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COMPOSITION OF CELL WALLS OF *UROMYCES PHASEOLI*

by

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TITLE

COMPOSITION OF CELL WALLS OF

UROMYCES PHASEOLI

BY

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INTRODUCTION

Considerable effort has been spent studying the chemistry of fungal cell walls in hopes of determining whether there are differences in wall composition in different taxonomic fungi. These investigations have shown that most fungal walls are composed of 80% to 90% carbohydrate with the remaining 10% to 20% as protein and lipid. Wide departures from these values are quite rare, such as the 40% protein reported in the cell walls of the yeast Saccharomycetes guttalata (67). In addition, fungal walls may have quantities of inorganic ions or pigments. The most common polysaccharides in fungal walls contain glucose, mannose, and N-acetyl glucosamine, but the relative proportions of these monosaccharides vary enormously from traces in certain organisms to principle components in others. Some of the monosaccharides less frequently found and with a somewhat characteristic distribution among certain fungi are galactose (Ascomycetes), glucosamine (Mucorales), and xylose (Basidiomycetes). The protein in fungal cell wall appears to be more than a structural component. With the discovery of enzymes in and on cell walls (39) (64) and cell wall glycoprotein complexes (12) (49) (50) (55) the protein may have a dynamic role in cell wall synthesis and function.

The information that is available about rust fungal walls is quite limited (41) (45) (59) (60). Further, most of these studies have been made only upon uredospores. The one examination made upon bean rust germ tube walls was concerned with lipid content (45). Aqueous extractions of wheat rust uredospores have removed polysaccharides which contain mannose, glucose, and, in one report (41), small quantities of galactose. The extracted polymers appear to be mainly

composed of mannose linked via β(1→3) and β(1→4) bonds in an alternating arrangement with traces of glucose reported at the nonreducing end of the polymer (59). This structure appears to be rather novel, although similar β-linked mannans have been found to be excreted by Rhodotorula glutinis (33), and possibly by Sporobolomyces roseus (31). A rather more common mannosidic linkage in fungal walls involves α-anomeric (1→2), (1→3), and (1→6) bonds. The finding of only glucose at the reducing end of the spore mannan also is unique since glucose is usually present in polymers bound together via β(1→3), β(1→6), (31), or, in certain Phycomycetes, β(1→4) linkages (10).

The possibility of wall glycoprotein or proteoglycan in the host walls is of considerable interest. With one exception (78), the literature suggests that there is no net protein synthesis during germination of rust uredospores (72) (68), although in vitro ribosomal incorporation of amino acids (29) (71) (84) has been observed. It has been suggested that failure of protein synthesis to occur in vivo during germination of uredospores is an important factor in the phenomenon of obligate parasitism (78). The presence of protein in the cell walls might explain the diverse results reported in the literature, especially in view of the fact that in earlier studies (72) (73) hot acid was used to precipitate protein. The use of acid precipitation may have resulted in error because it has been found that appreciable amounts of glycoprotein from germinated bean rust uredospores were soluble in hot acid (82).

In view of the limited knowledge of obligately parasitic fungal walls, the wall components of uredospore walls and germ tube walls of Uromyces phaseoli var. typica Arth. were investigated. In the research

to be described, the carbohydrate composition of the cell walls were obtained by acid hydrolysis. Using enzymatic and methylation procedures the identity of the linkages binding the sugars together was investigated in an attempt to determine the architectural structure of the polysaccharides comprising the different bean rust walls. Since no amino acid materials have yet been identified to be part of the walls in rust fungi, studies were designed to test this possibility.

The eventual goal for determining the nature of uredospore walls would be to provide information useful in understanding uredospore wall biosynthesis. Determining the structure and functions of the wall, and the control of its synthesis might have application in disease control. Similarly, the germ tube wall arising during germination of uredospores represents a major metabolic activity. Knowledge of the wall structure and its synthesis might further be useful in preventing spore germination. Additional information about the architecture of the germ tube wall and its components could be helpful in explaining rust infection of plants or even the nature of obligate parasitism in rust fungi.

MATERIALS and METHODS

Spore Germination. Production, isolation, and germination of uniformly ^{14}C labeled uredospores or Uromyces phaseoli (pers.) Wint. var. typica arth. has been previously described (28) (78). In most experiments 500 to 900 mg of ^{14}C labeled spores were used for preparation of spore and germ tube walls. Before germination, spores were washed in distilled water (1 mg of spore/1ml of water) for 15 min, filtered, and air dried on filter paper. Germ tubes were evident 2 hr after dispersal of the uredospores upon aqueous media, but removal and isolation of the germ tube walls were not carried out until after 18 to 20 hr. At this time more than 80% of the spores had germinated and germ tubes were at least five times longer than the diameter of the spore (Figure 1A).

Isolation of Germ Tube and Uredospore Walls. Isolation of germ tubes from germinated uredospores was achieved using the following method devised by Langenbach. Germinated spores were removed from the germination medium and separated into 50 to 75 mg lots. Each of the lots were subjected to ultrasonic oscillation for 30 to 45 sec in 15 ml of distilled water or in 0.05M Tris buffer, pH 7.5, with a Branson Instruments sonifier, Model LS-75, equipped with a 1,5 in titanium disruptor horn. The power settings were 6 on the range selector dial and 2.1 amperes. The initial ultrasonic treatment served primarily to detach the germ tube from the uredospore with little breakage of the germ tube and no breakage of the uredospore. The dispersed fungal materialwas transferred to a 15 ml conical centrifuge tube which was rapidly accelerated to 700 x g in a swinging bucket rotor head and centrifuged for 5 min. All but 1 ml of the supernatant then was removed from the conical tube. The white layer of material containing germ tubes on top of the brown germinated uredospore wall material (hull) was dispersed gently in the remaining 1 ml of supernatant and removed with a

capillary tipped eye dropper. The brown uredospore wall material again was dispersed in 15 ml of water or buffer and the entire process repeated on each 50 to 75 mg lot from 6 to 10 more times. The combined white layers were subjected a second additional time to sonification and centrifugation in order to remove the small amounts of hull material that was present. Both the hull and germ tube fractions were lyophilized.

Figures 1A and 1B illustrate uredospores 18 hr after germination. Figure 1C shows that the white layer on top of the red-brown uredospore layer obtained from the first sonic oscillation of the mat of germinated uredospores (Figure 1B) was nearly all germ tubes. This white layer was further shown to be fungal hyphæ using staining methods employing Cotton Blue. Subsequent sonic oscillations did of course cause germ tube materials to fragment to smaller particle sizes.

Since uredospore walls could not be broken by sonic oscillation, 20 to 30 mg quantities of the lyophilized hull walls and walls from ungerminated uredospores were placed in Mickle disintegrator vials containing 300 mg of Calaphor No. 203 glass beads and 5 ml of one of the following solvents: water, 0.05M Tris buffer pH 8.0, 0.05M citrate buffer pH 6.0, or 0.05M Tris buffer pH 8.0 containing 0.2M NaCl. In one instance a germ tube wall preparation was treated with the Mickle disintegrator in a similar fashion to the spore wall material. Aqueous suspensions of broken uredospore walls and germ tube walls were placed in 15 ml Corex tubes and centrifuged at 700 x g for 5 min using the swinging bucket rotor. The supernatants were decanted and the pellets washed with 5 ml of buffer or water and recentrifuged at 700 x g twice. Subsequent extractions of all cell wall materials were made using 5 ml (2 times) of the following solvents in the order given:

chloroform-methanol (2:1), diethyl ether, 80% ethanol, water (room temperature), 0.2M NaCl, and boiling water. The remaining cell wall preparations were then lyophilized. These lyophilized preparations were highly hydroscopic and weights were difficult to obtain, but 500 mg of dry ungerminated uredospores gave approximately 20 mg of lyophilized germ tube wall and about 85 mg of hull wall.

Hydrolysis of Cell Walls for Amino Acids. In acid hydrolysis, 3 mg portions of lyophilized hulls, germ tube walls, and ungerminated uredospore walls were placed in test tubes containing 1 ml of 6N HCl, evacuated, and heated at 100° C for 12 to 14 hr. For enzymatic hydrolysis, the preparations were incubated for 12 to 14 hr at 37° C with 5 ml of 0.05M Tris buffer, pH 8.0, which contained 1 mg each of chymotrypsin and trypsin. After centrifugation of the acid hydrolyzates, the supernatants and washings of the acid insoluble cell wall residues were appropriately pooled, dried at 40° C, and analyzed for amino acids using a Hitachi-Perkins Analyzer, Model KLA-3B. The enzymatic hydrolyzates and washings acquired from the protease treated cell wall preparations were assayed for solubilized fungal wall materials using radioactivity assays.

Acid Hydrolysis of Cell Walls for Carbohydrates. The insoluble cell walls that remained after treatment with proteolytic enzymes were suspended in 1 ml of 2N Trifloroacetic acid (TFA) and heated at 95° C to 100° C for 12 to 14 hr under vacuum. Materials remaining insoluble after 2N TFA treatment were washed with three 1 ml portions of water and suspended in 6N HCl for further hydrolysis at 100° C under vacuum for 12 to 14 hr. Each of the acid hydrolyzates were dried at 40° C *in vacuo*, dissolved in 0.2 to 0.3 ml of water, spotted on 4 x 60 cm Whatman No. 1

paper strips, and chromatographed by descending paper chromatography in 2-butanone, acetic acid, boric acid saturated water (9:1:1), (solvent A). When the solvent front had nearly reached the edge of the paper, the paper strips were scanned with a Tracerlab strip scanner. Any radioactive areas near the solvent fronts were cut from the paper strips and counted. The remaining paper strips were rechromatographed in solvent A for an additional 48 hr with the solvent dripping from the edge of the paper. The location of the radioactive materials were then determined with the Tracerlab strip scanner, the radioactive components eluted from the paper strips, and their identities checked by additional paper chromatography for 48 hr in ethyl acetate, pyridine, boric acid saturated water (60:25:20) (solvent B), and again in solvent A. In all cases common monosaccharides and disaccharides were employed as standards and spotted with the radioactive materials in order to determine the identity of the carbohydrates. Location of the sugars standards chromatographed with the radioactive sugars was by a $AgNO_3$ dip (77).

Replacing 2N TFA with 7% HCOOH resulted in partial rather than complete cell wall hydrolysis. Uredospores that were treated in this manner released oligosaccharides of various sizes which were partially separated by elution with 0.2N acetic acid from a Sephadex G-15 (2 x 150 cm) column pre-equilibrated with 0.2N acetic acid. Four ml fractions were collected and assayed for carbohydrate by the Anthrone method (81).

Acid Hydrolysis of Cell Walls for Glycoprotein Fragments. Release of proteoglycans from germ tube and germinated spore walls was achieved by modification of the method described by Wynn and Gajdusek (82). Milligram quantities of lyophilized hull and germ tube walls were placed in separate test tubes containing 5% TFA. After heating in a boiling water

bath for 15 min, the contents of the tubes were centrifuged at 10,000 x g for 10 min. The hot TFA treatments were repeated 4 times on the pellets of cell wall residue. The supernatant fluids from each treatment were combined and then extracted 5 times with equal amounts of diethyl ether. The remaining large volumes of aqueous solutions were lyophilized, dissolved in 5 ml of water, made 90% with respect to ethanol, allowed to stand for 20 hr at 4° C, and centrifuged at 10,000 x g for 10 min. These supernatants were decanted and the pellet washed two more times with 5 ml of 90% ethanol, before dispersing in 0.01M Tris buffer, pH 7.5. After centrifugation, the Tris supernatant was applied to a DEAE Cellulose column (1 x 15 cm) that was pre-equilibrated and initially eluted with 0.01M Tris buffer, pH 7.5, at a rate of 0.4 ml/min. The column was further eluted using 0.01M Tris buffer (pH 7.5) containing 0.2M NaCl (0.4 ml/min). Detection and location of the eluted proteoglycans in the 4 ml fractions was by radioactivity, Anthrone (81), and Lowry (48) assays. The glycoproteins chromatographed from the cellulose column were then spotted in a band at the center of 4 x 60 cm Whatman No. 1 paper strips. Electrophoresis was carried out using 0.2M sodium tetraborate buffer, pH 9.2. A current of 25 ma per strip and a potential of 2000 volts was employed. Development time was 90 min at 16° C. After drying the paper strips, the location of the radioactive materials was determined using a Tracerlab strip scanner. The appropriate radioactive regions were then eluted from the paper, hydrolyzed in 2N TFA or 6N HCl, and assayed for amino acids using the amino acid analyzer and carbohydrates via paper chromatography in solvent A.

Methylation of Cell Walls. Cell wall polysaccharides were methylated using a modification of the Hakomori procedure (35). Between

100 to 1000 mg of 50% oil-coated sodium hydride was placed in a three necked round-bottomed flask and washed three times with 10 ml portions of hexane. After the final wash, solvent was removed from the sodium hydride in vacuo (1 mm pressure) at room temperature, and 5 to 10 ml of dry dimethyl sulfoxide (DMSO) was added. The suspension of sodium hydride in DMSO was stirred under N_2 at 60° C for 1 hr to form the methyl sulfinyl carbanion.

For the preparation of the alkoxide of cell wall polysaccharides, 50 to 500 mg of lyophilized cell walls were placed in 5 to 10 ml of methyl sulfoxide and stirred under N_2 for 1 hr at 60° C. The DMSO solution containing the cell walls was added to the methyl sulfinyl carbanion solution and stirred under N_2 for an additional 8 hr at room temperature. Based on the maximum number of OH equivalents in the polysaccharide, a 25% excess of dry NaH was used. (It was assumed that the molecular weight of the polysaccharide equalled 181 mg per millimoles and that 5 millimoles of OH were present for every millimole of cell wall polysaccharide.)

For the methylation reaction, the mixture was maintained in a water bath at room temperature. Methyl iodide, at 1 and 1/2 times the amount of hydroxyl equivalents assumed for the sample, was added dropwise with continued stirring. The reaction mixture was kept at room temperature for an additional 12 hr. Ice water (20 ml) was added to the solution and extracted with chloroform (25 ml x 3) to remove the methylated polysaccharide. The combined chloroform extracts were washed with water (25 ml x 3) and evaporated to a syrup under diminished pressure. The water phase was combined with the washings of the chloroform extracts and dialyzed against large volumes of distilled water for 24 hr. The

solution inside the dialysis tubing was lyophilized and remethylated.

Methylated derivatives of mannose, glucose, galactose, isomaltose, and potato starch were also prepared using the modified Hakomori procedure (35). Exposure of the methylated carbohydrates to methanolic hydrochloric acid yielded the following glycosides which were isolated by thin layer chromatography: α-2, 3, 4, 6 tetra-o-methyl mannoside, α and β-2, 3, 4, 6 tetra-o-methyl glucoside, α and β-2, 3, 4, 6 tetra-o-methyl galactoside, α and β-2, 3, 4 tri-o-methyl glucoside, α and β-2, 3, 6 tri-o-methyl glucoside. (For methodology see section on Hydrolysis and Analysis of Methylated Polysaccharides.)

Fractional Separation of Methylated Cell Walls. The combined chloroform solutions from both methylations were evaporated and the syrups extracted with methanol. These fractions were designated as the methylated uredospore walls soluble in chloroform and methanol (U-I), the methylated germ tube walls soluble in chloroform and methanol (GT-I), the methylated uredospore walls soluble in chloroform but insoluble in methanol (U-II), and the methylated germ tube walls soluble in chloroform but insoluble in methanol (GT-II). Methylated germ tube or methylated uredospore walls insoluble in chloroform were abbreviated as GT-III and U-III respectively. A KBr pellet was made of $CHCl_3$ insoluble fractions, while the other fractions were dissolved in chloroform and assayed for the presence of free hydroxyl groups by IR spectroscopy.

Hydrolysis and Analysis of Methylated Polysaccharides. Methylated cell wall fractions were hydrolyzed with 5% methanolic acid (MeOH-HCl) at 100° C for 16 hr in sealed vials. If large amounts of insoluble material remained after this time, the solution was removed and the remaining insoluble residue was treated again with methanolic hydrochloric

acid, but for 36 hr at 100° C. Removal of the MeOH·HCl was accomplished by placing the acid hydrolyzates in a partially evacuated desiccator containing KOH pellets. After two days at room temperature, the samples were dry and free of HCl. The methylated glycosides were spotted as bands on plates of Silica Gel G with thicknesses of 0.5 mm and 0.25 mm. Also, known methylated sugars were spotted near the bands of cell wall glycosides before development of the thin layer plates with the organic layer of the mixture- benzene, ethanol, water, ammonia (200:47:15:1) (solvent C). Two ascents were used. The region on the Silica Gel G plates containing the chromatographed sugar standards and a small portion of the zones, associated with the separated wall glycosides, were sprayed with a 50% aqueous ethanol solution containing 5% H_2SO_4. The glass plates were heated at 100° C for 10 min in order to char the sugars exposed to the spray. The location of the charred spots of cell wall glycosides and known methylated sugars were compared with one another. Relative positions of these sugars (R_m) were determined with respect to the position of the α-2,3,4,6 tetra-o-methyl mannoside standard. Zones adjacent to the charred wall glycosides then were scraped from the plates and the sugars extracted three times with methanol (10 ml). The methanol extracts were combined and evaporated in vacuo. The residue was then re-extracted with ether (10 ml) and the ether extracts evaporated to dryness.

Methylated glycosides obtained from permethylated fungal walls hydrolyzates were further separated by gas chromatography using a F and M Model Instrument equipped with a hydrogen flame ionization detector. The relative retention times (RT_m) of the wall glycosides were determined with respect to the retention time of α-2,3,4,6 tetra-ó-methyl

mannoside. Teflon columns (12 x 1/8" and 5' x 1/8") packed with 2.3% neopentyl glycol succinate polyester (NGS) on Anakrom ABS (110 to 130 mesh) support were employed. Operating temperatures used for separating the methylated sugars were 125°, 150°, and 160°C with flow rates of 20 to 25 ml of N_2/min.

Formation of Methylated Glycoses. Glycoses were produced from glycosides by heating them in 1N NCl (5 ml) at 100°C in vacuo for 12 to 14 hr. The solution was brought to neutrality by adding Dowex 50 x 8 resin in the hydrogen form. After filtration, the resin was further washed with 25 ml of 95% ethanol. The filtrates were dried under partial vacuum at 35°C, dissolved in methanol, and chromatographed on 0.25 mm Silica Gel G plates using benzene, ethanol, water, ammonia (200: 47:15:1) (solvent C) and/or water saturated 2-butanone (solvent D).

Gas chromatography of the glycoses was carried out using a 5' x 1/8" Teflon column packed with 2.3% NGS on Anakrom ABS (110 to 130 mesh). Operating temperature = 160°C. Flow rate = 25 ml N_2/min. The relative retention time of the cell wall glucoses (RT_m') was determined with respect to 2, 3, 4, 6 tetra-o-methyl mannose.

Enzyme Preparations and Assays. Several sources were assayed for various carbohydrases. These sources were Cellulase 9x (Miles Laboratories), Helicase (Industrie Biologique Francaise), homogenized grasshoppers prepared according to Talmadge and Albersheim (76), and a partially purified enzyme preparation from Jack Bean meal isolated via $(NH_4)_2$ SO_4 precipitation and dialysis against several buffers as described by Yu-Teh (85).

Glycosidase activities were checked by using p-nitrophenylglycosides. The rates of hydrolysis were determined by measuring the increase in

optical activity at 400 mμ resulting from the release of p-nitrophenol. Assay mixtures contained 5 μmoles of the proper substrates and 4 to 7 mg of the crude enzyme preparation in 0.05M sodium acetate (pH 4.8) buffer, except for the Jack Bean meal preparation. Analysis of glycosidase activity in Jack Bean meal was done in 0.05M sodium acetate (pH 6.0) containing 0.1M NaCl, since large amounts of protein precipitated and loss of glycosidase activity, particularly α-mannosidase, occurred when the pH 4.8 acetate buffer was used. The identities and activities of glycosidases noted to be present in the Jack Bean meal preparation were α-mannosidase (8.5 units), β-glucosaminidase (1.6 units), and α-galactosidase (less than 1 unit) (1 unit of activity = Δ0.01 absorbance of p-nitrophenylglycoside substrate at 400 mμ/min/mg of protein).

Several glycosidases were found in the grasshopper preparation. Their identities and activities were β-glucosaminidase (3.7 units), α-glucosidase (3.5 units), β-glucosidase (1.2 units), α-mannosidase (1.0 units), β-mannosidase (1.6 units), α-galactosidase (< 1 units), and β-galactosidase (2.5 units). No glycosidases were observed to be present in Cellulase 9x and only β-mannosidase (1.4 units) was noted in Helicase.

Investigations for chitinase activity was made on Cellulase 9x, Helicase, grasshopper extracts, Jack Bean meal extracts, highly purified exo-β(1→3)-glycanase from Basidiomycetes species QM 806, and chitinase (Nutritional Biochemical Corporation) using 4 mg of colloidal chitin as the substrate (13) and 4 mg of the enzyme, except for 0.3 mg of β-glucanase. After incubations of the preparations (1 hr at 37° C) in buffer used in glycosidase assays, the supernatants were checked for release of N-acetyl glucosamine and N-diacetyl chitobiose via the ferrocyanide colorometric assay (13). The chitinase preparation was most