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The structure of the glucuronoxylomannan produced by culinary-medicinal yellow brain mushroom (*Tremella mesenterica* Ritz.:Fr., Heterobasidiomycetes) grown as one cell biomass in submerged culture

Evgeny Vinogradov,[a,*] Bent O. Petersen,[b] Jens Ø. Duus[b] and Solomon Wasser[c]

[a]*Institute for Biological Sciences, National Research Council, 100 Sussex Dr., Ottawa, ON, Canada K1A 0R6*
[b]*Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Copenhagen Denmark*
[c]*Institute of Evolution, Haifa University, Mt. Carmel, Haifa 31905, Israel*

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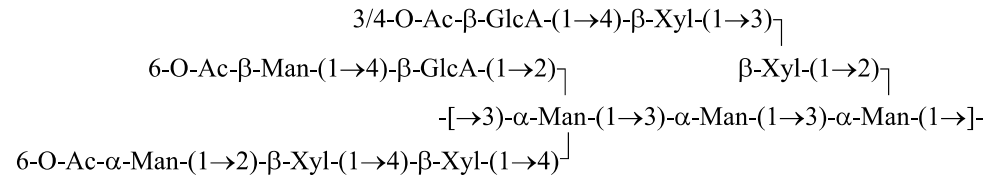
Abstract—The yellow brain mushroom *Tremella mesenterica* possesses a wide spectrum of medicinal properties, including immunostimulating, protecting against radiation, antidiabetic, anti-inflammatory, hypocholesterolemic, hepatoprotective, and antiallergic effects. A unique feature of *T. mesenterica* is that most of the above mentioned medicinal properties depend on glucuronoxylomannan (GXM) contained in fruiting bodies or produced in pure culture conditions. We developed a new strain of *T. mesenterica* CBS 101939, which grows in submerged culture and offers superior yields of one-cell biomass rich in exocellular heteropolysaccharide GXM. The structure of the GXM was analyzed by NMR spectroscopy and chemical methods. The polysaccharide has a defined repeating unit structure, which is O-acetylated at several points:



These results differ from previously published structure of *Tremella* extracellular polysaccharides, where mannan backbone was believed to be randomly glycosylated with xylan chains of different length.
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Keywords: *Tremella mesenterica*; Basidiomycetes; Fungi; Extracellular polysaccharide; Glucuronoxylomannan

1. Introduction

Higher Basidiomycetes mushrooms are unlimited sources of anticancer and immunostimulating polysaccharides.[1,2] Biologically active polysaccharides are widespread among higher Basidiomycetes mushrooms, and most of them have unique structures in different species. Moreover, different strains of a single higher Basidiomycetes species can produce polysaccharides with different properties. The development of antitumor polysaccharides from eight Japanese and Chinese medicinal mushrooms was reviewed by Mizuno.[3,4] For example, the proteoglucan krestin was developed in Japan from the strain of *Coriolus versicolor* CM-101, whereas polysaccharide–peptide (PSP) in China was

Abbreviations: GXM, glucuronoxylomannan

* Corresponding author. Tel.: +1-613-990-08-32; fax +1-613-952-90-92; e-mail: evguenii.vinogradov@nrc-cnrc.gc.ca

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developed from submerged cultures of the strain Cov-1 of the same species. Both proteoglucans have the same polysaccharide component but different protein molecules bound to the polysaccharide.[5]

The genus *Tremella* Pers. (Tremellales, Higher Basidiomycetes) belongs to the so-called 'Jelly Mushrooms' group that form gelatinous fruiting bodies. About 80 species have been recognized and none of them are known to be poisonous.[6,7] Jelly mushrooms from the genus *Tremella* have been used as food and folk medicines for centuries in many Asian countries.[8] During the past decades they have been intensively investigated for medicinal effects in in vivo and in vitro model system, and clinical applications were developed for different groups of patients.[9–14] *Tremella* species shows antitumor activity due to immunomodulating effects on both humoral and cellular immune factors in the body. *Tremella* species also stimulate vascular endothelial cells, possess pronounced antiradiation effects, stimulate hematogenesis, demonstrate antidiabetic, anti-inflammatory, hypocholesterolemic, antiallergic activities, and show hepatoprotective effects.[1]

A unique feature of *Tremella* mushrooms is that the majority of these medicinal properties depend on GXM contained in fruiting bodies, or those produced under pure culture conditions. GXM consists of a linear backbone of $(1\rightarrow3)$-linked α-D-mannose with mainly xylose and glucuronic acid in the side chains.

The cultivation of mushrooms to produce fruiting bodies is a long-term process requiring from one to several months for the first fruiting bodies to appear. The growth of mushroom cell cultures in submerged conditions in a liquid culture media accelerates the process, resulting in biomass yield within a few days. Further optimization of the culture medium composition and physicochemical conditions of growth allows regulation of fungal metabolism in order to obtain standardized nutriceutical substances in higher yield.

Recently, a process for *T. mesenterica* GXM production was developed for the first time.[2,15] The yield of crude precipitate (Tremellastin) containing *T. mesenterica* one-cell biomass and extracellular polysaccharides after 4–5 days of submerged cultivation attained 24–25 g/L of dry biomass. The composition of the crude product may be regulated in submerged culture conditions to alter the proportional content of GXM, whose amount can exceed 50% of the total yield.[15,16]

Tremellastin at a dose of 100 and 500 mg/kg by per oral administration for 15 consecutive days shows a statistically significant and dose-dependent reduction of intrinsic blood glucose levels after 15 treatment days, as well as significantly decreased triglyceride levels in the glucose-loading-assigned Tremellastin-treatment groups. It was shown that the Tremellastin preparation appeared to be an active interferonogen at the concentration of 10 mg/animal. At the same concentration, this compound was the most efficient activator of oxygen-dependent biocidactivity of macrophages. The activation of the oxygen-dependent macrophage biocide activity was associated with the induction of endogenous interferon by Tremellastin.[17] The biological activity of Tremellastin was shown to be due to the presence of GXM; other polysaccharides (glucan) demonstrated no activity.

GXM from *T. mesenterica* NRRL Y-6158 has been analyzed previously by chemical methods, mostly methylation and periodate oxidation, which led to the conclusion that it has α-$(1\rightarrow3)$-mannan main chain, randomly glycosylated with short xylan oligosaccharides and glucuronic acid.[18] NMR analysis was applied to the analysis of the polymeric product derived from *T. mesenterica* NRRL Y-6151 by Smith degradation.[19] We present here results of the detailed analysis of the GXM, isolated from culture grown *T. mesenterica* CBS 101939.

2. Experimental

2.1. NMR spectroscopy and general methods

NMR spectra were recorded at 40 or 50 °C in D_2O on a Varian UNITY INOVA 800 instrument at 799.96 MHz for proton and 201.12 MHz for carbon, using acetone as reference for proton (2.225 ppm) and 1,4-dioxane for carbon (67.4 ppm). Standard Varian programs tndqcosy, tnnoesy (mixing time of 100 ms), tntocsy (spinlock time 80 ms), gHSQC, gHSQCTOCSY, gHSQCNOESY, and gHMBC were used with digital resolution in F2 dimension <2 Hz/pt. Spectra were assigned using computer program PRONTO.[20]

For the monosaccharide analysis, hydrolysis was performed with 4 M CF_3CO_2H (110 °C, 3 h), monosaccharides were conventionally converted into the alditol acetates and analyzed by GLC on a Agilent 6850 chromatograph equipped with a DB-17 (30 m × 0.25 mm) fused-silica column and using a temperature gradient of 180 °C (2 min) → 240 °C at 2 °C/min. GC–MS was performed on a Varian Saturn 2000 system with an ion-trap mass spectral detector, using the same column.

Gel chromatography was carried out on Sephadex G-50 (2.5 × 95 cm) and Sephadex G-15 columns (1.6 × 80 cm) in pyridinium-acetate buffer, pH 4.5 (4 mL pyridine and 10 mL AcOH in 1 L water) and the eluate was monitored by refractive index.

For the determination of the absolute configuration of the monosaccharides products **1a**,**b** and 2 (1 mg each) were treated with 10:1 (*S*)-2-butanol–AcCl (0.25 mL, 2 h, 85 °C), dried under the stream of air, acetylated, and analyzed by GC in comparison with authentic standards, prepared with (*S*)- and (*R*)-2-butanol.

2.2. Preparation of GXM

The polysaccharide mixture (500 mg), obtained from *T. mesenterica* culture liquid by EtOH precipitation, was treated with ultrasound (Bransonic, at 50% cycle, power 6) in 3% H_2O_2 (300 mL) for 10 min without cooling, insoluble glucan was removed by ultracentrifugation at 40,000 rpm (3 h), solution dialyzed, and dried to give GXM (300 mg). The specific rotation of the GXM was $[\alpha]_D$ −15.3 (*c* 0.4, water).

2.3. Methylation analysis

2.3.1. Deacylating conditions. [21] The polysaccharide (5 mg) was dissolved in Me_2SO (1 mL) (5 min at 80 °C), pulverized NaOH (20 mg) was added, the mixture stirred for 20 min, methyl iodide (0.3 mL) was added, and the mixture stirred for 1 h. Methyl iodide was then removed under a stream of air. Water (10 mL) was added, the mixture passed through a SepPak C18 cartridge, washed with water, the product eluted with MeOH (5 mL), hydrolyzed, monosaccharides converted to 1*d*-alditol acetates by conventional methods, and analyzed by GC–MS. Part of the methylated polysaccharide was reduced with $NaBH_4$ in 80% EtOH (2 h, 25 °C) prior to hydrolysis, and material was recovered by SepPak adsorbtion as described above.

2.3.2. Nondeacylating conditions.[22] Polysaccharide (5 mg) was dissolved in trimethylphosphate (2 mL) at 60–80 °C (30–60 min required for complete dissolution, polymers were completely insoluble at room temperature), methyl triflate (0.3 mL) was added and the mixture kept at 40 °C for 1 h. Water (10 mL) was added, the mixture passed through a SepPak C18 cartridge, washed with water, the product eluted with MeOH (5 mL), hydrolyzed, monosaccharides converted to 1*d*-alditol acetates by conventional methods, and analyzed by GC–MS.

2.4. Treatment of GXM with Li in ethylenediamine[23–25]

GXM (200 mg) was dissolved in anhyd ethylenediamine (5 mL) and pieces of metallic lithium (about 3 cm of 19 × 0.75 mm lithium ribbon, Sigma, cut in small pieces) were added. The mixture was stirred until a deep blue color appeared (24 h), poured in water (200 mL), and dialyzed against water, dried, passed through a Sephadex G-50 column (2.5 × 80 cm), and the polysaccharide fraction was dried to give 80 mg of GXM-Li.

2.5. Deacetylation of GXM

GXM (50 mg) was dissolved in 5% NH_3 (20 mL), kept at 50 °C for 16 h, and dried to give GXM-deac (40 mg, part of the product became insoluble and was removed by centrifugation).

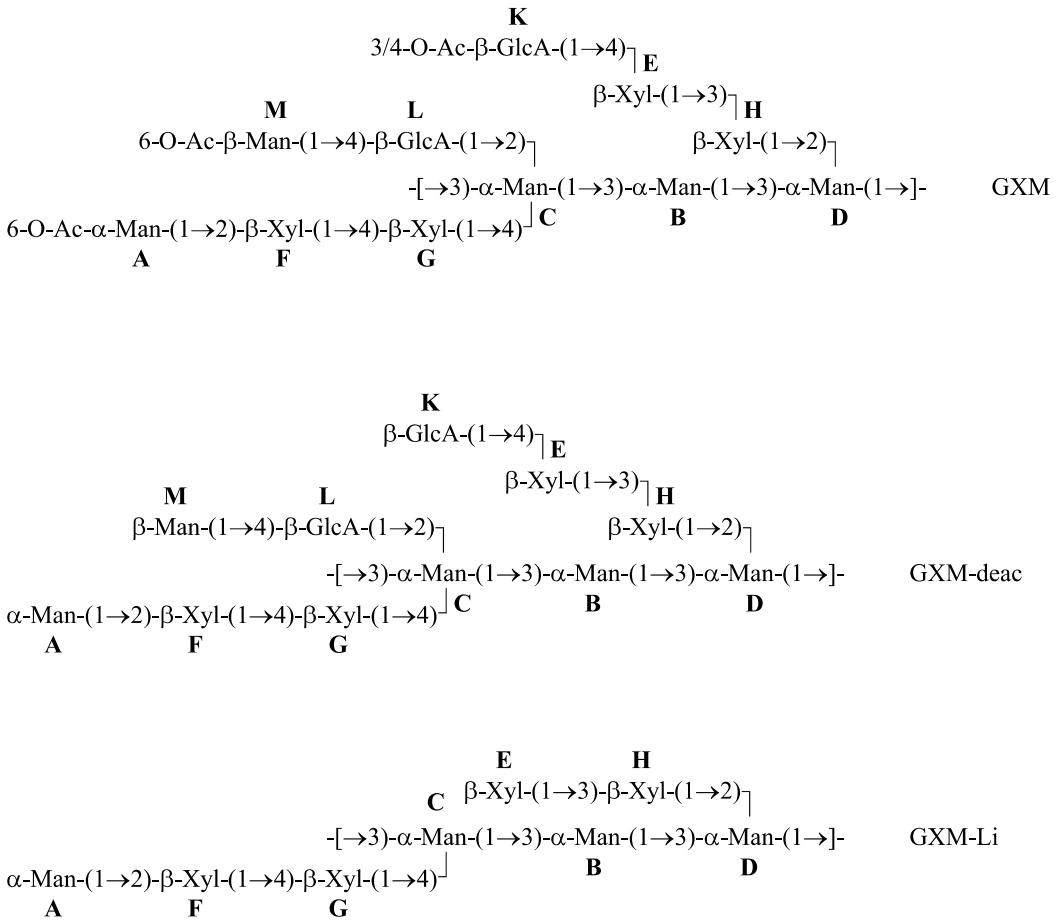
3. Results

Reduced molecular mass GXM was prepared from the mixture of extracellular polysaccharides, mostly GXM and glucan, produced by *T. mesenterica*, by ultrasound treatment in 3% H_2O_2 and ultracentrifugation, where GXM remained in solution and glucan precipitated. GXM obtained in this way gave moderately viscous transparent solutions at concentrations up to 20 mg/mL. It contained glucuronic acid, xylose, and mannose in the molar ratio of 0.36:0.75:1, as determined by GC of the acetylated products of methanolysis, or by combination of the data from GC determination of neutral monosaccharides as alditol acetates and a color test for GlcA. Glucose was also detected by GC analysis of the alditol acetates. It was the product of the partial reduction of the glucuronolactone, formed from GlcA after acid hydrolysis. This was confirmed by the monosaccharide analysis with the reduction of the hydrolyzate with deuterated borohydride, which led to the formation of 1,6,6-trideuteroglucitol. Thus glucan component was efficiently removed during purification.

Two derivatives of GXM were obtained (Scheme 1). GXM-Li was produced by the treatment of GXM with Li in anhyd ethylenediamine, which led to O-deacetylation, removal of uronic acids and the β-Man residue **M**. Another portion of GXM was deacylated with 5% ammonia to give GXM-deac.

NMR analysis of GXM-Li showed that it had a regular structure with an octasaccharide repeating unit. Spectra were well resolved, and the integral intensity of all anomeric signals was similar, which is characteristic of regular polysaccharides (Fig. 1). The identity of the monosaccharides was established on the basis of ^1H and ^{13}C NMR chemical shifts and $^3J_{H,H}$ coupling constants. The β-configuration of the Xyl residues was confirmed by the observation of the intraresidue NOEs between H-1 and H-3, H-1 and H-5. All expected transglycosidic NOE and most of the corresponding HMBC correlations, indicating monosaccharide sequence, were observed (Table 1). The structure was confirmed by methylation analysis, where the residues of terminal, 2-, 3-, and 4-substituted xylopyranose, terminal, 3-, 2,3-, and 3,4-substituted mannopyranose were identified. Alditols derived from 2- and 4-substituted xylopyranose residues differ only by deuteration and thus coelute in GC, but the presence of both of them could be detected in the mass spectra.

The NMR spectra of the GXM-deac were then assigned (Fig. 2). Proton NMR signals of mannose residues in the main chain partially overlap and gave weak COSY and TOCSY correlations due to fast relaxation, but assignment was possible with the use of the data for GXM-Li and the results of methylation analysis. This product differed from GXM-Li by the presence of additional two residues of GlcA, linked to Man **C** and



Scheme 1. Structure of the GXM, its deacylated derivative and a product of lithium-ethylenediamene degradation.

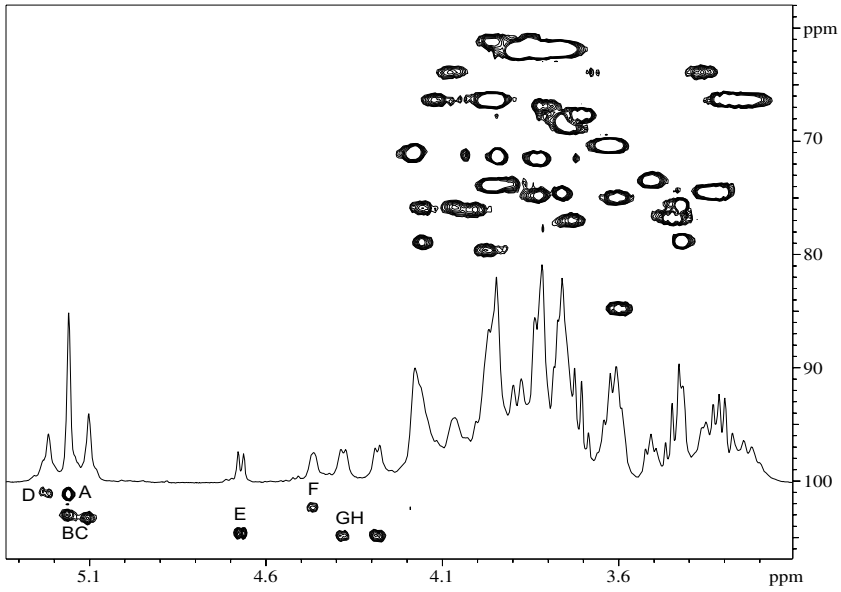


Figure 1. ^{1}H–^{13}C HSQC spectrum of the GXM-Li polysaccharide.

Xyl **E** and by the presence of β-Man **M**, which was lost after ethylenediamine-Li treatment together with GlcA **L**. Identification of the position of β-Man residue **M** presented some difficulties because of strong intraglycosidic

Table 1. NMR data for *T. mesenterica* GXM, GXM-deac, and GXM-Li polysaccharides

Unit	Nucleus	1	2	3	4	5(5a)	6a(5b)	6b	NOE	HMBC
A, GXM-Li	^{1}H	5.16	3.94	3.83	3.70	3.96	3.77	3.83	F1, F2, G4	F2
	^{13}C	101.2	71.4	71.6	67.6	73.9	62.1			
A, GXM-deac	^{1}H	5.17	4.00	3.89	3.72	4.01	3.80	3.86	F2	F2
	^{13}C	101.2	71.3	71.5	67.7	73.9	61.9			
A, GXM	^{1}H	5.14	4.01	3.89	3.76	4.20	4.31	4.40	F2	F2
	^{13}C	101.4	71.3	71.5	68.8	71.7	64.8			
B, GXM-Li	^{1}H	5.16	4.18	3.97	3.82	3.83	3.74	3.90	D3	
	^{13}C	103.0	71.1	79.8	67.0	74.9	62.0			
B, GXM-deac	^{1}H	5.28	4.21	4.04	3.88	3.83	3.75	3.87	D3	
	^{13}C	102.6	71.0	79.8	66.5	74.7	61.9			
B, GXM	^{1}H	5.28	4.20	4.03	3.87	3.84	3.75	3.87	D3	
	^{13}C	102.7	70.9	79.8	66.7	74.8	61.9			
C, GXM-Li	^{1}H	5.10	4.19	4.16	4.01	3.92	3.85	3.96	B3	B3
	^{13}C	103.3	71.1	76.0	76.2	73.9	61.2			
C, GXM-deac	^{1}H	5.18	4.28	4.21	4.01	3.94	3.93	3.96	B3	B3
	^{13}C	101.2	78.6	75.8	75.8	73.7	60.6			
C, GXM	^{1}H	5.19	4.28	4.22	4.03	3.94	3.93	3.95	B3	
	^{13}C	101.3	78.6	75.6	75.9	73.8	60.6			
D, GXM-Li	^{1}H	5.22	4.16	4.07	3.76	3.77	3.74	3.90	C3, H1	C3
	^{13}C	101.1	79.0	75.7	68.2	74.7	62.0			
D, GXM-deac	^{1}H	5.12	4.17	4.01	3.63	3.83	3.75	3.87	C3	
	^{13}C	102.2	79.1	75.9	69.3	74.7	61.9			
D, GXM	^{1}H	5.13	4.17	4.00	3.63	3.83	3.75	3.87	H1, C3	
	^{13}C	102.1	79.1	75.9	67.8	74.7	61.9			
E, GXM-Li	^{1}H	4.67	3.31	3.45	3.61	3.30	3.96		H3, H2w	H3
	^{13}C	104.7	74.6	76.8	70.4	66.4				
E, GXM-deac	^{1}H	4.73	3.39	3.62	3.85	3.40	4.12		H3	H3
	^{13}C	104.4	74.5	75.0	77.9	64.1				
E, GXM	^{1}H	4.72	3.39	3.63	3.84	3.41	4.12		H3	H3
	^{13}C	104.4	74.5	75.0	78.0	64.1				
F, GXM-Li	^{1}H	4.47	3.42	3.42	3.64	3.25	3.97		G4, G5	G4
	^{13}C	102.4	78.9	75.6	70.5	66.4				
F, GXM-deac	^{1}H	4.45	3.44	3.49	3.67	3.28	3.98		G4, G6	G4
	^{13}C	102.6	78.9	75.7	70.4	66.5				
F, GXM	^{1}H	4.45	3.41	3.49	3.67	3.28	3.99		G4	G4
	^{13}C	102.6	79.2	75.6	70.5	66.5				
G, GXM-Li	^{1}H	4.38	3.36	3.61	3.74	3.37	4.08		C4, C6	C4
	^{13}C	104.9	74.5	75.1	77.1	64.0				
G, GXM-deac	^{1}H	4.41	3.37	3.63	3.71	3.37	4.05		C4, C6	C4
	^{13}C	104.9	74.6	75.0	77.3	63.8				
G, GXM	^{1}H	4.41	3.36	3.63	3.71	3.37	4.05		C4, C6	C4
	^{13}C	104.9	74.6	74.9	77.3	64.0				
H, GXM-Li	^{1}H	4.28	3.51	3.60	3.75	3.22	4.13		D1, D2	
	^{13}C	104.9	73.6	84.8	68.8	66.4				
H, GXM-deac	^{1}H	4.27	3.54	3.63	3.77	3.23	4.15		D2	
	^{13}C	104.9	73.6	84.8	68.8	66.5				
H, GXM	^{1}H	4.28	3.53	3.63	3.77	3.23	4.16		D1, D2	D2
	^{13}C	104.9	73.6	84.8	68.8	66.5				
K, GXM-deac	^{1}H	4.53	3.31	3.51	3.51	3.73			E4, E6	E4
	^{13}C	102.2	73.9	76.5	72.9	76.8				
K, GXM	^{1}H	4.54	3.32	3.52	3.53	3.80			E4	E4
	^{13}C	102.3	73.9	76.5	72.8	76.6				
3-O-Ac-K, GXM	^{1}H	4.65	3.49	4.99	3.72	3.87			E4	E4
	^{13}C	101.7	72.2	78.0	71.2	76.4				
4-O-Ac-K, GXM	^{1}H	4.57	3.41	3.73	4.87	3.89			E4	E4
	^{13}C	102.0	73.9	74.6	73.6	74.6				
L, GXM-deac	^{1}H	4.45	3.46	3.62	3.83	3.74			C1, C2	C2
	^{13}C	102.6	73.3	75.0	81.5	76.8				
L, GXM	^{1}H	4.46	3.47	3.63	3.81	3.77			C1, C2	C2
	^{13}C	102.7	73.2	75.1	82.5	76.6				
M, GXM-deac	^{1}H	4.63	3.95	3.62	3.57	3.38	3.74	3.92	L4	L4
	^{13}C	101.1	71.9	73.8	67.9	77.5	62.1			
M, GXM	^{1}H	4.64	3.97	3.64	3.63	3.59	4.28	4.47	L4	L4
	^{13}C	101.9	71.7	73.7	69.1	75.0	64.8			

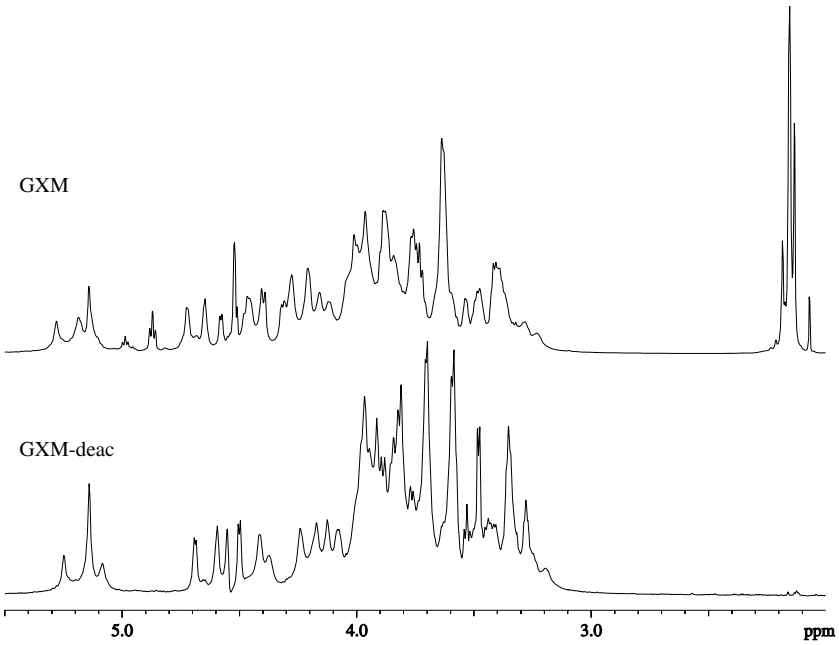


Figure 2. ^{1}H NMR spectra of the GXM and GXM-deac polysaccharides.

NOEs, overlapped with transglycosidic correlations. Combined NMR and methylation data show that all monosaccharides are present in stoichiometric amount and thus polymer is built up from defined repeating units.

In the methylation analysis of the GXM-deac the residues of 2-, 3-, and 4-substituted xylopyranose, and terminal, 3-, 2,3-, and 2,3,4-substituted mannopyranose were detected. Part of the sample of methylated GXM-deac was treated with $NaBH_4$ in order to reduce uronic acid esters. Subsequent hydrolysis, reduction, acetylation, and GC–MS analysis showed one new peak with the mass spectrum of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-1*d*-glucitol, derived from 4-substituted GlcA **L**. However, no products originating from the terminal GlcA **K** was detected for unknown reasons.

Finally, GXM was analyzed. NMR data (Table 1, Fig. 2) indicated that it contained the same sugar components present in GXM-deac and additionally several *O*-acetyl groups per repeating unit. Downfield shifts of the **A**6, **M**6, **K**3/**K**4 (in different variants of **K**) proton signals indicate acetylation positions, which were confirmed by the observations of NOEs from acetyl methyl groups to respective monosaccharide protons. *O*-Acetates at **A**6 and **M**6 were present stoichiometrically. The residue **K** was present in three variants: acetylated at O-3 $\sim$ 30%, acetylated at O-4 $\sim$ 50%, and nonacetylated $\sim$20%. Methylation of GXM by the Ciucanu–Kerek procedure (Me_2SO–NaOH–MeI) gave the same products as obtained by methylation of GXM-deac. Methylation in nonbasic conditions was then performed using methyl triflate in trimethylphosphate, which led to the same products as resulted in NaOH–Me_2SO–MeI methylation, except that the alditol derived from terminal mannopyranose residues disappeared and the product from 6-substituted mannopyranose was observed instead, indicating 6-*O*-acetylation of both residues **A** and **M**.

4. Discussion

GXM from *T. mesenterica* NRRL Y-6158 was analyzed previously[19] and its structure was described as being an α-(1 $\rightarrow$ 3)-mannan main chain randomly glycosylated with xylose oligosaccharides and glucuronic acid, although Smith degradation of the *T. mesenterica* Y-6151 led to the mannan with every third mannose substituted with glucuronic acid.[18] A similarly irregular structure was ascribed to GXM from *T. fuciformis* Berk[26] and *T. aurantia*;[27] these hypotheses were summarized in a recent review.[28] We were able to show here that GXM from *T. mesenterica* CBS 101939 has a defined oligosaccharide repeating unit, with two stoichiometric acetyl groups and one nonstoichiometric, distributed between two positions in the same monosaccharide. This is not easily visible because of the low quality NMR spectra of the native polymer. The crucial experiment was Li-EDA treatment of the polysaccharide, which led to simpler polymer with a clearly visible repeating unit. In the O-deacylated and native GXM signal intensities in 2D spectra were different for all monosaccharides due to different relaxation times, but no spin-system or transglycosidic NOE and HMBC correlation remained

unassigned and thus the possibility of a random structure is excluded. GXM is no less regular than O-chains of the lipopolysaccharides, whose regularity is accepted as a rule. These data may indicate that polymers from other fungi are more regular than was thought. In fact, most strains of *Cryptococcus neoformans* contain well defined structures,[29] which can change with spontaneous phenotypic variations.[30]

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