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A novel polysaccharide secreted by *pal/rim* mutants of the phytopathogen fungus *Ustilago maydis*

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ABSTRACT

Linear (1 → 3)-β-D-glucans and side-chain-branched β-D-glucans are major constituents of capsular materials, with roles in bacterial aggregation, virulence and carbohydrate storage; whereas branched (1 → 3),(1 → 6)-β-D-glucans constitute the most abundant components in the cell walls of fungi. In the present manuscript we describe the chemical characterization of a linear β-D-glucan secreted by mutants of the Basidiomycota biotrophic fungus *Ustilago maydis* affected in the Pal/Rim pathway involved in pH responses, that in contrast is not made by the wild type strain, at least in measurable amounts. The polysaccharide was obtained from the culture medium of a *Δrim13::Cbx* mutant of the fungus by ethanol precipitation, and analyzed using FT-IR spectroscopy, NMR spectroscopy in solid and liquid states, HPAEC-PAD chromatography, thin layer chromatography, enzymatic digestion and immunodetection. Our data revealed that, according to its characteristics, the polysaccharide is a (1 → 3)-β-D-glucan, and that the mutants used represent a relevant resource of this polysaccharide with wide applications.

1. Introduction

β-D-glucans made of glucose units bound by different linkages, with variable physical properties, and widely distinct roles, constitute an important source of polysaccharides in fungi. Accordingly, they may constitute intracellular reserve material, important structural component of the cell wall, or mucilaginous material secreted to the medium; but as a rule they are made of branched (1 → 3),(1 → 6)-β-D-glucans (for a review see Ruiz-Herrera, 1991), although the extent of the side chain substitutions can vary considerably (Schmid et al., 2001). For example, many of these polysaccharides including schizophyllan (also named sizofiran) from *Schizophyllum commune*, and scleroglucan from *Sclerotium glucanicum* both have a (1 → 3)-β-D-linked backbone, with an average of one (1 → 6)-β-D-glucose substitution every three backbone residues (Johnson et al., 1963; Kikumoto & Kimura, 1971); whereas on the other hand the cell wall of fungi and yeasts contains highly branched (1 → 3),(1 → 6)-β-D-glucans (Fleet & Manners, 1976; Manners, Mason, & Patterson, 1973). Many other fungal β-D-glucans have been described, but their detailed structure and branching frequencies are still mostly unclear (Seviour, Stasinopoulos, & Auer, 1992). Interestingly the only unbranched (1 → 3)-β-D-glucan was described to be synthesized under artificial conditions by *Saccharomyces cerevisiae* protoplasts (Kopecka & Kreger, 1986), or cell-free extracts (Larriba, Morales, & Ruiz-Herrera, 1981).

As occurs with other polysaccharides, (1 → 3)-β-D-glucans present a wide range of potential applications in various industries and notably in agronomy, cosmetics, therapeutic aspects, foods and others. In the food industry, beside classical applications of polysaccharides as thickening agents, (1 → 3)-β-D-glucans have an increasing interest in the areas of edible films, thus numerous patented applications have been directed to obtain capsules or "tablet-like" ingredients which can deliver their content further on after ingestion or during cooking, as food for domestic animals, and as low calorie supplement (Laroche & Michaud, 2007). From a biological point of view, it has been recognized for a long time that (1 → 3)-β-D-glucans belong to the group of biological response modifiers (BRM), that do not have direct cytotoxic activities, but are able to boost the natural defense mechanisms of the host (Williams et al., 1991). Their benefits to health include markedly stimulating the immune system from attack by pathogenic microbes and from harmful effects of environmental toxins and carcinogens (Brown & Gordon, 2005; Vetvicka & Yvin, 2004). In animal experiments, β-D-glucans have shown different activities against sarcomas, mammary cancer, some chemically induced cancers, adenocarcinoma, colon cancer and some leukemias (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1987; Jeannin et al., 1988). It has also been reported that they may reduce blood glucose concentrations after eating, possibly by delaying stomach emptying so that dietary glucose is

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absorbed more gradually (Lo, Tsai, Wasser, Yang, & Huang, 2006), they also appear to be effective in lowering blood cholesterol concentrations (Nicolosi et al., 1999), and control blood pressure (Tam, Yip, Fung, & Chang, 1986), as well as in helping patient recovery from chemotherapy and radiotherapy (Patchen & MacVittie, 1986).

Ustilago maydis is a Basidiomycota biotrophic pathogen fungus responsible for common smut in maize (*Zea mays* L.) and teozintle (*Z. mays* ssp. parviglumis). Although not an obligate parasite, the completion of the *U. maydis* life cycle requires invasion of the host (Christensen, 1963). The fungus displays an alternation of two morphologies during its life cycle: a yeast saprophytic stage and a virulent mycelial form (Martínez-Espinoza, García-Pedrajas, & Gold, 2002). This dimorphic transition can be reproduced *in vitro* by control of the external pH (Ruiz-Herrera, León, Guevara-Olvera, & Carabez-Trejo, 1995). In fungi, a pathway indistinctly named Pal or Rim (neutrally we use the term Pal/Rim), is known to be involved in the adaptation to changes in pH (reviewed by Arst & Peñalva, 2003; Peñalva & Arst, 2002; Peñalva, Tilburn, Bignell, & Arst, 2008). These results led us to analyze the mechanism that involved the Pal/Rim pathway in *U. maydis* dimorphic transition through the isolation of mutants in this pathway. Among the phenotypic characteristic in *ΔpacC/rim101*and *pal/rim* mutants was the secretion of high amounts of a viscous material to the medium that was tentatively identified as a polysaccharide, which was completely absent in the wild type strains analyzed. That this phenotype was due to mutation of the corresponding genes was confirmed by reintroducing the wild type copy (Aréchiga-Carvajal & Ruiz-Herrera, 2005; Cervantes-Chavéz, Ortiz-Castellanos, Tejeda-Sartorius, Gold, & Ruiz-Herrera, 2010). In the present communication we describe the characteristics and identification of the polysaccharide as a novel unbranched (1 → 3)-β-D-glucan.

2. Materials and methods

2.1. Organism

The *U. maydis* strain used in this study was AC401 (*a1b1Δrim13::Cbx*). The strain was maintained at −70 °C in 50% (v/v) glycerol. *U. maydis* cells were transferred to liquid complex or minimal media (CM or MM; Holliday, 1961) supplemented with 10 μM carboxin as selective agent, and shaken (180 rpm) at 28 °C for 40 h.

2.2. Purification of the polysaccharide

Liquid MM (100 ml) adjusted to pH 9 was inoculated with the corresponding strains at an OD at 600 nm (OD_{600}) of 0.01. The cultures were incubated for 40 h at 28 °C under shaking conditions. After cell elimination by centrifugation the polysaccharide was recovered by different protocols: precipitated with cold absolute ethanol, dried and weighed according to Aréchiga-Carvajal & Ruiz-Herrera (2005), or concentrated in a Rotavapor to one tenth of the initial volume and freeze dried or not, the later being kept at 4 °C.

2.3. Acid hydrolysis

The polysaccharide was hydrolyzed with 2 N HCl for 8 h under vacuum at 100 °C. Samples were recovered and placed in a sealed container over NaOH lentils, and HCl was evaporated under vacuum at room temperature. The dry material was resuspended in 0.005 N NaOH to pH 7.

2.4. Polysaccharide digestion with (1 → 3)-β-D-glucanase

The freeze dried polysaccharide was treated with 1 ml (0.4 mg per mg of polysaccharide) of Lyticase (Sigma) in 50 mM phosphate buffer pH 7, and incubated at 25 °C. After 5, 8 and 10 h of digestion, neutral sugars were determined with anthrone (Dimler, Schaeffer, Wise, & Rist, 1952).

2.5. Dionex chromatography

Simples of the polysaccharide hydrolyzed with HCl or (1 → 3)-β-D-glucanase were analyzed by HPAE chromatography in a Dionex instrument with a CarboPac PA-100 column at a temperature of 25 °C. The solvent system was: eluent A, 0.15 M NaOH, and eluent B, 0.5 M sodium acetate containing 0.15 M NaOH in a linear gradient, at a rate of 0.8 ml/min.

2.6. Thin layer chromatography (TLC)

Acid hydrolyzed samples were applied to thin layer plates of silica and developed with a mixture of isopropanol–chloroform–water (14:8:2, v/v) for 2–3 h, and revealed with 3% triphenyltetrazolium chloride (TTC) in methanol and 6 N NaOH 1:1 (v/v) at 95 °C.

2.7. Immunodetection of polysaccharide

We used a solution of the polysaccharide, and as control a hydrolyzate of cell walls of *Candida albicans* obtained with Zymolyase (Sigma). Dilutions of 1, 2, 4, 8, 16, 32, 64 and 128 of 100 μl were applied under vacuum on a nitrocellulose membrane. The samples were revealed as described by Kurien and Scofield (2006), with a specific polyclonal anti-(1 → 6)-β-D-glucan (diluted 1/1000) (courtesy of R. Sentandreu, University of Valencia, Spain) and as second antibody goat anti-rabbit bound to peroxydase (1/10,000 dilution).

2.8. FT-IR analysis

Spectrophotometric analysis of the dried polysaccharide as KBr pellets was recorded in a Perkin-Elmer spectrophotometer model FT 1600. The spectrum was determined from 4000 to 400 cm^{-1}.

2.9. NMR spectroscopy

We performed ^{1}H NMR in liquid state with polysaccharide solutions dissolved in D_2O. ^{13}C NMR in the solid state was performed with the dried polysaccharide. For both analysis a Varian 300 MHz spectrometer in a Fourier transformed mode, using 32 scans, at a temperature of 30 °C with an acquisition time of 3.5 s and a pulse of 90° was used.

3. Results and discussion

The *U. maydis pal/rim* mutant used secreted to the culture medium a material, which tentatively was considered to be a polysaccharide. As a first approach to the identification of such material, the ethanol-precipitated material was analyzed by the anthrone method, obtaining a positive result. As a second approach we analyzed hydrolyzates of the material by thin layer chromatography (TLC). As shown in Fig. 1, the hydrolyzed material gave a single spot that matched with the glucose standard.

In order to determine the type of bonding of glucose in the polysaccharide, and taking into considerations the description of (1 → 6)-β-D-glucans secreted by Basidiomycota species (Prokop, Rapp, & Wagner, 1992), a test was performed with an antibody that recognizes (1 → 6)-β-D-glucans (see Section 2). There was no sign of reactivity of the polysaccharide with the antibody at any of the dilutions used. By contrast, the hydrolyzate of cell walls of *C. albicans* used as positive control showed a strong reaction at all

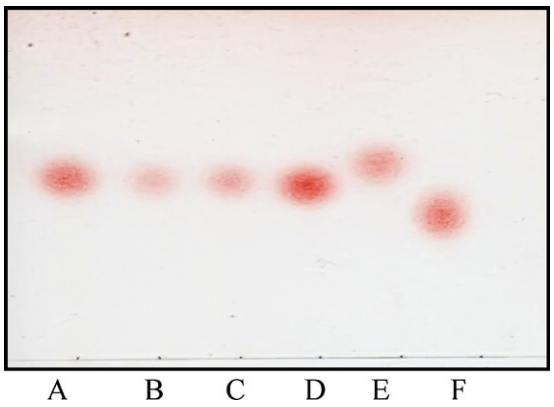

Fig. 1. Thin layer chromatography of three different purified samples of the hydrolyzed polysaccharide. The chromatogram was developed with isopropanol–chloroform–water (14:8:2, v/v) and the spots were visualized by treatment with 3% triphenyltetrazolium chloride (TTC) in methanol and 6 N NaOH 1:1 (v/v) at 95 °C. (A) Polysaccharide hydrolyzed with HCl with an Rf value of 0.43; (B) polysaccharide digested 10 h with Lyticase followed by HCl hydrolysis with an Rf value of 0.43; (C) polysaccharide digested with Lyticase for 10 h with an Rf value of 0.43; (D) glucose with an Rf value of 0.42; (E) mannose with an Rf value of 0.48; (F) galactose with an Rf value of 0.35.

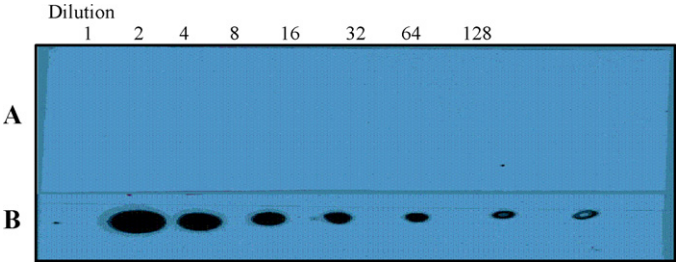

Fig. 2. Immunodetection of the polysaccharide. An anti-β-(1,6)-glucan (1/1000) was used. (A) Samples of polysaccharide at different dilutions and (B) positive control of a cell wall hydrolyzate of *C. albicans* with Zymolyase.

dilutions used (Fig. 2). This result strongly indicates that the *U. maydis* secreted polysaccharide does not contain (1 → 6)-β-D-bonded glucose.

In a further experiment the polysaccharide was digested with a (1 → 3)-β-D-glucanase ("Lyticase" Sigma). After 5, 8 or 10 h digestion, 70%, 89.4%, and 96% of the polysaccharide was digested as determined with anthrone. When the products of the 8 h of enzymatic or 10 h acid hydrolysis were subjected to anion-exchange chromatography (HPAEC), the only product detected showed a retention time of 5.53 min that corresponded to glucose (Fig. 3).

In order to obtain more solid evidence of the type of glucose linkage in the polysaccharide, we proceeded to its analysis by infrared spectroscopy. The infrared spectrum of the polysaccharide showed

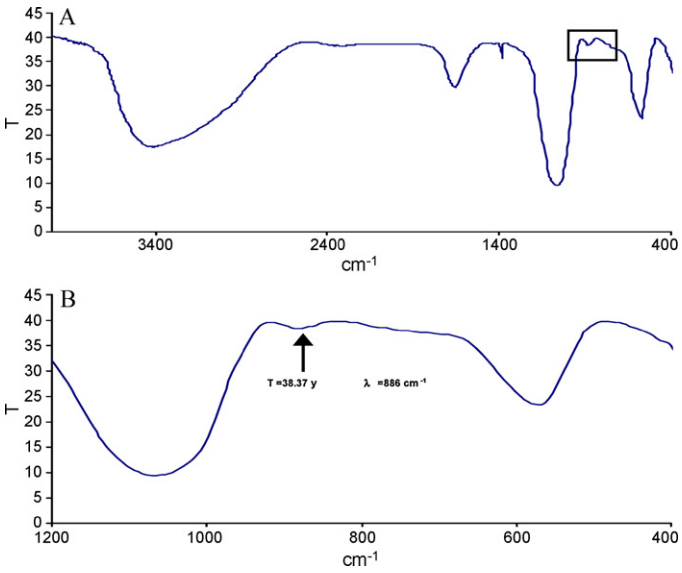

Fig. 4. Fourier-transform infrared (FT-IR) spectroscopy. (A) Spectrum of the purified polysaccharide and (B) amplification of the section in the box.

the presence of a band at 886 cm^{-1} characteristic of a glycosidic β type bond (Fig. 4). On the other hand, there was no band at 850 cm^{-1} that is characteristic for an α type glycosidic bond.

Further evidence that the secreted polysaccharide by the *pal/rim* mutants of *U. maydis* was an unbranched (1 → 3)-β-D-glucan, came from the results obtained by NMR. It has been reported that the anomeric signals for (1 → 3)-β-D-glucan in a ^{1}H NMR spectrum in the liquid state are between 4.7–4.8 and 3.0–3.2 ppm, whereas those of (1 → 6)-β-D-glucan appear between 4.4 and 4.6 ppm (Sugawara, Takahashi, Osumi, & Ohno, 2004). The ^{1}H NMR spectrum of the polysaccharide under study showed a double peak located at 4.7 ppm and a triple peak located at 3.2 ppm as shown in Fig. 5, indicating the presence of residues linked by a glucopiranosyl (1 → 3)-β-D-bond. In the same line, analysis of our polysaccharide by ^{13}C NMR in solid state showed that the chemical shifts of carbon atoms (C) were 103.76 ppm for C-1, 74.45 ppm for C-2 and C-5, 86.17 ppm for C-3, and 68.59 ppm for C-4, 68.33 C-6 (Fig. 5). These results agree with the reported anomeric carbon (C-1) of a glycoside which appears between 100 and 110 ppm, and the C-2, C-3, C-4, C-5 and C-6 glycosidic ring appearing between 60 and 80 ppm (Gonzaga, Ricardo, Heatley, & Soares, 2005). The values here obtained are very similar to those characteristic of (1 → 3)-β-D-glucans and finally the spectrum is a typical one of dry samples of (1 → 3)-β-D-glucans. Based on these results we can conclude that the polysaccharide secreted by the mutant *pal/rim* of *U. maydis* is a (1 → 3)-β-D-glucan, without (1 → 6)-β-D-branches (Table 1).

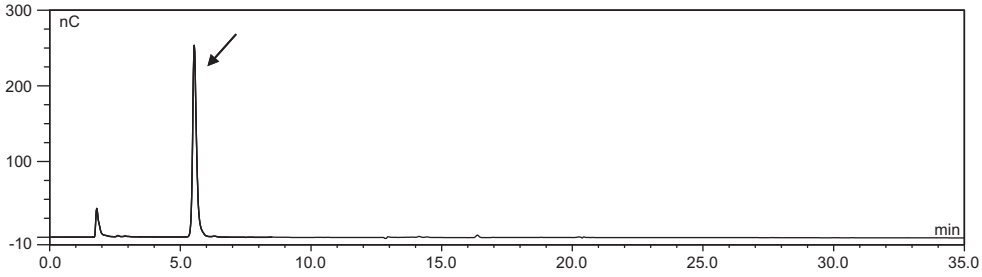

Fig. 3. HPAE chromatogram of the product obtained after 10 h of digestion of the polysaccharide with β-(1,3)-glucanase (Lyticase). The peak obtained (arrow), corresponded to the glucose standard.

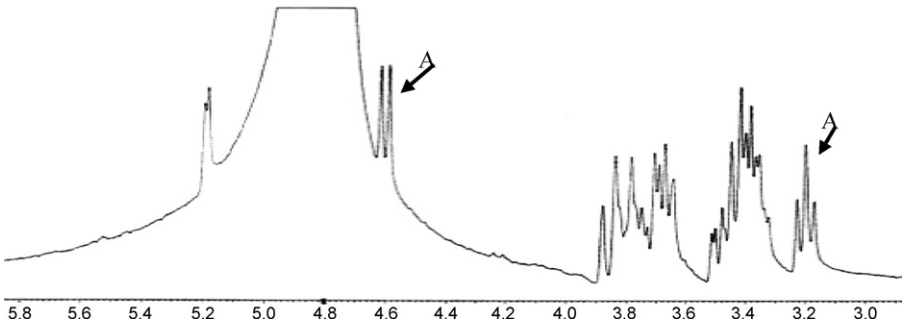

Fig. 5. ^{1}H NMR spectrum of the purified polysaccharide dissolved in D_2O. The arrows with the letter A correspond to the peaks of β-(1,3)-glucan.

Table 1
^{13}C NMR chemical shift data (ppm) of the polysaccharide.

Carbon number	ppm
C-1	103.755
C-2	74.447
C-3	86.170
C-4	68.585
C-5	74.447
C-6	62.333

It must be stressed that fungal cells do not secrete to the medium unbranched (1 → 3)-β-D-glucans in measurable amounts. The secretion of the polysaccharide by the *pac/rim11*, and *pal/rim* mutants is probably due to an alteration in the cell wall integrity mechanisms (Aréchiga-Carvajal & Ruiz-Herrera, 2005). To assess the secretion of polysaccharide in the process of growing the *ΔpacC/RIM101*, mutant and wild type strains in minimal medium at different pH values (Cervantes-Chavéz et al., 2010), and precipitated the polysaccharide secreted with cold ethanol. There was no polysaccharide secreted appreciable in several trials by the wild type and revertant strains. Accordingly the polysaccharide here described, appears to be a unique and peculiar product that is secreted as a side effect for the mutation.

Also, considering that many β-D-glucans appear to have potential for treating several diseases and for others important applications (Chen & Seviour, 2007) and that the unbranched (1 → 3)-β-D-glucan secreted by this mutant of *U. maydis* in the Pal/Rim pathway reaches a value 0.5 mg of polysaccharide for milliliter of culture medium (a value that possibly can be increased by controlling different culture conditions), this strain appears to be a good source for the high production of an unbranched (1 → 3)-β-D-glucan with different health and industrial applications. Also, of relevant importance is the fact that this polysaccharide is produced by an edible fungus, being therefore safe for animals and the human being.

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