New Structural Features of the Antigenic Extracellular Polysaccharides of *Penicillium* and *Aspergillus* Species Revealed with Exo- β -D-Galactofuranosidase

ANDREA W. VAN BRUGGEN-VAN DER LUGT,¹ HENRI J. KAMPHUIS,¹ GERHARD A. DE RUITER,¹ PETRA MISCHNICK,² JACQUES H. VAN BOOM,³ AND FRANK M. ROMBOUTS^{1*}

Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703 HD Wageningen,¹ and Department of Organic Chemistry, Gorlaeus Laboratory, 2300 RA Leiden,³ The Netherlands, and Department of Organic Chemistry, University of Hamburg, D-2000 Hamburg 13, Germany²

Received 14 April 1992/Accepted 23 July 1992

To study the structures of the epitopes of the extracellular polysaccharides from *Penicillium* and *Aspergillus* species, an exo- β -D-galactofuranosidase was purified from a commercial crude enzyme preparation from *Trichoderma harzianum*. Analysis of ring size and linkage position of the galactose residues of the extracellular polysaccharide of *Penicillium digitatum*, before and after enzymatic treatment, was determined by the reductive-cleavage technique. In addition to terminal and β (1-5)-linked galactofuranosides, β (1-6)-linked and β (1,5,6)-linked branched galactofuranose residues could be identified. After degradation with the purified exo- β -D-galactofuranosidase, all initial linkages of the galactofuranose residues were still present, but the amount of β (1-5)-linked galactofuranose residues had decreased considerably. Treatment of the extracellular polysaccharides of *Penicillium* and *Aspergillus* species with the purified exo- β -D-galactofuranosidase resulted in complete disappearance of the enzyme-linked immunosorbent assay reactivity of these polysaccharides, using immunoglobulin G antibodies raised against *P. digitatum*. Therefore, with the use of this enzyme, it was proved that the β (1-5)-linked galactofuranosyl residues only are responsible for the antigenicity of the extracellular polysaccharides of *Penicillium* and *Aspergillus* molds. A new structural model for the antigenic galactofuranose side chains of the galactomannan from *P. digitatum* is proposed.

Molds are able to excrete a large variety of polysaccharides, some of them with antigenic properties (22, 29). Several authors studied the chemical structure of polysaccharides isolated from mycelium of mold species belonging to the genera *Penicillium* and *Aspergillus* (3-5). *Penicillium* and *Aspergillus* species are important fungi which are distributed worldwide and are the cause of many cases of food spoilage. Medically, some members are significant because of their ability to cause aspergillosis in humans and the release of mycotoxins. The polysaccharides are constituted mainly of mannose, galactose, and glucose, with minor amounts of protein (3-5). Galactosamine has been found in the polysaccharides of several *Aspergillus* species (2, 13, 27). Extracellular polysaccharides (EPSs) produced by these molds appeared to have similar structures (12, 24).

The EPSs are characterized as galactomannans composed mainly of an α (1-6)- and α (1-2)-linked D-mannopyranosyl backbone and side chains of galactose residues, some of which are β (1-5)-linked D-galactofuranosyl sequences (3-5, 12). The presence of (1-4)-linked galactopyranosyl residues has also been demonstrated (3, 18). No detailed information is available about the glycosidic linkage of glucose.

The antigenicity of these EPSs can be used for detection of these molds in food by using immunoassays (17, 21). Polyclonal immunoglobulin G (IgG) antibodies raised against EPS derived from *Penicillium digitatum* were found to be specific for species of the closely related genera *Penicillium* and *Aspergillus* (22). Hapten inhibition studies revealed inhibition in both the enzyme-linked immunosorbent assay (ELISA) and latex agglutination assay, using these antibod-

ies with galactofuranose monomers and β (1-5)-linked oligomers (6, 17, 23). It was found that the tetramer and higher oligomers of β (1-5)-linked galactofuranose possess the greatest inhibitory effect.

Enzymatic hydrolysis of β (1-5)-linked galactofuranosyl residues from EPS of *P. charlesii* by an exo- β -D-galactofuranosidase produced by this fungus was reported (26). An endo- β (1-5)-D-galactofuranosidase from *P. oxalicum* and the enzymatic hydrolysis of β (1-5)-galactofuranosyl residues from polysaccharides obtained from *Eupenicillium crustaceum* and *Talaromyces flavus* have also been reported (25). However, the influence of the exo- β -D-galactofuranosidase on the antigenic properties of EPS has not been studied. Antigenicity of the EPS was lost by acid hydrolysis of the acid-labile galactofuranosyl residues (16). The question remains as to whether the β (1-5)-linked galactofuranose residues are the epitopes of *Aspergillus* and *Penicillium* species. To prove this proposition, specific enzymatic degradation of these sequences should result in complete loss of ELISA activity.

In this report, we describe the purification and characterization of an exo- β -D-galactofuranosidase isolated from a commercially available crude enzyme preparation of the mold *Trichoderma harzianum* (10). Also, we report on the degradation of EPS preparations derived from *Penicillium* and *Aspergillus* species by this purified enzyme in relation to their antigenicity. Reductive cleavage (15) was used for chemical characterization of the EPS before and after enzymatic hydrolysis. The results made it possible to prove that β (1-5)-linked galactofuranose sequences are uniquely responsible for the antigenicity of these EPS and to propose a new structural model for the antigenic galactofuranose side chains of the galactomannan from *P. digitatum*. In this

* Corresponding author.

model, β (1-6)-linked galactofuranosyl sequences are also present in these fungal polysaccharides. In addition, possible applications of this epitope-degrading enzyme are described.

MATERIALS AND METHODS

Enzyme preparation. The α -D-galactofuranosidase was purified from a crude enzyme preparation of fungal origin (*T. harzianum*), commercially available as Glucanex (Novo Ferment AG, Basel, Switzerland).

Isolation of EPS. Mold strains used in this study were *P. digitatum* RIVM H-236, *P. aurantiogriseum* CBS 342.51, *P. dierckxii* RIVM 90, *P. citrinum* CBS 117.64, *Aspergillus fumigatus* RIVM 51-1, and *A. niger* CBS 553.65. They were grown at 23°C in shaking cultures with yeast nitrogen base (Difco Laboratories, Detroit, Mich.) as the basal synthetic culture medium (6.7 g/liter) supplemented with glucose (30 g/liter) as the carbon source. Inoculation was performed with a spore suspension. The EPSs were isolated by ethanol precipitation from the culture fluid after removal of the mycelial pellets by filtration as described previously (9).

Determination of neutral sugar composition and protein content. The sugar composition of the EPS preparations was determined as alditol acetates, using gas-liquid chromatography (GLC). Sugars were released by pretreatment with 12 M sulfuric acid for 1 h at 30°C, followed by hydrolysis with 1 M sulfuric acid for 3 h at 100°C. Next, sugars were converted to their alditol acetates as described by Englyst and Cummings (11) and analyzed by GLC, using inositol as the internal standard.

Protein was determined by the methods of Sedmak and Grossberg (28) and Lowry et al. (19), with bovine serum albumin (BSA) as the standard.

Structural analysis of EPS by methylation analysis and reductive cleavage. The native and enzyme-treated EPS preparations of *P. digitatum* were permethylated by a modified Hakomori procedure, using lithium dimethyl sulfinyl anion and methyl iodide in dimethyl sulfoxide (7). The permethylated EPS was isolated by extraction with dichloromethane and purified on an LH-20 column. The methylated polymeric fraction was investigated further both by conversion of the fractions to partially methylated alditol acetates (1) and by reductive cleavage (15). The enzyme-treated sample was purified on a Sep-Pak C₁₈ cartridge.

Reductive cleavage of the permethylated samples was performed by dissolving 1 mg in 100 μ l of dry dichloromethane in a silylated screw-cap glass vial. To this solution, 25 equivalents of triethylsilane and trimethylsilyl methanesulfonate per glycosidic bond and 5 equivalents of borontrifluoride etherate were added, and the mixture was stirred for 17 h at room temperature. The reaction was quenched with methanol, treated with mixed-bed ion-exchange resin, and filtered. After evaporation of methanol, the residue was acetylated with 1-methyl imidazole and acetic anhydride. To prove that all galactose residues occur in the furanoside form, the acetyl groups of the reductive-cleavage samples were exchanged for methyl groups (20).

Quantitative chromatography was performed on a Carlo Erba Fractovap 4160 GLC equipped with an on-column injector, using a DB-5 capillary column (40 m by 0.25 mm). Two-dimensional GLC was performed on a Siemens Sicromat 2 instrument equipped with a CP Sil 5 CB column (25 m by 0.25 mm) and an octakis(2-O-methyl-3,6-di-O-pentyl)- γ -cyclodextrin capillary column (25 m by 0.25 mm). GLC-mass spectrometry was performed with a Hewlett-Packard 5840

A-5985 A instrument (chemical ionization, ammonia; electron impact, 70 eV).

Sandwich ELISA. Polyclonal IgG antibodies (332) were obtained by immunization of rabbits with EPS from *P. digitatum* as described by Notermans and Heuvelman (21). The sandwich ELISA was carried out by using polyvinyl microtiter plates as described previously (16). ELISA reactivity was expressed as the titer of the EPS, which is the reciprocal dilution of a solution of 100 μ g of EPS per ml in distilled water just giving a positive reaction, i.e., an extinction at 450 nm $\geq$ 0.1 above that of a blank containing no antigenic EPS.

Purification of α -D-galactofuranosidase. The purification of α -D-galactofuranosidase enzyme was carried out at 4°C; all buffers contained 0.01% (wt/vol) sodium azide to prevent microbial growth. Five grams of Glucanex was dissolved in 10 ml of 10 mM sodium acetate (pH 5.0) and centrifuged to remove solids. Desalting was carried out on a Bio-Gel P10 (100/200-mesh) column (82 by 3.5 cm) in the same buffer. Anion-exchange chromatography was done with a DEAE Bio-Gel A column (18 by 2 cm), using 10 mM sodium acetate (pH 5.0) as the buffer. The flow rate was maintained at 0.33 ml/min, and a gradient from 0 to 1 M NaCl was used to release the proteins subsequently. Chromatography with a Bio-Gel hydroxyapatite column (10 by 2 cm; Bio-Rad Laboratories, Richmond, Calif.) was performed with the same flow rate, using a gradient of 2 to 100 mM NaH₂PO₄-Na₂HPO₄ buffer (pH 6.8). Further purification was performed on an FPLC (fast protein liquid chromatography) system (Pharmacia LKB Biotechnology, Uppsala, Sweden) equipped with a Mono Q HR 5/5 anion-exchange column (50 by 5 mm) eluted with 20 mM bis-Tris buffer with a flow rate of 1 ml/min. During gradient elution from pH 7.0 to 5.5, peak control was used to elute protein peaks with a minimum amount of contamination by maintaining the composition of the eluent at a fixed value during elution of the peaks. Protein was determined by the assay of Sedmak and Grossberg (28). The purity of the fractions containing the α -D-galactofuranosidase was checked by sodium dodecyl sulfate (SDS)-gel electrophoresis on a 10 to 15% polyacrylamide gradient gel.

Determination of α -D-galactofuranosidase activity. The α -D-galactofuranosidase activity present in enzyme fractions was determined both by high-performance anion-exchange chromatography and by the sandwich ELISA. The amount of liberated galactose residues was estimated with the use of high-performance anion-exchange chromatography with a Dionex Bio-LC high-performance liquid chromatography system (Dionex, Sunnyvale, Calif.) equipped with a CarboPac PA1 column (4 by 250 mm) and pulsed-amperometric detection with a gold working electrode and an Ag-AgCl reference electrode as described previously (8). The epitope-degrading activity was measured by incubation of EPS from *P. digitatum* and respective enzyme fractions followed by a sandwich ELISA. Incubation was performed in a reaction mixture containing 175 μ l of EPS (1 μ g/ml) in 50 mM sodium acetate (pH 5.5) and 25 μ l of an enzyme fraction. After incubation for 16 h at 35°C, the enzyme was inactivated by heat treatment (5 min, 100°C).

Determination of side activities. The APIZYM (BIO Merieux SA, Lyon, France) was used for screening several side activities as instructed by the supplier.

Glucanase activities were measured after incubation of 100 μ l of the solutions of the respective glucan substrates (1 g/liter) in 90 μ l of 50 mM sodium acetate (pH 5.0) together with 10 μ l of the enzyme fraction at 35°C for 4 h. Laminaran

(Nutritional Biochemicals Co., Cleveland, Ohio), a $\beta(1-3,1-6)$ -glucan, and a $\beta(1-3,1-6)$ -glucan produced by *Botrytis cinerea* and isolated as described for the EPS of *Penicillium* and *Aspergillus* species were used as glucan substrates. After inactivation of the enzyme (5 min, 100°C), the release of glucose was measured by high-performance anion-exchange chromatography as described above.

The activity toward various *p*-nitrophenyl-glycosides (Sigma, St. Louis, Mo.) was measured spectrophotometrically at 405 nm, using the molar extinction coefficient $13,700 \text{ M}^{-1} \text{ cm}^{-1}$. One unit was defined as the amount of enzyme which was able to release 1 μmol of *p*-nitrophenol per min.

SDS-gel electrophoresis, isoelectric focusing, and titration curve. For determination of the molecular weight of the exo- β -D-galactofuranosidase, SDS-gel electrophoresis was performed on a 10 to 15% polyacrylamide gradient gel, using the PhastSystem (Pharmacia LKB Biotechnology) according to the instructions of the supplier. Standards in the range of 10 to 100 kDa were used. For determination of the isoelectric point of the exo- β -D-galactofuranosidase, isoelectric focusing and titration curves were performed on homogeneous polyacrylamide gels containing Pharmalyte carrier ampholytes, which generate a linear pH gradient from 3 to 9 in the gel. Standards with an isoelectric point in the range of 3 to 9 were used. Proteins were detected by the Coomassie brilliant blue R-250 staining and silver staining.

Temperature and pH optima, K_m , and V_{max} . Optimum pH and temperature were determined with 400 μl of a solution of EPS (0.5 mg/ml) of *P. digitatum* in 100 mM sodium acetate with pH in the range of 3 to 6 incubated with 100 μl of a partly purified enzyme fraction containing 25 μg of protein per ml (28) for 2 h at a temperature interval between 20 and 50°C. K_m and V_{max} were determined with 400 μl of a solution of *P. digitatum* EPS in 100 mM sodium acetate (pH 5.0) with a concentration in the range of 7.5 to 750 $\mu\text{g/ml}$ incubated with 100 μl of a partly purified enzyme fraction containing 25 μg of protein per ml (28) for 2 h at 35°C. After inactivation of the enzyme (5 min, 100°C), the release of galactose was measured by high-performance anion-exchange chromatography as described above.

Substrate specificity of the exo- β -D-galactofuranosidase. Dimers of β -D-(1-2)-, β -D-(1-3)-, β -D-(1-5)-, and β -D-(1-6)-linked galactofuranose (31) and a tetramer of β -D-(1-5)-linked galactofuranose (30) were used to test the specificity of the exo- β -D-galactofuranosidase. The release of galactose after incubation with the enzyme from the differently linked galactofuranosyl oligomers was determined by incubation of 100 μl (25 $\mu\text{g/ml}$) of oligomer in 50 mM sodium acetate buffer (pH 5.0) with 5 μl of the purified enzyme solution for 16 h at 30°C. The amount of galactose released was determined by high-performance anion-exchange chromatography as described above.

Degradation of the epitopes of EPS from *Penicillium* and *Aspergillus* species by the exo- β -D-galactofuranosidase. The epitope-degrading activity was studied by analysis of the reaction products after enzyme treatment of EPS from *P. digitatum*, *P. aurantiogriseum*, *P. citrinum*, *A. fumigatus*, and *A. niger*. An aliquot of 750 μl of EPS solution (1 mg/ml) and 100 μl of 1 M sodium acetate buffer (pH 5.0) with 0.01% sodium azide were incubated with 150 μl of the purified enzyme fraction (protein content of <5 $\mu\text{g/ml}$ [28]) at 35°C for 16 h. After inactivation (5 min, 100°C) the reaction products were analyzed by high-performance anion-exchange chromatography. The antigenicity of the EPS was measured by the sandwich ELISA.

FIG. 1. Final purification of the exo- β -D-galactofuranosidase on a Mono Q HR anion-exchange column, using FPLC with a pH gradient from 7.0 to 5.5 in bis-Tris buffer (see Materials and Methods). Symbols: —, A_{280} ; . . . , pH of the effluent; ---, ELISA reactivity of EPS incubated with each fraction.

RESULTS

Purification of the exo- β -D-galactofuranosidase. Several crude enzyme preparations were screened for the presence of exo- β -D-galactofuranosidase activity. Glucanex, a crude enzyme preparation isolated from the fungus *T. harzianum*, contained the highest activity. The exo- β -D-galactofuranosidase was isolated from this preparation after desalting of the crude preparation and anion-exchange chromatography. Most of the exo- β -D-galactofuranosidase activity appeared in the void volume of the DEAE Bio-Gel A anion-exchange column. However, some activity also eluted from the column with the gradient at 70 mM NaCl. Only the exo- β -D-galactofuranosidase present in the void volume was purified further on a hydroxyapatite column. The major part (55%) of the protein, which did not bind to this column, contained the exo- β -D-galactofuranosidase. This void fraction also contained most of the side activities present in the crude enzyme preparation (results not shown). Finally, the exo- β -D-galactofuranosidase was purified from the void fraction of the hydroxyapatite column on a Mono Q anion-exchange column (Fig. 1). To identify the enzyme fraction able to degrade the epitopes of *P. digitatum* EPS, each fraction eluting from this column was incubated with EPS and the ELISA reactivity thereof was measured (Fig. 1). The fraction eluting at pH 6.3 (arrow in Fig. 1) from this column contained the epitope-degrading enzyme, and its purity was checked by SDS-gel electrophoresis. One single protein band at 35 kDa, representing the molecular mass of the enzyme, was visualized after staining with Coomassie brilliant blue R-250. Silver staining revealed some additional minor protein bands. The titration curve of the purified exo- β -D-galactofuranosidase, indicating the electrophoretic mobility of the purified enzyme at different pH values, is shown in Fig. 2.

Incubation with *p*-nitrophenyl derivatives, naphthyl derivatives, and two glucans was performed to detect any side activity that might be present in the purified enzyme fractions. The crude Glucanex preparation contained enzyme activities toward the *p*-nitrophenyl derivatives of β -D-galactofuranoside, β -D-galactopyranoside, and β -D-glucopyranoside and to the 2-naphthyl derivatives of phosphate, butyrate, and caprylate. Most of the initially present side activities were removed with the purification. No activity of the purified enzyme toward *p*-nitrophenyl- β -D-galactofuranoside was detected after 1 h of incubation, but some release

FIG. 2. Titration curve of the purified exo- β -D-galactofuranosidase, using a pH gradient from 3 to 9. The protein on the gel was stained with silver.

of *p*-nitrophenol was observed after 24 h. The purified enzyme contained traces of glucanase activity. However, no release of glucose was detected after incubation of this purified exo- β -D-galactofuranosidase fraction with the EPS from *Penicillium* and *Aspergillus* species. Therefore, no further attempts were made to remove this glucanase activity.

Properties of the exo- β -D-galactofuranosidase. The purified exo- β -D-galactofuranosidase has an isoelectric point of about 4 (Fig. 2) and an optimal activity on EPS of *P. digitatum* between pH 4 and 4.5. The enzyme activity dropped above pH 5, and no activity could be detected at pH 7 or higher (results not shown). No galactose was released as a result of chemical hydrolysis due to low pH during incubation, as established in a separate experiment (results not shown). Maximum enzyme activity was obtained between 35 and 40°C. The V_{max} and K_m of the purified enzyme calculated for EPS of *P. digitatum* were 1 U/mg and 0.2 mg/ml, respectively.

Galactose was released from β -D-(1-5)-linked galactofuranoside tetramer but not from the β -D-(1-5)-dimer and the corresponding β -D-(1-2), β -D-(1-3), and β -D-(1-6) dimers. The enzyme did not liberate any sugars from EPS of *Fusarium* spp. or species belonging to the order *Mucorales*.

Degradation of the epitopes of *Penicillium* and *Aspergillus* EPS by the purified exo- β -D-galactofuranosidase. The degradation of β (1-5)-linked galactofuranosyl residues of *Penicillium* and *Aspergillus* EPS in relation to their antigenicity was studied by incubation of EPS from a number of *Penicillium* and *Aspergillus* species with the purified exo- β -D-galactofuranosidase. The antigenicity of these EPSs was determined by measuring their ELISA reactivities, expressed as the minimal amount of EPS which could be detected in this ELISA system, before and after enzyme treatment (Table 1). The antigenicity of the enzyme-treated EPS disappeared completely, as the minimal detectable quantity of EPS before enzyme treatment (<2 to 400 ng/ml) increased to more than 0.1 mg/ml after enzyme treatment.

The sugar composition of the initial EPS and the relative amount of galactose released as a result of enzymatic hy-

TABLE 1. Minimal detectable quantities of various EPS preparations of *Penicillium* and *Aspergillus* species in an ELISA with IgG antibodies raised against *P. digitatum* before treatment with exo- β -D-galactofuranosidase^a

Origin of EPS	Minimal detectable quantity (ng/ml)
<i>P. digitatum</i>	45
<i>P. aurantiogriseum</i>	<2
<i>P. dierckxii</i>	400
<i>P. citrinum</i>	10
<i>A. fumigatus</i>	70
<i>A. niger</i>	5

^a Incubation was performed for 16 h at 35°C. In all cases, the minimal detectable quantity in enzyme-treated samples was >10⁵ ng/ml.

drolysis are listed in Table 2. The EPS contained mainly mannose, galactose, and glucose and a minor amount of xylose. The amount of galactose initially present decreased after treatment with the exo- β -D-galactofuranosidase. Despite the fact that the antigenicity of EPS disappeared completely upon enzyme treatment, only 26 to 33% of galactose residues were released by the enzyme. Analysis of the products after enzyme treatment by high-performance anion-exchange chromatography with pulsed-amperometric detection showed that only monomers of galactose were liberated (Fig. 3).

Structural characterization of the initial EPS from *P. digitatum* by reductive cleavage followed by acetyl-methyl exchange showed that galactose occurs in the furanoside form. The glucose and mannose residues are exclusively linked as pyranosides. All products identified by reductive cleavage were confirmed by standard methylation analysis; the molar ratios of the galactose derivatives before and after enzymatic degradation are summarized in Table 3. As shown, β (1-5)-linked, β (1-6)-linked, and β (1,5,6)-linked galactofuranose residues could be identified in the native polysaccharides. After enzymatic degradation with the purified exo- β -D-galactofuranosidase, the relative amount of β (1-5)-linked galactofuranosides decreased considerably, whereas the amounts of β (1-6)-linked and β (1,5,6)-linked galactofuranose did not change. The apparent increase of the terminal galactofuranose residues after enzymatic treatment was not confirmed by standard methylation analysis; therefore this result can be considered not significant.

DISCUSSION

Exo- β -D-galactofuranosidase-catalyzed degradation of EPS from *Penicillium* and *Aspergillus* species was studied in relation to the antigenic properties of these polysaccharides. The exo- β -D-galactofuranosidase was purified by using anion-exchange and adsorption columns combined with FPLC. The enzyme was active on the tetramer of β (1-5)-linked galactofuranose, from which it released galactose. The enzyme acted as an exoenzyme on EPS, as only monomers of galactose were released. A low activity was found on synthetic *p*-nitrophenyl- β -galactofuranoside, indicating that the enzyme needs more than one galactofuranose residue for binding. The purified enzyme was checked for a large number of side activities and showed only some glucanase activity. The influence of pH on the activity of the exo- β -D-galactofuranosidase appeared to be similar to that described by Rietschel-Berst et al. (26) for the exo- β -D-galactofuranosidase from *P. charlesii*; its temperature optimum was slightly lower.

TABLE 2. Neutral sugar composition of native EPS of several *Penicillium* and *Aspergillus* species and the percentage of galactose released by exo- β -D-galactofuranosidase (16 h, 35°C)

Origin of EPS	Sugar composition ^a (mol%)				% of galactose released ^b
	Xyl	Man	Gal	Glc	
<i>P. digitatum</i>	4	35	46	15	26
<i>P. aurantiogriseum</i>	2	22	30	46	33
<i>P. dierckxii</i>	16	33	18	33	30
<i>P. citrinum</i>	6	30	37	27	30
<i>A. fumigatus</i>	4	18	20	58	32
<i>A. niger</i>	6	46	34	14	32

^a The sugar content of the EPS preparations varied between 40 and 70% (wt/wt).

^b Amount of galactose released from the EPS preparations after enzyme treatment, expressed as percentage of the initial galactose content.

Structural characterization of the EPS preparation of *P. digitatum* revealed that the galactose residues occur in the furanose form, as previously established for galactomannans derived from *A. niger* (5). Galactopyranose residues as previously found in the cell wall of *A. niger* (3) and in the extracellular slime of *A. fumigatus* (18) were probably absent in EPS of *P. digitatum*, as shown by the reductive-cleavage procedure.

In addition to the earlier reported β (1-5)-linked galactofuranose residues (4, 12), the presence of β (1,5,6)-linked and β (1-6)-linked residues was also demonstrated. The latter compound was previously found in a cell wall arabinogalac-

TABLE 3. Relative amounts of differently linked galactose residues in EPS of *P. digitatum* before and after treatment with exo- β -D-galactofuranosidase

Galactose linkage	Relative amt	
	Native	Enzyme treated
t-Galactofuranose ^a	0.3	0.5
1,5-Galactofuranose	2.1	1.0
1,6-Galactofuranose	1.0	1.0
1,5,6-Galactofuranose	0.1	0.1

^a Nonreducing terminal galactofuranose residue.

tan derived from *Mycobacterium smegmatis* (14). To our knowledge, this is the first time that β (1,5,6)-linked galactofuranoses have been identified in fungal polysaccharides.

The antigenicity of the EPS disappeared completely after treatment with the purified exo- β -D-galactofuranosidase, indicating that all of the epitopes were degraded. Only monomers of galactose were liberated; no complete hydrolysis of the galactofuranoses, initially present in the EPS, could be obtained with the exo- β -D-galactofuranosidase. In fact, after enzymatic degradation of EPS of *P. digitatum*, the molar ratios of β (1-5)-linked and β (1-6)-linked galactofuranose residues decreased from 2:1 to 1:1. With these results, we can propose a new structural model for the antigenic galactofuranose side chains of the extracellular galactomannans from *P. digitatum* and possibly from all *Penicillium* and *Aspergillus* species (Fig. 4). Our model is composed of a branched side

FIG. 3. High-performance anion-exchange chromatogram of the reaction products obtained after incubation of EPS from *P. digitatum* with exo- β -D-galactofuranosidase compared with the chromatogram of a standard mixture of sugar residues (inset). PAD, pulsed-amperometric detection.

FIG. 4. Proposed structural model for the immunologically active galactofuranose side chains of the extracellular galactomannans of *Penicillium* and *Aspergillus* species. The values for K, L, and M can vary from zero to eight residues.

chain attached to the mannan core. The side chain is a $\beta(1-5)$ -linked galactofuranose sequence which, in turn, carries a $\beta(1-6)$ -linked galactofuranose sequence attached to a $\beta(1,5,6)$ -galactofuranose branch point. On the basis of the amount of galactose liberated and of the $\beta(1-5)$ -linked galactofuranose residues still present in EPS after enzyme treatment, the values of K, L, and M as indicated in Fig. 4 can vary from zero to eight residues. This model replaces the older model of Gander and coworkers (12), which was constituted of unbranched $\beta(1-5)$ -galactofuranose chains attached to a mannan backbone.

The exo- β -D-galactofuranosidase attacks the $\beta(1-5)$ -linked sequence from the nonreducing end and is stopped near the branch point. It can hydrolyze neither the $\beta(1-6)$ -linked galactofuranose sequence nor the interior $\beta(1-5)$ -linked galactofuranose sequence. Since the antigenicity disappears upon enzyme treatment, it can also be concluded that the antibodies used in this study bind only to sequences of $\beta(1-5)$ -linked galactofuranose residues, with an unsubstituted nonreducing galactofuranose terminus.

In conclusion, this epitope-degrading enzyme appeared to be very useful for structural analysis of the epitopes of these antigenic polysaccharides. The unique epitope-degrading properties of the exo- β -D-galactofuranosidase enable specific application of this enzyme. It can be useful for the detection of false-positive reactions in immunological methods such as ELISA and the latex agglutination assay, since it provides an alternative to the use of synthetic $\beta(1-5)$ -linked galactofuranoside epitopes as specific blocking agents (17, 23). Also, the purification and availability of the exo- β -D-galactofuranosidase allow the specific removal of the nonreducing β -D-(1-5)-galactofuranose sequences, down to close to the β -D-(1-6) branch points, leaving the intact β -D-(1-6)-galactofuranose sequences freely exposed. As a result, it may be possible to raise antibodies against this enzymatically modified EPS and to study their specificities toward *Aspergillus*, *Penicillium*, and other mold species.

ACKNOWLEDGMENTS

We thank K. Dörreich (Novo Ferment AG, Basel, Switzerland) for the gift of the Glucanex enzyme preparation and R. A. Samson (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) for providing several *Penicillium* and *Aspergillus* strains.

These investigations were supported by the Netherlands' Foundation for Chemical Research with financial aid from the Netherlands' Technology Foundation and by the Deutsche Forschungsgemeinschaft.

REFERENCES

- Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. 1967. A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. *Carbohydr. Res.* 5:340-345.
- Bardalaye, P. C., and J. H. Nordin. 1976. Galactosaminogalactan from cell walls of *Aspergillus niger*. *J. Bacteriol.* 125:655-669.
- Bardalaye, P. C., and J. H. Nordin. 1977. Chemical structure of the galactomannan from the cell wall of *Aspergillus niger*. *J. Biol. Chem.* 252:2584-2591.
- Barreto-Bergter, E. M., P. A. J. Gorin, and L. R. Travassos. 1981. Cell constituents of mycelia and conidia of *Aspergillus fumigatus*. *Carbohydr. Res.* 95:205-218.
- Barreto-Bergter, E. M., L. R. Travassos, and P. A. J. Gorin. 1980. Chemical structure of the D-galacto-D-mannan component from hyphae of *Aspergillus niger* and other *Aspergillus* spp. *Carbohydr. Res.* 86:273-285.
- Bennett, J. E., A. K. Bhattacharjee, and C. P. J. Glaudemans. 1985. Galactofuranosyl groups are immunodominant in *Aspergillus fumigatus* galactomannan. *Mol. Immunol.* 22:251-254.
- D'Ambra, A. J., M. J. Rice, S. G. Zeller, P. R. Gruber, and G. R. Gray. 1988. Analysis of positions of substitution of O-methyl or O-ethyl groups in partially methylated or ethylated cellulose by the reductive cleavage method. *Carbohydr. Res.* 177:111-116.
- De Ruiter, G. A., H. A. Schols, A. G. J. Voragen, and F. M. Rombouts. Carbohydrate analysis of water-soluble uronic acid containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.*, in press.
- De Ruiter, G. A., A. W. Van der Lugt, A. G. J. Voragen, F. M. Rombouts, and S. H. W. Notermans. 1991. High-performance size-exclusion chromatography and ELISA detection of extracellular polysaccharides from Mucorales. *Carbohydr. Res.* 215:47-57.
- Dubourdieu, D., C. Desplanques, J.-C. Villettaz, and P. Ribereau-Gayon. 1985. Investigations of an industrial β -D-glucanase from *Trichoderma harzianum*. *Carbohydr. Res.* 144:277-287.
- Englyst, H. N., and J. H. Cummings. 1984. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* 109:937-942.
- Gander, J. E., N. H. Jentoft, L. R. Drewes, and P. D. Rick. 1974. The 5-O- β -D-galactofuranosyl-containing exocellular glycopeptide of *Penicillium charlesii*. Characterization of the phosphogalactomannan. *J. Biol. Chem.* 249:2063-2072.
- Gomez-Miranda, B., and J. A. Leal. 1981. Extracellular and cell wall polysaccharides of *Aspergillus alliaceus*. *Trans. Br. Mycol. Soc.* 76:249-253.
- Gruber, P. R., and G. R. Gray. 1990. Isolation and analysis by the reductive-cleavage method linkage positions and ring forms in the *Mycobacterium smegmatis* cell-wall arabinogalactan. *Carbohydr. Res.* 203:79-90.
- Jun, J.-G., and G. R. Gray. 1987. A new catalyst for reductive cleavage of methylated glycans. *Carbohydr. Res.* 163:247-261.
- Kamphuis, H. J., G. A. De Ruiter, G. H. Veeneman, J. H. Van Boom, F. M. Rombouts, and S. H. W. Notermans. 1992. Detection of *Aspergillus* and *Penicillium* extracellular polysaccharides (EPS) by ELISA: using antibodies raised against acid hydrolysed EPS. *Antonie van Leeuwenhoek* 61:323-332.
- Kamphuis, H. J., S. Notermans, G. H. Veeneman, J. H. Van Boom, and F. M. Rombouts. 1989. A rapid and reliable method for the detection of molds in foods: using the latex agglutination assay. *J. Food Prot.* 52:244-247.
- Latgé, J.-P., J.-P. Debeauvais, M. Moutaouakil, M. Diaquin, J. Sarfati, M. C. Prévost, J. M. Wieruszkeski, Y. Leroy, and B. Fournet. 1991. Galactomannan and the circulating antigens of *Aspergillus fumigatus*, p. 143-155. In J.-P. Latgé and D. Boucias (ed.), *Fungal cell wall and immune response*. NATO ASI series, vol. H53. Springer-Verlag, Berlin.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mischnick, P. 1991. Determination of the substitution pattern of cellulose acetates. *J. Carbohydr. Chem.* 10:711-722.
- Notermans, S., and C. J. Heuvelman. 1985. Immunological

- detection of moulds in food by using the enzyme-linked immunosorbent assay (ELISA); preparations of antigens. *Int. J. Food Microbiol.* **2**:247–258.
22. Notermans, S., and P. S. S. Soentoro. 1986. Immunological relationship of extra-cellular polysaccharide antigens produced by different mould species. *Antonie van Leeuwenhoek* **52**:393–401.
 23. Notermans, S., G. H. Veeneman, C. W. E. M. Van Zuylen, P. Hoogerhout, and J. H. Van Boom. 1988. (1-5)-Linked β -D-galactofuranosides are immunodominant in extracellular polysaccharides of *Penicillium* and *Aspergillus* species. *Mol. Immunol.* **25**:975–979.
 24. Preston, J. F., E. Lapis, and J. E. Gander. 1969. Isolation and partial characterization of the exocellular polysaccharides of *Penicillium charlesii*. III. Heterogeneity in size and composition of high molecular weight exocellular polysaccharides. *Arch. Biochem. Biophys.* **134**:324–334.
 25. Reyes, F., C. Alfonso, M.-J. Martinez, A. Prieto, F. Santamaria, and J.-A. Leal. 1992. Purification of a new galactanase from *Penicillium oxalicum* catalysing the hydrolysis of β -(1-5)-galactofuran linkages. *Biochem. J.* **281**:657–660.
 26. Rietschel-Berst, M., N. H. Jentoft, P. D. Rick, C. Pletcher, F. Fang, and J. E. Gander. 1977. Extracellular exo- β -D-galactofuranosidase from *Penicillium charlesii*. Isolation, purification and properties. *J. Biol. Chem.* **252**:3219–3226.
 27. Ruperez, P., and J. A. Leal. 1981. Extracellular galactosaminogalactan from *Aspergillus parasiticus*. *Trans. Br. Mycol. Soc.* **77**:621–625.
 28. Sedmak, J. J., and S. E. Grossberg. 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* **79**:544–552.
 29. Suzuki, S., and N. Takeda. 1975. Serologic cross-reactivity of the D-galacto-D-mannans isolated from several pathogenic fungi against anti-*Hormodendrum pedrosoi* serum. *Carbohydr. Res.* **40**:193–197.
 30. Veeneman, G. H., P. Hoogerhout, P. Westerduin, S. Notermans, and J. H. Van Boom. 1987. Synthesis of a cell-wall component of *Aspergillus niger* containing four β -(1-5)-interlinked D-galactofuranosyl residues. *Recl. Trav. Chim. Pays-Bas* **106**:129–131.
 31. Veeneman, G. H., S. Notermans, P. Hoogerhout, and J. H. Van Boom. 1989. Synthesis of an immunologically active component of the extracellular polysaccharide produced by *Aspergillus* and *Penicillium* species. *Recl. Trav. Chim. Pays-Bas* **108**:344–350.