EXTRACELLULAR POLYSACCHARIDES OF *Candida mucifera* STRAIN CCY 29-170-1

Alzbeta KARDOSOVA and Vladimir PATOPRSTY

Institute of Chemistry,
Slovak Academy of Sciences, 842 38 Bratislava, Slovak Republic

Received September 4, 1995
Accepted November 9, 1995

Extracellular polysaccharides produced by *Candida mucifera* strain CCY 29-170-1 in a liquid medium containing malt extract have been investigated by chemical, enzymic, and NMR spectroscopic methods. Mild acid hydrolysis of the native polysaccharide, followed by enzymic treatments of the polymeric residue, afforded two low-molecular neutral homopolysaccharides. The dominant one was a (1→4)-α-D-glucan with approximately each fifth chain unit branched on O-6. The other polysaccharide was a linear (1→4)-β-D-xylan. The results of linkage analysis evidenced that, originally, some xylan-chain units bore on O-3 substituents, which were split off during the hydrolytic steps.
Key words: *Candida mucifera*; Extracellular polysaccharides.

In our previous papers[1,2] we reported on production of extracellular polysaccharides by *Candida mucifera* strain CCY 29-170-1 and described the structure of a representative phosphogalactomannan recovered from the medium with glucose as the carbon source. The present work provides results on extracellular polysaccharides produced in the medium containing malt extract.

EXPERIMENTAL

Materials and Methods

The type strain *Candida mucifera* CCY 29-170-1 was maintained on malt agar in the Slovak Collection of Yeasts at the Institute of Chemistry, Slovak Academy of Sciences, Bratislava. The cultivation conditions of the yeast in medium containing malt extract (Sigma Chemicals) and isolation of polysaccharides were described[1]. α-Amylase from Pancreas (1,4-α-D-Glucan-glucanohydrolase; EC 3.2.1.1, 25 IU/mg) was a product of Fluka AG and *endo*-1,4-β-Xylanase (1,4-β-D-Xylan-xylanohydrolase; EC 3.2.1.8) was isolated from *Aspergillus niger*[3]. The substrates used to check the activity of enzymes were Amylose (AVEBE, The Netherlands) and 4-*O*-methyl-D-glucurono-D-xylan[4]. The reference compounds maltotriose, xylobiose, and xylotriose were commercial products (Sigma Chemicals).

Concentrations were performed under reduced pressure at bath temperature not exceeding 45 °C. Optical rotations (1 ml cells) were measured at 20 ± 1 °C with a Perkin–Elmer Model 141 polarimeter. Infrared spectra of the methylated products were recorded with a Nicolet magna 750 spec-

trometer. HPGPC was performed using a commercial instrument (Laboratorni pristroje, Prague, Czech Republic) equipped with two Tessek Separon HEMA BIO-100 exclusion columns (8 × 250 mm) and aqueous 0.1 M $NaNO_3$ as solvent and eluent (0.4 ml/min). The eluate was monitored by refractometry. A set of pullulan standards P-5, P-10, P-20, and P-50 (Shodex Standard, P-82, Macherey–Nagel, Germany) was used for calibration of the columns. Therefore, the molecular weight parameters shown are relative to the pullulan reference material. A computing procedure[5] based on linear effective calibration curve was applied to obtain the molecular weight distribution.

Descending paper chromatography (PC) was performed on Whatman No. 1 and 3MM papers in the solvent system ethyl acetate–pyridine–water (8 : 2 : 1) and TLC (Merck silica plates) in 1-propanol–methanol–water (2 : 1 : 1). Sugars were detected with anilinium hydrogen phthalate and silver nitrate. The mobilities of oligosaccharides (R_{Xyl}, R_{Glc}) are expressed relative to those of D-xylose and D-glucose. Carbohydrates were determined by the phenol–sulfuric acid assay[6] and protein by the method of Lowry et al.[7], using bovine serum albumin as standard. Total phosphate was determined by the method of Ames and Dubin[8], using KH_2PO_4 as standard.

Gas chromatography of alditol trifluoroacetates was conducted on a Hewlett–Packard 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m) at the temperature program of 110–125 (2 °C min^{-1})–165 °C (20 °C min^{-1}) and flow rate of hydrogen 20 ml min^{-1}. GC/MS analyses of partially methylated alditol acetates were effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm × 30 m) at 80–240 °C (6 °C min^{-1}), 70 eV, 200 μA, and ion-source temperature 150 °C. Chemical ionization was performed at the same conditions using protonated pyridine as the reagent gas[9].

Proton-decoupled FT ^{13}C, ^{31}P, and ^{1}H NMR spectra were recorded with a Bruker AM-300 spectrometer at 75, 121, and 300 MHz, respectively, for solutions in D_2O at 25 °C. ^{13}C chemical shifts were referenced to internal MeOH (δ 50.15), ^{31}P chemical shifts to external 85% orthophosphoric acid (δ 0.00), and ^{1}H chemical shifts to HOD (δ 4.80).

Methylation analysis was performed on 30–50 mg samples applying the method of Ciucanu and Kerek[10]. The polysaccharide sample was dissolved in dimethyl sulfoxide (3–5 ml) under stirring at ambient temperature. The solution was then treated with pulverized NaOH (100–200 mg) and methyl iodide (0.8–1.3 ml) for 1 h. Completeness of methylation was checked by IR absorption for hydroxyl. The permethylated product was isolated by partition with $CHCl_3$ (8–13 ml) and, after conversion into partially methylated alditol acetates by hydrolysis, reduction, and acetylation, subjected to linkage analysis by GC-MS.

Degradation of the Intact Polysaccharide (PS) with Mild Acid

Intact polysaccharide[1,2] (PS, 300 mg) was treated with 1 M TFA (180 ml) for 30 min at 100 °C and then freed from low-molecular products by water irrigation from a Biogel P-2 column (2.5 × 130 cm). The polymeric portion (PS1) was subjected to further hydrolysis under the same conditions. The residue (PS2), recovered from the hydrolysate, was, on repeated column chromatography, resolved to two distinct fractions (PS2-1, PS2-2) which were subjected to compositional and linkage analyses. Reduction and distribution of M_w of the polymeric residues (PS1, PS2) was followed by HPGPC. The low-molecular products, collected from both steps, were separated by gel filtration on the Biogel P-2 column to monosaccharides and oligomers, which were subsequently purified by preparative PC. The structure of the oligomers were elucidated by ^{13}C and ^{1}H NMR spectroscopy.

α-Amylase Digestion of the Degraded Polysaccharide (PS2-2)

PS2-2 (100 mg) was digested with α-amylase (2 mg) in sodium acetate buffer of pH 5.6 (100 ml). The reaction was allowed to proceed at 26 °C and was monitored by TLC. After 25 h, the solution

was boiled for 10 min, deionized with Dowex 50 Wx4 (H^+), and loaded on the column of Biogel P-2 (2.5 × 130 cm). The polymeric residue was analyzed for sugar composition and by NMR spectroscopy. The oligomers were further purified by preparative PC and subjected to structure analysis.

endo-1,4-Xylanase digestion of PS2-2 was effected at the same conditions, except that the pH of the acetate buffer was 4.8. It was ascertained in separate experiments that α-amylase was not contaminated with xylanase and, vice versa, *endo*-xylanase did not contain any amylase activity.

RESULTS AND DISCUSSION

The extracellular polysaccharides produced in the liquid medium containing malt extract were recovered from the culture filtrate as an off-white, water-soluble product (PS). Its general characteristics are presented in Table I. As seen from the table, of the five sugar components, D-glucose dominates. This sugar representation is completely different from that of the phosphogalactomannan[2] produced in the medium with D-glucose as the only carbon source. Phosphorus, found in very low amount, is probably associated with mannose, as the ^{1}H-decoupled ^{31}P NMR spectrum of PS displayed signals at the same field (δ –1.0 and –1.5 ppm) as the phosphogalactomannan[2], and as phosphorus was not detected in the individual glucan and xylan species, recovered from subsequent degradation steps.

HPGPC analysis revealed a molecular heterogeneity of PS (Table II). Attempts to separate the two fractions of orderly different molecular masses by employing gel permeation chromatography on Sephadex G-50 and G-75 were unsuccessful. Therefore, controlled acid degradation under mild conditions (Scheme 1) was conducted. The data in Table II show that molecular homogeneity was achieved upon two degradation steps, when the high-molecular fraction, representing 9% of the intact PS, completely disap-

TABLE I
General characteristics of the intact polysaccharide (PS)

Yield	0.3[a]
Protein, %	3.0
Phosphorus, %	0.4
$[\alpha]_D$, °	+65.4
Sugar composition (mole ratio)	
D-Glc	12.3
D-Gal	0.8
D-Man	1.0
L-Ara	0.8
D-Xyl	1.0

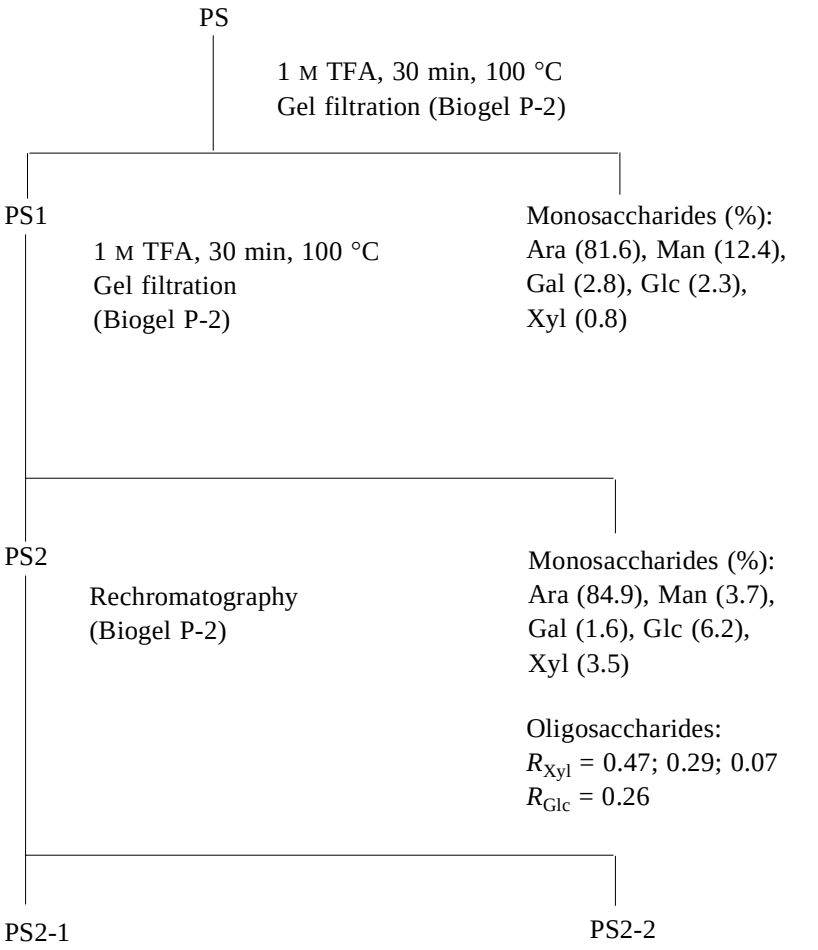
[a] In g per 500 ml culture medium.

peared. The molecular mass of the main fraction was reduced by about 9% only. Treatment of PS with 1 M TFA for 30 min (see Scheme 1), followed by gel filtration on Biogel P-2, yielded, besides the residue eluted in the void volume (PS1), a mixture of monosaccharides, the main component of which was L-arabinose. PS1, subjected to the second short hydrolysis, produced, in addition to the above mentioned monosaccharide mixture, three oligosaccharides based on xylose and one based on glucose. The polymeric residue (PS2), recovered from gel filtration, was resolved upon rechromatography to two fractions (PS2-1, PS2-2) differing in sugar composition. While the glucose component predominating (93%) over four other sugars pointed to a glucan structure of PS2-1, the composition of PS2-2 indicated either a heteropolysaccharide composed of glucose (86%) and xylose (14%), or a mixture of two homopolysaccharides. To ascertain this, enzyme hydrolyses were employed. Treatment of PS2-2 with α-amylase generated glucose, a glucooligosaccharide, and an enzyme-resistant polymer composed of xylose only. On the other hand, the end products of the *endo*-xylanase hydrolysis of PS2-2 were two xylooligosaccharides and a resistant polymeric residue consisting of glucose only. The two residual homopolymers and the fact that neither enzyme hydrolysis yielded hetero(xylogluco)oligosaccharides evidenced that the native extracellular polysaccharide was composed of at least two distinct polysaccharide species, a glucan and a xylan.

The oligomeric products, generated on acid hydrolysis of PS and enzyme hydrolyses of PS2-2, were recovered from gel filtration of their mixtures. Their structures were identified on the basis of their ^{13}C NMR spectra (Table III). The assignment of signals was effected on the basis of comparison with the data for xylooligosaccharides[11,12], maltotriose[13], and starch polysaccharides[14,15]. Both acid and α-amylase hydrolyses afforded a glucooligomer of R_{Glc} 0.26. Its mobility corresponded to that of the reference maltotriose. The doublet at δ 5.40 with $J(1,2)$ = 3.8 Hz in its ^1H NMR spectrum pointed to α-glycosidic links between the sugar units[14]. The positions of the anomeric signals in

TABLE II
Molecular weight distribution on HPGPC chromatograms of the intact (PS) and degraded (PS1, PS2) polysaccharides

Sample	M_w	M_w/M_n	Area, %
PS	8 170	1.30	91
	147 800	1.02	9
PS1	7 728	1.34	97
	128 800	1.06	3
PS2	7 614	1.43	100



SCHEME 1
Acid degradation of the intact polysaccharide

the ^{13}C NMR spectrum (Table III) at 100.90 ppm (nonreducing unit) and 100.65 ppm (central unit) and those of the downfield shifted resonances of C-4 at 78.02 ppm (reducing unit) and 77.81 ppm (central unit), reflected the α-(1→4)-glycosidic links. The other signals in the spectrum also corresponded to the carbon resonances of maltotriose[13].

endo-1,4-Xylanase hydrolysis produced two xylooligosaccharides of R_{Xyl} 0.47 and 0.29. Their mobilities were identical with those of *O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose and *O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose standards, respectively. The positions of the anomeric signals in the ^{13}C NMR spectra at 102.94 ppm (nonreducing unit) of the dimer and 102.90 ppm (nonreducing unit) and 102.75 ppm (central unit) of the trimer, as well as the resonances of C-4 at 77.70 ppm (reducing unit, α-anomer) and 77.54 ppm (reducing unit, β-anomer) for the dimer and at 77.48 ppm (central and reducing units) for the trimer proved the β-(1→4) links between the xylose residues. The β configuration was confirmed also by the coupling constant $J(1,2)$ = 7.8 Hz in the ^{1}H NMR spectra of both compounds, and by the $[\alpha)_D$ values –22.0° for the dimer (ref.[16] gives –23.0°) and –44.5° for the trimer (ref.[16] gives –47.7°). Mild acid hydrolysis of PS yielded, in addition to the above described two oligomers, a higher xylooligomer of R_{Xyl} 0.07. On the basis of the linear relationship of log (R_F/1 – R_F)) plotted vs the degree of polymerization it was identified as a xylopentaose. The β-configuration of the linkages in this oligomer was indicated by the

TABLE III
^{13}C NMR chemical shifts (ppm) of oligomers recovered from the enzyme hydrolysates of PS2-2

Compound	Residue[a]	C-1	C-2	C-3	C-4	C-5	C-6
Xylobiose[b]	α	93.12	72.48	72.06	77.70	59.97	–
β-(1→4)	β	97.60	75.10	75.03	77.54	64.11	–
	nr	102.94	73.88	76.71	70.30	66.32	–
Xylotriose[b]	α	93.20	72.50	72.00	77.48	59.70	–
β-(1→4)	β	97.50	75.00	75.00	77.48	64.07	–
	int	102.75	73.82	74.74	77.48	64.07	–
	nr	102.90	73.82	76.70	70.28	66.31	–
Maltotriose[c]	α	92.99	72.41	74.10	78.02	71.07	61.60
α-(1→4)	β	96.87	75.12	77.33	77.81	75.68	61.86
	int	100.65	72.78	74.34	77.81	72.41	61.60
	nr	100.90	72.78	73.97	70.45	73.79	61.60

[a] Internal (int), nonreducing (nr), and reducing sugar under α- or β-configuration. [b] *endo*-1,4-Xylanase digestion. [c] α-Amylase digestion.

$[\alpha]_D$ value –68.6°, which is close to –70.1° given in ref.[16] for (1→4)-β-D-xylopentaose. Due to shortage of the material further evidence for the indicated linkage could not be obtained.

The intact (PS) and the degraded polysaccharides (PS2-1, PS2-2) were analyzed for their glycosidic linkages after converting them into the corresponding *O*-acetyl-*O*-methylalditols by subsequently adopting the methods of Ciucanu[10] and Jansson[17]. The

TABLE IV
Methylation analysis data of intact (PS) and degraded (PS2-1, PS2-2) polysaccharides

Derivative[a]	Mole %			Linkage indicated
	PS	PS2-1	PS2-2	
2,3,5-Me_3-Ara	3.9	0.4	–	Araf-(1→
2,3,4-Me_3-Xyl	1.6	0.4	0.9	Xylp-(1→
2,3-Me_2-Xyl	2.9	3.7	13.5	→4)-Xylp-(1→
2-Me-Xyl	0.3	0.5	–	→3,4)-Xylp-(1→
Total:	4.8	4.6	14.4	
2,3,4,6-Me_4-Man	4.5	–	–	Manp-(1→
3,4,6-Me_3-Man	1.3	0.7	–	→2)-Manp-(1→
3,6-Me_2-Man	–	0.3	–	→2,4)-Manp-(1→
Total:	5.8	1.0	–	
2,3,4,6-Me_4-Gal	1.8	0.7	–	Galp-(1→
2,4,6-Me_3-Gal	0.4	–	–	→3)-Galp-(1→
2,6-Me_2-Gal	0.5	0.3	–	→3,4)-Galp-(1→
3,6-Me_2-Gal	3.5	–	–	→2,4)-Galp-(1→
2,4-Me_2-Gal	0.4	–	–	→3,6)-Galp-(1→
Total:	6.6	1.0	–	
2,3,4,6-Me_4-Glc	9.5	18.6	19.3	Glcp-(1→
2,4,6-Me_3-Glc	1.6	1.0	–	→3)-Glcp-(1→
2,3,4-Me_3-Glc	5.0	–	2.8	→6)-Glcp-(1→
2,3,6-Me_3-Glc	52.7	55.1	51.8	→4)-Glcp-(1→
3,4,6-Me_3-Glc	–	0.9	–	→2)-Glcp-(1→
2,3-Me_2-Glc	10.1	17.2	11.7	→4,6)-Glcp-(1→
Total:	78.9	92.8	85.6	

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-methylarabinose, etc., determined as partially methylated alditol acetates.

results are presented in Table IV. As seen from the table, the sugar composition of PS corresponded with the results obtained by GC (Table I). The formation of only 2,3,5-tri-*O*-methylarabinose derivative indicated that all arabinose were present as terminal nonreducing furanosyl residues. The decreased content of this derivative in PS2-1 and its absence in PS2-2 confirmed the splitting of these acid-labile units on degradation.

The mannopyranose derivatives evidenced that about 77% of this sugar occupied terminal position and the remaining part was linked through O-2. The splitting of this sugar during degradation was reflected in loss of mainly the tetramethyl derivative.

Formation of five galactose derivatives pointed to engagement of this sugar in variety of linkages. The 3,6-di-*O*-methylgalactose derivative, arising from 1,2,4-linked pyranosyl residues, accounted for 3.5% of total 6.6% galactose component of PS. Nonreducing ends were indicated by 1.8% tetramethyl derivative, and the remaining portion represented the sum of approximately equal proportions of 1,3-, 1,3,4-, and 1,3,6-linked residues. Some terminal and 1,3,4-linked units were still present after degradation, as evidenced by the respective derivatives in PS2-1.

Xylose was shown to occur in pyranosyl form, occupy nonreducing end position, and be also 1,4- and 1,3,4-linked. PS2-2 contained only end units and residues linked through O-4. This sugar was, besides glucose, the most resistant component to acid degradation.

The derivatives of the main component of the intact polysaccharide, glucose, pointed to pyranosyl form of this sugar and to its involvement in five types of linkages. However, terminal nonreducing units, residues linked through O-4, and 1,4,6-linked units evidently dominated. Approximately each fifth 1,4-linked chain-unit was substituted on O-6. Only 6% of total glucose derivatives indicated 1,6- and 2% 1,3-linked residues. The data for PS2-2 evidenced that all 1,3-linked and part of the 1,6-linked residues were cleaved on degradation.

The above presented data supported the results of enzyme hydrolyses in that the proportion (14%) and the kind of the xylose derivatives well corresponded with the α-amylase-resistant xylan residue (12%) of PS2-2 and, likewise, the proportion of 85.6% glucose derivatives was comparable to that of the *endo*-xylanase-resistant glucan residue (84%) of PS2-2.

The ^{13}C NMR spectrum of the *endo*-xylanase-resistant polymeric portion of PS2-2, an α-D-glucan, is presented in Fig. 1 and that of the α-amylase-resistant portion, a β-D-xylan, is in Fig. 2. The signals were assigned on the basis of comparison with the literature data for amylopectin[15], glycogen[15,18], and xylan[11]. The spectrum of the α-D-glucan showed three signals in the anomeric region. The signals at 101.05 and 100.90 ppm were assigned to C-1 linked α-(1→4). The simple resonance at 99.68 ppm is clearly due to C-1 engaged in an α-(1→6) linkage. The signals at 78.90 and 78.11 ppm arose from the resonance of C-4 in an α-(1→4) link. The presence of two resonances for C-1 and C-4, respectively, was explained by the sensitivity at these positions to the nature

of this linkage[18]. Another diagnostic signal in the spectrum is that at 68.41 ppm for C-6 involved in α-(1→6) linkage. The other signals observed were assigned to carbons not engaged in linkages as follows: 74.44–73.85 ppm (C-3), 72.83–72.38 ppm (C-2), 71.48 ppm (C-5), 70.45 ppm (C-4), and 61.62 ppm (C-6).

The spectrum of the β-D-xylan (Fig. 2) is a simple one, showing five signals of equal intensity. Diagnostic for the structure determination of the polymer is the anomeric signal at 102.70 ppm and that at 77.48 ppm due to the resonance of C-4. They ascertain the involvement of these carbons in β-(1→4) links. The other resonances were observed at 73.84 (C-2), 74.79 (C-3), and 64.10 ppm (C-5). The additional signal of low intensity at 66.30 ppm arose from the resonance of C-5 of the nonreducing residue. The above presented spectral data supported the results of chemical linkage analysis.

It can be concluded that the extracellular polysaccharides produced by *Candida mucifera* in the medium with malt extract comprise a low-molecular (1→4)-α-D-glucan

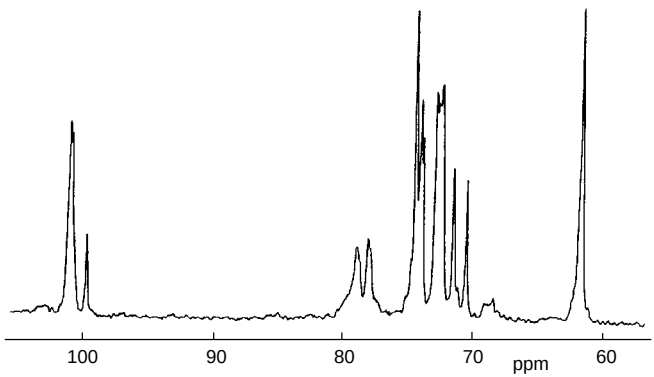


FIG. 1
^{13}C NMR spectrum of α-D-glucan

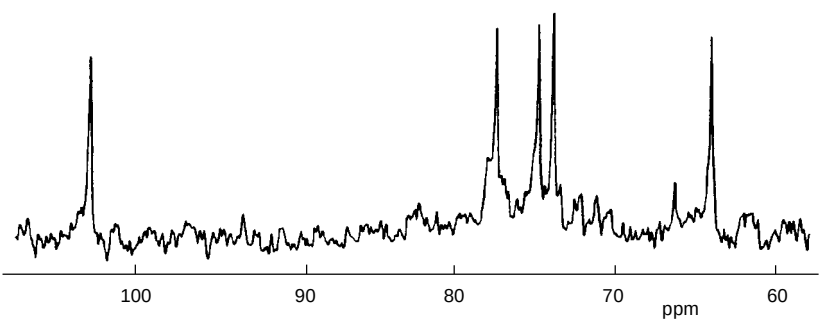


FIG. 2
^{13}C NMR spectrum of β-D-xylan

(more than 70%) with approximately each fifth chain-unit branched on O-6 and a low-molecular (1→4)-β-D-xylan (about 4%). It is known that the average chain-length of branches in glycogen is much shorter than in amylopectins[19]. Dais and Perlin[15] found in the ^{13}C NMR spectra of glycogen and amylopectins a difference in the distribution of chemical shifts within the group of C-1 and C-4 signals and stated that it reflects the variations in the arrangements of branchings within these two classes of polymers. The ^{13}C NMR spectral pattern of the glucan studied herein revealed a fine structure which can be classified as a glycogen-type rather than an amylopectin-type one. Comparison of these polysaccharides with the phosphogalactomannan[2], produced in a liquid medium with D-glucose as carbon source, revealed the ability of the yeast to respond to the nutrient.

The authors are indebted to Dr E. Machova (Institute of Chemistry, Slovak Academy of Sciences, Bratislava) for HPGPC analysis.

REFERENCES

1. Danis J., Kardosova A., Slavikova E.: Chem. Papers *46*, 123 (1992).
2. Kardosova A., Danis J., Alfoldi J.: Chem. Papers *48*, 189 (1994).
3. Gorbachev I. V., Rodionova N. A.: Biochim. Biophys. Acta *484*, 79 (1977).
4. Kardosova A., Capek P., Rosik J.: Chem. Papers *43*, 705 (1989).
5. Soltes L., Alfoldi J., Sandula J.: Appl. Polym. Sci. *48*, 1313 (1993).
6. Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A., Smith F.: Anal. Chem. *28*, 350 (1956).
7. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
8. Ames B. N., Dubin D. T.: J. Biol. Chem. 235, 769 (1960).
9. Patoprsty V., Kovacik V., Karacsonyi S.: Rapid Commun. Mass Spectrom. *9*, 840 (1995).
10. Ciucanu I., Kerek F.: Carbohydr. Res. *131*, 209 (1984).
11. Kovac P., Hirsch J.: Carbohydr. Res. *85*, 177 (1980).
12. Lahaye M., Jegou D., Buleon A.: Carbohydr. Res. *262*, 115 (1994).
13. Morris G. A., Hall L. D.: Can. J. Chem. *60*, 2431 (1982).
14. Gidley M. J.: Carbohydr. Res. *139*, 85 (1985).
15. Dais P., Perlin A. S.: Carbohydr. Res. *100*, 103 (1982).
16. Hirsch J., Kovac P., Petrakova E.: Carbohydr. Res. *106*, 203 (1982).
17. Jansson P. E., Kenne L., Liedgren H., Lindberg B., Lönngren J.: Chem. Commun. Univ. Stockholm *8*, 1 (1976).
18. Colson P., Jennings H. J., Smith I. C. P.: J. Am. Chem. Soc. *96*, 8081 (1974).
19. Manners D. J.: Adv. Carbohydr. Chem. *17*, 371 (1962).