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Influence of exo β-D-galactofuranosidase inhibitors in cultures of *Penicillium fellutanum* and modifications in hyphal cell structure

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Abstract

We have examined β-D-galactofuranosidase production by *Penicillium fellutanum* in the presence of D-galactono-1,4-lactone or 4-aminophenyl 1-thio-β-D-galactofuranoside, two potent in vitro inhibitors of the enzyme. Activity of the enzyme in the culture filtrate was increased by 35% when glucose was replaced by D-galactose as the carbon source, and the activity diminished 80% of the control value when the inhibitors were added. Significant alterations of the culture were observed: (a) the medium became increasingly opalescent due to the secretion of a protein aggregate (PA) which contained 15% neutral sugar, mainly ribose; (b) the peptidophosphogalactomannan (pPGM) containing galactofuranose, normally produced by *P. fellutanum*, could not be obtained from the culture medium in the presence of the inhibitors; (c) the content of galactofuranose in the cell wall was significantly decreased in the presence of D-galactono-1,4-lactone. The influence on the mycelia growth was investigated by light microscopy (LM) and transmission electron microscopy (TEM) showing important alterations.

Keywords: *Penicillium fellutanum*; Galactofuranose; Galactofuranosidase; Inhibitors

1. Introduction

The glycobiology of D-galactofuranose is a topic of increasing interest, as the sugar in this configuration is a component of pathogenic bacteria, protozoa and fungi.[1] Particularly important are the arabinogalactan of mycobacteria,[2] the lipopolysaccharide of *Klebsiella pneumoniae*,[3] and the glycoinositolphospholipids of *Trypanosoma cruzi*[4] and *Leishmania* species.[5] The synthesis of cell-wall core galactofuranan is essential for growth of mycobacteria.[6] The absence of galactofuranose in mammal glycoconjugates suggests that the enzymes involved in their metabolism in infectious microorganisms are good targets for chemotherapy. However, there are few reports related to these enzymes. A specific exo β-D-galactofuranosidase was first purified from the culture medium of *Penicillium fellutanum*[7] and later described in *Helminthosporium sacchari*[8] and *Penicillium* and *Aspergillus* species.[9]

Although the enzyme was first described[7] in 1977, its structure and catalytic mechanism are not known. Interest in the enzymes involved in the metabolism of D-galactofuranose has increased in recent years. In *Escherichia coli*[10] and *Klebsiella sp.*,[11] it was shown that the precursor UDP-galactofuranose is formed by a unique mutase action on UDP-galactopyranose. The UDP-galactopyranose mutase was very recently crystallized and its structure elucidated.[12] The gene which encodes the novel galactofuranosyl transferase of *M. tuberculosis* has been described.[13]

In previous work, we developed the synthesis of 1-thio-β-D-galactofuranosides and showed that 4-aminophenyl 1-thio-β-D-galactofuranoside and D-galactono-1,4-lactone are good in vitro inhibitors of the exo β-D-galactofuranosidase purified from *P. fellutanum*.[14]

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An affinity chromatography system for purification of the enzyme using the thiogalactofuranoside as ligand and D-galactono-1,4-lactone as eluent was described.[15] In a recent publication, it was shown that D-galactono-1,4-lactone is also a good inhibitor for the binding of a galactofuranose containing glycoconjugate in *Aspergillus niger* with a specific monoclonal antibody.[16]

P. fellutanum also secretes a peptidophosphogalactomannan (pPGM) which contains 5-*O*-β-D-galactofuranosyl residues.[17,18] The pPGM may provide sufficient quantities of carbohydrates, amino acids, phosphate, and choline to prevent starvation during nutritionally stressful conditions.[19] *P. fellutanum*, easily cultivated and secreting good amounts of the enzyme, is a good model for studying the in vivo influence of inhibitors of β-D-galactofuranosidase. It has been described that 2-deoxy-D-glucose inhibits extracellular galactofuranosidase formation, although, it has no effect on the synthesis of the pPGM.[20] We now report the effect of structural analogues of the substrate, namely, D-galactono-1,4-lactone and 4-aminophenyl 1-thio-β-D-galactofuranoside, on the enzyme activity and on secretion of the pPGM. Alterations in the mycelia were observed by light microscopy (LM) and transmission electron microscopy (TEM) when the inhibitor was present in the medium.

2. Results and discussion

When *P. fellutanum* is cultured in the modified Raulin–Thom medium,[21] the sequence of events is: (a) the exocellular pPGM is released from the organism; (b) the modification of the medium, with depletion of the monosaccharide carbon source, causes the release of exo β-D-galactofuranosidase, which degrades the galactan region of the secreted pPGM providing the carbohydrate for survival of the *Penicillium*.[7,22] Production of glycosidases in many microorganisms is susceptible to catabolic suppression.[23] However, in our case, activity of the enzyme in the culture filtrate was increased by 35% at the maximum point, when D-glucose was replaced by D-galactose as the carbon source, and it was accelerated by using a concentration ten times lower of D-glucose or D-galactose (0.5%) with no significant decrease in the activity. A maximum of β-D-galactofuranosidase activity is attained in the 8-day culture (Fig. 1). On the other hand, the medium contained less proteins and polysaccharides, thus facilitating analysis of the enzyme and its purification (data not shown). The enzyme activity dropped abruptly after this time and almost disappeared after 12 days.

Addition of 0.4 mM D-galactono-1,4-lactone or 4-aminophenyl 1-thio-β-D-galactofuranoside caused a dramatic alteration of the culture medium, which became consistently opalescent after 2–3 days from addition of the inhibitor. In contrast, after filtration of the mycelium, the control culture medium remained clear. An increase in A_{600} nm of the filtered cultures (Fig. 2) showed the extent of increase in opalescence. β-D-Galactofuranosidase activity was determined in the clear medium after centrifugation and dialysis to re-

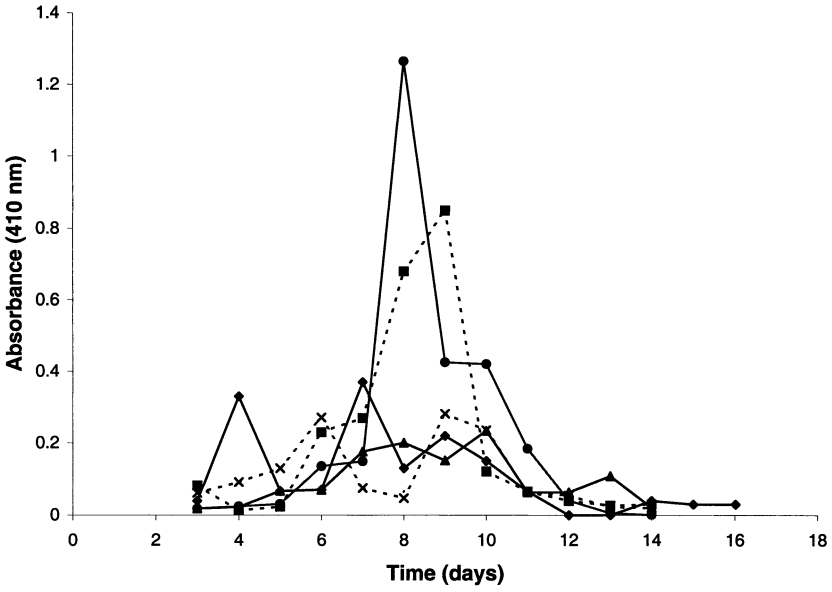

Fig. 1. Activity of exo β-D-galactofuranosidase in cultures of *P. fellutanum* with galactose or glucose as carbon source, supplemented or not with 1 mM D-galactono-1,4-lactone. Activity is expressed by the absorption at 410 nm of the 4-nitrophenolate anion,[14] and determined as described in Section 3. (●-●) 0.5% D-galactose; (■-■) 0.5% D-glucose; (▲-▲) 0.5% Gal + 1 mM D-galactono-1,4-lactone; (× - ×) 0.5% Glc + 1 mM D-galactono-1,4-lactone; (◆-◆) 0.5% Gal + 0.4 mM 4-aminophenyl 1-thio-β-D-galactofuranoside. All activities were reproducible within 5%.

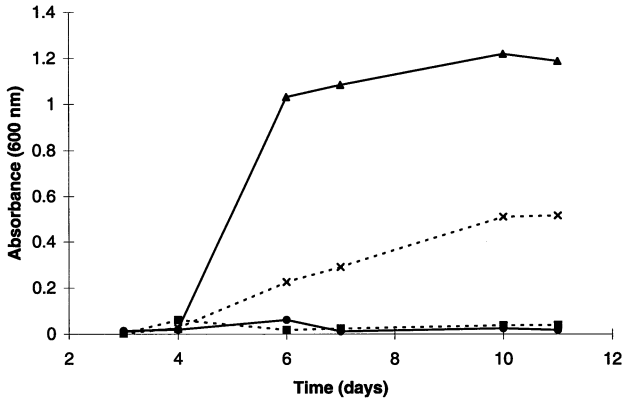

Fig. 2. Influence of D-galactono-1,4-lactone on the appearance of a protein aggregate in the culture medium of *P. fellutanum*. (●-●) 0.5% galactose ; (■-■) 0.5% D-glucose; (▲-▲) 0.5% D-Gal + 1 mM D-galactono-1,4-lactone ; (× - ×) 0.5% Glc + 1 mM D-galactono-1,4-lactone. All activities were reproducible within 5%.

move the inhibitors. The control cultures were subjected to the same treatments. Growing in the presence of the inhibitors reduced the activity of β-D-galactofuranosidase by 80% of the maximum activity in the control cultures (Fig. 1). The same effect was obtained using D-galactose or D-glucose as the carbon source (not shown).

Aldonolactones, mainly D-glucono-1,5-lactone, have been used as inhibitors of glycopyranoside hydrolases.[24] It is a competitive inhibition due to the similarity between the 1,5-lactone conformation with that of the glycosyloxocarbonium ion intermediate. For that reason, the isomeric 1,4-lactones are poor inhibitors of the glycopyranosidases. It was suggested that the inhibition of β-D-galactopyranosidase by D-galactono-1,4-lactone was in fact caused by a small proportion of the 1,5-lactone formed in solution. In our case, D-galactono-1,4-lactone has the same ring size as the β-D-galactofuranoside substrates, and we previously proved that the lactone as well as the thiogalactofuranoside, were good inhibitors, in vitro, of the exo β-D-galactofuranosidase.[14] Accordingly, it was very recently described that D-galactono-1,4-lactone was a better inhibitor than the galactopyranosides for the inhibition of binding of galactofuranose epitopes with a monoclonal antibody.[16]

Whereas the pPGM could be precipitated by addition of ethanol to the filtrates of the control cultures of *P. fellutanum*, no pPGM could be obtained from the cultures with the inhibitors after separation of the opalescence by centrifugation. The percentage of galactofuranose with respect to total neutral sugar determined in a cell-wall fraction was significantly decreased in the presence of the lactone (Table 1). Thus, the inhibitor may be also affecting the enzymes responsible for the incorporation of β-D-galactofuranose into the macromolecules. Galactofuranosyl transferases of *P. fellutanum* have not been described.

The other striking feature was the release of an insoluble product which caused the opalescence in cultures with the inhibitors. The product was insoluble in water, even by heating at 80 °C, in organic solvents or dimethyl sulfoxide. It could be dissolved in 2 M NaOH after stirring for 24 h at 25 °C. Protein (40% by Lowry) is the main component and several bands were shown by Coomassie Blue in SDS-PAGE (Fig. 3, lane 1). The main band was shown at 40 kDa and also bands at 105, 70, and 50 kDa were evident. A broad zone at 35–50 kDa was also detected with PAS reagent for sugars (not

Table 1
Analysis of neutral sugars in cell walls and in the protein aggregate in *P. fellutanum*

Monosaccharides [a]	Cell wall and membranes		Secreted protein aggregate
	Control cultures	Cultures with 1 mM D-galactono-1,4-lactone	
Rhamnose		3.7	25.1
Fucose		3.8	
Ribose	11.5	11.7	53.7
Arabinose		4.6	
Xylose		3.9	
Mannose	11.0	11.6	4.5
Galactose [b]	22.7	15.8	3.3
Galactofuranose [c]	21.5	11.2	traces
Glucose	54.8	44.9	13.4

[a] Abundance of sugars is given in molar proportions.
[b] Total galactose.
[c] The percentage of galactose originally present as furanose was determined in a partial acid hydrolysate (20 mM TFA, 2 h, 100 °C).

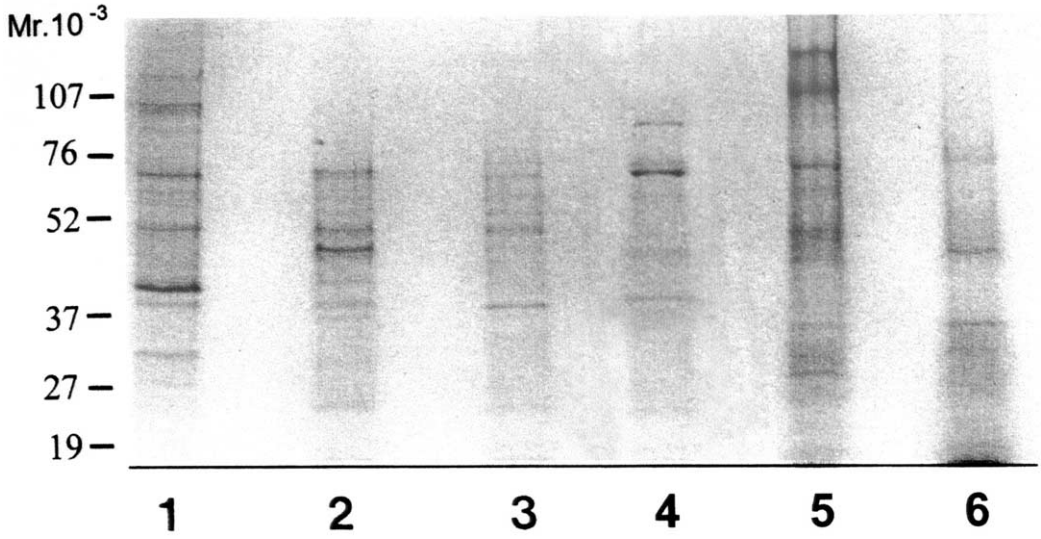

Fig. 3. Analysis of the protein aggregate from the medium of *P. fellutanum* by SDS-PAGE (10% polyacrylamide) stained with Coomassie Blue. Lanes: (1) protein aggregate (PA) from cultures with 1 mM D-galactono-1,4-lactone; (2) supernatant after separation of PA; (3) control culture filtrate (without inhibitor); (4) β-D-galactofuranosidase (70 kDa) partially purified by affinity column chromatography;[15] (5 and 6) same as (1) and (2) but without 2-mercaptoethanol.

shown). A sample of partially purified β-D-galactofuranosidase[15] was analyzed for comparison (Fig. 3, lane 4). It can be seen that a protein with a similar M_W is part of the PA, but it is not the main protein. An aliquot of the control culture medium was analyzed. A different profile for this sample (Fig. 3, lane 3), and also for the supernatant remaining after separation of the protein aggregate (PA) from the cultures with inhibitors (Fig. 3, lane 2) was obtained. In the absence of 2-mercaptoethanol, larger proteins were evident in PA (Fig. 3, lane 5) indicating that intermolecular disulfide bonds could also contribute to the stability of PA.

The total amount of neutral sugars in PA was 15%. Values of 21.3 and 25.7% were determined in cell wall fractions from cultures with or without the inhibitor. The monosaccharides were analyzed by GLC as alditol acetates after acid hydrolysis with 2 M TFA and identified by their retention times and cochromatography with authentic standards. They were confirmed by GLC–mass spectrometry. The composition is shown in Table 1. Data for cell-wall preparations with or without the inhibitor are also given. It can be seen that ribose is the main component in the PA, with minor amounts of galactose and mannose, indicating that an insoluble form of pPGM is not part of it. An intriguing feature is the presence of ribose and rhamnose in the aggregate. None of these monosaccharides are present in the pPGM.[18] ADP-ribosylation of proteins, with a variety of effects on their activities, has been described.[25] The nature of the ribose modification in the PA was not investigated in *P. fellutanum*. Protein aggregates have been observed as inclusion bodies in bacteria and attributed to defective folding of newly synthesized polypeptide chains. Inter-chain interactions result in the formation of aggregates, as opposed to intrachain interactions in the native proteins.[26] In our case, the inhibitor, interacting with the active site of the enzyme may alter its folding, promoting aggregation with other proteins, and yielding a very insoluble product.

The striking alterations caused in the cultures of *P. fellutanum* by the inhibitors, prompted us to perform light (LM) and transmission electron microscopy (TEM) studies. Mycelia from control cultures with D-glucose or D-galactose as the carbon source (Fig. 4(A, B)) were compared by LM with mycelia grown in the presence of inhibitors (Fig. 4(C, D)). The mycelia aggregate in pellets, which are more compact and hard in the cultures with the inhibitors. In controls (Fig. 4(A, B)), the diameter of the hyphae was uniform attaining approximately 3 μm after 6 days of growth. In the presence of D-galactono-1,4-lactone after the same time, the diameter of the cell hyphae was variable (Fig. 4(C, D_2)), especially in the D-galactose-containing medium (arrow, Fig. 4(D_2)). The presence of small unicellular forms (< 1 μm diameter), shown with arrow heads in Fig. 4(C and D_1), characterizes the cultures with the inhibitor.

Ultrastructural observations (Fig. 5) were made on hyphae from the peripheral regions of the pellet in control cultures and in cultures with the β-D-galactofuranosidase inhibitors. The same alterations were observed with both sugars (glucose or galactose) in the presence of anyone of the inhibitors, however, we only show the action of D-galactono-1,4-lactone in cultures with D-galactose as the carbon source, as more replica-

tions of these experiments were done. The 4-aminophenyl 1-thio-β-D-galactofuranoside, has to be specially synthesized in the laboratory.[14] In all control preparations well defined organella, such as, mitochondria, endoplasmic reticulum, ribosomes, and vacuoles are distinguished (Fig. 5(A)). In contrast, in cultures of the same age, but containing the inhibitor (Fig. 5(B–F)) a variable pattern was obtained. In some cases, the center of the cell was occupied by a large vacuole (V) with an electron-dense content and inner vesicles (Ve) sometimes projecting towards the cell membrane were observed (Fig. 5(C)). In other preparations several translucent vacuoles were evident (Fig. 5(B and E)). Small cells are shown close to a hypha in Fig. 5(D). The striking feature was the appearance of a dense sheath surrounding the cell wall before separation of the PA into the medium (Fig. 5(B and F)). It could be seen that the sheath became electronically dense after the double staining. This material was shown in the cytoplasm

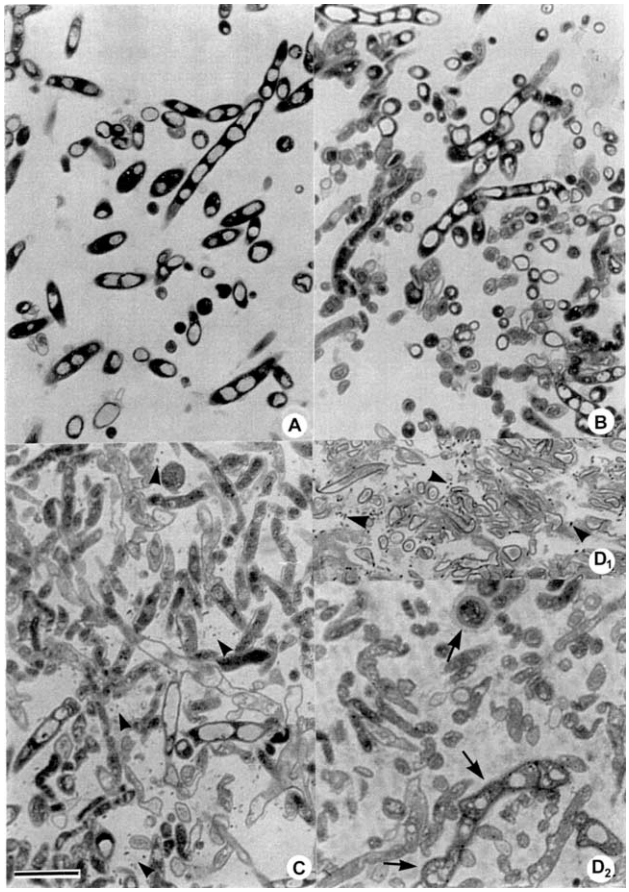

Fig. 4. Light microscopy of mycelia from liquid cultures of *P. fellutanum* (A–D), 8-day cultures were used in all cases. Control cultures with D-galactose (A) or D-glucose (B) as the carbon source. Cultures with glucose plus D-galactono-1,4-lactone as inhibitor (C) and D-galactose plus inhibitor (D_1, D_2) show irregular hyphae (arrow) and small forms (arrow head). Bar (all panels) = 20 μm.

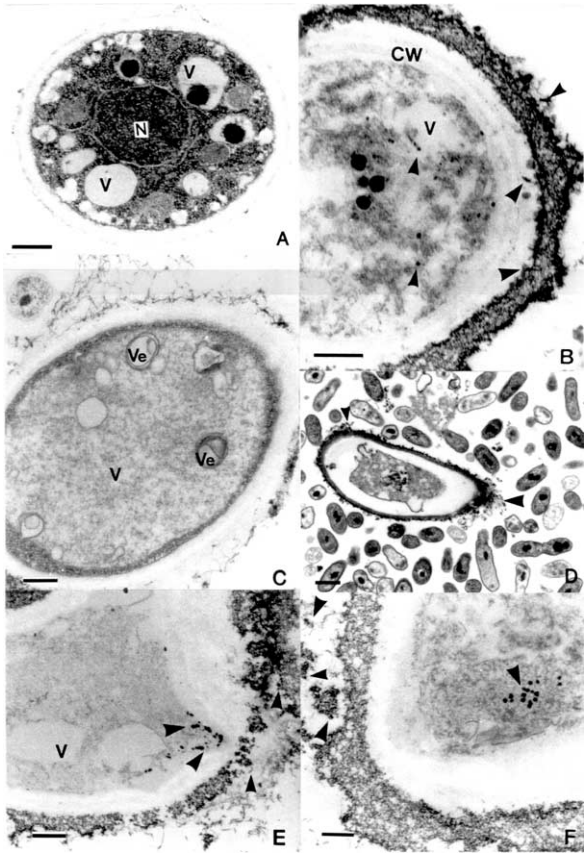

Fig. 5. Transmission electron micrograph (TEM) of a hypha from a control culture (A) with D-galactose as the carbon source; mitochondria, rough endoplasmic reticulum, nucleus (N) and vacuoles with electronically-dense material were seen (V). TEM of hyphae from cultures grown with the inhibitor: (B–F). An electron-dense sheath was present in all the cultures containing the inhibitor (B–F). In (C), a dense vacuole occupies the center of the cell. The vacuole contains several vesicles (Ve) which were originated from the peripheral cytoplasm. In (B) and (E), several electronically-translucent vacuoles were observed. Unicellular forms appear in the cultures with inhibitor (C, D). Arrow heads show the protein aggregates. For all panels, except (D), bars = 0.5 μm. For (D), bar = 1 μm.

(arrow head) and was transported through the cell wall to the external space, without any membrane structure (Fig. 5(B, E, F)). The ultrastructural changes suggest that modification of the polymers of the cell wall may induce perturbations in the cross linking events, causing the secretion to the culture medium of the protein aggregate.

In conclusion, we have shown that inhibitors of β-D-galactofuranosidase affect the secretion of the enzyme, the production of galactofuranose containing glycoconjugates, and strongly disturb the cell structure. The influence of these inhibitors in the biological characteristics of *P. fellutanum* suggests their use for in vivo experiments with pathogenic microorganisms for which galactofuranose play an important role.

3. Experimental

Culture conditions.—An inoculum of *P. fellutanum* Biouge was kindly provided by Dr J.E. Gander (University of Florida, USA) and maintained in Czapek–Dox agar medium.[27] *P. fellutanum* was grown as described previously[21] except that 0.5% D-glucose or D-galactose was used as the carbon source. For inhibition studies, on day 3 the medium was supplemented with 0.4 or 1 mM of D-galactono-1,4-lactone. In some experiments 4-aminophenyl 1-thio-β-D-galactofuranoside (0.4 mM) was used as inhibitor.[14]

Isolation of a protein aggregate from cultures of P. fellutanum *grown in the presence of* D-*galactono-1,4-lactone.*—The mycelium, from a 6-day old culture of *P. fellutanum* grown in the presence of the inhibitor D-galactono-1,4-lactone (0.4 mM) added after 3 days of inoculation, was separated by filtration through Whatman No. 1 filter paper. From the opalescent filtrate, a fine precipitate called protein aggregate (PA) was separated by centrifugation at 8000*g* for 20 min at 10 °C.

Cell-wall preparation.—The mycelium of *P. fellutanum* was ground in a mortar with 2:1 $CHCl_3$–MeOH, sonicated for 10 min and centrifuged for 20 min at 4 °C and 8000*g*. The extraction was repeated three times and the delipidated pellet was dried, suspended in 0.1 M citrate buffer (pH 6), 2 M NaCl, sonicated for 10 min, and stirred for 1 h at rt. The extract was then separated by centrifugation and the treatment was repeated twice. The remaining pellet was washed with water, dried and used for chemical analyses.

Analytical methods.—Protein concentration was determined by the method of Lowry[28] or estimated by spectrometry at 280 nm. Neutral carbohydrate was measured by the phenol–H_2SO_4 method[29] using glucose as standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels[30] under reducing (2-mercaptoethanol) or nonreducing conditions, and stained alternatively with Coomassie Brilliant Blue R-250[31] or Schiff reagent.[30] Monosaccharides were analyzed after acid hydrolysis of the glycoconjugates with 2 M trifluoroacetic acid (TFA) at 105 °C for 3 h. For the analysis of galactofuranose, the sample (1 mg) was hydrolyzed with 20 mM TFA for 2 h at 100 °C. After centrifugation at 5000*g* at rt, the solution was evaporated to dryness, sugars were converted into the alditol acetates and analyzed by gas–liquid chromatography (GLC). Capillary GLC was performed with a Hewlett–Packard 5890 gas chromatograph with nitrogen as the carrier gas and an SP 2340 column (0.20 mm × 30 m, Supelco). The following conditions were used: flow rate, 1.4 mL/min, column temperature 220 °C, injector temperature 230 °C, and detector temperature 250 °C. The monosaccharides were identified by comparison with the retention time of standards and cochromatography. Their nature was confirmed by GLC–MS using a Shimadzu QP-5050 spectrometer at 70 eV and an SP 2330 column; the following conditions were used: initial column temperature, 180 °C; rate, 2 °C/min; final column temperature, 230 °C; injector temperature, 240 °C; detector temperature, 240 °C; and helium, 0.5 mL/min.

*Assay of β-*D-*galactofuranosidase.*—The enzymatic activity was assayed using 4-nitrophenyl β-D-galactofuranoside as substrate,[14] synthesized in our laboratory as previously reported.[32] Briefly, activity was measured using 31 μL of 5 mM 4-nitrophenyl β-D-galactofuranoside, 100 μL of 66 mM acetate buffer, pH 4.0 and the culture filtrate in a final volume of 0.5 mL. After 1.5 h at 37 °C, the reaction was stopped by the addition of 0.25 M Na_2CO_3–0.25 M $NaHCO_3$ (1 mL) and the absorbance at 410 nm was measured.

Light and transmission electron microscopy.—Small blocks of mycelium were fixed for 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 6.8 at 4 °C. Both, fresh unfixed tissues and tissues that had been embedded for TEM were used for light microscopy. The staining procedures[33] included Toluidine Blue O, 8-anilino-1-naphtalene sulfonic acid and Coomassie Brilliant Blue for proteins and periodic acid–Schiff reagent (PAS) for polysaccharides. For TEM, tissues were postfixed in 1% OsO_4 for 1 h, dehydrated in a graded EtOH–acetone series, and embedded in Spurr's resin. Sections were mounted on grids, stained with uranyl acetate followed by lead citrate, and examined by a Turbo Zeiss EM 109 instrument.

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