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Carbohydrates present in the glycoprotein from conidia of the opportunistic pathogen *Scedosporium prolificans*

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ABSTRACT

Hot aqueous extraction of conidia of *Scedosporium prolificans* gave a heterogeneous glycoprotein (RMP-Sp-Coni) with 41% protein and 2MeRha, Rha, Ara, Man, Gal, Glc, and $GlcNH_2$ in a 2:18:3:47:9:15:6 M ratio, the first report of 2-*O*-methylrhamnose in fungi. Methylation analysis showed nonreducing end- (10%), 2-*O*- (11%), and 3-*O*-substituted Rha*p* (7%), nonreducing end- (8%), 2-*O*- (12%), 3-*O*- (16%), and 2,6-di-*O*-substituted Man*p* (9%), nonreducing end- (4%), 3-*O*- (7%), and 4-*O*-substituted Glc*p* (7%), and nonreducing end-units of Gal*p* (9%). Mild reductive β-elimination of RMP-Sp-Coni cleaved *O*-linked structures to give a mixture of oligosaccharides, of which 2MeRha capping groups were present in $2MeRhaRha_2Hex_2Hex$-ol, $2MeRhaRha_2Hex$-Hex-ol, $2MeRhaRha_2HexHex$-ol, and $2MeRhaRha_2Hex$-ol (ESI-MS–MS). The mixture was fractionated by Biogel P-2 column chromatography and the two predominant isolates were β-D-Gal*p*-(1 → 6)-[2Me-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-D-Man*p*-(1 → 2)]-D-Man-ol, and another lacking the β-Gal*p* unit. Neither was formed from mycelial glycoprotein, although β-D-Gal*p*-(1 → 6)-[α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-D-Man*p*-(1 → 2)]-D-Man-ol was a common component.

1. Introduction

Scedosporium prolificans is a common fungus occurring in soil and plant residues and is an opportunistic pathogen, capable of infecting immunocompetent, as well as immunocompromised patients. Hot aqueous extraction of its mycelium furnished a heterogeneous polymer (RMP-Sp) with 35% protein and 62% carbohydrate. Mild reductive β-elimination provided an oligosaccharide mixture and a resistant polymer, the former consisting mainly of β-D-Gal*p*-(1 → 6)-[α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-D-Man*p*-(1 → 2)]-D-Man-ol, a pentasaccharide lacking β-D-Gal*p* side-chain units, and β-D-Gal*p*-(1 → 6-[α-D-Man*p*-(1 → 2)]-D-Man-ol in a 16:3:1 w/w ratio (Barreto-Bergter et al., 2008). A preliminary report on analysis of a glycoprotein from conidia of *S. prolificans* (RMP-Sp-Coni) described some structural features and the presence of 2-*O*-methylrhamnose residues (Gorin et al., 2008). The analysis is now described in more detail, as well the structures of oligosaccharide epitopes formed on mild reductive β-elimination.

2. Materials and methods

2.1. Microorganism and growth conditions

A culture of *S. prolificans* was supplied by Dr. J. Guarro, Unitat de Microbiologia, Facultat de Medicina e Institut d'Estudis Avançats, Réus, Spain. It was grown in Erlenmeyer flasks containing 200 mL of Sabouraud modified medium (g/L) peptone, 10; yeast extract, 5; glucose, 40; and incubated at room temperature for 7 days with shaking (pre-inoculum). Conidia were grown on Petri plates containing modified Sabouraud medium at room temperature. After 7 days, conidia were obtained by washing the plate surface with phosphate-buffered saline and hyphal fragments and debris were removed by filtration through gauze.

2.2. Extraction of conidia

In a typical experiment, conidia were extracted with 0.05 M phosphate buffer, pH 7.2, at 100 °C for 2 h, and the mixture was then dialyzed. Centrifugation of retained material provided a supernatant, which was evaporated to a small volume and freeze-dried to give crude glycoprotein (541 mg). An aqueous solution was then dialyzed to give retained material (RMP-Sp-Coni, 119 mg).

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2.3. Analytical methods

RMP-Sp-Coni was analyzed using methods employed with the glycoprotein isolated from mycelia of *S. prolificans* (Barreto-Bergter et al., 2008). These were (1) determination of carbohydrate, hexosamine, and protein contents, (2) quantitative and qualitative monosaccharide GC–MS analyses, (3) HPSEC with molar mass (M_w) determination, (4) methylation-GC–MS analyses, and (5) NMR spectroscopy, following conditions described in the Bruker manual, although DEPT was used to enhance –O$\underline{C}$H$_3$ signals and HSQC spectra were obtained with fractions that provided weak ^{13}C NMR spectra, and (6) ESI-MS and ESI-MS–MS of sodiated and lithiated ions, following pre-treatment with traces of NaCl and LiCl, respectively.

2.4. Preparation and fractionation of β-eliminated oligosaccharides on Biogel P-2

According to the method of Yen and Ballou (1974), RMP-Coni (201 mg) was treated with aqueous $NaBH_4$–NaOH at 25 °C for 40 h and following neutralization (HOAc), the solution was treated with Amberlite IR-120 (H^+ form), which was filtered off, and the filtrate freeze-dried. The residue was dissolved in MeOH, and the solution evaporated to remove boric acid. An aqueous solution of the residue was dialyzed through a membrane (Barreto-Bergter et al., 2008). An eluted β-eliminated oligosaccharide mixture (97 mg) was obtained, and was applied to a Biogel P-2 column (140 × 2.8 cm i.d.; v_o 275 mL), which was eluted at 0.9 mL/min to give fractions of 4.5 mL. Thirteen fractions were obtained, ranging in yields from 1.0 to 2.9 mg. Each was assayed colorimetrically for carbohydrate using the phenol–H_2SO_4 reagent (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.5. Controlled Smith degradation of Fractions 1 and 2

Added to each sample (50 μg) in water (2 mL) was added $NaIO_4$ (100 mg), and after 18 h, the solution was treated with a mixture of Amberlite IR-120 (H^+ form) and Amberlite IR-400 (OAc^- form) exchange resins. Filtration and evaporation gave a residue, to which MeOH was added, and the solution was evaporated, a process that removed boric acid and trimethyl borate. The product was partially hydrolyzed in aqueous TFA with pH 2.0 (5 mL) for 30 min at 100 °C (Gorin, Horitsu, & Spencer, 1965). The residue obtained on evaporation was examined by ESI-MS and ESI-MS–MS.

3. Results

3.1. Preliminary analysis of glycoprotein RMP-Sp-Coni

Hot phosphate buffer extraction of conidia provided a crude glycoprotein (RMP-Sp-Coni), which contained 41% protein and 62% carbohydrate with 2MeRha (2-*O*-methylrhamnose), Rha, Ara, Man, Gal, and $GlcNH_2$ in a 2:18:3:47:9:15:6 M ratio. The neutral monosaccharides were analyzed as their derived alditol acetates, which had typical retention times and GC–MS electron impact profiles. Glucosamine was also identified, but quantified colorimetrically.

Unlike the glycoprotein obtained from mycelium (Barreto-Bergter et al., 2008), Cetavlon-borate treatment did not provide a precipitate.

HPSEC, using a refractive index detector, showed RMP-Sp-Coni to be a mixture (Fig. 1) with main components having 18 and 21 kDa.

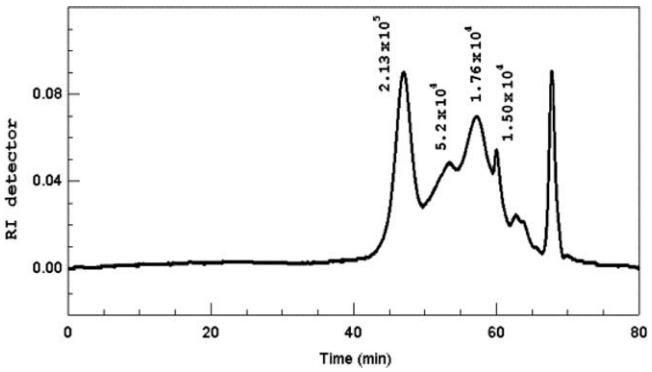

Fig. 1. HPSEC of RMP-Sp-Coni with M_w values.

3.2. Methylation and NMR analysis of RMP-Sp-Coni

Methylation analysis of RMP-Sp-Coni and GC–MS examination of partially *O*-methylated alditol acetates (Table 1) showed a complex structure with nonreducing end- (10%), 2-*O*- (11%), and 3-*O*-substituted Rha*p* (7%), nonreducing end- (8%), 2-*O*- (12%), 3-*O*- (16%) and 2,6-di-*O*-substituted Man*p* (9%), nonreducing end- of Gal*p* (9%), and nonreducing end- (4%), 3-*O*- (7%), and 4-*O*-substituted Glc*p* units (7%). This composition resembled closely the structures present in the extract from mycelia of RMP-Sp (Barreto-Bergter et al., 2008).

RMP-Sp-Coni gave a complex anomeric region in its ^{13}C NMR spectrum (Fig. 2A), with eight main signals ranging from δ 96.5 to 103.6. Its HMQC spectrum (not shown) contained an H-1/C-1 region with a signal at δ 4.43/103.6, arising from a β-pyranosyl structure. All other H-1 signals were from δ 5.10 to 5.46, indicating α-anomers. The ^{13}C spectrum of RMP-Sp-Coni differed from that of the glycoprotein from mycelial RMP-Sp (Fig. 2B) (Barreto-Bergter et al., 2008), but with some signals in common. The structural complexity of RMP-Sp-Coni was shown by its COSY spectrum, which had H-6/H-5 correlations of Rha*p* and 2MeRha units, with signals at δ 1.17/3.61, 1.22/3.70, 1.25/4.03, and 1.27/3.79 (Fig. 2C), arising from four different environments. That of RMP-Sp from mycelium contained three correlations (Barreto-Bergter et al., 2008).

3.3. Analysis of mixture of oligosaccharide epitopes formed on β-elimination of RMP-Sp-Coni

In order to determine structural sequences in RMP-Sp-Coni and some of their structures, it was subjected reductive β-elimination with aqueous $NaBH_4$–NaOH at 25 °C, which liberated carbohydrate *O*-linked to protein, furnishing nonreducing oligosaccharides (Yen & Ballou, 1974). The solution was neutralized (HOAc) and dialyzed, which allowed retention of polymer, probably *N*-linked, and the passage of nonreducing oligosaccharides.

This mixture was shown by ESI-MS to be a complex mixture (Fig. 3A), with main sodiated molecular ions of up to *m/z* 967 (Rha_3-Hex_2Hex-ol) and 981 (2MeRhaRha_2Hex_2Hex-ol), the latter not being formed from mycelia (Fig. 3B; Barreto-Bergter et al., 2008). MS–MS of the *m/z* 967 ion (Fig. 3C) showed removal of *m/z* 146 of Rha and 162 of Hex terminal units from a branched structure. As can be seen, successive fragments showed removal of other units to form a Hex_2Hex-ol core (*m/z* 529). MS–MS of the *m/z* 981 ion indicated removal of a 2MeRha capping group with *m/z* 160 to give a fragment with *m/z* 821 (Fig. 3D). Fig. 3B and C shows sequential removal of the monosaccharide units, starting from the capping groups.

The β-eliminated mixture was treated with cationic exchange resin to remove any compounds containing free amino groups.