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AN EXTRACELLULAR FUNGAL POLYSACCHARIDE COMPOSED OF 2-ACETAMIDO-2-DEOXY-D-GLUCURONIC ACID RESIDUES*

PAUL R. WATSON, PAUL A. SANDFORD, KERMIT A. BURTON, MARTIN C. CADMUS, AND ALLENE JEANES

Northern Regional Research Laboratory†, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604 (U. S. A.)

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ABSTRACT

The black yeast-like fungus NRRL YB-4163, now tentatively identified as *Rhinocladiella elatior* Mangenot, has been found to produce an extracellular microbial polysaccharide composed mainly of 2-acetamido-2-deoxy-D-glucuronic acid residues. Polysaccharide (PS) YB-4163, when isolated in good yield as the neutral potassium salt, dissolves readily in water to produce extremely viscous solutions, which form stable foams and emulsions. By depolymerizing PS YB-4163 with [^{14}C]methanol–HCl, the polysaccharide can be both identified and quantitated radiochemically by determining the individual [^{14}C]methyl glycosides after their separation by paper chromatography. When the methyl glycosides of PS YB-4163 were reduced with NaB^3H_4, only the methyl glycosides of 2-acetamido-2-deoxy-D-[6-^{3}H]glucose were found. Analysis of the monosaccharide released from carboxyl-reduced PS YB-4163 by acid hydrolysis or methanolysis also showed 2-acetamido-2-deoxy-D-glucuronic acid to be the main constituent. Previously, the only polysaccharides known to be composed entirely or hexosaminuronic acid have been cellular products from pathogens. Of these, the antigenic polysaccharide (SPSA) from *Staphylococcus aureus* is composed entirely of 2-amino-2-deoxy-D-glucuronic acid, but its amino groups are substituted equally with acetyl and *N*-acetylalanyl groups. The specific optical rotation of PS YB-4163, $[\alpha]_D^{20}$ −75° (*c* 0.5, water), is similar to that of SPSA (−91°), and suggests β-D-linkages that must be either (1→3) or (1→4).

INTRODUCTION

In a search for new types of extracellular microbial polysaccharide, a unique acidic polysaccharide (PS) composed mainly of 2-acetamido-2-deoxy-D-glucuronic acid (GlcNAcA) residues was obtained from the black yeast-like fungus strain

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NRRL YB-4163, now tentatively classified as *Rhinocladiella elatior* Mangenot[1]. PS YB-4163 can be isolated in good yield and dissolves readily in water to produce highly viscous solutions, which readily form stable foams and emulsions.

The identity and the percentage of GlcNAcA in PS YB-4163 was established by use of radiochemical methods[2] previously used for another black-yeast polysaccharide[3]. A hexosaminuronic acid was first identified in a polysaccharide in 1958. Since that time, several cellular products from pathogens have been found to contain various hexosaminuronic acids[4–12]. From among all of these products, the composition of PS YB-4163 resembles most closely the cellular staphylococcal polysaccharide antigen (SPSA) isolated from *Staphylococcus aureus*[13,14]. Although both SPSA and PS YB-4163 are composed mainly of 2-amino-2-deoxy-D-glucuronic acid residues, in PS YB-4163 the amino groups are substituted solely with acetyl groups, whereas in the SPSA the amino groups are substituted equally with acetyl and *N*-acetylalanyl groups. PS YB-4163 also differs from SPSA in being produced extracellularly.

EXPERIMENTAL

Materials. — NRRL YB-4163, a black yeast-like fungal organism, was isolated from a "slime outbreak" at a pulp and paper mill by Dr. Joseph R. Sanborn, National Aluminate Corp., and sent to this laboratory for characterization. [^{14}C]-Methanol (58 mCi/mmole) and [^{3}H]$NaBH_4$ (249 mCi/mmole) were purchased from New England Nuclear Corporation and 2,5-diphenyloxazole (PPO, scintillation grade) from Beckman Instruments, Inc. [^{14}C]Methanol–HCl (1.0M, 600 μCi/ml) was prepared as described previously[2].

Professor Kurt Heyns, University of Hamburg, graciously supplied an authentic sample of 2-amino-2-deoxy-D-glucuronic acid.

Culture methods. — The methods used were the same as described previously for another black yeast-like fungus[15]. Although conditions for experimental production of PS YB-4163 have not been thoroughly investigated thus far, satisfactory results were obtained by use of a medium containing D-glucose and bovine serum albumin (BSA) previously found suitable for another black fungus[15]. Systematic investigation of fermentation conditions may be expected to lead to improvement in yield and possibly reduction in fermentation time.

When grown in Fernbach flasks, the culture liquor (500 ml) reached maximum viscosity after shaking (rotary shaker) for 7 days at 25°. As the concentration of extracellular polysaccharide increased near the end of the fermentation, the culture medium developed into a dense foam. When centrifuged in 250-ml bottles (1,000 × g, 30 min), this foam broke down and allowed the culture fluid to be seen as a gray-to-black, highly cohesive liquid (like an egg white).

Recovery of PS YB-4163. — Seven-day cultures, having a viscosity about 3,500–5,000 centipoises (Brookfield viscometer, Model LVT, spindle No. 3 or 4, 30 rev/min), were diluted with 3 vol. of water to about 315 centipoises and then

centrifuged (35,000 × *g*, 30 min) to remove cells. By addition of solid potassium chloride (1%) and ethanol (95%, 2 vol.), the total polysaccharide product separated as a stringy precipitate that wound around the stirrer. After dissolving it in water and by addition of 1% hexadecyltrimethylammonium bromide (Eastman Organic Chemicals), the major fraction (acidic) was precipitated as a tough, stringy precipitate that wound around the stirrer; the neutral fraction, about 15% of the total product, remained in solution and has not been investigated further. After this step, the precipitate of the acidic fraction was dissolved in 2M potassium chloride and, after dilution by addition of 1 vol. of water, it was reprecipitated by addition of 2 vol. of ethanol. This product, which was the potassium salt form of the acidic polysaccharide, was redissolved and dialyzed in deionized water until free from extraneous salt. The retentate was adjusted to pH 6.3 with dilute potassium hydroxide and then freeze dried. The yield of polysaccharide in the K^+ salt form was 0.5 g/100 ml of culture fluid, or 10% based on D-glucose converted.

Pigment adhering to the polysaccharide was removed by the Sevag procedure[16]. To a water solution of PS YB-4163 or a cell-free culture fluid (1% with potassium chloride, viscosity 50 centipoises) was added 1/4 vol. of chloroform and 1/10 vol. of 1-butanol, and the mixture was stirred rapidly for 5 min. The resulting emulsion was then centrifuged (1,000 × *g*, 15 min), which left denatured protein and pigment at the water–chloroform interface. The water layer containing PS was withdrawn and given two additional Sevag treatments, after which the PS YB-4163 was precipitated by alcohol (2 vol.), dissolved in water, and freed of salt by dialysis in water. The retentate was adjusted to pH 6.3, filtered through a 5-μm filter (Millipore), and lyophilized. The pigment-free product was isolated in 75% yield. Previously, we have shown that a pigment-free polysaccharide could be obtained from another black yeast merely by including BSA in the medium. Inclusion of BSA in the medium for PS YB-4163, however, was less effective, as the pigment was not removed during the normal isolation procedure. The presence of BSA in the medium for YB-4163 did, however, result in improved removal of pigment by the Sevag procedure.

Neutral equivalent weight. — A solution of PS YB-4163 (0.01%) was decationized by passing it through a column (1.5 cm diameter × 200 cm) of AG-50 × 4 (200–400 mesh, Bio-Rad) and titrating the effluent with standardized potassium hydroxide (0.1M) and a pH meter as the indicator.

Methanolysis. — Methanolysis procedures[2] previously established for a polysaccharide composed of GlcNAcA and GlcNAc were used, with minor changes. A 2% mixture of predried PS YB-4163 (free carboxyl form) in anhydrous [^{14}C]-methanol–hydrogen chloride (M) was refluxed (75–77°) for 48 h and re-*N*-acetylated before radiochromatography.

Methanolysis products were de-esterified with 0.025M potassium hydroxide under nitrogen for 3 h at room temperature[2].

Paper chromatography. — Chromatograms (Whatman No. 1 paper) were developed (descending) with the following solvents (v/v): A, 1-butanol–acetic acid–water (19:6:25, upper layer); B, pyridine–ethyl acetate–water (2:5:5, upper layer);

C, 1-butanol–ethanol–water (4:1:5, upper layer); D, water-saturated butanone; and E, 1-butanol–ethanol–water (4:1:1).

The following dips or sprays were used to locate compounds on paper chromatograms: sodium hypochlorite–potassium iodide–amylose sprays[17] for amino sugars and their glycosides, both free and *N*-acetylated forms; hydroxylamine and ferric chloride sprays[18] for glycosides containing methyl-esterified uronic acids; ninhydrin dip[19] for amino sugars; Elson–Morgan spray reagents[19] for 2-amino-2-deoxyhexoses; and alkaline silver nitrate (dip)[19] for reducing sugars (glycosides of amino sugars gave weak spots).

Radiochromatography. — Radiochromatograms, after development on Whatman paper (No. 1, 2 × 46 cm) in solvent C or D, were cut into 1-cm segments, each of which was placed in a scintillation vial and counted in 10 ml of scintillation fluid (PPO/toluene, 4 g/l) in a liquid scintillation-counter (Beckman LS-250).

RESULTS

Characterization of PS YB-4163. — Lack of appreciable absorption at 260 or 280 nm, failure to detect amino acids in acid hydrolyzates (6M hydrochloric acid, 24 h at reflux), negative biuret test[20], and failure to detect phosphorus ruled out the presence either of protein or nucleic acid in PS YB-4163. It does not contain simple uronic acids such as glucuronic acid (carbazole method[21]), neutral sugars (less than 5% as glucose in the phenol–sulfuric acid test[22]), sialic acids (Warren procedure[23]), or free amino groups (ninhydrin[24] or Fluram®[25]). PS YB-4163 did react positively in the sodium hypochlorite–amylose–potassium iodide procedure[17,26], which detects acylated and free amino groups. Absorption in the i.r. region at 1730 cm^{-1} indicated that PS YB-4163 (acidic form) contained free carboxyl groups, whereas absorption at 1650 cm^{-1} (Amide I band) and 1540 cm^{-1} (Amide II band) indicated the presence of secondary amide groups in PS YB-4163. All these tests suggested that PS YB-4163 contains an *N*-acylated amino sugar.

Chromatography of PS YB-4163 hydrolyzates. — Hydrolysis (M hydrochloric acid, 2–16 h) results in extensive degradation, as evidenced by the formation of black humin. This behavior[2,3,9] is characteristic of polysaccharides containing 2-amino-2-deoxyhexuronic acids. After short-term hydrolysis (M hydrochloric acid, 1 h) of PS YB-4163 trace amounts of glucose, galactose, and mannose were detected on paper chromatograms developed with solvents A and B. The major component migrated as authentic 2-amino-2-deoxy-D-glucuronic acid [R_{GlcN} 0.75 (solvent A), 0.26 (solvent B)]. Further evidence for a 2-amino-2-deoxyhexuronic acid was the positive reaction of the major component to silver nitrate dip[19], Elson–Morgan spray[19], and *o*-aminobiphenyl spray[27] (test for uronic acids). Also, with ninhydrin (dip)[19], the major component initially gave a brown color that gradually turned purple. Again, this behavior is characteristic of 2-amino-2-deoxyhexuronic acids[4,9]. 2-Amino-2-deoxyhexoses were not detected in hydrolyzates.

Chromatography of PS YB-4163 methanolyzates. — After application of the

methanolysis procedure[2] to PS YB-4163, the major component-glycosides, when separated by paper chromatography (Solvents C and D), migrated like the methyl glycosides of GlcNAcA. This mixture of α and β glycosides showed migration rates (R_{GlcNAc}) of 1.76, 2.19, and 2.59 in solvent C, and R_{GlcNAc} of 3.90, 4.41, and 6.10 in solvent D. Traces of methyl glycosides of glucose and mannose also appeared on the chromatograms. Components that would correspond to the methyl glycosides of GlcNAc, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-mannose, *N*-acetylmuramic acid, or *N*-acetylneuraminic acid were not found in methanolyzates of PS YB-4163.

Use of [^{14}C]methanol[2] in the methanolysis procedure allowed quantitation of individual methyl glycosides. As methanolysis of GlcNAcA produced an undefined mixture of lactone and methyl ester forms of its methyl glycosides, the glycosidic mixture from PS YB-4163 was treated with dilute alkali[2] (25mM potassium hydroxide, 3 h, 25°), which converted the mixture into a single, potassium salt form (Solvent C, R_{GlcNAc} 0–0.19) that was amenable to quantitation by radiochromatography. Quantitating the amount of [^{14}C]methyl 2-amino2-deoxy-α (and β)-D-glucopyranosiduronic acids (K^+ salts), which migrated as a single spot in solvent C, from PS YB-4163 on paper chromatograms by scintillation counting of radioactivity, accounts for at least 92% of PS YB-4163 as GlcNAcA.

The identity of the 2-amino-2-deoxyhexuronic acid as GlcNAcA was further established by reducing with NaB^{3}H$_4$ the methyl ester–lactone glycosides[2] from PS YB-4163 to give methyl 2-acetamido-2-deoxy-α (and β)-D-[6-^{3}H]glucopyranosides.

Analysis of carboxyl-reduced PS YB-4163. — After esterification[28] with methanolic hydrogen chloride (0.1M, 24 h at 25°), PS YB-4163 was reduced[2,28] with NaB^{3}H$_4$ (0.5M dissolved in 0.1M sodium hydroxide). The decrease in carboxyl groups was monitored by infrared analysis. Esterification and reduction with NaB^{3}H$_4$ were repeated to give approximately 90% reduction. Hydrolysis (M hydrochloric acid, 2–16 h) of reduced PS YB-4163 and paper chromatography (solvents A and B) of the products gave a [^{3}H]amino sugar that migrated like 2-amino-2-deoxy-D-glucose but not like either 2-amino-2-deoxy-D-galactose or 2-amino-2-deoxy-D-mannose. This [^{3}H]amino sugar was isolated as its hydrochloride from paper chromatograms (Solvent B), and had an observable optical rotation ($[\alpha]_D^{20}$ +70.9°, *c* 0.2, water) similar to that of 2-amino-2-deoxy-D-glucose hydrochloride ($[\alpha]_D^{20}$ +72.0°, *c* 0.5, water). Deamination of this [^{3}H]amino sugar with ninhydrin[29] and separation of the deamination product(s) by paper chromatography (solvent E) revealed a single [^{3}H]pentose that migrated like D-arabinose but not like D-lyxose, D-xylose, or D-ribose. The results from this series of reactions also indicated that the parent, acidic amino sugar in PS YB-4163 is D-GlcNAcA. Treatment of NaB^{3}H$_4$-reduced PS YB-4163 with unlabeled or [^{14}C]methanol–hydrogen chloride gave labeled glycosides that migrated on paper chromatograms (solvents C and D) like authentic samples of methyl 2-acetamido-2-deoxy-α and β-D-glucopyranoside.

Our data, as summarized in Table I, agree with the conclusion that the extracellular polysaccharide from the black fungus NRRL YB-4163 is composed mainly

of D-GlcNAcA. The complete inertness of PS YB-4163 to sodium periodate[30] at 20° indicates linkages through the 3- or 4-positions, or both. The highly negative optical activity for the salt-form, $[\alpha]_D^{20}$ −75° (*c* 0.5, water), suggests the β-D configuration.

TABLE 1

COMPOSITION AND SOME PROPERTIES OF POLYSACCHARIDE YB-4163

Carbon (%)	*Hydrogen* (%)	*Nitrogen* (%)	*Acetyl* (%)	*Residues of*[a] (%)	*Neutral sugar*[b] (%)	*Neutral equivalent weight* (g)	$[\alpha]_D^{22}$ (°)
Determined for PS YB-4163 (K^+ salt form)							
36.54	3.93	5.33	14.9	92[c]	5.0	263[d]	−75[e]
Calculated for repeat unit [-*GlcNAcA*-] (K^+ salt form)							
37.65	3.92	5.49	16.82	100	0	255	—

[a]GlcNAcA calculated as free acid, % by wt. [b]Calculated as D-glucose, % by wt., in the phenol–sulfuric acid procedure[22]. [c]Determined by ^{14}C-methanolysis. [d]pKa = 3.67. [e](*c* 0.5, water).

DISCUSSION

Although methanolysis of PS YB-4163 does not appear to proceed as smoothly as with PS Y-6272 (ref. 2), as evidenced by traces of acidic oligomers on paper chromatograms, more than 92% of YB-4163 can be accounted for as methyl glycosides of GlcNAcA. The neutral-sugar portion (5%) of PS YB-4163 appears to be a contaminant associated with the black pigment; fractions that had been depigmented by the Sevag technique had less neutral sugar than pigmented preparations.

An outstanding property of PS YB-4163 solutions that has practical implications is their ability to stabilize emulsions. Stable emulsions are observed when aqueous solutions are shaken with chloroform–1-butanol, as in the Sevag procedure. The ability of YB-4163 cultures to form a dense foam in later stages of growth may also have practical significance.

Initial observations also show that solutions of PS YB-4163 possess an elasticity higher than usual: when solutions (1%) of PS YB-4163 are poured or stirred, the entire solution tends to cling together.

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ADDENDUM

Animal (mice and guinea pigs) pathogenicity tests have been completed on the fungus culture *Rhinocladiella elatior* NRRL YB-4163 at the Center for Disease

Control, Atlanta, Georgia. It appears that, under the conditions of the tests, the culture (mycelium and spores) is not pathogenic.

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