The Polysaccharides of Yeasts and Yeast-like Microorganisms (II) The Extracellular Mannans of *Candida albicans* BERKHOUT

D. ŠIKL, L. MASLER, Š. BAUER

Institute of Chemistry, Slovak Academy of Sciences, Department of Biochemistry of Saccharides, Bratislava

Dedicated to Academician J. Vašátko on the occasion of his seventieth birthday

In the culture liquid from the cultivation of five strains of *Candida albicans* BERKHOUT on the glucose substrate the high branching extracellular α-mannans of DP 150—630 were isolated.

The extracellular mannans belong to two structural types as on the base of methylation analysis, partial hydrolysis, periodate oxidation and Smith degradation was found out. The first one, less branched has the main linkage composed from mannose units linked through α-1,6 glycosidic linkages. To every second mannose unit two to five-unit side chains containing α-1,2 glycosidic linkages are attached. The side chains are bound to main chain with α-1,2 linkage. In the second more branched type every unit of main chain is the branching point, where the four-unit side chains through α-1,2 glycosidic linkages are attached. In the main chains α-1,6 linkages, in the side chains α-1,2 linkages are present.

In the previous paper [1] we have described the isolation and characterisation of a water soluble branched extracellular surface mannan with α-1,6 and α-1,2 linkages isolated from the surface of cellular membranes of yeast *Candida albicans* BERKH. Continuing the elucidation of structural features of polysaccharides of *Candida albicans* we studied the extracellular homogenous mannans in order to determine the possibility of production the polysaccharides with the same or different primary chemical structures by the different strains.

The choice of the strains was made on the base of their virulence [2], and we elected two slightly virulent strains Nos 29—3—18 and 29—3—21, the medium virulent strain 29—3—19, and two strong virulent strains 29—3—65 and 29—3—109.*

The individual strains were cultivated in usual manner using glucose as carbohydrate substrate. The polysaccharides were isolated from culture medium by the method described in experimental part in 2 — 3 % yields calculated on the dry weight of yeasts.

On acidic hydrolysis of the crude polysaccharides D-mannose and D-glucose were identified. By moving boundary electrophoresis in borate buffer, there were indicated two components, mannan with electrophoretic mobility $u =$

* The designation of strains according to [3].

$= 9.1 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and glucan with $u = 3.3 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. The ratio of mannan to glucan in extracellular polysaccharides is listed in Table 1.

Table 1

Yields and mannan/glucan ratio in extracellular polysaccharides of *Candida albicans*

Polysaccharides of strains	Yield		Ratio mannan/glucan
	g	%	
C. albicans 18	3.9	1.92	1 : 0.53
19	4.5	3.12	1 : 0.39
21	3.2	1.99	1 : 0.21
65	4.3	1.73	1 : 0.52
109	3.6	2.40	1 : 0.71

The separation of mannans from glucans was done by fractionation with Fehling's solution in the form of mannan-copper water insoluble complexes. After the releasing of mannans from these complexes the mannans were precipitated with alcohol and freeze dried. Thus obtained amorphous white polysaccharide samples (0.5 — 1 % yield based on dried cells) were slightly soluble in water. The values of specific optical rotation of these mannans ranged between $+51°$ to $+63°$. After the hydrolysis in 1 N-HCl the specific rotation changed to $+13°$, which indicated the presence of α-glycosidic linkages in mannans.

This fact was confirmed by infrared spectrum in which there were present no absorption bands at 893 cm^{-1} [4]. The physico-chemical data are listed in Table 2.

Table 2

The physico-chemical constants of extracellular mannans of *Candida albicans*

Physico-chemical constants	Mannan of strain				
	18	19	21	65	109
Optical Rotation $[\alpha]_D$ (Water)	$+51°$	$+51°$	$+52°$	$+63°$	$+57°$
Diffusion Coefficient $D^{20} \cdot 10^7$	4.92	6.43	8.55	3.42	3.91
Sedimentation Constant $S^{20} \cdot 10^{13}$	4.16	3.87	4.37	4.86	4.71
Partial Specific Volume ϱ^{20}	0.5878	0.6361	0.6598	0.6592	0.6381
Molecular Weight $\overline{M}_{s,D} \cdot 10^{-3}$	50.2	23	36.5	102	99.7

The isolated homogenous extracellular mannans were characterized by methylation analysis, partial hydrolysis, periodate oxidation and Smith degradation.

By gas-liquid partition chromatography (GLC) of the methanolysis pro-

ducts of methylated mannans of the strains No 18, 19, 21 and 65 the following *O*-methyl mannosides were determined:

methyl-2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (2 moles),
methyl-3,4,6-tri-*O*-methyl-α-D-mannopyranoside (5 moles),
methyl-2,3,4-tri-*O*-methyl-α-D-mannopyranoside (1 mole),
methyl-3,4-di-*O*-methyl-α-D-mannopyranoside (2 moles).

In the products of methanolysis of methylated mannans of strain No 109:

methyl-2,3,4,6-tetra-*O*-methyl-α-D-mannopyranoside (1 mole),
methyl-3,4,6-tri-*O*-methyl-α-D-mannopyranoside (2 moles),
methyl-3,4-di-*O*-methyl-α-D-mannopyranoside (1 mole) were found. The relative retention times of *O*-methyl ethers of mannopyranosides are listed in Table 3.

Table 3

The molar ratio and relative retention times of methylmannosides obtained after hydrolysis of methylated extracellular mannans of *Candida albicans*

Methylmannosides	RT	Moles Strains	
		18, 19, 21, 65	109
Methyl-2,3,4,6-tetra-*O*-methyl-α-D-manno-pyranoside	1.00	2	1
Methyl-3,4,6-tri-*O*-methyl-α-D-manno-pyranoside	2.04	5	2
Methyl-2,3,4-tri-*O*-methyl-α-D-manno-pyranoside	3.14	1	—
Methyl-3,4-di-*O*-methyl-α-D-mannopyranoside	4.60	2	1

In the mannans of strains No 18, 19, 21 and 65 after partial hydrolysis with HCOOH 2-*O*-α-D-mannopyranosyl-D-mannose was isolated by preparative paper chromatography. The partial hydrolysis resulted in the formation of homologous series of oligosaccharides (Figure 1) with the structure *O*-α-D--manp(1→[2)-*O*-α-D-manp(1→]$_n$2)--D-manp where $n = 0 - 4$. The proof of

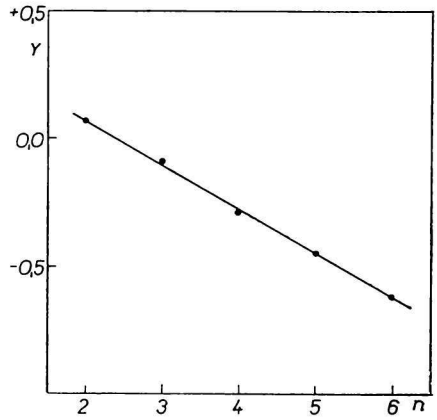


Figure 1. The homologous series of oligosaccharides based on D-mannose. n = number of units of D-mannose;

$$y = \log \frac{R_G}{1 - R_G}.$$

homologues series was brought forward on the basis of linear dependence between $\log \frac{R_F}{1-R_F}$ and the number of units n in the molecule of oligosaccharide [5]. After the partial hydrolysis of mannans from strain 109 by paper chromatography a similar homologous series of oligosaccharides of D-mannose (where $n = 0 - 3$) was established.

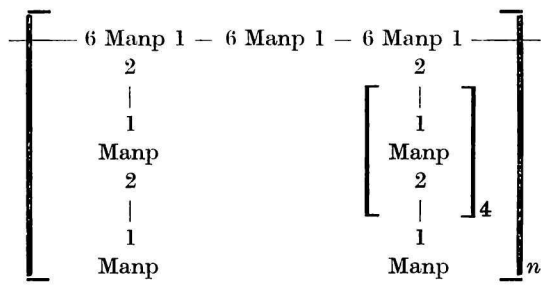
The results of periodate oxidation, the course of which is listed in Table 4, were in agreement with the results of methylation analysis. In accordance with the number of branching points, average lenght of side chains and due to the fact that higher oligosaccharides were isolated, the existence of side linkages with varying lenght can be assumed.

Table 4

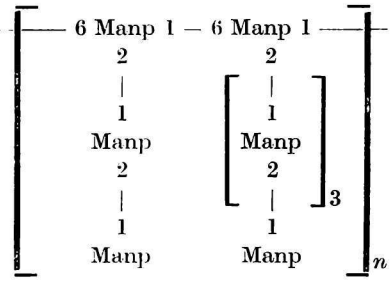
Periodate oxidation of extracellular mannans of *Candida albicans*

Mannan of strain	24 h		48 h		72 h		96 h	
	HCOOH	$NaIO_4$	HCOOH	$NaIO_4$	HCOOH	$NaIO_4$	HCOOH	$NaIO_4$
C. albicans 18	0.27	1.15	0.28	1.17	0.30	1.20	0.31	1.31
19	0.28	1.14	0.28	1.15	0.30	1.30	0.31	1.30
21	0.30	1.13	0.30	1.13	0.31	1.30	0.32	1.31
65	0.27	1.16	0.28	1.16	0.29	1.30	0.32	1.30
109	0.19	0.93	0.21	1.11	0.23	1.19	0.26	1.25

Therefore for the extracellular mannans of strains 18, 19, 21 and 65 the following tentative structure formulas are proposed:



and for extracellular mannan of strain 109 the tentative structure:



The described strains of *Candida albicans* produce two types of extracellular polysaccharides, the first one with lower degree of branching and with longer side linkages. This is typical for strains 18, 19, 21 and 65, while the second type with higher degree of branching and with shorter side linkages is typical for strain 109. The occurence of two structural types of mannans with different degree of branching in different strains of *Candida albicans* is in agreement with their immunochemical behaviour as described by H. F. Hasenclever [6, 7].

Experimental Part

Materials and Methods

The following strains of *Candida albicans* were used: *Candida albicans* (ROBIN) BERKHOUT 29—3—18 and 29—3—21 (slightly virulent strains), 29—3—19 (medium virulent strain), 29—3—65 and 29—3—109 (strongly virulent strains). The specific names and characterisation of these strains are given according to ref. [3].

Moving boundary electrophoresis in 1 % solutions of polysaccharides were carried out in 0.05 M borate buffer in a Tiselius-type apparatus Zeiss Model 35. Before the experiment the solutions were dialysed 1 hour against the borate buffer. The infrared spectra of the polysaccharides in potassium bromide discs were obtained on Zeiss UR 10 Spectrophotometer. The optical rotations were measured at ambient temperature with Automatic polarimeter Bendix—Ericsson, type 143 A.

GLC of *O*-methylethers of D-mannopyranoside were done with Gas liquid chromatograph Chrom I, Laboratorní přístroje, Prague, equipped with flame ionisation detector. The columns were packed with 5 % (w/w) butandiol succinate polyester on Celite 545. Temp. = 180 °C, carrier gas N_2, the flow rate 50 ml/min. Paper chromatography was carried out on Whatman paper No 1 (for analytical separation of saccharides) and Whatman 3 (for preparative separation) in the following solvent systems (v/v):

S_1 = *n*-butanol—pyridine—benzene—water 7 : 3 : 1 : 2,

S_2 = *n*-butanol—acetic acid—water 4 : 1 : 5 (three fold chromatography was used, 3 × 16 hours). The solvent system,

S_3 = ethyl acetate—pyridine—water 7 : 2 : 1 for the chromatography of polyols was used.

The paper electrophoresis of saccharides was done on Whatman paper No 1 in 0.05 M borate buffer pH 9.23, using a potential gradient of 25 V/cm for 2 hours.

Spraying reagents aniline phthalate [8], diphenylamine aniline [9], 2,3,5-triphenyltetrazolium chloride [10], silver nitrate—sodium hydroxide [11] and sodium metaperiodate—benzidine [12] were used.

Optical density of developed spots were measured by ERI 10, C. Zeiss, Jena densitometer.

The periodate consumption was determined by Fleury—Lange arsenite method [13]. The determination of formic acid was performed by iodometric titration using amperometric indication [14].

The diffusion coefficients in 0.05 M borate buffer pH 9.23 solution were carried out on Tiselius-type apparatus Zeiss model 35.

The sedimentation constants were determined by ultracentrifuge MOM G 110 at $1.82 \cdot 10^5$ *g*. The partial specific volumes were determined pycnometrically.

Cultivation

The strains were cultivated on medium containing glucose, in similar manner as described in our previous work [15].

Isolation of Polysaccharides

After a week cultivation the cells were separated from cultivating medium washed with cold water and repeatedly centrifuged. The cells were worked up separately.

The cultivating medium (volume 40 l) and washings were combined and evaporated under reduced pressure to a small volume which was poured into five times its volume of 95 % ethanol. The precipitated polysaccharide protein complex was centrifuged and deproteinized by Sevage method [16]. The deproteinized polysaccharides were freeze-dried. In Table 1 the yields of polysaccharides (based on dried cells), the ratio mannan/glucan in polysaccharides (determined by combination of Gauss and interference curves obtained from moving boundary electrophoresis) are listed.

Hydrolysis of Polysaccharides

Polysaccharide (20 mg) was hydrolysed with 1 ml 1 N-HCl in a sealed tube at 100 °C for 6 hours. The hydrolysates were analysed by paper chromatography in S_1 and by paper electrophoresis. The molar ratio glucose/mannose was determined by quantitative evaluation of chromatograms. The results are listed in Table 5.

Table 5

The molar mannose/glucose ratio in the products of hydrolysis of extracellular polysaccharides of *Candida albicans*

Polysaccharides of strains	Molar ratio mannan/glucan
18	1 : 0.47
19	1 : 0.41
21	1 : 0.33
65	1 : 0.61
109	1 : 0.67

Isolation of Mannans

From the crude polysaccharides the mannans were precipitated with Fehling's solution as their copper complexes. The precipitated complexes were throughly washed with cold water, suspended in water and decomposed by gradual addition of 1 N-HCl until the dissolution was complete. From the solution the mannan was precipitated with the 4 volumes of ethanol, centrifuged and washed with acetone and ether, dissolved in small amount of water and freeze-dried. The hydrolysis of the mannans was carried out as the hydrolysis of polysaccharides. On paper chromatography mannose was the only detectable sugar. The homogenity of mannans was confirmed by moving boundary electrophoresis and their electrophoretic mobility $u = 9.1 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. In Table 6 the yields of mannans calculated on the weight of dried cells are given.

Table 6

The yields of extracellular mannans of *Candida albicans*

Mannan of strain	Yield	
	g	%
18	1.3	0.64
19	1.5	1.04
21	1.7	1.06
65	1.3	0.52
109	1.1	0.73

Methylation Analysis of Mannans

Mannan (400 mg) was methylated three times by the Haworth procedure [17] with dimethyl sulphate and sodium hydroxide. The partial methylated mannan was then methylated according to R. Kuhn [18] with methyl iodide and silver oxide in dimethylformamide. The infrared spectrum of the methylated products showed no hydroxyl absorption. The methylated mannans were hydrolysed with 90 % formic acid one hour at 100 °C and then with 0.25 M-H_2SO_4 24 hours at 100 °C. After the neutralization with $BaCO_3$ and evaporation the residual mixture was refluxed for 6 hours with 2 % methanolic hydrogen chloride and solution neutralized with Ag_2CO_3. The mixture of *O*-methyl ethers of D-mannopyranosides was deionized with Zerolite 225 (H^+ form) and resolved by GLC. The ratio of the components was calculated from the peak areas (Table 3).

Partial Hydrolysis of Mannans

Mannan (100 — 200 mg) was hydrolyzed 6 hours with 10 ml of 90 % HCOOH at 100 °C. The mixture was concentrated to 2 ml, 10 ml of water was added, solution was heated 1 hour and concentrated to a sirup. The mixture was separated using paper chromatography in S_2. R_G values of obtained oligosaccharides are listed in Table 7. The first member of the homologous serie, 2-*O*-α-D-mannopyranosyl-D-mannose was received in form of sirup-like substance, $[\alpha]_D^{23} = +43°$ ($c = 1$; water) and give no colour reaction with 2,3,5-triphenyltetrazolium chloride.

Table 7

R_G-values of oligosaccharides obtained by partial hydrolysis of extracellular mannans *Candida albicans* in S_2

O-α-D-Manp(1 ——→ 2)-D-Manp	0.54
O-α-D-Manp(1 ——→ 2)-*O*-α-D-Manp(1 ——→ 2)-D-Manp	0.45
O-α-D-Manp(1 —[→ 2)-*O*-α-D-Manp(1 —]$_2$→ 2)-D-Manp	0.34
O-α-D-Manp(1 —[→ 2)-*O*-α-D-Manp(1 —]$_3$→ 2)-D-Manp	0.26
O-α-D-Manp(1 —[→ 2)-*O*-α-D-Manp(1 —]$_4$→ 2)-D-Manp	0.19

Periodate Oxidation

Mannan (82 mg) was dissolved in 50 ml of distilled water. 50 ml of 0.06 M sodium periodate was added to the solution and oxidation was carried out at 25 °C in dark (blank tests as well). For estimation of HCOOH and $NaIO_4$ 10 ml and 5 ml samples were taken in time intervals (Table 3). For periodate estimations 10 ml of saturated aqueous solution of $NaHCO_3$, 50 ml of 0.01 N-Na_3AsO_3, 1 ml of 20 % KI and 5 ml starch indicator were added and titrated with 0.01 N iodine solution. For estimation of formic acid the excess of periodate in a 5 ml aliquot was destroyed by 1 ml ethylenglycol and after 30 min. 5 ml 0.4 M-KI and 5 ml 0.01 N-$Na_2S_2O_3$ were added and the excess of $Na_2S_2O_3$ was titrated with 0.01 N iodine solution. The end point was amperometrically indicated. The results in moles per mannose unit are given in Table 3.

Smith Degradation

After completed periodate oxidation 40 ml aliquots were taken and excess of periodate removed with $Pb(CH_3COO)_2$. The obtained precipitate was centrifuged and washed with distilled water. The washings were combined with filtrate and evaporated under reduced pressure at 35 °C to 5 ml volume. The oxidized mannan was reduced with 80 mg $NaBH_4$ and hydrolyzed with 2 ml of 1 N-HCl at 100 °C in a sealed tube. The hydrolyzate was filtered through Zerolit G (OH^- form) and analyzed by paper chromatography in S_3. In hydrolyzates only glycerol was found.

POLYSACHARIDY KVASINIEK A KVASINKOVITÝCH MIKROORGANIZMOV (II)
EXTRACELULÁRNE MANÁNY *Candida albicans* BERKHOUT

D. Šikl, L. Masler, Š. Bauer

Oddelenie biochémie sacharidov Chemického ústavu Slovenskej akadémie vied, Bratislava

Po kultivácii piatich kmeňov *C. albicans* BERKHOUT sa zo živného média izolovali vysoko vetvené extracelulárne α-manány s DP 150—630.

Na základe metylačnej analýzy, parciálnej hydrolýzy, jodistanovej oxidácie a Smithovej degradácie sa zistilo, že extracelulárne manány patria k dvom štrukturálnym typom: prvý typ, menej vetvený, má hlavný reťazec zložený z manózových jednotiek spojených α-1,6-glykozidickými väzbami, ktorého každá druhá manózová jednotka je substituova-

ná dvojčlenným a päťčlenným bočným reťazcom s α-1,2-glykozidickými väzbami. Bočné reťazce sa pripájajú k hlavnému reťazcu α-1,2-väzbami. Druhý typ manánu je viacej vetvený, každá jednotka hlavného reťazca predstavuje bod vetvenia. V bodoch vetvenia sa pripájajú α-1,2-glykozidickými väzbami dvojčlenné a štvorčlenné bočné reťazce. V hlavnom reťazci sú väzby α-1,6 a v bočných reťazcoch α-1,2.

ПОЛИСАХАРИДЫ ДРОЖЖЕЙ И ДРОЖЖЕПОДОБНЫХ МИКРООРГАНИЗМОВ (II)
ВНЕКЛЕТОЧНЫЕ МАННАНЫ РОДА *Candida albicans* BERKHOUT

Д. Шикл, Л. Маслер, Ш. Бауэр

Отдел биохимии сахаридов Химического института Словацкой академии наук, Братислава

После выращивания культур пяти штаммов *C. albicans* BERKHOUT были изолированы из питательной среды сильно разветвленные внеклеточные α-маннаны с DP от 150 до 630.

На основании метилирующего анализа, частичного гидролиза, периодатного окисления и расщепления по Смиту было определено, что внеклеточные маннаны относятся к двум структуральным типам: первый тип, в меньшей степени разветвленный, главная цепь которого состоит из маннозных единиц соединенных α-1,6-глюкозидными связями, каждая вторая маннозная единица которого замещена двух- и пятичленной боковой цепью с α-1,2-глюкозидными связями. Боковые цепи присоединены к главной цепи α-1,2-связями. Второй тип маннана является более разветвленным, каждая единица главной цепи представляет собой точку разветвления. В точках разветвления присоединены α-1,2-глюкозидными связями двух- и четерехчленные боковые цепи. В главной цепи имеются α-1,6-связи а в боковых цепях α-1,2.

Preložil M. Fedoroňko

REFERENCES

1. Šikl D., Masler L., Bauer Š., *Chem. zvesti* ***19***, 21 (1965).
2. Šandula J., Kocková-Kratochvílová A., Zámečníková M., *Folia microbiologica* ***8***, 313 (1963).
3. *Catalogue of Cultures of Czechoslovak Collections of Microorganisms*, Brno 1964.
4. Miyazaki T., *J. Pharm. Soc. Japan* ***82***, 1678 (1963).
5. French D., Wild D. M., *J. Am. Chem. Soc.* ***75***, 2612 (1953).
6. Hasenclever H. F., Mitchell W. O., *J. Bacteriol.* ***82***, 570 (1961).
7. Hasenclever H. F., Personal communication.
8. Patridge S. M., *Nature* ***164***, 443 (1949).
9. Schwimmer S., Bevenue A., *Science* ***123***, 543 (1956).
10. Wallenfells K., *Naturwiss.* ***37***, 491 (1950).
11. Trevelyan W. E., Procter D. P., Harrison J. S., *Nature* ***166***, 444 (1950).
12. Viscontini M., Hoch D., Karrer P., *Helv. Chim. Acta* ***38***, 642 (1955).
13. Fleury P. F., Lange J., *J. Pharm. Chim.* ***17***, 1966 (1933).
14. Babor K., Kaláč V., Tihlárik K., *Chem. zvesti* ***18***, 913 (1964).
15. Masler L., Šikl D., Bauer Š., Šandula J., *Folia microbiologica* ***11***, 373 (1966).

16. Sevag M. G., *Biochem. Z.* ***273***, 419 (1934).
17. Haworth W. N., *J. Chem. Soc.* ***107***, 13 (1915).
18. Kuhn R., Trischmann H., Löw I., *Angew. Chem.* ***67***, 32 (1955).

Received June 24th, 1966

The adress of the authors:
Ing. Dobroslav Šikl, CSc., Ing. Ladislav Masler, CSc., Ing. Dr. Štefan Bauer, CSc., Oddelenie biochémie sacharidov Chemického ústavu SAV, Bratislava, Dúbravská cesta.