatography[16].

The storage glucans were isolated from the mycelium by extraction with buffer, dialysis, and ion-exchange chromatography. At the end of culture, the freeze-dried mycelium contained 26% of storage glucans (Table IV), the yield of which could be enhanced by fed-batch fermentation. At day 2.5, when the sucrose approached a concentration of 10 g/L (Fig. 1), 600 mL of culture medium was removed and replaced (3 mL/min) by 600 mL of fresh medium which contained 50 g of D-glucose, 15 g of asparagine, 2.5 g of KH_2PO_4, 1.25 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of $CaCl_2$, and 5 mg of thiamine. The sucrose concentration measured at the end of

TABLE IV

Quantitative evaluation (%) [a] of the analysis of storage and cell-wall polysaccharides in the mycelium of *P. aphanidermatum*

Mycelium	100			
Storage glucans	**26**			
Cell-wall material	**6**	100		
Cell-wall polysaccharide	**4**	**65**	100	
Cell-wall polysaccharide hydrolysed with 2 M CF_3CO_2H	3.1	**51**	78	100
Cell-wall glucans solubilised by treatment with CF_3CO_2H				
0.2 M	1.8	**30**	46	59
0.1 M	1.6	**26**	40	51
0.05 M	1.3	**22**	34	44
Hot-water-soluble cell-wall glucans	1	**17**	26	33
Polysaccharide in 2 M CF_3CO_2H-residue hydrolysed with H_2SO_4	0.9	**14**	22	
Content of cellulose determined by the Updegraff method in				
cell-wall material	0.7	**12**	18	
2 M CF_3CO_2H-residue	0.7	**11**	17	
0.1 M CF_3CO_2 H-residue	0.7	**12**	18	

[a] Numbers in bold type represent data obtained by analysis, and all other numbers are calculated therefrom.

culture at day 4 was still 6 g/L. The yield of freeze-dried mycelium increased up to 9.5 g/L with a content of storage glucans of 38%.

Maintaining the sucrose concentration at high levels avoids the consumption of storage glucans for further growth and as an energy source. The turnover of mycolaminarans during cellular differentiation and utilisation of these special types of storage glucans at glucose deficiency have been described for *Phytophthora* sp.[3,35,36].

(c) The cell-wall composition. The residue after extraction of the mycelium with buffer was used for the isolation of cell-wall material by extraction with water and organic solvents (delipidation). The mycelium contained ~ 6% of cell-wall material, of which only 65% (4% of the mycelium) was polysaccharide (Table IV). The non-polysaccharide material was not investigated further. Previous studies of the cell wall of *Pythium* showed that it consists mainly of glucans (80%), lipids (8%), proteins (2%), and some sugars[37]. The non-cellulosic polysaccharides of the cell-wall material should be susceptible to hydrolysis with 2 M trifluoroacetic acid, leaving the cellulose[19], which can then be hydrolysed by the Saeman method[20]. GLC of the alditol acetates derived from the products in the hydrolysates revealed that the cell-wall material was composed of 51% of non-cellulosic polysaccharides and 14% of cellulose (Table IV).

The cellulose content was also determined by the Updegraff method[21] after hydrolysis of the non-cellulosic cell-wall polysaccharides.

Cellulose determination was performed on cell-wall material and on the residues after hydrolysis with 2 or 0.1 M trifluoroacetic acid. Independent of the starting material, the cellulose content of the cell-wall material was estimated to be 11–12% or 17–18%, respectively, of the cell-wall polysaccharides (Table IV). These data accord with values (14 or 22%) obtained by sequential CF_3COOH–H_2SO_4-hydrolysis (Table IV). A similar content (11%) of cellulose-type glucose residues was found in the cell walls of *P. acanthicum*[38], but it was supposed that the microfibrils visible by electron microscopy were composed of mixed linked (1 → 3),(1 → 4)-β-D-glucans containing long stretches of (1 → 4) linkages. The strong conditions of hydrolysis (2 M trifluoroacetic acid or Updegraff method) used in the present investigation indicate, however, that the acid-resistant material is cellulose, since we have found that (1 → 3),(1 → 4)-glucans are susceptible to such treatment. There seems to be general agreement on the glucan–cellulose nature of the cell wall of Oomycetes[2,39], which, together with other characteristics, indicate their isolated position within the fungi and raises the question as to whether Oomycetes really belong to the fungi[2].

(d) Yield of extractable cell-wall glucans. Hot-water extraction (121°) solubilised 17% of the cell-wall material, 26% of the cell-wall polysaccharides, or 33% of the non-cellulosic cell-wall polysaccharides (Table IV)

Pretreatment of the cell-wall material with 0.05, 0.1, and 0.2 M trifluoroacetic acid at 100°, before extraction with hot water (121°), increased the solubilisation of the cell-wall material up to 30%, of the cell-wall polysaccharides up to 46%, and of

TABLE V

Sugar composition (mol%) of cell walls, hot-water-soluble and acid-soluble cell-wall glucans, as well as storage and medium glucans from *P. aphanidermatum* determined by acid hydrolysis and GLC of the derived alditol acetates

Sugar	Sequential hydrolysis of cell-wall material with		Cell-wall glucans solubilised with		Storage glucans	Medium glucans
			water at 121°	0.1 M CF_3CO_2H		
	CF_3CO_2H	H_2SO_4				
Rha	trace	1				
Ara	trace	2				
Xyl	trace	4		2		
Man	2	9	1	1	1	1
Gal	trace	3		2		
Glc	97	81	99	95	99	99

the non-cellulosic cell-wall polysaccharides up to 59%. (Table IV). This enhanced release of cell-wall glucans was due probably to limited hydrolysis of insoluble glucans (Table II) and/or to breakdown of cross-links between glucans.

Characterisation of storage and solubilised cell-wall glucans.—(a) Sugar composition. Hydrolysis combined with GLC of the derived alditol acetates showed that the principal component of the cell-wall material, solubilised cell-wall polysaccharides, and the storage and medium polysaccharides was glucose (Table V). Significant amounts of other sugars (xylose, galactose, and mannose) were detected only in the H_2SO_4-hydrolysate of cell-wall material and, to a lesser extent, in the fraction solubilised with 0.1 M trifluoroacetic acid. The existence of small proportions of mannans, glucomannans, or mannose-, xylose-, galactose-, and arabinose-containing glycoproteins in the cell wall of *P. aphanidermatum* is possible. Thus, the cell wall mainly consists of glucans, most of which are of non-cellulosic nature.

(b) Molecular weights. The MW of the soluble glucans were determined by gel-permeation chromatography in comparison with standard pullulans. All fractions were polydisperse. The MW of the storage glucans was 20000 (elution maximum) ranging from 8000–40000, that of hot-water-soluble cell-wall glucans was 10000 ranging from 5000–30000, and that of the acid-soluble gluans was 4000–7000 ranging from 2000–10000, respectively (Table II).

(c) Structure. The occurrence of only β linkages in the glucan preparations was demonstrated by ^{13}C-NMR spectroscopy (C-1 resonances at 102.9 ppm), as exemplified by the ^{13}C-NMR spectrum of the storage glucan shown in Fig. 2. The linkage positions in the soluble glucan preparations were determined by methylation analysis (Table III). Each glucan was shown to be (1 → 3),(1 → 6)-linked. The ratios of (1 → 3)- to (1 → 3,6)-linked residues were ~ 11 : 1 in the storage glucans, ~ 4 : 1 in the hot-water-soluble cell-wall glucans, and ~ 2 : 1 in the acid-soluble cell-wall and medium glucans. The proportion of (1 → 6) linkages in storage glucans was negligible, whereas, in cell-wall and medium glucans, it was in the

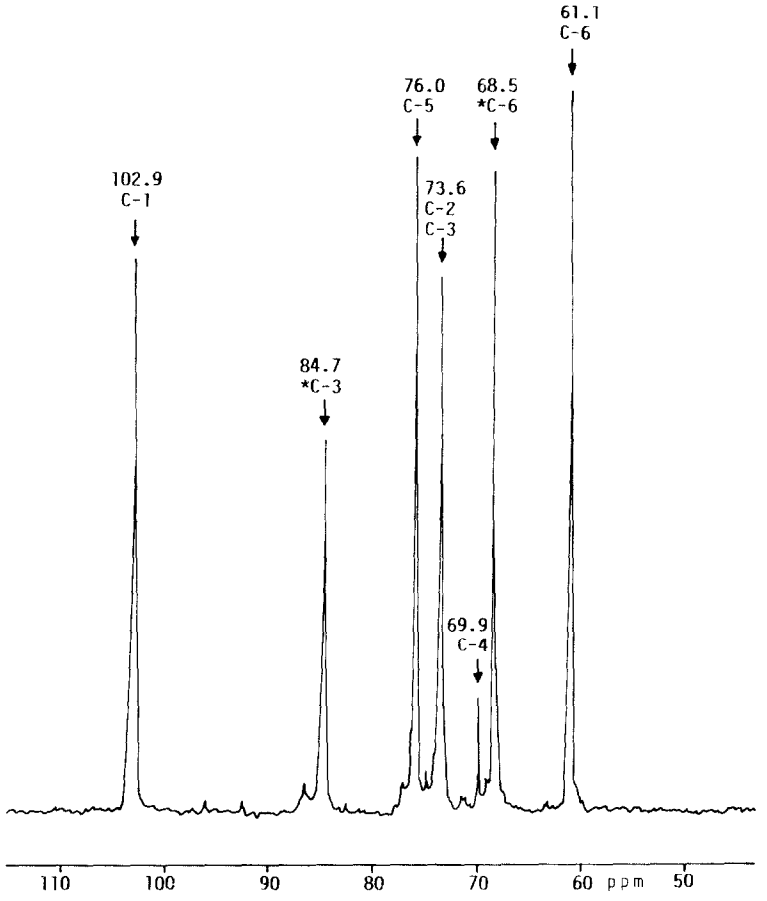

Fig. 2. ^{13}C-NMR spectrum of the storage glucans from *P. aphanidermatum*: * denotes C atoms involved in (1 → 3,6) linkage.

range 6–16%. Up to 10% of (1 → 4) linkages were found in the acid-soluble cell-wall and medium glucans. By sequential Smith degradation combined with methylation analysis[14,16,40], it was demonstrated that, in the hot-water-soluble cell-wall glucans, the side chains consisted of (1 → 3)-linked glucose residues with a preponderance of single residues. Thus, the glucans probably are composed of a (1 → 3)-linked β-D-glucan backbone with various degrees of branching at positions C-6 and rather short side chains. It is still uncertain whether (1 → 6)- and (1 → 4)-linked residues, when present, are inserted exclusively into the backbone either randomly or in blocks, or whether they also are present in the side chains.

Fungal cell-wall model.—The foregoing data show that at least 3 different glucans are involved in the construction of the cell-wall material of *P. aphanidermatum*. Investigations on the hyphal walls of *P. acanthicum*, including electron microscopy, showed that only the inner surface of the cell wall is microfibrillar[38].

Microfibrils are covered with an amorphous matrix material that is partly soluble in aqueous hydroxide[38]. With these results in mind, the following model for the cell wall of *Pythium* may be proposed. Cellulose microfibrils, which amount to ~ 20% of the cell-wall glucans, are covered with (1 → 3),(1 → 6)-β-D-glucans with relatively high proportions of (1 → 4) and (1 → 6) linkages (8 and 15%, respectively) and have a high degree of branching. Assuming that the (1 → 4) linkages in these glucans are arranged in blocks, the formation of many hydrogen bonds may be responsible for their tight connection to the microfibrils. These glucans become soluble only after mild treatment with acid, which favours hydrolysis of some (1 → 3) linkages, thus decreasing the MW, and correspond to the xyloglucans that cover the microfibrils in the cell walls of plants. These glucans may be linked through the highly branched regions to the residual (1 → 3),(1 → 6)-β-D-glucans with a lower degree of branching, a lower proportion of (1 → 6) linkages (6%), and without (1 → 4)-linked residues. These glucans, which are at least partially soluble in hot water, comprise the major portion of the amorphous matrix material, and probably are interlinked mainly by hydrogen bonds, the melting of which at elevated temperatures results in solubilisation. When the isolated hot-water-soluble cell-wall glucans were treated with water at 121° for 30 min, there was no alteration in structure or MW. Therefore, the original solubilisation of the hot-water-soluble glucan fraction should not have caused dramatic breakdown of these cell-wall glucans. The extent of interlinkage of the various glucans by covalent bonds remains to be investigated. Oomycetes seem to synthesise their cell-wall network mainly of glucans by varying the type of linkage, the degree of branching, and the MW.

Antitumour activity of the glucans.—(a) LPS-contamination. Some of the best known immunomodulators are lipopolysaccharides (LPS) from the cell walls of Gram-negative bacteria. LPS show marked biological effects at the ng-level[41]. Therefore, the LPS-content of the glucan preparations to be assayed for in vivo antitumour activity was examined by a gel-clotting system with limulus amoebocyte lysate (LAL). Pyrogent^R was used since it contains no glucan-inducible factors (factor G), thus being specific for LPS. With other test kits (e.g., Pyrogel^R, Concept^R) which contain factor G, the storage glucans of *P. aphanidermatum*, for example, induced gel clotting at concentrations as low as a few ng/ml. The Pyrogent^R test kit showed that the glucan preparations contained 20–100 ng of LPS/mg of glucan. By chromatography on agarose-bound Polymyxin-B or treatment with sodium hydroxide (see Experimental), The LPS-contamination could be reduced to < 0.5 ng of LPS/mg of glucan, and these glucans were used in the antitumour tests. Methylation analysis and gel-permeation chromatography showed that removal of the LPS caused no structural changes in the glucans.

(b) Activity against the Sarcoma 180 in vivo. The cell-wall glucans, which were obtained in high yields by treatment with 0.1 M trifluoroacetic acid without excessive reduction in MW (Table II), were inactive (Table VI). However, the hot-water-soluble cell-wall glucans and the storage glucans each caused $> 90\%$

TABLE VI

Antitumour activity of storage and solubilised cell-wall glucans from *P. aphanidermatum* against Sarcoma 180

Glucan	Dose (mg/kg)	Average tumour weight (g)	Inhibition [a] ratio (%)	Complete [b] regression	Significance [c] (p) <
Hot-water-soluble cell-wall glucan	Control	10.87		0/12	
	0.2	5.10	53	2/9	0.05
	1	0.90	92	2/9	0.0001
	5	0.05	99	2/9	0.0001
Storage glucan	Control	5.68		0/10	
	5	0.09	99	4/10	0.002
0.1 M CF_3COOH-soluble cell-wall glucan	Control	5.95		0/10	
	1	6.50	−9	0/10	ns [d]
	5	3.50	41	0/10	ns [d]

[a] $(C - T/C) \times 100$, where C is the average-tumour weight of the control group, and T of the treated group. [b] Number of tumour-free mice/number of treated mice. [c] Significant difference of tumour weight compared to control. [d] Not significant.

inhibition of tumour growth at concentrations of 1 and 5 mg/kg, respectively (Table VI).

(c) Activity against the DBA / 2-MC.SC-1 fibrosarcoma in vivo. The time courses of tumour growth of the groups treated with the hot-water-soluble cell-wall glucans and the control are shown in Fig. 3. There was a dose-dependent antitumour effect and ~ 90% inhibition ($p < 0.001$) at a dose of 5 mg/kg. The inhibition rates and significances were calculated at day 40. There was no effect at 1 mg/kg.

The antitumour activity of (1 → 3)-β-D-glucans with branches at positions C-6, isolated from a wide variety of microbial organisms, especially fungi[5], have been investigated intensively. Their antitumour activity is not due to direct cytotoxic effects, but to modulation of the immune system of the host[12,13]. It has been assumed that the antitumour activity of (1 → 3),(1 → 6)-β-D-glucans is associated with a high MW (> 50000) and an ordered structure, e.g., triple helices[42]. Recently, Kraus et al.[15] demonstrated that (1 → 3),(1 → 6)-β-D-glucans with an average MW < 20000 and no ordered structure show high antitumour activity if the degree of branching is < 25%. Gomaa et al.[43] found that the antitumour activity of glomerellan, a (1 → 3),(1 → 6)-β-D-glucan with a high MW (~ 670000) isolated from the culture filtrate of *Glomerella cingulata*, was independent of the presence of ordered structures, since aggregates of triple helices, free triple helices, and a single helical conformation showed comparable activity against Sarcoma 180.

The results obtained with the (1 → 3),(1 → 6)-β-D-glucans from *P. aphanidermatum* support recent findings[15,43]. The antitumour-active hot-water-soluble cell-wall and storage glucans had MW of 10000 and 20000, and degrees of branching of 20 and 8%, respectively, but no ordered structures.

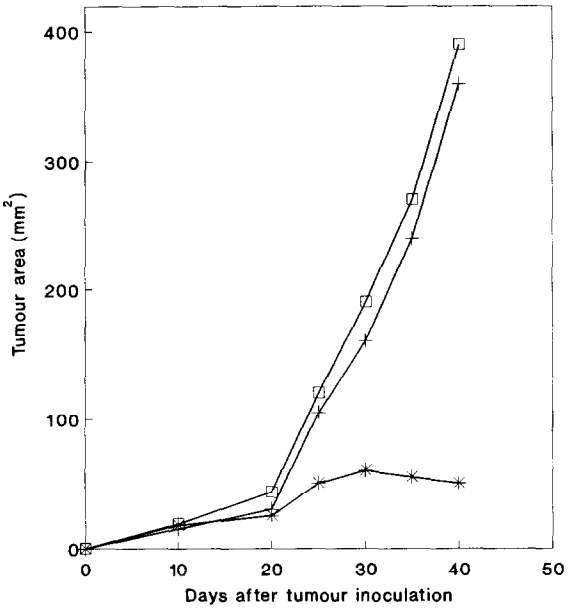

Fig. 3. Antitumour effect of hot-water-soluble cell-wall glucans from *P. aphanidermatum* at 1 (+) and 5 (*) mg/kg against DBA/2-MC.SC-1 fibrosarcoma (□, control).

The absence of ordered structures in glucan preparations from *P. aphanidermatum* was investigated on the basis of NaOH-induced absorption shifts[23] of glucan–Congo Red complexes (Fig. 4). For the triple helical (1 → 3),(1 → 6)-β-D-glucan schizophyllan, conversion into single helices was indicated by an increase of the λ_{max} to 510 nm effected by 0.05–0.1 M NaOH. At higher concentrations of alkali, random coils were formed and the λ_{max} moved to the value for alkaline solutions of Congo Red. This bathochromic shift was not observed for the storage and cell-wall glucans from *P. aphanidermatum*. Nevertheless, the *Pythium* glucans were active against the Sarcoma 180 and the DBA/2-MC.SC-1 fibrosarcoma.

In contrast, the cell-wall glucans obtained by mild acid hydrolysis (0.1 M trifluoroacetic acid), with 8% of (1 → 4) and 14% of (1 → 6) linkages, did not inhibit tumour growth. Thus, interruptions in the (1 → 3)-linked backbone by appreciable proportions of (1 → 6) and/or (1 → 4) linkages are disadvantageous for antitumour activity. Moreover, the acid-soluble glucan fraction should contain some hot-water-soluble glucans, which showed significant antitumour activity. However, these glucans seem to have been degraded, since the MW of the original hot-water-soluble glucans was 5 000–30 000, whereas that of the acid-soluble glucan fraction was 3 000–8 000 (Table II).

These results are in good agreement with the findings of Demleitner et al.[44,45], who demonstrated that synthetic branched derivatives of the (1 → 3)-linked β-D-glucan curdlan were highly active against Sarcoma 180, whereas branched deriva-

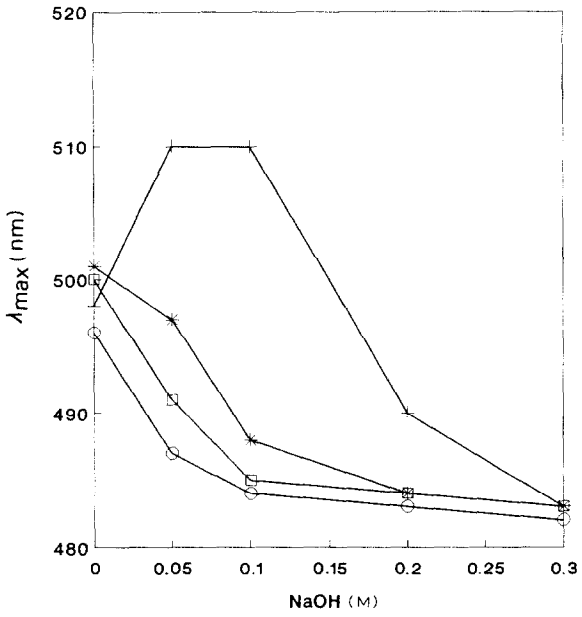

Fig. 4. The dependence of the λ_{max} of Congo Red/glucan complexes on the concentration of NaOH. Solutions of (1 mg of glucan/mL of 0.38 μM Congo Red): ○, Congo Red; +, schizophyllan; *, storage glucan; □, acid-soluble glucan.

tives of the original linear (1 → 3),(1 → 4)-linked β-D-glucan lichenin showed no significant activity. Investigation of the mode of action of the antitumour-active glucans from *P. aphanidermatum* are continuing.

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