Isolation and Characterization of Glycoproteins from the Yeast *Cryptococcus laurentii* I. Cell-Wall Glycoproteins

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Received 14 December 1993

Cell-wall glycoproteins were isolated by extraction with anhydrous ethylenediamine of the cell wall of the yeast *Cryptococcus laurentii*. The extraction yielded a water-soluble fraction A and a water-insoluble fraction B. The fraction A contained 43.3 % saccharides and 43.0 % proteins. Only 23.1 % of serine and 14.3 % of threonine residues of this fraction was glycosylated by *O*-linked oligosaccharides. The water-soluble glycoproteins were separated into a low-molecular mass glycoprotein containing galactose and mannose in the ratio (x_r = 5:1) and a high-molecular mass glycoprotein fraction containing galactose, glucose, mannose, xylose, and rhamnose in the mole ratio 0.3:1.0:0.75:0.5:0.03, with traces of arabinose. *N*-Glycosidically linked oligosaccharides released from the high-molecular mass fraction by endoglycosidase H were characterized by molar proportion mannose:xylose:galactose:glucose:rhamnose = 2.17:0.43:0.86:1.0:0.28. The *N*-glycosidically linked oligosaccharides released by alkaline cleavage were characterized by molar proportion of neutral monosaccharides — mannose, xylose, galactose, glucose, and arabinose in the ratio 0.08:0.18:0.025:1.0:0.15.

Cryptococcus laurentii is an encapsulated, yeast-like organism that is classified among the Fungi Imperfecti, and is closely related to the human pathogen *Cryptococcus neoformans*, the cause of cryptococcal meningitis.

C. laurentii has a complex cell envelope containing a β-linked glucan, acidic capsular polysaccharides composed of mannose, xylose, and glucuronic acid, and neutral polysaccharides containing mainly mannosyl, xylosyl, and galactosyl residues [1].

Sentandreau and *Northcote* [2] showed that glycopeptide from the yeast has carbohydrate linked to the peptide moiety by two types of linkages: *a*) alkali-labile linkages to serine and threonine (mainly mannose residues) and *b*) alkali-stable linkages to asparagine (high-molecular mass mannan).

N-Linked oligosaccharides have been shown to play important roles in a number of cellular recognition reactions [3—7].

Free or to protein linked oligosaccharides are not prior products of genes such as proteins. They are synthesized by enzymes called glycosyltransferases, without presence of template. Detailed enzymology of the pathway is poorly understood. One of the reasons for this paucity of information is lack of pure protein-linked oligosaccharide species that can be used as substrates to assay these glycosyltransferases.

In this communication we describe the isolation and characterization of protein-linked oligosaccharides of the cell wall of *C. laurentii*, containing galactose, to be used after exoglycosidases digestion as substrate for galactosyltransferases.

EXPERIMENTAL

Cryptococcus laurentii CCY 17-3-5 from the Culture Collection of Yeasts and Yeast-Like Microorganisms, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, were grown at 28 °C in semisynthetic liquid medium at the concentration (g dm^{-3}): 2 glucose, 0.3 $(NH_4)_2SO_4$, 0.1 $MgSO_4$, 0.05 KH_2PO_4, 0.3 yeast autolysate. After 48 h yeast cells were harvested by centrifugation and washed twice with distilled water. The cell walls were fractionated by anhydrous ethylenediamine extraction according to *Korn* and *Northcote* [8]. Further separation was done according to Scheme 1.

Analytical Methods

Neutral carbohydrate was detected by the phenol—sulfuric acid method [9]. Protein content was estimated by the method of *Lowry* [10], using bovine serum albumin as a standard. Glucosylamine was estimated by the method of *Cessy* and *Piliego* [11].

Nitrogen analysis was performed on Perkin—

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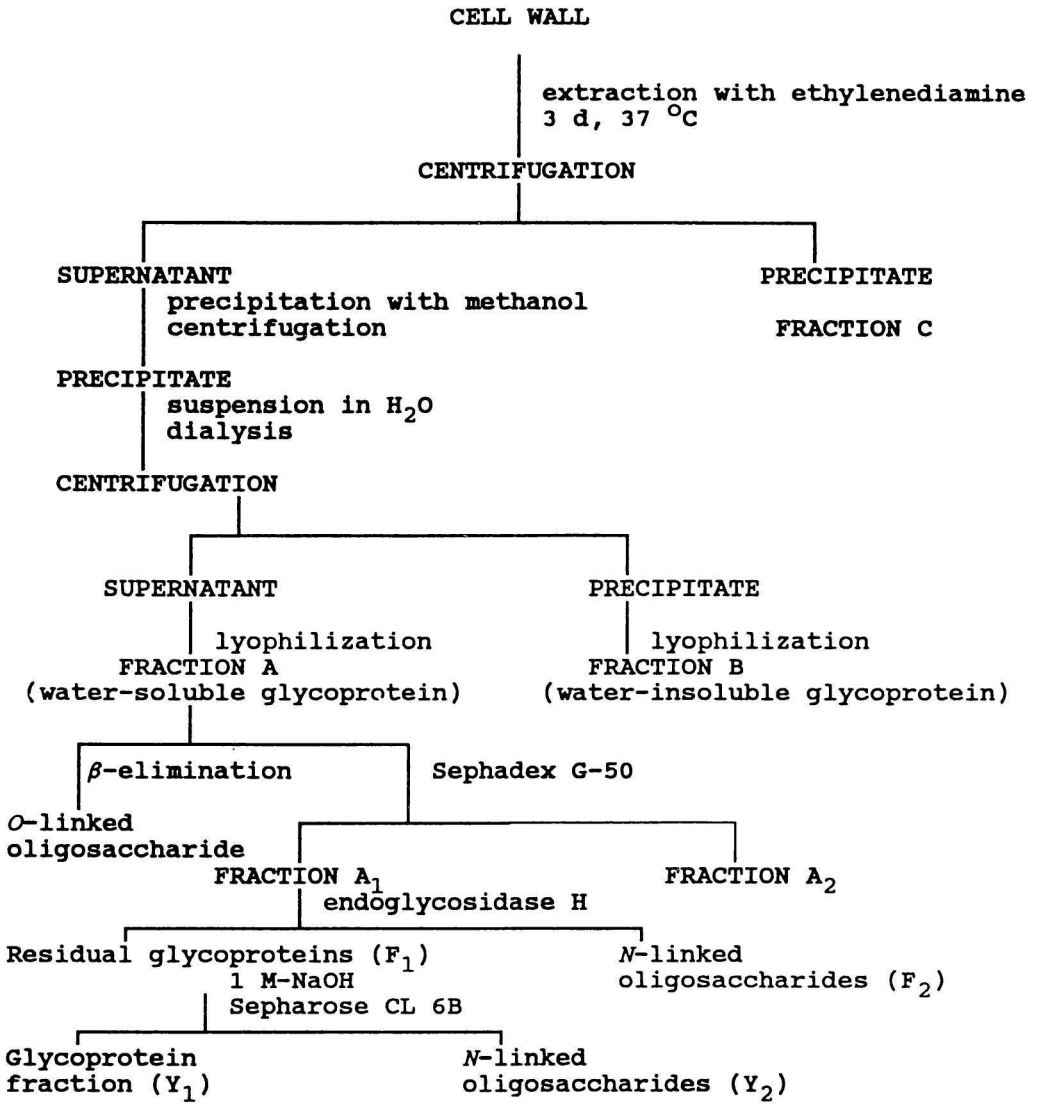

Scheme 1

Elmer analyzer. The constituent monosaccharides were identified after hydrolysis (1 M-HCl, 100 °C, 8 h), reduction ($NaBH_4$), and acetylation (pyridine—acetic anhydride, φ_r = 1:1, 16 h, room temperature). The resulting alditol acetates were analyzed by GLC using a Hewlett—Packard 5890 model programmed to hold a temperature of 125 °C for 2 min, then to increase it to 165 °C for 20 min; and column PAS 1701 (250 cm × 0.32 cm). The carrier gas was H_2 (15 cm^3 min^{-1}).

HPLC analysis of β-elimination products was performed with an SGX-NH_2 column (0.4 cm × 25 cm) in a Hewlett—Packard 1050 liquid chromatograph equipped with differential refractometer. The solvent system was water—acetonitrile (45:55) pumped at the rate 1.0 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).

The number average molecular mass ($\overline{M}_r$) was determined osmometrically at 30 °C, using a Knauer Vapour Pressure Osmometer.

Column Chromatography

A portion of glycoprotein fraction A (70 mg) was dissolved in 0.1 M-acetic acid (2 cm^3) and applied to a column (65 cm × 2.5 cm) of Sephadex G-50 equilibrated with the same solution of acetic acid. Fractions (3.5 cm^3) were collected at 20 min intervals and analyzed by measuring the absorbance at λ = 280 nm to give high-molecular fraction A_1 (60 mg) and low-molecular fraction A_2 (2 mg). The carbohydrate content was determined by phenol—sulfuric acid methods [9].

Gel filtration of endoglycosidase H digest of glycoprotein (fraction A_1) was performed as that of fraction A.

Alkali-treated fraction F_1 (10 mg) was dissolved in 0.05 M-Tris-HCl buffer (0.1 cm^3), pH 7.8, and applied

to a column (1.4 cm × 88 cm) of Sepharose CL-6B equilibrated with the same buffer. Elution was conducted at the flow rate of 16.5 $cm^3\ h^{-1}$

Alkali-Induced β-Elimination

Glycoproteins were treated with 0.1 M-NaOH in the presence of 1 M-$NaBH_4$ at 40 °C for 16 h. The materials were neutralized with HCl, desalted by Biogel P-4 column chromatography and analyzed for amino acid composition.

Alkaline Cleavage of *N*-Glycosidic Linkages

Glycoprotein fraction F_1 was dissolved in 2 M-NaOH—1 M-$NaBH_4$ solution (50 mg cm^{-3}). The mixture was heated under reflux for 8 h at 100 °C. The solution maintained in an ice-bath was neutralized with 50 % acetic acid to a pH value of 6. After neutralization the products were separated on a column of Sepharose CL 6B.

Enzymatic Cleavage of *N*-Glycosidic Linkages

Digestion of glycoprotein fraction A_1 (100 mg) with endoglycosidase H (Sigma, USA) was done in a phosphate buffer (1 cm^3), pH 5.5, using 1 Unit of enzyme and incubation at 37 °C for 36 h. At the end of the incubation giving fractions F_1 (35 mg) and F_2 (50 mg) the digest was chromatographed on Sephadex G-50.

RESULTS AND DISCUSSION

Extraction of the cell wall of *Cryptococcus laurentii* by anhydrous ethylenediamine (Scheme 1) yielded water-soluble fraction A and water-insoluble fraction B. The chemical composition of these fractions is summarized in Table 1. While the saccharide part of fraction B contained only glucose, GLC analysis of fraction A showed the presence of xylose, mannose, glucose, galactose, and the traces of rhamnose and arabinose (Table 2).

To test for the presence of *O*-glycosidic bonds, the fraction A was treated with 0.1 M-NaOH. The effect of β-elimination reaction is shown in Table 3. The loss of serine and threonine showed that only 23.1 % of serine and 14.3 % of threonine residues of water-soluble cell-wall glycoproteins of *C. laurentii* are glycosylated. Mannose was only a constituent of saccharides released by the β-elimination reaction. The released *O*-glycosidic oligosaccharides were chromatographed using HPLC. The profile of released oligosaccharides is shown in Fig. 1. The retention times of released saccharides were approximately 5.72 min and 6.72 min, corresponding to mannobiose and mannose, respectively. Mannobiose was the major product released from the glycoprotein fraction A. Fractionation of water-soluble fraction A afforded two fractions, A_1 and A_2 (Fig. 2). Fraction A_1 contained 53 % of saccharide with the monosaccharide composition: galactose, mannose, glucose, and xylose. Low-molecular mass fraction A_2 contained only 11.6 % of saccharide. Saccharide part of fraction A_2 was composed mainly of galactose (82.5 %) and minor proportion of mannose (17.5 %) (Table 4). It was impossible to closer characterize the fraction A_2 for its very low yield. Gel filtration of endoglycosidase H digest of the glycoprotein

Table 1. Chemical Analysis of Water-Soluble Fraction A and Water-Insoluble Fraction B

	w_i(A)/%	w_i(B)/%
Protein	43.0	8.0
Saccharides	43.3	8.8
Nitrogen	7.9	1.3
Phosphorus	1.5	0.8
Glucosamine	1.5	0.2

Table 2. Content of Monosaccharides (Glc = 1) of Fraction A and Fraction B

Saccharide	Mole ratio $x_r = x_i/x(\mathrm{glc})$	
	Fraction A	Fraction B
Galactose	0.39	
Glucose	1.0	1.0
Mannose	1.03	–
Xylose	0.206	–
Rhamnose	Traces	–
Arabinose	Traces	

Table 3. Changes in Concentrations of Amino Acids Present in Fraction A after β-Elimination Reaction

Amino acid	before β-elimination $m/(\mu\mathrm{mol\ g^{-1}})$	after β-elimination $m/(\mu\mathrm{mol\ g^{-1}})$
Asp	22.37	22.69
Thr	13.13	11.25(−14.3 %)
Ser	14.09	10.56(−23.1 %)
Glu	22.09	21.68
Pro	10.63	10.00
Gly	19.36	20.69
Ala	18.65	22.24
Val	13.89	13.72
Met	1.47	1.55
Ile	12.22	12.34
Leu	18.55	18.68
Tyr	6.37	7.13
Phe	8.47	9.25
His	6.45	6.92
Lys	14.75	15.08
Arg	Traces	Traces

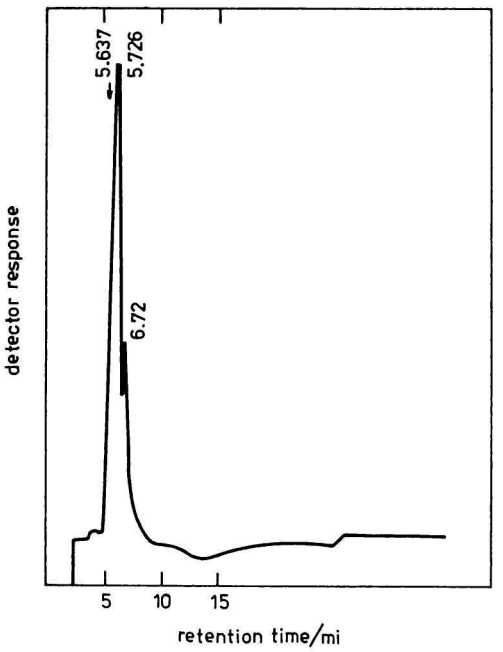

Fig. 1. HPLC profile of the saccharides released by the alkali-induced β-elimination reaction. Arrow indicates the elution position of cellobiose. Numerals above peaks refer to the retention time.

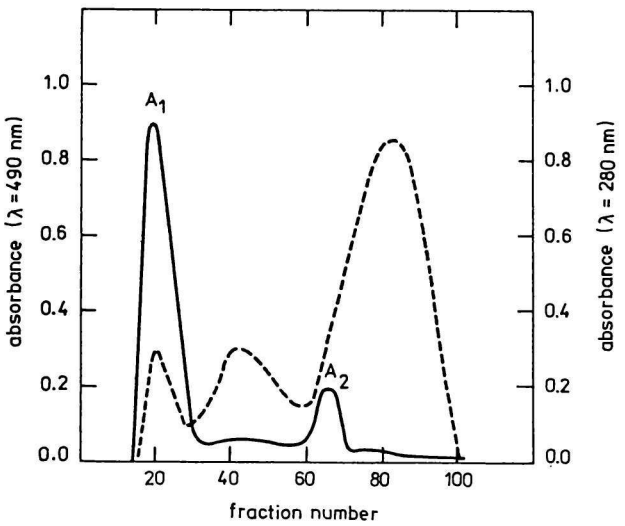

Fig. 2. Chromatography of the water-soluble fraction A on Sephadex G-50. (Each fraction contained 2.5 cm^3 of eluate collected at 10 min intervals.) —— at λ = 490 nm; - - - at λ = 280 nm.

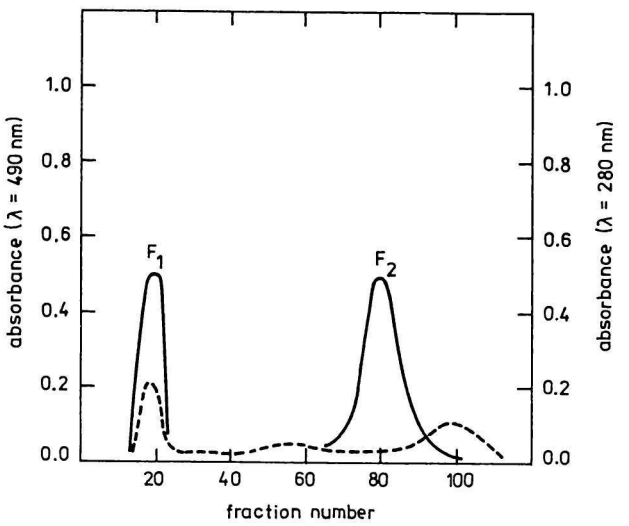

Fig. 3. Chromatography of fraction A_1 after digestion with endoglycosidase H on Sephadex G-50. (Each fraction contained 2.5 cm^3 of eluate collected at 10 min intervals.) —— at λ = 490 nm; - - - at λ = 280 nm.

Table 4. Mole Content of Monosaccharides (Glc = 1) of Fractions A_1 and A_2

Saccharide	Mole ratio $x_r = x_i/x(glc)$	
	Fraction A_1	Fraction A_2
Galactose	0.30	1.00
Glucose	1.00	
Mannose	0.75	0.21
Xylose	0.5	–
Rhamnose	0.03	

Table 5. Monosaccharide Content of *N*-Linked Oligosaccharides (Glc = 1) Released by Endoglycosidase H and by 2 M-NaOH

Saccharide	Mole ratio $x_r = x_i/x(glc)$	
	Release by endoglycosidase H (Fraction F_2)	by NaOH (Fraction Y_2)
Xylose	0.43	0.18
Mannose	2.17	0.08
Glucose	1.0	1.0
Galactose	0.86	0.025
Arabinose	–	0.15
Rhamnose	0.28	–

A_1 (Fig. 3) yielded residual glycoprotein F_1 and saccharides fraction F_2 ($M_r \approx 10000$).

Fraction F_2 enzymatically released from cell-wall glycoproteins of *C. laurentii* represents *N*-glycosidically linked saccharides portion of the glycoprotein. Mannose is dominating in these oligosaccharides (Table 5). This observation is in a good agreement with the knowledge of endoglycosidase H. It is known that this enzyme releases high mannose structures from the glycoproteins.

Fraction F_1 possessed a form not sensitive to endoglycosidase H. A similar fraction was found in the yeast *Schizosaccharomyces pombe* [12].

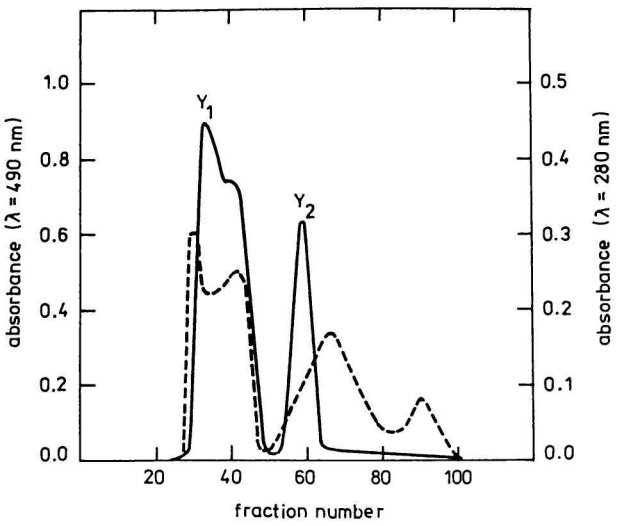

Fig. 4. Chromatography of fraction F_1 after alkali digestion with 2 M-NaOH on Sepharose CL 6B. (Each fraction contained 3 cm^3 of eluate collected at 20 min intervals.) —— at $\lambda = 490$ nm; - - - at $\lambda = 280$ nm.

The residual glycoprotein F_1 was treated under drastic alkaline conditions. The released components were fractionated on column of Sepharose CL 6B. The fractionation yielded fraction Y_1 ($M_r \geq 100000$) containing 9 % of protein and fraction Y_2 ($M_r = 92000$) representing an alkali-released *N*-glycosidically linked glycan (Fig. 4). The alkali-released glycan fraction Y_2 was contaminated with 3 % of protein. The neutral sugars of this fraction determined by GLC are represented in Table 5. Fraction Y_2 was composed mainly of glucose, xylose, arabinose, and traces of mannose and galactose. Possible explanation for the resistance of *N*-glycosidically linked oligosaccharide of the fraction Y_2 to endoglycosidase H is a low content of mannose.

At present, a glycoprotein cannot be separated into its individual glycoforms by chromatographic, electrophoretic or mass spectrometric method. This is largely because of the sheer number of individual glycoforms constituting most glycoproteins and the relatively small difference in the physical and chemical properties between most glycoforms [13].

Relatively high content of galactose, and no contamination with protein was supposed to be a reason for choosing fraction F_2 after digestion with exoglycosidases as a natural acceptor of yeast galactosyltransferases, which are studied in our laboratory. Due to a low amount of mannogalactan fraction A_2, however, it could not be used as the acceptor for galactosyltransferases.

Acknowledgements. This research was supported by the Slovak Grant Agency for Science.

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Translated by N. Kolarova