EXTRACELLULAR POLYSACCHARIDE OF *Aspergillus flavus*

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A crude extracellular polysaccharide was isolated by precipitation with ethanol from the culture medium of *Aspergillus flavus*, where the sole carbon cource was D-galactose; purification afforded a homogeneous water-soluble polysaccharide in 0·05% yield on the weight of the employed carbon source. This polysaccharide had the relative molar weight 55 000 and $[\alpha]_D^{22}$ −4·2° (*c* 0·5, H_2O); upon total hydrolysis it afforded D-mannose and D-galactose in a 1 : : 0·44 ratio. The products of hydrolysis of the methylated polysaccharide and also the course of partial acid and enzymic hydrolyses of the polysaccharide showed that the main chain was formed by (1→2) β-linked D-mannose units, of which each second, on average, was substituted by monomeric D-galactose units at $C_{(6)}$.

Recently, the conditions of polysaccharide production by microorganisms and structure elucidation of these compounds, particularly those having similar properties as the polysaccharides of plant gums, have been intensively investigated. Except utilization of these polysaccharides in industry, the problem is important also from the standpoint of the host-pathogen relations in plant pathology, where polysaccharides play a significant role. So far, more papers have dealt with the examination of the host resistance than with the metabolism and nutrition requirements of the parasite.

Microorganisms produce polysaccharides of three distinct types[1]: extracellular polysaccharides, structural components and intracellular storage polysaccharides. Extracellular polysaccharides can further be classified into two forms: capsules integral with the cell wall and structurally demonstrable microcapsules, and slimes accumulating outside the cell wall and diffusing constantly into the culture medium. Microbial synthesis of the majority of homopolysaccharides requires a specific saccharide, since a single enzyme, or a very simple enzymic system is involved in their production. On the other hand, heteropolysaccharides are formed of every utilizable carbon source, since a multienzymic system is encountered here. Extracellular polysaccharides probably protect microorganisms against various adverse environmental factors. Their considerably high moisture-holding capacity enables microorganisms to maintain at least a minimum of moisture in their immediate environment even after an extended exposure to low humidity. In general, these

polysaccharides do not appear to function as a reserve energy source. There are indications, however, that some fungi degrade and reutilize their own capsular polysaccharides.

Heteropolysaccharides containing galactose and mannose as main saccharide units are widespread in the nature. They have been isolated from yeast cell walls[2,3], leguminous plants[4], as well as from cell walls and extracellular secretions of various fungi belonging to *Aspergillus*[5–7], *Penicillium*[5,8], *Trichophyton*[5,9], *Histoplasma*[10], *Cladosporium*[11] and *Hormodendrum*[12] species. Some of these microorganisms have galactomannans present in complexes with phosphate and protein. These polysaccharides probably do not play an important structural role, as they little contribute to the composition of cell walls. However, many hyphal fungi contain galactomannans and peptidogalactomannans which show surface-active properties as antigen determinants[5,10,12,13].

When studying the gummosis evoked by pathogens and determining the structure of gum polysaccharides of fruit-trees belonging to *Prunus* genus, we isolated *Aspergillus flavus* as an incidental infection in the aqueous solution of the peach gum polysaccharide. This microorganism was, in turn, utilized for degradation of the substrate[14] under investigation. Later, also the isolated extracellular glycanohydrolases of the same microorganism[15,16] have been used for the above-mentioned degradation. It was of interest to ascertain the relation of the determined structure of the peach gum polysaccharide to the structure of the extracellular polysaccharide of *A. flavus*. Therefore, we isolated the polysaccharides produced by the microorganism during cultivation on a medium containing D-galactose as a sole carbon source. The choice of this substrate has been motivated by the fact that D-galactose was found to be the main component of the peach gum polysaccharide. This paper deals with the course of production, isolation and structural characterization of the extracellular polysaccharide of *A. flavus*.

EXPERIMENTAL

Material and Methods

Isolation and characterization of the mixture of extracellular glycanohydrolases has already been described[15]. *A. flavus*, employed in this project, was identified at the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague. Paper chromatography was carried out on a Whatman No 1 paper in the solvent systems S_1 ethyl acetate–pyridine–water 8 : 2 : 1 and S_2 ethyl acetate–pyridine–acetic acid–water 5 : 5 : 1 : 3. Saccharides were detected with anilinium hydrogen phthalate[17] and alkaline silver nitrate solution[18]. Optical rotations of aqueous solutions were measured with a Perkin-Elmer, model 141, apparatus, aqueous solutions were concentrated under diminished pressure at 40°C, and proteins were removed by a procedure according to Sevag[19]. The monosaccharide constituents were quantitatively determined as alditol acetates by gas chromatography. The amount of reducing groups present

in the incubation mixture was read from the calibration graph for D-galactose. Absorption was recorded with a Beckmann DB-GT spectrophotometer at 530 nm. The IR spectrum of the methylated polysaccharide in chloroform was taken with a Perkin-Elmer, model 457, spectrometer. The instrument Electrophorese (Zeiss, Jena) was employed for electrophoresis in a borate buffer of pH 9·3. The relative molecular weight of the polysaccharide was determined by ultracentrifugation using an MOM 3170 B (Hungary) instrument at 180 000 *g* by a sedimentation equilibrium method. Mass spectra of the partially methylated alditol acetates were run with a JMS-100 (Jeol) spectrometer at an ionizing electron energy 23 eV, trap current 100 μA and 150—180°C ionization chamber temperature. For gas chromatography/mass spectrometry the JGC-20K apparatus was employed; injection port temperature 220°C, column temperature 170—210°C (4°/min), inlet He pressure 0·1 MPa, stationary phase 3% OV-225. Hewlett–Packard, model 5711 A was used for gas chromatography (carrier gas N_2, 30 ml/min, 170°C). The ^{13}C-NMR spectrum of the polysaccharide was measured with a Jeol FX-100 apparatus operating at 25·2 MHz (5% solution in D_2O, 1,4-dioxane internal reference).

Cultivation Conditions

Aspergillus flavus was cultivated in a solution consisting of $NaNO_3$ (2 g), $KH_2PO_4.12\ H_2O$ (1 g), KCl (0·5 g), $MgSO_4.7\ H_2O$ (0·5 g), $FeSO_4.7\ H_2O$ (0·01 g), and D-galactose (15 g) in 1 000 ml. The initial pH of the cultivation solution was adjusted with tartaric acid or sodium hydroxide to 4·5. The liquid medium (200 ml) was inoculated with a suspension of conidia *A. flavus* in a sterile distilled water (2 ml) after sterilization. The cultivation proceeded statically at 28°C.

Isolation of the Extracellular Polysaccharide

After cultivation (maximum amount of the mycelium) the mycelium was removed first by filtration through paper and then through a sintered glass filter S_1. The concentrated filtrate was dialyzed against water for 3 days. The polysaccharide was precipitated with 4 volumes of ethanol and suspended in water; the water-insoluble portion was removed by centrifugation and the supernatant was lyophilized. Dissolution in water, centrifugation, and lyophilization was repeated three times. The accompanying proteins were removed according to[19]. Yield of the final water-soluble polysacchairde after a 21-day cultivation was 0·05% on the weight of the D-galactose used.

Total Hydrolysis

The polysaccharide (10 mg) was hydrolyzed with 0·5M-H_2SO_4 (10 ml) in a sealed test-tube at 105°C for 20 h. The solution was then neutralized with barium carbonate and the excessive Ba^{2+} ions were removed on a Dowex 50WX4 (H^+) column. The solution was concentrated and chromatographed in the above-mentioned solvent systems.

Partial Acid Hydrolysis

The polysaccharide (100 mg) dissolved in water (100 ml) was hydrolyzed with Dowex 50 WX4 (H^+) (10 g, washed with water and dried on a sintered glass filter) in a stoppered flask at 100°C. Liberation of reducing groups during the hydrolysis was monitored by the Somogyi[20] method and the products were analyzed by paper chromatography in solvent systems S_1 and S_2. The ion exchanger was filtered off and washed with water after a 5 h hydrolysis. The supernatant and washings were concentrated to a small volume, the low-molecular portion up to relative

molecular weight 1 000 was separated from the high-molecular residue by ultrafiltration through an M2 filter. The filtrate was concentrated and analyzed by paper chromatography. The precipitate of the high-molecular portion was dissolved in water and lyophilized.

Enzymic Hydrolysis

The polysaccharide (80 mg) was incubated at 30°C by the mixture of extracellular glycanohydrolases of *A. flavus*[15] in a 0·05M acetate buffer solution of pH 4·4 (20 ml) containing toluene to prevent infection. Liberation of reducing groups during hydrolysis was monitored by the Somogyi method[20] and the products of hydrolysis were checked by paper chromatography.

Methylation, Hydrolysis, Reduction and Acetylation of the Polysaccharide

The polysaccharide was methylated with dimethyl sulfate in the presence of sodium hydroxide according to Haworth[21] and with methyl iodide and silver oxide according to Purdie[22]. A part of the methylated product (35 mg) was hydrolyzed with 90% formic acid (12 ml) at 100°C for 1 h. Dilute sulfuric acid (0·13M, 18 ml) was added to the concentrated solution and hydrolysis continued at 100°C for additional 10 h. The solution was then neutralized with barium carbonate, filtered, and the methylated saccharides (aqueous solution, 4 ml) were reduced with sodium hydridoborate (150 mg) 2 h under strirring. The solution was acidified with Dowex 50WX4 (H^+) to pH 3·5, filtered, and evaporated to dryness. Boric acid was removed by codistillation with methanol (3 × 25 ml). The product was acetylated with acetic anhydride–pyridine (1 : 1, 4 ml) at 100°C for 1 h; the excess reagents were removed by distillation with toluene, and the product was dissolved in dichloroethane, and concentrated.

RESULTS AND DISCUSSION

The greatest amount of the deproteinized polysaccharide, *i.e.* 0·05% yield with regard to the weight of the employed carbon source, was obtained after a 21-day cultivation when the yield of the mycelium was maximum, too. The isolation process is reproducible.

TABLE I

Relative retention times and characteristic peaks (m/z) of partially methylated alditol acetates

Derivative	R_t[a]	m/z			
1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol	1·15	87	101	117	129
		145	161	205	
1,2,5-Tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol	1·61	71	87	99	101
		129	145	161	189
1,2,5,6-Tetra-O-acetyl-3,4-di-O-methyl-D-mannitol	2·88	43	87	99	129
		189			

[a] Retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

The relative molar weight of the polysaccharide, homogeneous at free electrophoresis and ultracentrifugation, was found to be 55 000, specific optical rotation $[\alpha]_D^{22}$ −4·2° (*c* 0·5, H_2O). The paper chromatographic analysis of products resulting from acid hydrolysis displayed the presence of D-mannose and D-galactose as main components and a negligible amount of D-glucose in the given solvent systems. Detection of the chromatogram with ninhydrine was negative. Quantitative determination of the polysaccharide components by gas chromatography in form of alditol acetates showed the molar ratio of D-mannose to D-galactose to be 1 : 0·44.

It was ascertained that the courses of partial acid and enzymic hydrolyses were identical. D-Galactose as a monomeric unit was released from the polysaccharide during both processes; liberation of D-mannose was observed only in the latest phase of the hydrolyses. The remaining high-molecular portion afforded D-mannose and traces of D-galactose after total hydrolysis.

In order to find out the nature of bonds in the polysaccharide, this was methylated, hydrolyzed, reduced, and acetylated. The products, partially methylated alditol acetates, were identified by gas chromatography and electron impact mass spectrometry. The results listed in Table I show that D-galactose forms only non-reducing terminal units represented by the tetramethyl derivative. D-Mannose units bound by (1→2) glycosidic bonds form the backbone of the polysaccharide, as indicated by the main product of methylation - 3,4,6-tri-O-methyl-D-mannose. Further identified product of methylation, 3,4-di-O-methyl-D-mannose, proved that D-mannose formed also branching points. The molar ratio of D-mannose to D-galactose and the fact that D-galactose was found exclusively as a nonreducing terminal unit entitle to conclude that almost each second D-mannose unit, on average, bound in the main chain, is substituted by monomeric D-galactose units at $C_{(6)}$.

The results of partial acid and enzymic hydrolyses together with those of methylation analysis led to the assumption that the polysaccharide resembles a "comb" having a mannan core with (1→2) glycosidic bonds, whilst monomeric D-galactose units are attached to D-mannose units at $C_{(6)}$. The low value of specific optical rotation of the polysaccharide (−4·4°) and signals in its ^{13}C-NMR spectrum at 107·95 and 101·30 ppm indicate a β-anomeric character of glycosidic bonds in the polysaccharide[23,24]. A polysaccharide with a similar mannan backbone, but with longer oligomeric side chains consisting of D-galactose units was isolated by Miyazaki and coworkers[24] from the fungus *Cordyceps sinensis*.

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