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ORIGINAL ARTICLE

Structure elucidation of a galactofuranose-rich heteropolysaccharide from aciduric fungus *penicillium purpurogenum* JS03-21

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Abstract The exopolysaccharide-producing fungus *Penicillium purpurogenum* JS03-21 from the acidic purple clay was screened to develop greater insights into microbial exopolysaccharide resources from extremophilic microorganisms. An exopolysaccharide JS1-1 was purified from the fermented broth of the aciduric fungus using a combination of ethanol precipitation, anion-exchange and size-exclusion chromatography. JS1-1 was found to be a neutral heteropolysaccharide composed of galactose, mannose and glucose in the ratio of 7.9:2.1:1.0, with a molecular weight of about 26 kDa. JS1-1 was subjected to a series of methylation and partial acid hydrolysis reactions, as well as being analyzed by one- and two-dimensional nuclear magnetic resonance, and the results of these analyses revealed JS1-1 to be a galactofuranose-rich polysaccharide. The main chain of JS1-1 contained two regions, including a galactose part composed of a repeating trisaccharide unit [*i.e.*, →5)-β-D-Gal*f*(1→5)-β-D-Gal*f*(1→6)-β-D-Gal*f*(1→], and a mannose section consisting of (1→6)-linked α-D-Man*p* residues. Two further segments [*i.e.*, α-D-Glc*p*(1→2)-α-D-Gal*f* (1→and α-D-Gal*f*(1→2)-α-D-Man*p*(1→] existed as branches that were linked to the *O-2* atoms of the (1→6)-β-D-Gal*f* and (1→6)-α-D-Man*p* regions of the main chain, respectively. The results of the current study demonstrate that JS1-1 is a unique polysaccharide that is rich in both α and β galactofuranose units and therefore different from most of the other known extracellular polysaccharides. The function of this polysaccharide is currently being researched in our laboratory.

Keywords Aciduric fungus · *Penicillium purpurogenum* · Exopolysaccharide · Structure

Introduction

Organisms that live at the extremes of pH (pH >8.5, pH <5.0), temperature (>45 °C, <15 °C), pressure (>500 atmospheres), salinity (>1.0 M NaCl) and in high concentrations of recalcitrant substances or heavy metals are known as extremophiles [1]. Extremophiles are well known to be an impeccable source of biomaterials such as polysaccharides, proteins and other industrially important biomolecules, and therefore represent a rich resource of materials for biotechnological and industrial discovery [2–4]. Most applications involving extremophiles are based on their biomolecules, with particular interest in their enzymes, although some of their other proteins have also been reported to reach their optimal activities under extreme temperature and pH conditions, and these proteins have been used extensively in the food, textile, pulp and chemical industries. One of the most well-known examples of the successful application of the extremophile is that of Taq DNA polymerase, which was derived from a geothermal spring [5]

Besides enzymes and proteins, extremophilic microorganisms also produce and secrete exopolysaccharides (EPSs), which protect them from the pressures of the harsh environment in which they live and make up a substantial component of their extracellular polymers. Some extremophilic microorganisms can produce EPSs with novel structures, unusual properties and functional activities under extreme conditions, and some of these materials have potential applications

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in other areas of research [6, 7]. For example, biocompatible nanofibers based on the extremophilic bacterial polysaccharide Mauran (MR), which is an extremophilic sulfated exopolysaccharide isolated from *Halomonas maura*, have been reported to enhance cell growth *in vitro*. These materials could also be used in a variety of other biomedical applications, such as tissue engineering and drug delivery [8].

Compared with prokaryotic life, much less is known about the EPSs produced by eukaryotic organisms under extreme conditions, and fungal species from extreme environments represent an underexploited resource. In certain acidic environments, eukaryotes have a higher level of biodiversity than prokaryotes [9]. In this study, the exopolysaccharide-producing fungus *Penicillium purpurogenum* JS03-21 was isolated from the acidic purple clay, which is used for the production of the famous purple pottery in Jianshui, Yunnan Province, China. This material was subsequently analyzed and its primary structure elucidated to allow for the development of a deeper understanding of the biological properties of EPS resources from extreme environments and the potential use of these materials in industrial and medical applications.

Materials and methods

Growth of the microorganism The aciduric fungus *Penicillium purpurogenum* JS03-21 was isolated from the acidic purple clay, which is a kaolinite-rich type of high-quality pottery clay, and purified through gradual dilution and the spread plate method on potato dextrose-agar (PDA) medium (pH= 2). The fungus was identified using traditional colony morphology, Internal Transcribed Spacer (ITS) and 18 s rRNA sequence analyses. Working stocks were prepared on PDA slants and stored at 4 °C. The fungus was activated to inoculate the correct amount of fungus to fresh PDA slants, as mentioned above, and cultured for 3 days at 28 °C. After activation, the mycelia were inoculated into conical flasks (1000 mL) containing 300 mL of the culture medium (*i.e.*, maltose (20 g), mannitol (20 g), glucose (10 g), monosodium glutamate (10 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.3 g), yeast-extract paste (3 g) and maize paste (1 g) dissolved in 1 L water (pH 2.0)). *Penicillium purpurogenum* JS03-21 was grown under static conditions at 22 °C for 35 days in the culture medium. The whole fermented broth (40 L) was obtained.

Isolation and purification of the extracellular polysaccharide The fermented broth was filtered through cheese cloth to separate the supernatant from the mycelia. The supernatant was concentrated to 1/20 original volume under reduced pressure at 40 °C. The protein in the concentrated solution was removed by the method of Sevag [10] and a threefold of the volume of 95 % (v/v) cold ethanol was added to the aqueous phase to precipitate the EPS. The resulting precipitate was recovered by centrifugation at 3600 g for 10 min. Then, the precipitate was resuspended with distilled water and dialyzed in a cellulose dialysis membrane (flat width 44 mm, molecular weight cut off 3500) against distilled water for 2 days, changed four times a day. After vacuum-dried of the retained fraction, the crude polysaccharides were obtained.

The fractions eluted with water were pooled and further purified on a Superdex^{TM} 75 column (GE, USA, 70×2 cm) eluted with 0.2 mol/L aqueous NH_4HCO_3 at a flow rate of 0.3 mL/min. The major polysaccharide fractions were pooled, freeze-dried, and designated JS1-1.

Composition analysis Total sugar content was determined by the phenol–sulfuric acid method using a mixture of mannose and galactose as the standard [11]. The protein content was assayed using the method described by Lowry [12]. Phosphate content was determined after hydrolysis by ammonium molybdate spectrophotometric method [13]. Sulfate ester content was estimated according to the method of Therho and Hartiala [14]. After totally hydrolysis with 2 mol/L trifluoroacetic acid (TFA) (1 mg/ml) at 105 °C for 6 h in a sealed tube, the monosaccharide compositions were determined by reversed-phase high performance liquid chromatography (HPLC) coupled with Eclipse XDB-C18 column (Agilent Technologies, USA, 4.6 μm×250 mm) after pre-column derivatization with PMP (1-phenyl-3-methyl-5-pyrazolone) and UV detection [15]. Sugar identification was done by comparison with reference sugars (L-rhamnose, L-arabinose, L-fucose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, D-galacturonic acid, D-mannuronic acid and *N*-acetyl-β-D-glucosamine; Ultra Pure, Sigma). Calculation of the molar ratio of the monosaccharides was carried out on the basis of the peak area of each monosaccharide.

Homogeneity and Mw determination The homogeneity and Mw was determined by high-performance size-exclusion chromatography (HPSEC) [16], which was performed on a Agilent HPLC system fitted with one Shodex Ohpak SB-804 HQ column (7.8 mm ID×30.0 cm L, Shodex, Japan) and a Agilent RID-10A detector. The mobile phase was 0.2 % Na_2SO_4, and the flow rate was 0.5 mL min^{-1} at 40 °C. The samples were applied in 1 % solutions in the mobile phase, and the injected volume was 20 μL.

The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular mass (M_w: 788, 404, 112, 47.3, 22.8, 11.8 and 5.9 kDa; Ultra Pure, Fluka).

Infrared spectrum (IR) analysis The polysaccharide was mixed with KBr powder, grounded and then pressed into a

1 mm pellets for FTIR measurement on the Nicolet Nexus 470 instrument in the frequency range of 4000–500 cm^{-1} at the resolution of 4.0 cm^{-1} with background scanning frequency of 32. FTIR spectrum of the polysaccharide was measured by using the Nicolet Omnic software.

Methylation analysis Methylation analysis was performed according to a modified version of the method described by Hakomori [17]. Briefly, the polysaccharide in dimethyl sulfoxide was methylated using NaH and iodomethane. The completeness of methylation was confirmed by Fourier transform infrared (FTIR) spectroscopy. After total hydrolysis with 2 mol/L trifluoroacetic acid at 105 °C for 6 h, the methylated sugar residues were converted to partially methylated alditol acetates by reduction with $NaBH_4$, followed by acetylation with acetic anhydride. The derivative sugar residues were extracted into dichloromethane and evaporated to dryness, dissolved again in 100 μL dichloromethane, and then analyzed by gas chromatography–mass spectrometric (GC–MS) on a HP6890II/5973 instrument using a DB 225 fused silica capillary column (0.25 mm × 30 m) (Agilent Technologies, USA). The partially methylated alditol acetates were separated using a temperature program from 100 °C, 5 °C /min up to 220 °C, and then 20 min at 220 °C. The injector temperature was set to 250 °C.

Identification of partially methylated alditol acetates was carried out on the basis of the retention time and its mass fragmentation patterns [18].

Partial acid hydrolysis The partial acid hydrolysis of heteropolysaccharide JS1-1 was performed to determine the distribution of monosaccharides and the linkages between the main chain and the branches. The exopolysaccharide was degraded with 0.01 mol/L aqueous trifluoroacetic acid at 105 °C for 2.0 h, and the resulting hydrolysate was dialyzed, separated by centrifugation and dried under vacuum at 40 °C. The dried sample was subjected to monosaccharide and methylation analysis [19].

NMR spectroscopy analysis Following deuterium exchange by two successive freeze-drying steps in 99.98 % D_2O, the resulting polysaccharide (70 mg) was analyzed by nuclear magnetic resonance (NMR) in 99.98 % D_2O solution at 23 °C using a JEOL JNM-ECP 600 MHz spectrometer (JEOL, Japan).

Results

Preparation and purification of the exopolysaccharides The acid-tolerant fungus *P. purpurogenum* JS03-21 can also exist in neutral culture medium, although it tends to produce larger amounts of the more prominent exopolysaccharides under acidic culture conditions. The concentration of exopolysaccharides was found to be about 0.6 g/L in the acidic fermented broth (pH=2). The crude exopolysaccharides were purified by Q Sepharose Fast Flow column chromatography and fractionated into three fractions (Fig. 1a). Preliminary analysis of the three fractions, which were named JS1, JS2 and JS3, revealed that they all had similar characteristics, and that they were all composed of galactose (D-Gal), mannose (D-Man) and glucose (D-Glc). Galactose was determined to be the major monosaccharide component in all three of the fractions, although the ratio of this material to the other monosaccharides varied considerably across the three different fractions. JS1 possessed the highest amount of D-Gal of all three fractions, and the average molecular weights of JS2 and JS3 were smaller than that of JS1 at about 12 kDa. Analysis of the three different fractions revealed that they were composed of similar linkage styles, and that the only major difference between the three fractions was their protein contents. For example, the protein content of JS1 was found to be 3 %, where the protein content of JS3 was two-fold higher at 6 %. This difference in the protein content of the fractions provided some explanation as to why the crude exopolysaccharides had been separated into three fractions by anion-exchange chromatography. JS1 was the largest of the three fractions, and gave a yield of 65 % following purification of the crude polysaccharides over a Superdex 75 column. The major peak was collected as shown in Fig. 1b and afforded the polysaccharide fraction JS1-1, which gave a yield of 45 % of the crude polysaccharides and was used for the structural analysis experiments.

Homogeneity and chemical composition of JS1-1 Good purity and homogeneity characteristics are crucial for effective structure elucidation. Pleasingly, JS1-1 gave a single and symmetrical peak in its high performance gel chromatogram (Fig. 1c), which indicated its high homogeneity. Its average molecular weight was estimated to be about 26.0 kDa, and therefore similar to most other fungal exopolysaccharides, which generally have molecular weights of less than 100.0 kDa.

JS1-1 contained 90.8 % total carbohydrate and several other minor proteins (2.14 %), and did not contain any other substituent groups, such as sulfate esters. Reversed-phase HPLC analysis showed that JS1-1 consisted of D-Gal, D-Man and D-Glc in a molar ratio of 7.9:2.1:1.0. Based on the monosaccharide composition results, JS1-1 was determined to be composed of neutral monosaccharides, with Gal being identified as the predominant component. No acidic or amino sugars were detected in JS1-1, which indicated that JS1-1 was a neutral heteropolysaccharide rich in D-Gal. Exopolysaccharides composed of D-Man, D-Glc and D-Gal were very common. Especially D-Man was usually the major

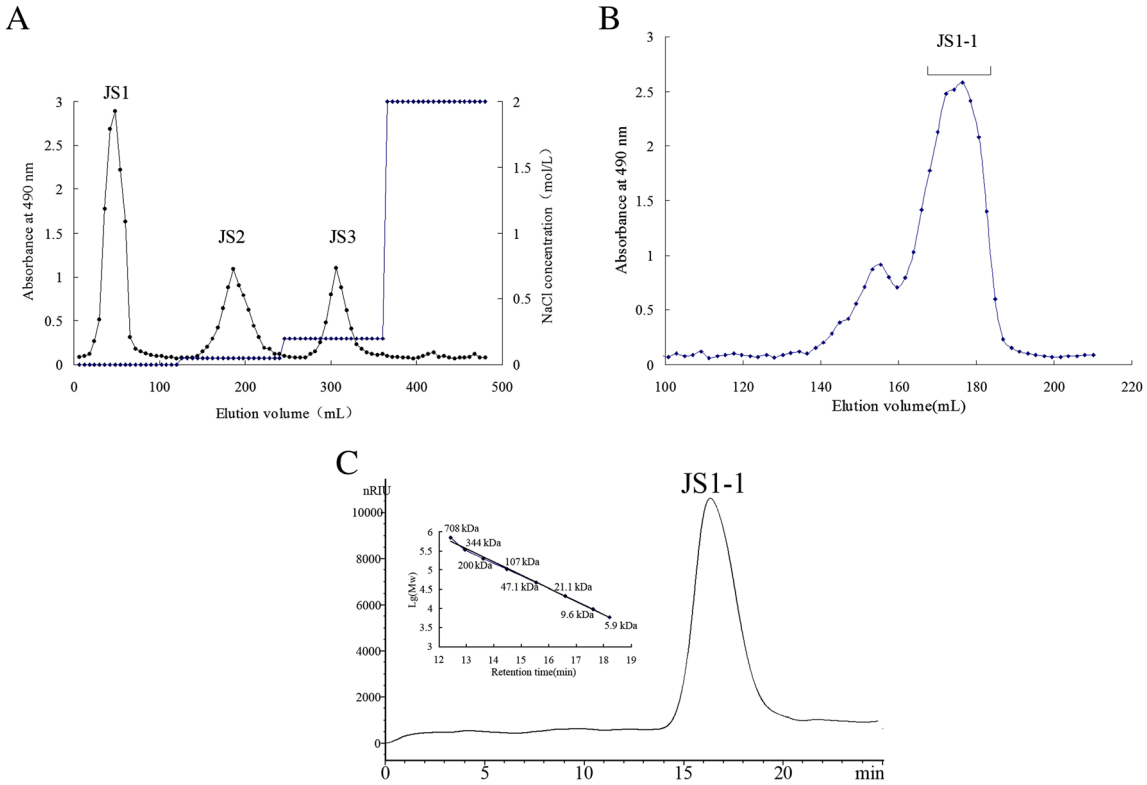

Fig. 1 Isolation and HPGPC chromatograms of the exopolysaccharide produced by aciduric fungus *Penicillium purpurogenum* JS03-21. **a** The crude polysaccharides obtained from the aciduric fungus were applied to a Q Sepharose Fast Flow column. The fractions eluted with water were pooled and denoted JS1, **b** The fraction JS1 was further purified over a Superdex^{TM} 75 column. The purified fraction was named JS1-1 and **c** HPGPC chromatogram of JS1-1 on a Shodex Ohpak SB804 column (0.8×30 cm) with a standard molecular weight curve

monosaccharide. But D-Gal as the predominant component was relatively rare [20, 21].

Methylation analysis JS1-1 was subjected to methylation analysis to determine the linkage patterns of the sugar residues. Data pertaining to the types of linkage and the proportion of methylated alditol acetate residues were obtained by comparing the retention times of the samples with specific standards, analyzing the breakage modes of the MS using reference data from the Complex Carbohydrate Research Center's Spectral Database for Partially Methylated Alditol Acetates and peak area integration. The linkage patterns of the different monosaccharides are shown in Table 1. Gal was the main monosaccharide found in JS1-1. All of the Gal existed as (1→)-linked, (1→2)–, (1→5)- and (1→2,6)-linked galactofuranose residue in a molar ratio of 1.0:2.3:4.3:2.1. The mannose portion of JS1-1 was composed of (1→2)–, (1→6)- and (1→2,6)-linked mannopyranose residues in a molar ratio of 1.1:1.0:1.0. The Glc in the JS1-1 only existed in the form of (1→)-linked glucopyranose units. Glc was therefore located at the terminal of the polysaccharide. Based on these data, it is clear that JS1-1 is a unique heteropolysaccharide that is rich in galactofuranose units.

IR spectroscopy IR spectroscopy is a convenient method that can be used to develop a deeper understanding of the basic structural features of polysaccharides, including their conformation and the presence of unusual functional groups. As shown in Supplemental Fig. S1, the FTIR spectrum of JS1-1 was typical of that of a polysaccharide. The broad and intense band at 3385 cm^{-1} was attributed to the stretching vibrations of the O–H groups, whereas the signal at 2933 cm^{-1} was attributed to the stretching vibrations of the C–H bonds. The band at 1648 cm^{-1} was assigned to the bending vibration of the O–H groups, and the signal at 1537 cm^{-1} was attributed to the vibrations of the C–O bonds. The band at 1412 cm^{-1} was assigned to C–H bending vibrations, and the signal at 1053 cm^{-1} was attributed to the stretching vibrations of the C–O–C linkages of Mannan. The characteristic absorption band at 871 cm^{-1} suggested the presence of a furanose ring. Furthermore, the absorption at 814 cm^{-1} was indicative of the mannose units existing in the α-anomeric configuration [22–25].

IR spectroscopy can also be used as a characterization tool during methylation procedures, where it can provide an indication as to whether a methylation process has progressed to completion. These data can also be related to the correctness

Table 1 GC–MS analysis result of partial O-methylated alditol acetates of JS1-1

Methylated alditol acetate	MS (m/z)	Linkage type	Molar ratio
1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-d-glucitol	87,101,117,145,161,205	Glc*p*-(1→	1.8
1,4-di-O-acetyl-2,3,5,6-tetra-O-methyl-d-galactitol	89,101,117, 161,205,277	Gal*f*-(1→	1.2
1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-d-mannitol	129,161,189	→2)-Man*p*-(→	1.1
1,2,4-tri-O-acetyl-3,5,6-tri-O-methyl-d-galactitol	89,101,129,139,189,305	→2)-Gal*f*-(1→	2.8
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-d-galactitol	87,101,113,117,131,161,173,233	→5)-Gal*f*-(1→	5.1
1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-d-mannitol	87,101,117,129,161,189,233	→6)-Man*p*-(1→	1.0
1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-d-mannitol	129,189	→2,6)-Man*p*-(1→	1.0
1,2,4,6-tetra-O-acetyl-3,5-di-O-methyl-d-galactitol	101,117,129,189,233	→2,6)-Gal*f*-(1→	2.5

of the methylation analysis. After methylation, the stretching vibrations of the O–H bonds at 3385 cm^{-1} disappeared completely, whereas the intensity of the signal at 2933 cm^{-1}, which was attributed to the stretching vibrations of the C–H bonds, increased significantly. This result indicated that the methylation reaction had proceeded to a significant extent.

Partial acid hydrolysis The fraction JS1-1 was subjected to partial acid hydrolysis. After partial acid hydrolysis and dialyzation, the still retained part of the polysaccharide was used to analyze. Analysis of the monosaccharide composition following the partial acid hydrolysis of the polysaccharide revealed that the composition of the hydrolysate was very different from that of the original polysaccharide JS1-1. Analysis of the hydrolysate after dialyzation revealed that it was composed almost entirely of Man, whereas JS1-1 was rich in Gal. From the results of the methylation analysis, it was clear that the Gal in JS1-1 existed in its furanose ring form, which can be readily hydrolyzed under acidic conditions. This provided some explanation as to why the hydrolysate of JS1-1 was composed almost exclusively of Man, and also suggested that Gal and Man were located on independent domains of the polysaccharide chain. The results of the methylation analysis also revealed similar variations. The materials in the hydrolysate were predominantly (1→2,6)- and (1→6)-linked Man*p*, together with some smaller amounts of (1→2)- and (1→)-linked Man*p*. These results indicated that the Man in JS1-1 had a backbone composed of the following linkage structure: →6)-Man*p*(1→, the side chains that were composed of→2)-Man*p*(1→branched at the *O*-2 of the backbone.

NMR analysis The ^{1}H NMR spectrum of JS1-1 was recorded at 600 MHz (Fig. 2a) and revealed seven signals in the anomeric proton region in the range of 5.0–5.4 ppm. These signals were designated **A**–**G** in decreasing order according to their chemical shifts. The signals of the other protons were observed in the range of 3.5–4.4 ppm. The ^{13}C spectrum of JS1-1 (Fig. 2b) revealed numerous signals in the range of 60–110 ppm. Compared with the seriously overlapping signals in the corresponding ^{1}H NMR spectrum, it was possible to acquire more useful information from the ^{13}C spectrum. The signals at 109.3 and 106.9 ppm were characteristic of the anomeric carbons of a β-galactofuranose moiety because of their extremely low field shifts. Only the anomeric carbon signals of galactofuranose and arabinofuranose can typically reach up to 105 ppm. Based on the same reasoning, the signals at 88.39 and 83.21 ppm could be attributed to the C2 and C4 positions of β-galactofuranose, because the carbon signals of normal sugar residues would not normally exceed 82.1 ppm.

The structure of JS1-1 was subsequently elucidated using a mixture of 2D homo- and hetero-NMR experiments (Fig. S2a, H-H COSY; Fig. S2b, HMQC), which allowed for almost all of the proton and carbon signals in the main residues of the JS1-1 polysaccharide to be assigned (Table 2). Furthermore, a comparison of the chemical shift values of JS1-1 with those of model polysaccharides bearing similar linkages, as well as consideration of the methylation results, allowed for the glycosylation sites of the residues to be determined: **A**, (1→2)-linked α-D-Gal*f* [26], the furanoid ring form was confirmed by the three-bond correlations observed in the ^{1}H-^{13}C HMBC spectrum between H1 and C4 of the residue. The configuration of the anomeric protons of the furanosides can be determined from their ^{13}C chemical shifts and homonuclear coupling constants. The ^{13}C chemical shift of the anomeric carbon at 100.2 ppm and the small coupling constant of the anomeric proton (*i.e.*, $J_{H1,H2}$= 4.14 Hz) proved that **A** was in the α configuration. Anomeric signal **B** was assigned to (1→2,6)-linked β-D-Gal*f* based on the higher chemical shifts of the C2 and C6 carbons, which had moved to 88 and 70 ppm, respectively, compared with the corresponding monosaccharide [27]. Anomeric signal **C** was determined to be (1→2)-linked α-D-Man*p* because the signal for C2 had shifted downfield (δ 78 ppm) of the resonance of C-2 compared with the parent α-D-Man*p* (δ 70–72 ppm). Signal **D** was assigned to (1→)-linked α-D-Gal*f* based on the 4.14 Hz coupling constant of the anomeric proton [28, 29]. Anomeric signal **E** was determined to be that of a (1→2,6)-linked α-D-Man*p* residue because of the downfield shift of the C-2 (δ 78 ppm) and C-6 (δ 67 ppm) carbons compared with those of the parent α-D-Man*p* residue (C-2, δ 70–72 ppm; C-6, δ 61 ppm). Signal **F** was assigned to (1→5)-linked β-D-Gal*f*

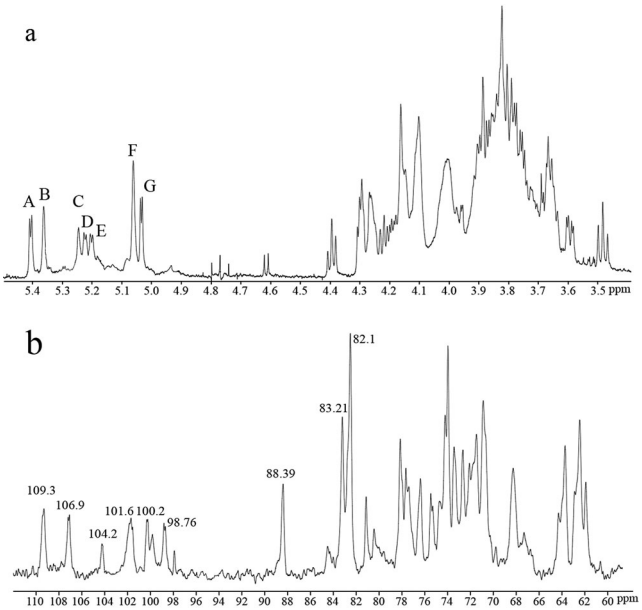

Fig. 2 NMR spectra of JS1-1. **a** ^{13}C NMR, **b** ^{1}H NMR spectrum, **a**: (1→2)-α-D-Gal*f*; **b**: (1→2,6)-β-D-Gal*f*; **c**: (1→2)-α-D-Man*p*; **d**: α-D-Gal*f*(1→; **e**: (1→2,6)-α-D-Man*p*; **f**: (1→5)-β-Gal*f*; G: α-D-Glc*p*(1→

based on the shift of the anomeric carbon at 109 ppm and the downfield shift of the C-5 carbon to 76.6 ppm [30]. Anomeric signal **G** was determined to represent a (1→)-linked α-D-Glc*p* based on the H2 chemical shift, which gave the smallest coupling constant of all of the anomeric protons at 3.54 Hz [31].

The sequence of the sugar residues in the repeating units of JS1-1 was determined by ^{1}H-^{13}C HMBC (Fig. S2c) and NOESY (Fig. S2d) analyses. The strong inter-residue H1(**A**)/C2(**B**) cross peak in the HMBC spectrum of JS1-1, as well as the H1(**A**)/H2(**B**) signal in its NOESY spectrum, indicated that the (1→2)-linked α-D-Gal*f* was linked to the C2 of the (1→2,6)-linked β-D-Gal*f*. Furthermore, the H1(**F**)/H6(**B**) signals in the NOESY spectrum of JS1-1 indicated that the (1→5)-linked β-D-Gal*f* was linked to C6 of the (1→2,6)-linked β-D-Gal*f*, whereas the H1/H5 signal in **F** confirmed the presence of an→5)-β-D-Gal*f*(1→5)-β-D-Gal*f* (1→segment. The H1(**G**)/H2(**A**) signal confirmed that the terminal α-D-Glc*p* unit was linked to C2 of the (1→2)-linked α-D-Gal*f* unit. The H1(**C**)/C2(**E**) and H1(**D**)/H2(**C**) cross signals confirmed that the (1→2)-linked α-D-Man*p* was linked to C2 of the (1→2,6)-linked α-D-Man*p* and that the terminal α-D-Gal*f* was linked to C2 of the (1→2)-linked α-D-Man*p*, respectively.

Taken together with the results above, these data suggest that the structure of the exopolysaccharide JS1-1 from *P. purpurogenum* JS03-21 was composed of two parts. One of these parts was the backbone structure, which consisted of the following repeating trisaccharide unit: →5)-β-D-Gal*f*(1→5)-β-D-Gal*f*(1→6)-β-D-Gal*f*(1→, branching occurred at *O-2* of the (1→6)-β-D-Gal*f* unit and was composed of a α-D-Glc*p*(1→2)-α-D-Gal*f* (1→segment. The second part was composed predominantly of Man, and had a backbone structure consisting of (1→6)-linked α-D-Man*p* residues, where the *O-2* position was substituted with the following branch segment: α-D-Gal*f*(1→2)-α-D-Man*p*(1→. It was envisaged that these two parts could be linked together through the *O-2* position of the (1→2,6)-linked α-D-Man*p* residue. The proposed repeating structure of JS1-1 is shown in Fig. 3.

Discussion

Several galactofuranose-containing polysaccharides have also been isolated from the fermented broths and cell walls of different fungi [32, 33]. Several species of *Penicillium*, in

Table 2 ^{1}H and ^{13}C NMR data for the residues of JS1-1

Sugar residue	^{1}H/^{13}C					
	1	2	3	4	5	6
→2)-α-D-Gal*f*(1→**A**	5.41	4.29	4.40	3.89	3.80	–
	100.23	82.1	73.1	72.0	–	–
→2,6)-β-D-Gal*f*(→ **B**	5.37	4.30	4.28	4.12	3.78	3.67/3.83
	106.92	88.39	76.38	83.21	72.11	70.29
→2)-α-D-Man*p*(1→**C**	5.26	4.12	4.03	3.70	3.86	3.93/3.77
	101.6	78.0	70.6	68.2	72.22	62.4
α-D-Gal*f*(1→**D**	5.24	4.22	4.25	–	–	–
	104.2	78.21	74.4	82.3	–	–
→2,6)-α-D-Man*p*(1→**E**	5.20	4.22	3.99	3.70	3.83	3.79/3.71
	99.7	78.7	70.25	68.2	–	67.27
→5)-β-D-Gal*f*(→ **F**	5.08	4.18	4.12	4.17	3.88	3.71/3.84
	109.3	82.5	77.4	82.5	76.6	63.4
α-D-Glc*p*(1→**G**	5.05	3.61	3.82	3.49	3.86	3.78/3.67
	98.76	72.14	74.11	70.40	72.37	61.88

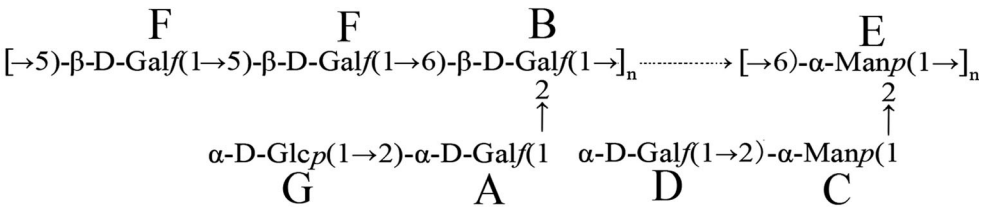

Fig. 3 The proposed structure of JS1-1 (n≈14), Gal*f*: galactofuranose, Man*p*: mannopyranose, Glc*p*: glucopyranose

particular, possess the necessary characteristics to produce water-soluble polysaccharides containing galactofuranose with different linkages and configurations. The types of structure formed in these cases can be divided into six major groups based on their structural characteristics. Based on their structural diversity, it has been proposed that polysaccharides could be distinguished on the basis of their chemotaxonomic characteristics [34]. One of the most remarkable features of the heteropolysaccharide JS1-1 from *P. purpurogenum* JS03-21 was the presence of galactofuranose as the major component. The observation that both the α and β configurations of galactofuranose are present in this structure is also very rare. Although the structure of JS1-1 is complex, it also belongs to the same structural class of polysaccharides derived from *Penicillium* species that contain galactofuranose units. However, the exopolysaccharide compounds derived from the same group were different because they had a different ratio of monosaccharides. JS1-1 had a higher Gal content than any of the other polysaccharides belonging to this group and had more (1→5)-β-D-Gal*f* and (1→2)-α-D-Man*p* type linkages. Based on this result, it was determined that although heteropolysaccharides rich in galactofuranose from the same group may have similar structure characteristics, their structures may vary in terms of their chain-length monosaccharide composition, degree of branching and the ratio of their different linkage styles. It is possible that genetic factors may have played a role in production of similar polysaccharides by different fungal species, but further work would be needed to thoroughly explore this possibility. The significance of the link between the nature of the different linkages and configurations of the galactofuranose units and the different types of *Penicillium* species also needs to be explored in greater detail.

Although galactofuranose-containing glycoconjugates have been found in various microorganisms, they have never been reported to exist in mammals. Galactofuranose residues with a variety of linkages are highly immunogenic [35] and essential for the survival or virulence of some pathogenic microorganisms. The absence of galactofuranose residues and galactofuranosidases in mammalian host cells makes these molecules potentially useful as specific targets for the treatment of parasitic and fungal diseases [36, 37]. The addition of a monoclonal antibody to structures of this type could also be useful for the design of effective tools for the diagnosis of *Aspergillus* infections [38]. Furthermore, it has been reported that some lectin has a high affinity for galactofuranose residues as well as terminal galactofuranose units [26, 39]. There is therefore a possibility that these galactofuranose-binding lectins or receptors could possess some innate immunity function. Galactofuranose-containing glycoconjugates are highly potent hydrogen-bonding compounds with a high level of stereodiversity. Compounds of this type could therefore also be used as antimycobacterial or antitumor agents [40]. Galactofuranosyltransferases involved in biosynthesis of galactofuranose of Galactofuranose-containing glycoconjugates. Disruption of the genes of galactofuranosyltransferases, the cells of microorganisms exhibited an abnormal morphology characterized by poor hyphal extension, hyphal curvature and limited formation of conidia [41]. The modified products could explored as microbial inhibitors through inhibiting the bioactivity of galactofuranosyltransferases.

The exopolysaccharide JS1-1, which is produced by the aciduric fungus *Penicillium purpurogenum* JS03-21, is a unique heteropolysaccharide that contains large amounts of galactofuranose with different configurations. Although the structure of JS1-1 has been elucidated in the current study, the function of galactofuranose has not yet been studied. JS1-1 may be a potential source of galactofuranose oligosaccharides with novel structures, and further investigations aimed at better understanding the characteristics of galactofuranose oligosaccharides derived from the controlled acid hydrolysis of JS1-1 are currently underway in our laboratory. It is envisaged that the potential biological functions of these unique galactofuranose oligosaccharides from JS1-1 will attract considerable interest from researchers working in numerous fields.

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References

1. Morozkina, E.V., Slutskaya, E.S., Fedorova, T.V., Tugay, T.I., Golubeva, O.V., Koroleva, L.I.: Extremophilic microorganisms: biochemical adaptation and biotechnological application. Appl. Biochem. Micro. **46**, 1–14 (2010)
2. Podar, M., Reysenbach, A.-L.: New opportunities revealed by biotechnological explorations of extremophiles. Curr. Opin. Biotechnol. **17**, 250–255 (2006)

3. Wilson, Z.E., Brimble, M.A.: Molecules derived from the extremes of life. Nat. Prod. Rep. **26**, 44–71 (2009)
4. Buommino, E., Schiraldi, C., Baroni, A., Paoletti, I., Lamberti, M., De Rosa, M., Tufano, M.A.: Ectoine from halophilic microorganisms induces the expression of hsp70 and hsp70B' in human keratinocytes modulating the proinflammatory response. Cell Stress Chaperones **10**, 197–203 (2005)
5. Antranikian, G., Vorgias, G.E., Bertoldo, C.: Extreme environments as a source for microorganisms and novel biocatalysts. Adv. Biochem. Eng. Biotechnol. **96**, 219–262 (2005)
6. Nicolaus, B., Schiano moriello, V., Lama, I., Poli, A., Gambacorta, A.: Polysaccharides from extremophilic microorganisms. Orig. Life Evol. Biosph. **34**, 159–169 (2004)
7. Poli, A., Anzelmo, G., Nicolaus, B.: Bacterial exopolysaccharides from extreme marine habitats: production, Characterization and Biological Activities. Mar. Drugs **8**, 1779–1802 (2010)
8. Raveendran, S., Dhandayuthapani, B., Nagaoka, Y., Yoshida, Y., Maekawa, T., Kumar, D.S.: Biocompatible nanofibers based on extremophilic bacterial polysaccharide, Mauran from halomonas Maura. Carbohydr. Polym. **92**, 1225–1233 (2013)
9. Johnson, D.B.: Biodiversity and ecology of acidophilic microorganisms. FEMS Microbiol. Ecol. **27**, 307–317 (1998)
10. Wang, X., Yuan, Y., Wang, K., Zhang, D., Yang, Z., Xu, P.: Deproteinization of gellan gum produced by *Sphingomonas paucimobilis* ATCC 31461. J. Biotechnol. **128**, 403–407 (2007)
11. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F.: Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**, 350–356 (1956)
12. Bensadoun, A., Weinstein, D.: Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**, 241–250 (1976)
13. Towns, T.G.: Determination of aqueous phosphate by ascorbic acid reduction of phosphomolybdic acid. Anal. Chem. **56**, 223–229 (1986)
14. Therho, T.T., Hartiala, K.: Method for determination of the sulfate content of glycosamino glycans. Anal. Biochem. **41**, 471–476 (1971)
15. Chen, Y., Mao, W.J., Yang, Y.P., Teng, X.C., Zhu, W.M., Li, H.Y., Qi, X.H., Chen, Y.L., Zhao, C.Q., Hou, Y.J., Wang, C.Y., Li, N.: Structure and antioxidant activity of an extracellular polysaccharide from coral-associated fungus, *Aspergillus versicolor* LCJ-5-4. Carbohydr. Polym. **87**, 218–226 (2012)
16. Liu, X., Sun, Z.L., Zhang, M.S., Meng, X.M., Xia, X.K., Yuan, W.P., Feng, X., Liu, C.H.: Antioxidant and antihyperlipidemic activities of polysaccharides from sea cucumber *Apostichopus japonicus*. Carbohydr. Polym. **90**, 1664–1670 (2012)
17. Ciucanu, I., Kerek, F.: A simple and rapid method for the permethylation of carbohydrate. Carbohydr. Res. **131**, 183–196 (1984)
18. Hung, W.T., Wang, S.H., Chen, Y.T., Yu, H.M., Chen, C.H., Yang, W.B.: MALDI-TOF MS analysis of native and permethylated or benzimidazole-derivatized polysaccharides. Molecules **17**, 4950–4961 (2012)
19. Asker, M.M.S., Shawky, B.T.: Structural characterization and antioxidant activity of an extracellular polysaccharide isolated from *Brevibacterium otitidis* BTS 44. Food Chem. **123**, 315–320 (2010)
20. Tulskaya, E.M., Senchenkova, S.N., Evtushenko, L.I., Shashkov, A.S., Naumova, I.B.: A new neutral polymer from the cell wall of actinomycete *kineosporia aurantiaca* VKM Ac-702. Carbohydr. Res. **340**, 1247–1251 (2005)
21. Chen, Y.L., Mao, W.J., Wang, J.F., Zhu, W.M., Zhao, C.Q., Li, N., Wang, C.Y., Yan, M.X., Guo, T., Liu, X.: Preparation and structural elucidation of a glucomannogalactan from marine fungus *Penicillium commune*. Carbohydr. Polym. **97**, 293–299 (2013)
22. Shingel, K.I.: Determination of structural peculiarities of dextran, pullulan and γ-irradiated pullulan by Fourier-transform IR spectroscopy. Carbohydr. Res. **337**, 1445–1451 (2002)
23. Galichet, A., Sockalingum, G.D., Belarbi, A., Manfait, M.: FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype. FEMS Microbiol. Lett. **197**, 179–186 (2001)
24. Kacurakova, M., Mathlouthi, M.: FTIR and laser-Raman spectra of oligosaccharides in water: characterization of the glycosidic bond. Carbohydr. Res. **284**, 145–157 (1996)
25. Joo, J.H., Yun, J.W.: Structural and molecular characterization of extracellular polysaccharides produced by a new fungal strain, *Trichoderma erinaceum* DG-312. J. Microbiol. Biotechnol. **15**, 1250–1257 (2005)
26. Gerwig, G.J., de Waard, P., Kamerling, J.P., Vliegenthart, J.F.G.: Novel O-Linked Carbohydrate Chains in the Cellulase Complex (Cellulosome) of *Clostridium thermocellum*. J. Biol. Chem. **264**, 1027–1035 (1989)
27. Ahrazem, O., Prieto, A., Leal, J.A., Gimćnez-Abián, M.I., Jimćnez-Barnero, J., Bernabć, M.: Fungal cell wall polysaccharides isolated from *Discula destructiva* spp. Carbohydr. Res. **342**, 1138–1143 (2007)
28. Gerwig, G.J., Kamerling, J.P., Vliegenthart, J.F.G., Morag, E., Lamed, R., Bayer, E.A.: Novel oligosaccharide constituents of the cellulase complex of *Bacteroides cellulosolvens*. Eur. J. Biochem. **205**, 799–808 (1992)
29. Ahrazem, O., Prieto, A., San-Blas, G., Leal, J.A., Jimćnez-Barbero, J., Bernabć, M.: Structural differences between the alkali-extracted water-soluble cell wall polysaccharides from mycelial and yeast phases of the pathogenic dimorphic fungus *Paracoccidioides brasiliensis*. Glycobiology **13**, 743–747 (2003)
30. Chen, Y., Mao, W.J., Wang, B.F., Zhou, L.N., Gu, Q.Q., Chen, Y.L., Zhao, C.Q., Li, N., Wang, C.Y., Shan, J.M., Yan, M.X., Lin, C.: Preparation and characterization of an extracellular polysaccharide produced by the deep-sea fungus *Penicillium griseofulvum*. Bioresour. Technol. **132**, 178–181 (2013)
31. Shibata, N., Okawa, Y.: Chemical structure of β-galactofuranose-containing polysaccharide and O-linked oligosaccharides obtained from the cell wall of pathogenic dematiaceous fungus *Fonsecaea pedrosoi*. Glycobiology **21**, 69–81 (2011)
32. Heiss, C., Skowyra, M.L., Liu, H., Klutts, J.S., Wang, Z., Williams, M., Srikanta, D., Beverley, S.M., Azadi, P., Doering, T.L.: Unusual galactofuranose modification of a capsule polysaccharide in the pathogenic yeast *Cryptococcus neoformans*. J. Biol. Chem. **288**, 10994–11003 (2013)
33. Cordeiroa, L.M.C., de Oliveirab, S.M., Buchib, D.F., Iacominia, M.: lichen *Ramalina gracilis* and its effect on macrophage activation. Int. J. Biol. Macromol. **42**, 436–440 (2008)
34. Leal, J.A., Gómez-Miranda, B., Prieto, A., Domenech, J., Ahrazem, O., Bernabć, M.: Possible chemotypes from cell wall polysaccharides, as an aid in the systematics of *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Mycol. Res. **101**, 1259–1264 (1997)
35. Notermans, S., Dufrenne, J., Wijnands, L.M., Engel, H.W.B.: Human serum antibodies to extracellular polysaccharides (EPS) of moulds. J. Vet. Intern. Med. **26**, 41–48 (1988)
36. Tefsen, B., Ram, A.F.J., Die, I.V., Routier, F.H.: Galactofuranose in eukaryotes: aspects of biosynthesis and functional impact. Glycobiology **22**, 456–469 (2012)
37. Shibata, N., Saitoh, T., Tadokoro, Y., Okawa, Y.: The cell wall galactomannan antigen from *Malassezia furfur* and *Malassezia pachydermatis* contains β-1,6-linked linear galactofuranosyl residues and its detection has diagnostic potential. Microbiology **155**, 3420–3429 (2009)
38. Stynen, D., Sarfati, J., Goris, A., Prevost, M.C., Lesourd, M., Kamphuis, H., Darras, V., Latge, J.P.: Rat monoclonal antibodies against aspergillus galactomannan. Infect. Immun. **60**, 2237–2245 (1992)
39. Soares, R.M.A., Angluster, J., De Souza, W., Alviano, C.: Carbohydrate and lipid components of hyphae and conidia of the

human pathogen *Fonsecaea pedrosoi*. Mycopathologia **132**, 71–77 (1995)

40. Peltier, P., Euzen, R., Daniellou, R., Nugier-Chauvin, C., Ferrières, V.: Recent knowledge and innovations related to hexofuranosides: structure, synthesis and applications. Carbohydr. Res. **343**, 1897–1923 (2008)
41. Komachi, Y., Hatakeyama, S., Motomatsu, H., Futagami, T., Kizjakina, K., Sobrado, P., Ekino, K., Takegawa, K., Goto, M., Nomura, Y., Oka, T.: GfsA encodes a novel galactofuranosyltransferase involved in biosynthesis of galactofuranose antigen of O-glycan in *Aspergillus nidulans* and *Aspergillus fumigatus*. Mol. Microbiol. **90**, 1054–1073 (2013)