Isolation and Characterization of Glycoproteins from the Yeast *Cryptococcus laurentii* var. *laurentii* II. Extracellular Glycoproteins

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The extracellular polysaccharide mixture of *Cryptococcus laurentii* var. *laurentii* was fractionated into three chemically distinct heteroglycans by differential precipitation. The compositional analysis of glucuronoxylomannan (GXM) revealed D-mannose (48.8 %), D-xylose (43.8 %), and D-glucuronic acid (7.4 %). It contained 2.3 % protein, 2.1 % phosphorus, and had relative molecular mass 2.4 × 10^6. The glucomannan (GM) contained 6.5 % protein, 1.0 % phosphorus, and consisted of D-mannose (94.2 %) and D-glucose (5.2 %). The relative molecular mass was determined to be 4.5 × 10^4. The galactoglucoxylomannan (GalGXM) was composed of D-mannose (70.1 %), D-galactose (18.0 %), D-glucose (5.7 %), and D-xylose (6.3 %). It contained 6.5 % protein, 0.7 % phosphorus, and had relative molecular mass 1.9 × 10^4. The results of structural analyses of the extracellular glycoproteins showed 1,6-linked α-D-mannopyranan backbone of GM and GalGXM, and 1,3-linked backbone of GXM.

The cell envelope of the yeast *Cryptococcus laurentii* is composed of a rigid cell wall and capsules. Capsule is the most outstanding characteristic of *Cryptococcus* genus. In addition to forming capsules, several species excrete extracellular polysaccharides [1]. The major glycoprotein isolated from cell envelope of *C. laurentii* was composed of D-mannose, D-galactose, D-xylose, L-arabinose, and D-glucuronic acid [2, 3].

From the cell wall of *Cryptococcus laurentii* var. *laurentii* two water-soluble glycoprotein fractions were isolated and characterized. High-molecular-mass water-soluble glycoprotein fraction contains D-galactose, D-glucose, D-xylose, and D-mannose with traces of L-arabinose. Low-molecular-mass fraction contains only D-galactose and D-mannose [4].

The capsular polysaccharides are similar in composition to extracelullar polymers that can be isolated from the culture medium. Previous studies have been directed on production and characterization of extracellular polysaccharide mixture produced by *C. laurentii* [5—7]. One of the polysaccharides isolated from the culture medium of *C. laurentii* var. *laurentii* was highly branched glucomannan with an α-(1→6)-linked mannan backbone. In contrast to structurally similar mannan from pathogenic yeast *Cryptococcus neoformans*, the polysaccharide isolated from *Cryptococcus laurentii* var. *laurentii* was modified with single glucose residues in the side chains [8, 9].

Present work provides results on isolation of extracellular polysaccharide mixture from *Cryptococcus laurentii* var. *laurentii*, its purification into homogeneous polymers and their physicochemical characterization.

EXPERIMENTAL

Cryptococcus laurentii CCY 17-3-5 from the Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, was grown in semisynthetic liquid medium at the concentration (ρ(g dm^{-3}) (i)): 2 (glucose), 0.3 ($(NH_4)_2SO_4$), 0.1 ($MgSO_4$), 0.05 (KH_2PO_4), 0.3 (yeast autolysate). Culture was incubated at 23°C in flask on reciprocal shaker. After 48 h yeast cells were harvested by centrifugation for 20 min at 8 000 *g*. Further separation was done according to Scheme 1.

Carbohydrates were detected by phenol—sulfuric acid assay [10]. Protein content was estimated by the method of Lowry using bovine serum albumin as a standard [11].

The relative molecular mass was determined by HPLC chromatography on columns of HEMA BIO 100 and 1000, eluted with 0.1 M-Tris-HCl buffer, pH 8.0. Free-boundary electrophoresis of a 1 % solution of extracellular polymer was carried out in a Zeiss 35 apparatus, using 0.05 M sodium tetraborate buffer (pH

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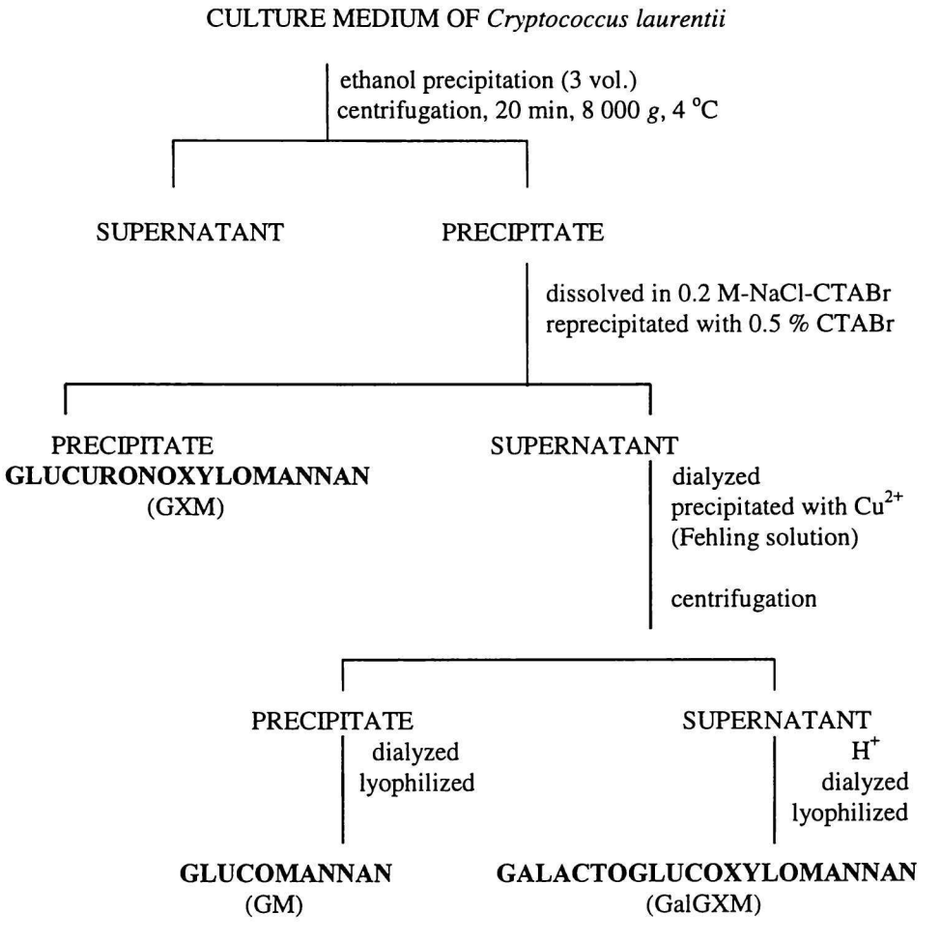


Scheme 1. Isolation of extracellular polysaccharide.

9.2) at 150 V cm^{-1} and 6 mA for 30 min.

The uronic acid content was determined spectrophotometrically with the 3-hydroxybiphenyl reagent [12]. The constituent monosaccharides of the extracelullar glycoproteins were identified after hydrolysis (2 M trifluoroacetic acid, 2 h, 100 °C) and reduction, in the form of their trifluoroacetates [13] by gas chromatography on a Hewlett—Packard Model 5890 Series II instrument equipped with a PAS 1701 column (0.32 mm × 25 m) at the temperature program of 110—125 °C (2 °C min^{-1})—165 °C (20 °C min^{-1}) and flow rate of nitrogen 20 cm^3 min^{-1}. The amino acid composition was established with an automatic amino analyzer, type 6020 (Mikrotechna, Prague), after hydrolysis of the glycoprotein (6 M-HCl, 20 h, 100 °C).

β-Elimination reaction: Glycoproteins were treated with 0.1 M-NaOH in the presence of 0.8 M-$NaBH_4$ at 37 °C for 72 h. The samples were neutralized with HCl, desalted on a column of Bio-Gel P-4 and analyzed for amino acid composition.

The MALDI mass spectrometric experiments were carried out on a Kompact MALDI III ToF (Shimadzu Kratos Analytical, Manchester, UK) equipped with a nitrogen laser (λ = 337 nm, pulse width 3 ns). Positive ions were accelerated from the target in the continuous mode to a final potential of 19 kV. All samples were measured in the reflectron mode. For each spectrum recorded, 50 single laser shots were accumulated. Calibration of the instrument was done externally with the [M + Na^+] ions of standard oligosaccharides. Matrix solution was prepared by dissolving 25 mg of gentisic acid (2,5-dihydroxybenzoic acid) in 1 cm^3 of acetonitrile and water (φ_r = 1:1). 1 mm^3 of the mixture of sample and matrix solution (φ_r = 1:3) was placed in the centre of one sample well of the stainless-steel 20-positions target. After air-drying of droplet the analyte—matrix mixture was transferred into the mass spectrometer.

Thin-layer chromatography (TLC) was carried out on Kieselgel 60 in the solvent system S, 1-butanol—formic acid—water (φ_r = 2:3:1). The saccharides were visualized by spraying the plates with 20 % ammonium sulfate and heating.

A portion of glycoprotein (20 mg) was dissolved in distilled water and applied to a column (30 cm × 2 cm) of Sepharose 2B equilibrated with the distilled water.

Fractions (1 cm^3) were collected and analyzed for

the carbohydrate content by the phenol—sulfuric acid method [10].

^{13}C NMR spectra of the polysaccharides were measured in D_2O at 25 °C on an FT NMR Bruker AVANCE DPX 300 spectrometer. Chemical shifts in the ^{13}C NMR spectra were referenced to external acetone at $\delta = 31.07$.

RESULTS AND DISCUSSION

The extracellular glycoproteins of *C. laurentii* var. *laurentii* were obtained by ethanol precipitation of the growth medium. The extracellular mixture was successively fractionated by cetyltrimethylammonium bromide (CTABr) [14] and Fehlings solution [15] into three crude heteroglycans: an acidic glucuronoxylomannan (GXM), neutral glucomannan (GM), and galactoglucoxylomannan (GalGXM) (Scheme 1).

The crude GXM was further purified by size-exclusion chromatography to give two fractions (I and II), differing in composition of the constituent sugars and molecular mass (Fig. 1). The fraction eluted first (peak I) represents the GXM. It was shown to be homogeneous by free-boundary electrophoresis. GXM contained 65.3 % of neutral carbohydrate, which was present as mannose : xylose in the mole ratio 1:0.62. This fraction contained also 7.3 % of glucuronic acid, 2.1 % of phosphorus, and 2.3 % of protein (Tables 1 and 2) and had relative molecular mass 2.4×10^6. This high-molecular-mass polysaccharide forms viscous solution. Peak II (Fig. 1) (≈ 18 % yield) accounted only for 4 % of polysaccharide applied. This minor fraction was not characterized further.

Size-exclusion chromatography of crude GalGXM afforded high (I) and low (II) molecular mass fractions, differing in composition of the constituent monosaccharides (Fig. 2). Peak I represented only 10 % of the crude glycoprotein fraction applied on the column and had relative molecular mass 1.9×10^4. Its monosaccharide composition was similar to GXM. The dominant fraction II represented about 90 % of the crude polymer, it was homogeneous by gel filtration and free-boundary electrophoresis, had the relative molecular mass 1.9×10^4 and contained phosphorus (0.7 %) and protein (6.5 %) as well. The carbohydrate content was 85.7 % and the mole ratio of monosaccharides mannose : galactose : glucose : xylose x_r = 1.00:0.26:0.08:0.09 (Table 2).

Extracellular glucomannan, isolated *via* its insoluble copper complex, was essentially free from another neutral polysaccharide (GalGXM) which was present in polysaccharide mixture obtained by ethanol precipitation of the growth medium (Scheme 1). Gel filtration of crude GM on columns Sepharose 2B (Fig. 3) afforded the pure GM fraction (≈ 95 % of crude GM). It was shown to be homogeneous by free-boundary electrophoresis, containing 91 % saccharides, 6.5 % protein, 1 % phosphorus (Table 1). The carbohydrate

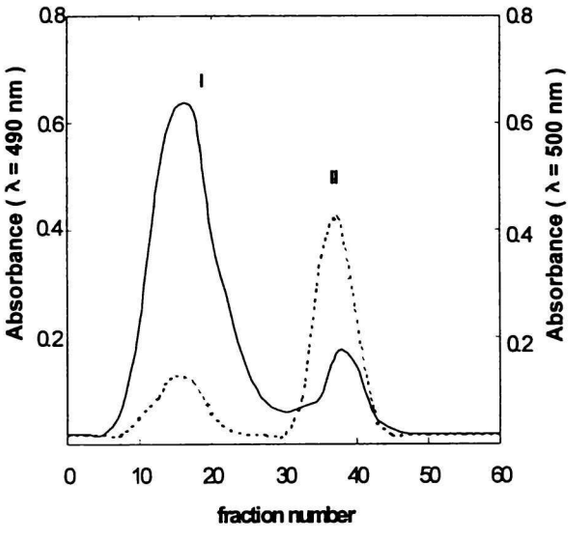


Fig. 1. Chromatography of glucuronoxylomannan (GXM) on SEPHAROSE 2B. (Each fraction contained 1 cm^3 of eluate collected at 5 min intervals.) ——λ = 490 nm; - - - λ = 500 nm.

Table 1. Chemical Composition of Extracellular Polysaccharides of *Cryptococcus laurentii*

	w_i/%		
	GXM	GalGXM	GM
Protein	2.3	6.5	6.5
Saccharides	65.3	85.7	91.0
Nitrogen	0.3	1.1	1.3
Phosphorus	2.1	0.7	1.0

Table 2. Content of Monosaccharides of Extracellular Polysaccharides of *Cryptococcus laurentii*

Saccharide	Mole ratio $x_y = x_i/x$(Man)		
	GXM	GalGXM	GM
Mannose	1.00	1.00	1.00
Galactose	–	0.26	–
Glucose	–	0.08	0.06
Xylose	0.62	0.09	–
Glucuronic acid	0.11	–	–

portion was composed of mannose and glucose in the mole ratio 1:0.06 (Table 2). It had relative molecular mass 4.5×10^4.

To test the presence of *O*-glycosidic bonds in GXM, GM, and GalGXM, all the glycoproteins were treated with 0.1 M-NaOH. Under β-elimination reaction, *O*-glycosidically substituted serine and thre-

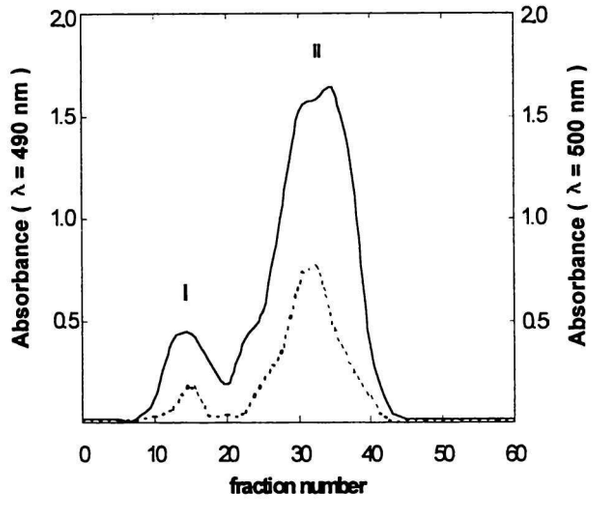


Fig. 2. Chromatography of galactoglucoxylomannan (GalGXM) on SEPHAROSE 2B. (Each fraction contained 1 cm^3 of eluate collected at 5 min intervals.) —— λ = 490 nm; - - - λ = 500 nm.

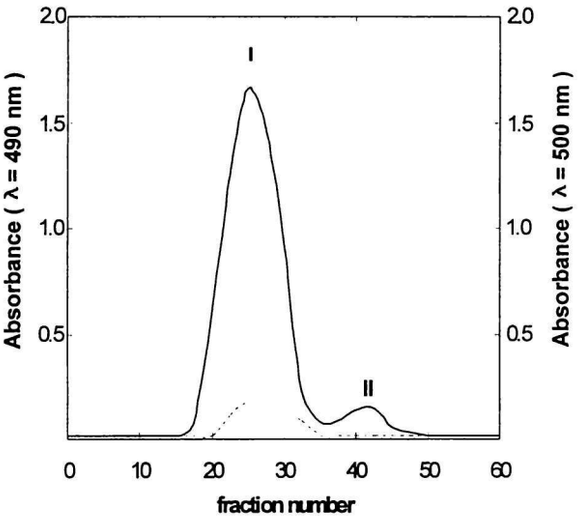


Fig. 3. Chromatography of glucomannan (GM) on SEPHAROSE 2B. (Each fraction contained 1 cm^3 of eluate collected at 5 min intervals.) —— λ = 490 nm; - - - λ = 500 nm.

onine residues are converted into alanine and α-aminobutyric acid, respectively. Quantitative analysis of the loss of serine and threonine showed that 87 % serine and 83 % threonine residues of GM and 44 % serine and 64 % threonine of GalGXM were glycosylated (Table 3). The fact that no significant changes in serine and threonine content of the protein part of GXM before and after β-elimination reaction were observed indicated that these amino acids were not *O*-glycosylated. Analysis of the products of β-elimination reaction of GM and GalGXM showed that D-mannose was the only monosaccharide constituent of all oligosaccharides released. Alkali treatment of GM afforded mannose, mannobiose, and mannotriose (Fig. 4*a*). Analysis of oligosaccharides released after β-elimination reaction of GalGXM showed that protein part is *O*-glycosylated by mannose, mannobiose, mannotriose, and mannotetraose (Fig. 4*b*).

Compositional analysis of the extracellular glucuronoxylomannan (GXM) from the strain *C. laurentii* var. *laurentii* revealed approximately equal proportions of D-mannose and D-xylose, and lower content of

Table 3. Content ($\gamma/(\mu mol\ g^{-1})$) of Amino Acids Present in GXM, GalGXM, and GM Fractions of *Cryptococcus laurentii* before and after β-Elimination Reaction

Amino acid	GXM		GalGXM		GM	
	before	after	before	after	before	after
Asp	12.2	12.78	15.01	15.22	29.64	26.05
Ser	15.54	13.00	**41.46**	**23.32**	**68.47**	**8.81**
Thr	22.21	19.70	**28.47**	**10.28**	**76.08**	**13.05**
α-Aminobutyric acid	–	–	–	7.82	–	60.81
Glu	8.22	8.46	18.36	18.68	30.72	28.68
Pro	10.27	10.11	Traces	Traces	18.41	16.92
Gly	7.77	7.54	14.92	15.69	18.47	20.69
Ala	9.71	13.21	22.78	59.24	28.58	82.24
Val	5.81	5.43	23.11	25.72	24.11	25.72
Met	2.34	1.52	7.55	6.82	1.47	1.95
Ile	Traces	Traces	7.07	7.34	8.77	7.34
Leu	7.79	7.86	6.61	6.08	9.29	10.68
Lys	0.85	0.9	7.33	6.98	5.23	5.31
Tyr	1.23	1.14	8.57	7.13	8.57	7.13
Phe	0.31	0.18	6.92	7.25	6.22	7.25
Arg	Traces	Traces	9.45	8.12	Traces	Traces
His	1.55	1.62	Traces	Traces	3.94	6.98

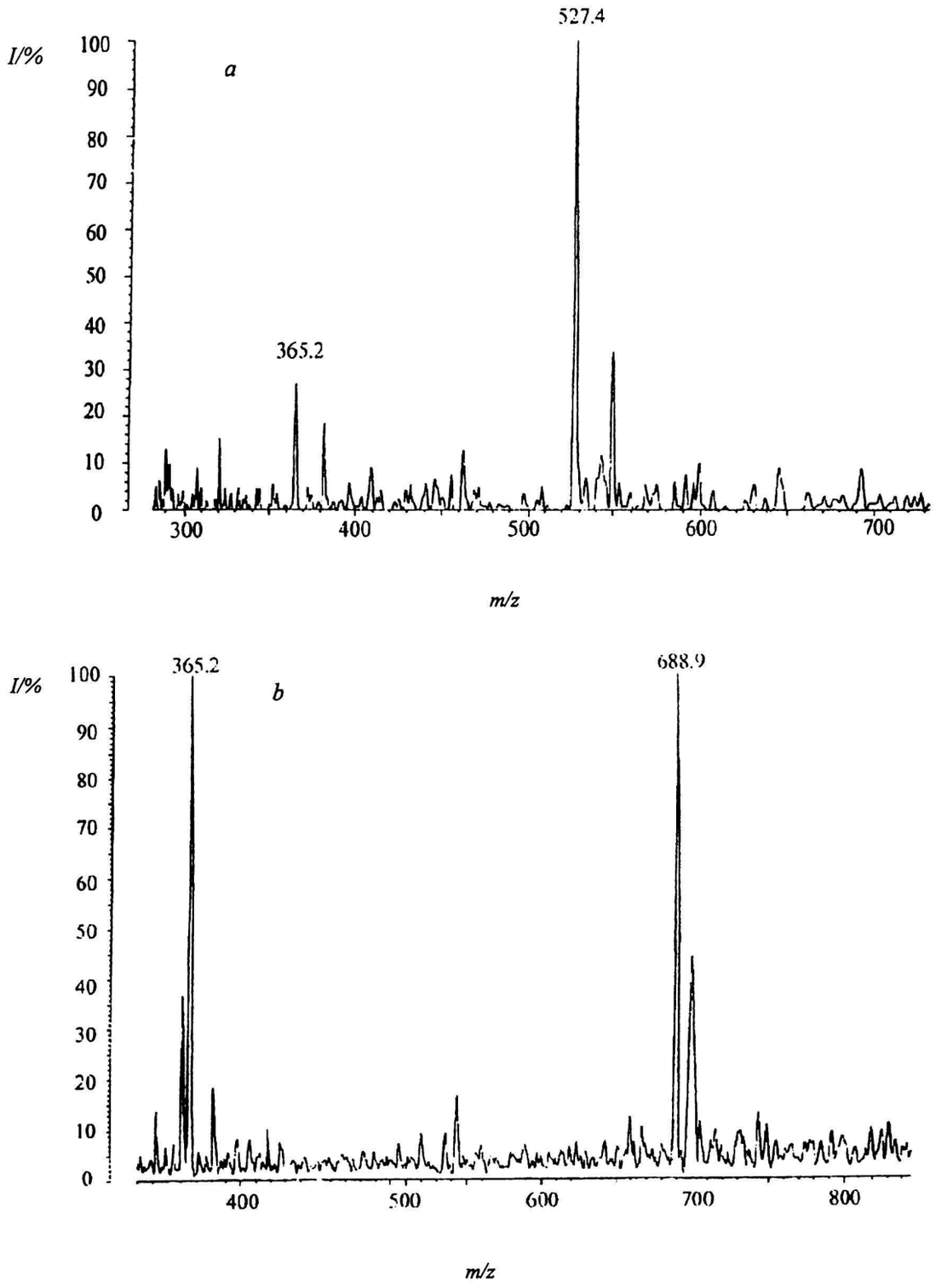
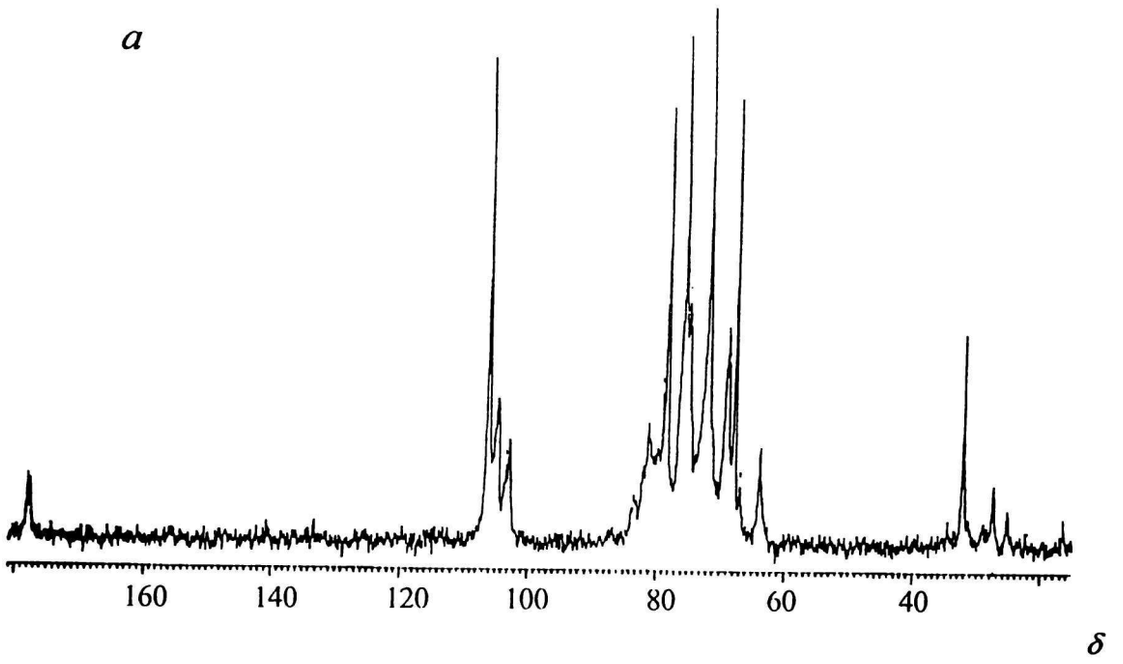


Fig. 4. Positive ion MALDI mass spectra of saccharides released from *a*) glucomannan (GM) by the alkali-induced β-elimination reaction, *b*) galactoglucoxylomannan (GalGXM) by the alkali-induced β-elimination reaction.

D-glucuronic acid. No acetyl groups were determined in this polymer. On the contrary, the capsular acidic polysaccharide (GXM) produced by the strain *C. laurentii* (NRRL Y-1401) was acetylated and composed of D-mannose, D-xylose, and D-glucuronic acid in the mole ratio 3:1:1 [5]. The polysaccharide had 1,3-linked α-D-mannopyran backbone to which β-D-glucuronic acid and β-D-xylose residues are linked in positions O-



2 and O-6, respectively [5]. The ^{13}C NMR spectrum of the GXM (Fig. 5*a*) from the strain *C. laurentii* var. *laurentii* supported the ratio of the monosaccharide components in the polymer. The assignment of signals was based on comparison with ^{13}C NMR chemical shift data from related oligo- and polysaccharides [16—18]. The two signals in the lowest magnetic field at δ = 178.09 and 177.69 were attributed to C-6 carbon atoms and confirmed the presence of uronic acid linked in different environments of the backbone. The five signals in anomeric region reflected a branched structure of the polysaccharide. The main signal observed at δ = 106.34 was assigned to C-1 of the terminal β-D-xylose residues [16, 17]. The occurrence of the anomeric signals δ = 104.88, 104.61, and 102.91 could be attributed to α-linked D-mannose residues variously substituted in the backbone. The lowest intensity signal at δ = 103.48 was attributed to C-1 of the D-glucuronic acid linked by β-glycosidic linkage [16—19]. Very weak resonance observed in the spectrum at δ = 63.96 arising from C-6 atoms of not substituted D-mannose residues indicated that the prevailing part of D-mannose units was branched in position O-6 (chemical shift for substituted C-6 atom was observed at δ = 68.01). In GXM from the strain *C. laurentii* (NRRL Y-1401) about one third of D-mannose units was branched in position O-6 by single D-xylose units and in position O-2 (of the same D-mannose unit) by single D-glucuronic acid [5], while in GXM produced by pathogenic *C. neoformans* 1,3-linked α-D-mannopyran backbone was branched predominantly in positions O-2 and O-4 by single D-xylose and D-glucuronic acid residues [16, 17].

The equal proportions of the constituent saccharides D-mannose and D-glucose in the polymer and the finding that the prevalent part of C-6 atoms of D-mannose residues was involved in branched points, indicated the structural variabilities of GXM isolated from different microbial sources.

Chemical analysis of the neutral extracellular glucomannan (GM) showed that polysaccharide was composed of D-mannose as the dominant sugar constituent and D-glucose was occurring only in small amount. The ^{13}C NMR spectrum (Fig. 5*b*) of the GM showed nine anomeric (C-1) resonances of nonequivalent areas, which indicates that the polysaccharide is composed of saccharides units of which C-1 atoms are involved at least in nine modes of linkages. The results of methylation analysis showed nine types of linkages of constituent saccharides and pointed at a highly branched structure of the polymer [8]. Weak and not completely resolved resonance in the anomeric region at δ = 103.5—103.7 could be assigned to 3- and 2,3-linked α-D-mannopyranose residues [18]. The dominant C-1 signal observed in the spectrum at δ = 102.8 is characteristic of terminal nonreducing D-mannose residues linked to position O-2 of the neighbouring unit. Further, the signals at δ = 100.1—101.8 arise from the resonance of C-1 atoms of internal 2-linked α-D-mannose units occurred in different environments [20, 21]. The other anomeric signals characteristic of α-D-mannose moieties at δ = 99.0—99.2 were not completely resolved, but they comprised the resonance of C-1 atoms of 6-, 2,6-, and 3,6-linked saccharide units [20]. Very weak signals in this spectrum at δ = 97.8—98.2 represent the resonance of C-1 atoms of termi-

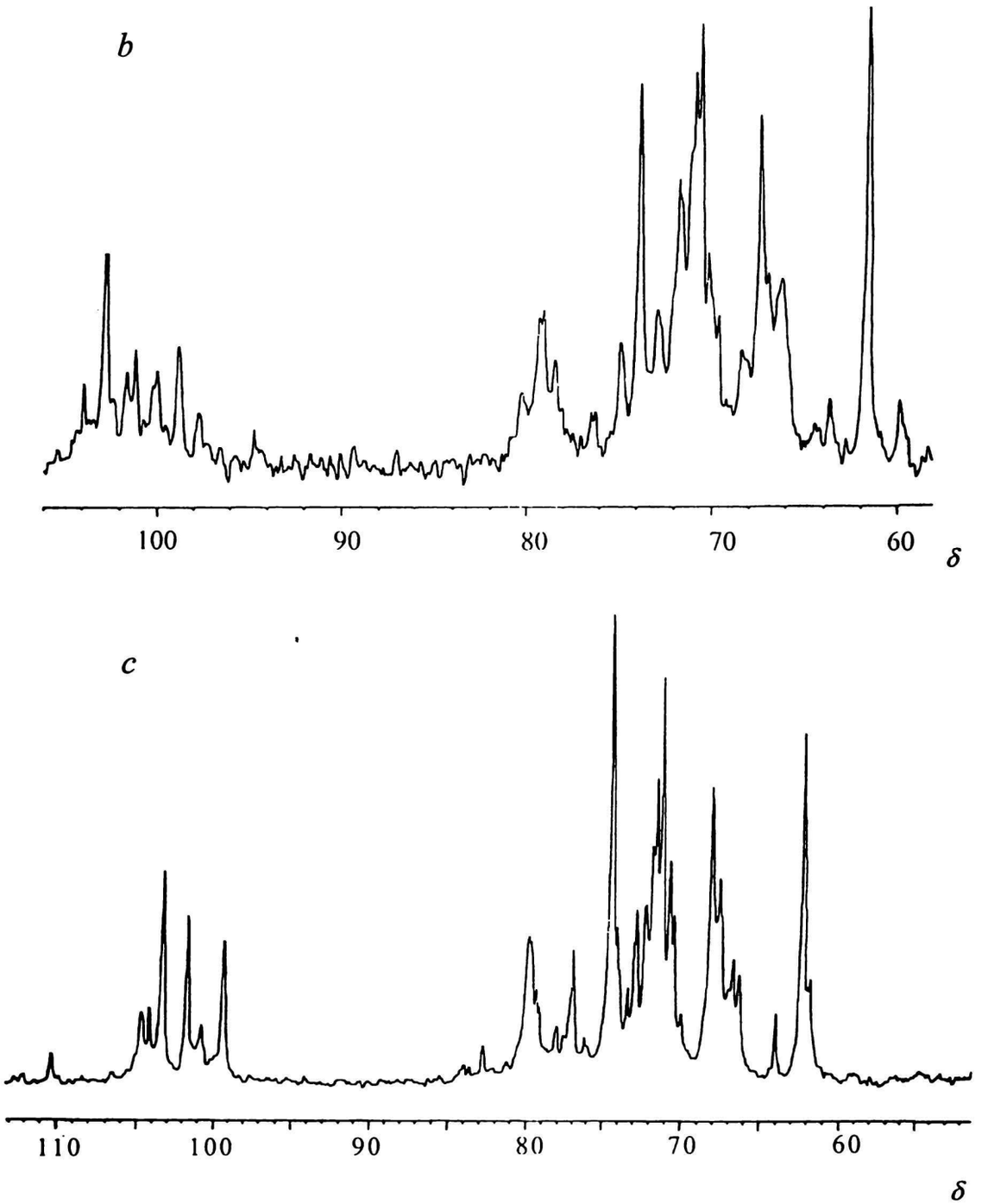


Fig. 5. ^{13}C NMR spectra of *a*) glucuronoxylomannan, *b*) glucomannan, *c*) galactoxyloglucomannan.

nal nonreducing α-D-glucose units [22]. Not very well resolved signals for carbon atoms of mannose units involved in glycosidic linkages were observed at δ = 80.2, 80.1 (C-2), 79.4, 79.2, and 78.4 (C-3), and 67.0 (C-6) [22].

Chemical and spectroscopic studies of the extracellular GM from *C. laurentii* var. *laurentii* showed a highly branched comb-like structure of the polymer with 1,6-linked α-D-mannopyran backbone to which side chains are linked in position O-2 [8]. It seems that the basic structural features of the extracelullar GM are similar to those of isolated yeast mannans [23, 24] and galactoxylomannan from *C. neoformans* [25].

Sugar analysis of a novel galactoglucoxylomannan (GalGXM) form of *C. laurentii* var. *laurentii* showed D-mannose as the prevalent saccharides constituent and the sugars D-galactose, D-xylose, and D-glucose were found in smaller proportions. The

proton-decoupled ^{13}C NMR spectrum of the GalGXM is shown in Fig. 5c. A comparative examination of the ^{13}C NMR spectra of the GalGXM and GM indicated a close similarity between these biopolymers (Fig. 5*b* and *c*). The six resolved signals in the anomeric region indicated a branched structure of the polysaccharide. The resonance at $\delta = 104.6$ and 104.1 could be attributed to the β-forms of D-galactose and D-xylose residues, respectively [19]. The absence of these signals in the ^{13}C NMR spectrum of glucomannan confirms this assumption. The resonance in the spectrum at $\delta = 103.3$ arises from C-1 atoms of nonreducing and 3-substituted D-mannose residues [25]. The anomeric signals characteristic of 2-linked D-mannose moieties were found at $\delta = 101.7$ and 100.7. The last resonance observed in the anomeric region at $\delta = 99.3$ (in the spectrum of the GM it was found at $\delta = 99.1$) is assigned to 6-linked D-mannose moieties in the polymer [25]. Preliminary structural studies on GalGXM showed that the polymer has the same 1,6-linked α-D-mannopyran backbone as those of GalXM from *C. neoformans* [25], yeast mannan [23, 24], and extracellular GM originating from the same source [8].

The results of our studies on extracellular polysaccharide mixture produced by *C. laurentii* var. *laurentii* showed that the culture medium contains at least three different glycoproteins, one acidic and two neutral. The acidic GXM differed from neutral polymers GM and GalGXM by very high molecular mass. Its molecular mass was much higher than molecular masses of some extracellular GXMs produced by pathogenic yeast-like fungus *C. neoformans* [18]. It seems that the 1,3-linked α-D-mannopyran cores of GXM isolated from different strains of *C. laurentii*, resemble each other. The differences are only in the size of the chains, mole ratio of monosaccharide constituents, and in the degree of branching of the core sugars. In GXM from *C. neoformans* serotypes D-mannose residues are branched in positions O-2 and O-4 while in GXM isolated from zoopathogenic yeast-like fungus *C. laurentii*, the saccharide residues of the backbone are branched predominantly in positions O-6 and O-2. The common feature of GXM from other strains of *C. laurentii*, and different serotypes of *C. neoformans* are *O*-acetyl groups which are together with uronic acids an important determinant of antigenic specificity of these polysaccharides, however, in GXM from *C. laurentii* var. *laurentii* *O*-acetyl groups are not determined. Two neutral extracellular polysaccharides GM and GalGXM represent about 40 % of the extracellular mixture. There was not a significant difference in molecular masses of these biopolymers. Both polymers revealed the similar structural features of the main chain which consisted of 1,6-linked α-D-mannose residues some of which were substituted by side oligosaccharide chains.

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