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Carbohydrate Research 307 (1998) 177–183

Note

Structural analysis of the intact polysaccharide mannan from *Saccharomyces cerevisiae* yeast using ^{1}H and ^{13}C NMR spectroscopy at 750 MHz

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Received 28 November 1997; accepted 30 January 1998

Abstract

The mannan from *Saccharomyces cerevisiae* yeast was studied by high field NMR spectroscopy in an attempt to deduce the structure of the polysaccharide and to assess the ratio of different side chains. The results show that all structural information, agreeing with previously published data, can be extracted by analysis of the NMR spectra of the intact and modified mannan.

Keywords: Yeast; Mannan; MALDI; HPAE

Mannans constitute a major part of the glycoproteins of the cell envelope of almost all yeasts. They are composed of heterogenous polymannose chains N-linked through core oligosaccharide to different proteins. The mannans interact with mannan-binding proteins of higher animals and elicit specific immune response with the formation of antibodies, which recognize oligosaccharide fragments of the mannose polymer [1–3]. Mannans are also involved in yeast cells aggregation, termed flocculation [4,5]. Flocculation is based on a Ca^{2+} dependent interaction of mannans with lectin-like proteins (flocculins) on the yeast cell surface. Although flocculation is important for many applications of yeast in food technology, particularly beer brewing, its mechanism is still not well understood.

Mannans from most kinds of yeasts have a similar overall architecture, consisting of α-1→6-linked mannose main chain, substituted with side chains of different composition and structure [6]. Usually the structure of a mannan is deduced from the structure of the oligosaccharide products, obtained after acetolysis, which cleaves selectively 1→6-linkages in the main chain, experiments using enzymatic cleavage, or by studies of mutant strains with defective mannan biosynthesis. Conventional acetolysis cleaves selectively the α-1→6- or eventually β-linkages between the mannose residues, and the glycosyl phosphate linkages. Where such linkages are absent in the side chains, oligosaccharides

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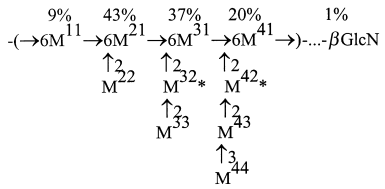
0008-6215/98/$19.00
PII S0008-6215(98)00042-1

representing the intact side chains are isolated. Otherwise more time consuming and complicated experiments are necessary to determine the structural details. NMR spectra of several mannans were analyzed previously [7–11], and the ratio of side chains was determined by NMR methods in the case of *Candida catenulata* mannan [7]. Usually only the signals of H-1,2 and C-1,2 were assigned. In the present study an attempt to deduce the structure of the whole mannan and to assess the ratio of different side chains on the basis of high field NMR spectroscopy data have been made for the *Saccharomyces cerevisiae* mannan, the structure of which has previously been suggested [6,12,13].



M is α-D-mannopyranosyl residue.

* indicates the presence of the following substituents at O6:

for mannan-1 (M1): 30% H, 20% P, 20% $M^{11P}P\rightarrow$, 30% $M^{22P}\rightarrow 3M^{21P}P\rightarrow$;

for mannan-2 (M2): 30% H, 70% P;

for mannan-3 (M3): H

Scheme 1.

1. Results and discussion

Three samples of *S. cerevisiae* mannan were studied by NMR: mannan-1, prepared by proteinase K digestion of conventionally isolated mannoprotein fraction, followed by anion exchange chromatography on DEAE column; mannan-2, obtained from mannan-1 by mild acid hydrolysis to cleave glycosyl phosphate linkages without removal of phosphate residues from the polymer; mannan-3, completely dephosphorylated mannan by HF hydrolysis and deproteinized by alkaline degradation. ^{1}H, ^{13}C, COSY, TOCSY, ROESY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMQC–TOCSY, ^{1}H-^{13}C HMBC spectra were recorded for each sample and analysed using the Pronto program [14]. For mannans 1 and 2 ^{31}P spectra and one-dimensional ^{31}P-^{1}H HMQC spectra were also measured. Based on the results from NMR spectroscopy the following structure of *S.cerevisiae* mannan was established (Scheme 1).

In order to discuss the structural details the two digit numbering of sugar residues in the mannan structure is proposed describing the array of oligosaccharides which can be obtained after complete cleavage of the main chain of the mannan. The first digit indicates the number of the side chain, the second refers to the position of the sugar in the oligosaccharide counting from reducing end.

A 750 MHz NMR (Fig. 1) spectrum of mannan-**1** showed a variety of mannose signals of different intensity and linewidth. It was not possible to assign all the signals to sugar residues presented in the formula. The residues of main polymeric chain have broad signals and are therefore the most difficult to identify. Furthermore, no difference was observed in spectra for the residues of type M^{33} attached to Man or Man-6-phosphate, but for M^{43} and the following residues (M^{44}) different data depending on the phosphorylation of M^{42} were observed. It was impossible to distinguish the residues carrying phosphate and glycosyl phosphate substituents. Several minor variants of structures of some mannose residue are present (marked as "minor" in the Tables 1 and 2). These monosaccharides may be present in the core oligosaccharides, at the end of polymeric chain, or may show different spectral data due to specific arrangement of neighboring substituents or conformational preferences.

The use of HMQC–TOCSY spectra allowed the identification of the carbon signals of the sugar residue, even though not all protons are assigned (Fig. 2). This makes it possible to exclude structures with chemically labile substituents, which otherwise is difficult to conclude from other experiments and is of critical importance for the identification of overall structure of the polymer. The present results show that no additional substituents are present.

Quantification of the different oligosaccharide fragments was performed by integration of the anomeric signals in the ^{1}H spectra and H-1,2-correlations in the TOCSY spectra, and averaging of the results. Setting the amount of trisaccharide to 1, a ratio of mannan components Man:Man_2:Man_3:Man_4 = 0.24:1.2:1:0.54 was calculated. The ratio of phosphodiesters to phosphomonoesters was obtained from the integration of ^{31}P spectra (Scheme 1).

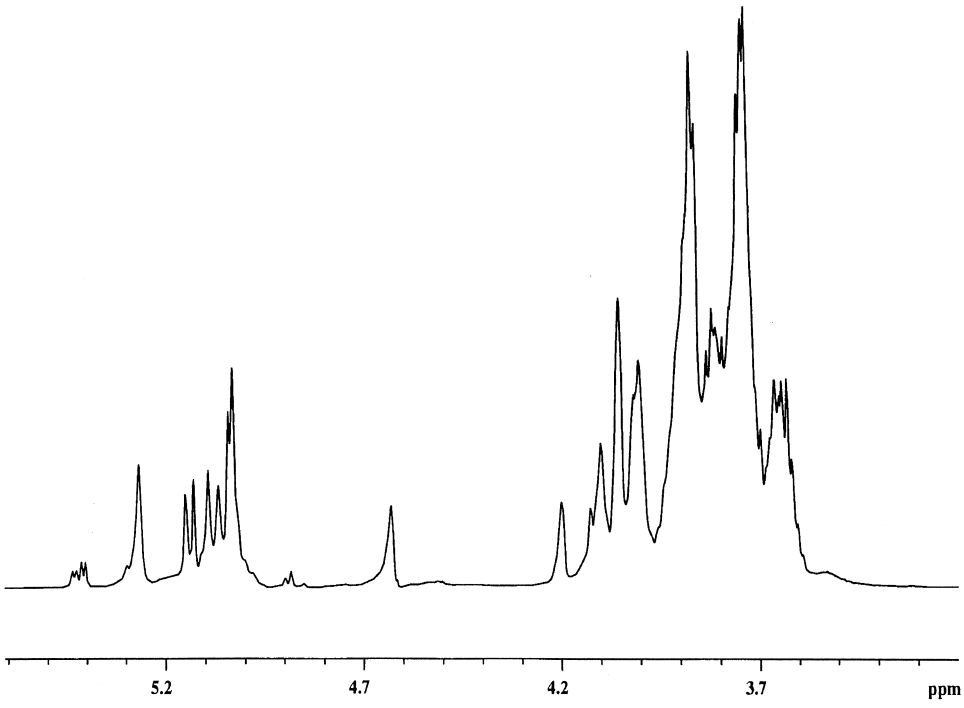


Fig. 1. 750 MHz ^{1}H NMR spectrum of mannan-1.

For comparison, the mannan structural analysis was performed using acetolysis experiments with the identification of the products by HPAE chromatography (Fig. 3) and MALDI mass spectrometry (Fig. 4).

The following oligosaccharide products were isolated after preparative acetolysis. The structural assignments were based on NMR and MS spectroscopy:

1 M6P→2M
2 M→2M6P→2M
3 M→3M→2M6P→2M
4 M→2M
5 M→3M
6 M→2M→2M
7 M→3M→2M
8 M→3M→2M→2M

Responce factors in HPAE were determined for the products **4**–**8**. Integration of peaks and using the responce factors gives the ratio of acetolysis products Man:Man_2:(M–2M–2M):(M–3M–2M):Man_4 = 0.23:1.9:1:0.15:0.9 for the dephosphorylated mannan-2 and 0.74:3.71:1:0.19:0.72 for the intact mannan-1 (disaccharides **4** and **5** are not separated under the experimental conditions used). The amount of Man and disaccharides in the products from intact mannan includes the products from the glycosyl phosphate cleavage. The increased amount of disaccharides compared to the NMR data is most likely due to degradation products from the core. Phosphorylated oligosaccharides were isolated but not quantified by HPAE because insufficient amounts and purity of isolated products were available to determine the responce factors accurately.

Two of the structures obtained after acetolysis, **1** and **7**, were not identified in the NMR spectra. However, fragments of the structure M–3M–2M are present in the polymeric chains of *S. cerevisiae* mannans in variable amounts and in some strains they are more abundant than M–2M–2M fragments [3,6,15,16], which reflects the activity of a 1-3-mannosyl transferase. The strain used in the current work synthesizes it in minor amount. These products are also expected to be formed from the core oligosaccharide [12,17]. The phosphorylated trisaccharide M–3M–2M6P was previously postulated to be present at the end of the polymeric chain and in the core [12].

MALDI mass spectra of the acetolysis products may be used to determine the molecular mass of neutral and phosphorylated oligosaccharides, obtained by acetolysis, however meaningful results are only obtained with oligosaccharides larger than

Table 1
^{1}H NMR data

Substance	Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
M 1	M^{11}	4.880	3.972	3.814				
		4.893	3.969	3.826				
		4.897	3.988					
M 2	M^{11}	4.879	3.973	3.815				
		4.894	3.965	3.829				
		4.898	3.983					
M 3	M^{11}	4.872	3.865	3.808				
		4.885	3.954	3.818				
		4.888	3.975					
M 1	M^{21}	5.090	4.018	3.905			4.00	3.66
M 2	M^{21}	5.091	4.020	3.906			4.00	3.66
M 3	M^{21}	5.087	4.015	3.897			4.00	3.66
M 1	M^{31} or M^{41a}	5.065	3.998	3.90			4.00	3.66
	M^{31} or M^{41b}	5.075	3.920					
M 2	M^{31} or M^{41}	5.065	4.000	3.900			4.00	3.66
	M^{31} or M^{41b}	5.075	3.920					
M 3	M^{31} or M^{41}	5.065	3.993	3.893			4.00	3.66
1	M^{21}	5.418	3.931	3.941	3.690	3.788	3.752	3.853
2	αM^{31}	5.377	3.881	3.906	3.661	3.740	3.820	
3	αM^{41}	5.410	3.897	3.919	3.673	3.760	3.836	
M 1	M^{22}	5.026	4.056	3.787	3.664	3.74	3.87	3.72
M 2	M^{22}	5.027	4.047	3.788	3.661	3.74	3.87	3.72
M 3	M^{22}	5.016	4.049	3.777	3.646	3.734	3.87	3.72
M 1	M^{32} or $M^{42\,a}$	5.267	4.096	3.895	3.71	3.71	3.87	3.72
	^{b}	5.267	4.108	3.912			4.09	4.09
	minor	5.267	4.085	3.921				
M 2	M^{32} or $M^{42\,a}$	5.267	4.097	3.895	3.71	3.71	3.87	3.72
	^{b}	5.267	4.107	3.912		3.81	4.09	4.09
	minor	5.267	4.085	3.921				
M 3	M^{32} or M^{42}	5.267	4.090	3.888	3.69	3.71	3.87	3.72
1	M^{22}	5.022	4.075	3.850	3.688	3.889	4.013	4.150
2	M^{32}	5.242	4.089	3.935	3.735	3.847	3.994	4.103
3	M^{42}	5.280	4.105	3.944	3.752	3.868	4.025	4.137
M 1	M^{33}	5.038	4.046	3.825	3.629	3.74	3.87	3.72
M 2	M^{33}	5.038	4.047	3.827	3.626	3.74	3.87	3.72
M 3	M^{33}	5.025	4.040	3.818	3.610	3.742	3.87	3.72
M 1	$M^{43\,a}$	5.014	4.201	3.954				
	$M^{43\,b}$	5.027	4.197	3.936	3.73	3.78	3.87	3.72
	minor	4.995	4.213					
M 2	$M^{43\,a}$	5.019	4.206	3.958	3.717			
	$M^{43\,b}$	5.026	4.199	3.934	3.744	3.77	3.87	3.72
	minor	5.014	4.197	3.921	3.740			
M 3	M^{43}	5.014	4.194	3.926	3.728	3.786	3.87	3.72
2	M^{33}	5.011	4.036	3.832	3.582	3.702	3.767	3.860
3	M^{43}	5.020	4.206	3.988	3.712	3.870	3.820	
M 1	$M^{44\,a}$	5.124	4.048	3.866	3.632	3.761	3.89	3.73
	$M^{44\,b}$	5.142	4.065	3.871				
M 2	$M^{44\,a}$	5.124	4.051	3.866	3.630	3.762	3.884	3.733
	$M^{44\,b}$	5.137	4.059	3.869	3.628	3.76		
M 3	M^{44}	5.114	4.042	3.857	3.610	3.754	3.87	3.72
3	M^{44}	5.150	4.051	3.864	3.615	3.720	3.770	
M 1	M^{11P}	5.429	3.990	3.900	3.697	3.80	3.87	3.72
M 1	M^{21P}	5.409	4.125	4.006	3.82	3.84	3.87	3.72
M 1	M^{22P}	5.146	4.062	3.874	3.640	3.76	3.87	3.72
M 1	GlcN	4.505	3.311	3.485	3.442	3.613		
M 2	GlcN	4.506	3.312	3.485	3.445	3.616	3.88	3.73
M 3	GlcN	4.500	3.300	3.475	3.440	3.610		

[a] Non-phosphorylated chains.
[b] Phosphorylated chains.

Table 2
^{13}C NMR data

Substance	Unit	C-1	C-2	C-3	C-4	C-5	C-6
M 1	M^{11}	99.8	70.2				
M 2	M^{11}	99.8	70.4	70.8			
M 3	M^{11}	99.7	70.3				
M 1	M^{21}	98.7	79.1				66.0
M 2	M^{21}	98.6	79.1				66.0
M 3	M^{21}	98.6	79.1				66.0
M 1	M^{31} or M^{41a}	98.7	79.1				66.0
	M^{31} or M^{41b}	99.2	79.1				
M 2	M^{31} or M^{41a}	98.6	79.1				66.0
	M^{31} or M^{41b}	99.2	79.1				
M 3	M^{31} or M^{41}	98.6	79.1				66.0
M 1	M^{22}	102.4	70.4	70.8	67.3	73.7	61.5
M 2	M^{22}	102.4	70.4	70.8	67.3	73.6	61.5
M 3	M^{22}	102.5	70.4	70.8	67.3	73.6	61.5
M 1	M^{32} or M^{42}	100.9	78.8	70.5	67.3	73.7	61.5
	M^{32}6P or M^{42}6P	100.9	78.8	66.7	72.8	64.4	
M 2	M^{32} or M^{42}	100.9	78.8	70.5	67.3	73.7	61.5
	M^{32}6P or M^{42}6P	100.9	78.8	70.5	66.7	72.8	64.4
M 3	M^{32} or M^{42}	100.9	78.8	70.5	67.3	73.7	61.5
M 1	M^{33}	102.4	70.4	70.8	67.3	73.6	61.5
M 2	M^{33}	102.4	70.4	70.8	67.3	73.6	61.5
M 3	M^{33}	102.5	70.4	70.8	67.3	73.6	61.5
M 1	M^{43}	102.4	70.0	78.3	66.6	73.6	61.3
M 2	M^{43}	102.4	70.0	78.3	66.6	73.7	61.3
M 3	M^{43}	102.5	70.0	78.3	66.6	73.7	61.3
M 1	M^{44}	102.5	70.4	70.8	67.3	73.6	61.5
M 2	M^{44}	102.5	70.4	70.8	67.3	73.6	61.5
M 3	M^{44}	102.5	70.4	70.8	67.3	73.7	61.5
M 1	M^{11P}	96.5	70.9	70.5	66.8	74.3	61.2
M 1	M^{21P}	96.4	70.5	77.9	66.2	74.3	61.0
M 1	M^{22P}	102.5	70.4	70.8	67.3	73.6	61.5

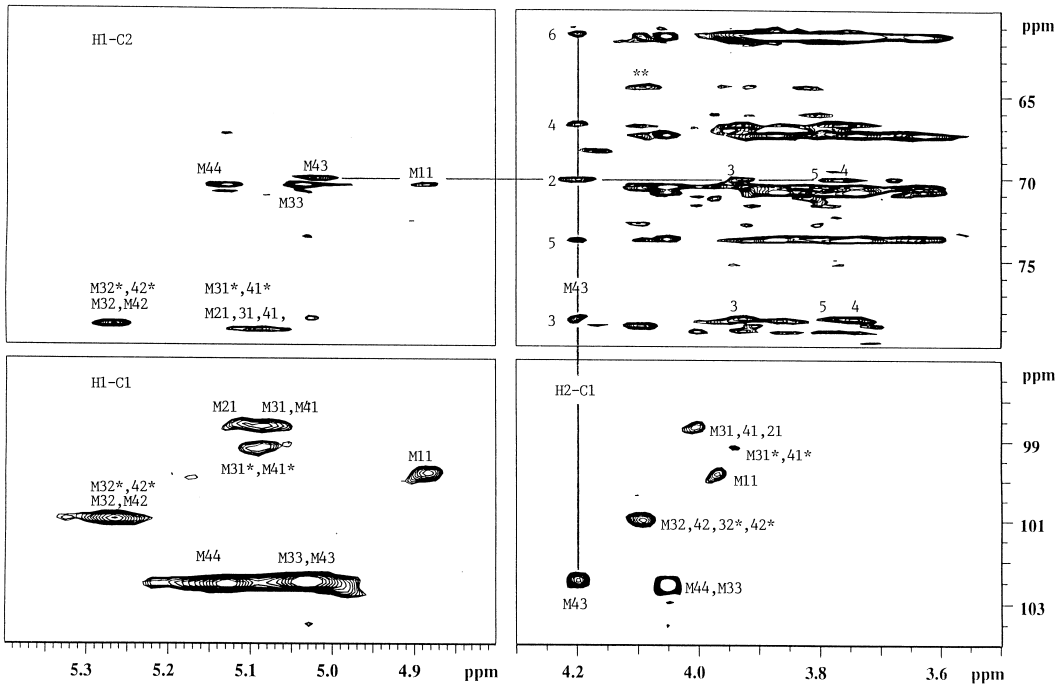


Fig. 2. ^{1}H-^{13}C HMQC–TOCSY spectrum of mannan-2. Vertical line connects correlations of H2 of M^{43} to C1-C6 of this residue, horizontal line connects correlations of C2 of M^{43} to its H1-H5; numbered are atoms of M^{43} residue. * indicates residues belonging to phosphorylated chains, ** indiates correlation between H2 and C6 of phosphorylated at O6 residues M^{32}, M^{42}.

disaccharide. The peak of disaccharide ($[M+Na]^+$ 365 amu) is lower than expected, and the mannose peak (203 amu) is not visible (Fig. 4A and C). The signals between the peaks of neutral oligosaccharides in the positive mode spectra originate from phosphorylated oligosaccharides. Negative mode spectra contain a single peak for each phosphorylated oligosaccharide at $[M-H]^-$.

Thus all structural information, agreeing with the data from other experimental methods, can be extracted by analysis of the high field NMR spectra of the mannan from *S. cerevisiae*. Important questions about the presence of labile linkages get clear answers using NMR spectroscopy. However, the determination of the sequential arrangement of the different side chains, as also concluded by other experimental methods, is not possible at this stage.

2. Experimental

Preparation of mannan derivatives.—Mannan was extracted from wild type strain X2180 yeast cells by autoclaving with 50 mM Na-citrate buffer pH 7.5 for 1 h, followed by precipitation with ethanol, redissolved in water, and charged substances were removed by precipitation with Cetavlon. Finally the mannan was precipitated with 1% borate, redissolved in 2% AcOH and precipitated with ethanol. This material was treated with Proteinase K (50 °C, 3 h), dialysed, chromatographed on DEAE TSK 650 M column using a gradient of NaCl from 0 to 1 M and dialysed to give the mannan-1 (M1) preparation. Hydrolysis of M1 with 2% AcOH (100 °C, 3 h) followed by separation of the products by gel chromatography on TSK HW40 column gave mannan-2 (M2), mannose and α-D-mannopyranosyl-(1→3)-D-mannopyranose due to hydrolysis of glycosyl phosphate linkages. The mannan was also dephosphorylated with 48% HF (0 °C, 2 days), diluted with water, dialysed, and subsequently treated with 1M NaOH–1M $NaBH_4$ (100 °C, 3 h) to remove protein, and desalted by gel chromatography to give mannan-3 (M3).

Acetolysis of acetylated mannan was carried out in an AcOH–Ac_2O–H_2SO_4 mixture (10:10:1) for 3 days at room temperature. The product were deacetylated by MeONa and separated on a DEAE column, the neutral and acidic fractions were further separated by gel chromatography and the neutral fraction separated by HPAE in 0.1 M NaOH.

NMR spectroscopy.—^1H, ^{13}C, COSY, TOCSY, ROESY, ^1H-^{13}C HMQC, ^1H-^{13}C HMQC–TOCSY, ^1H-^{13}C HMBC spectra were recorded on

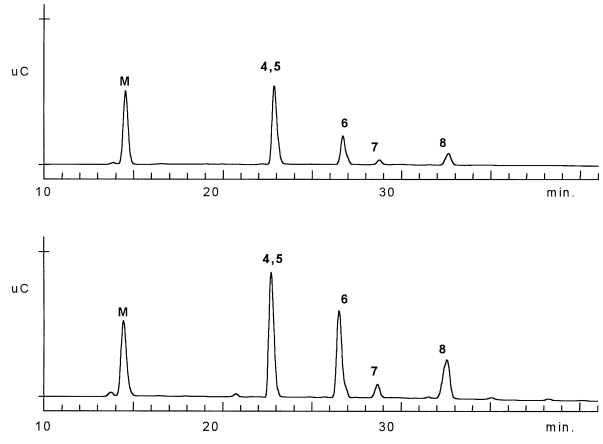


Fig. 3. Separation of the neutral oligosaccharides obtained after acetolysis of *S. cerevisiae* mannan on Carbopac PA-100 column. Numbers correspond to compound number; M, mannose. Upper trace, products from mannan-1; lower, products from mannan-3.

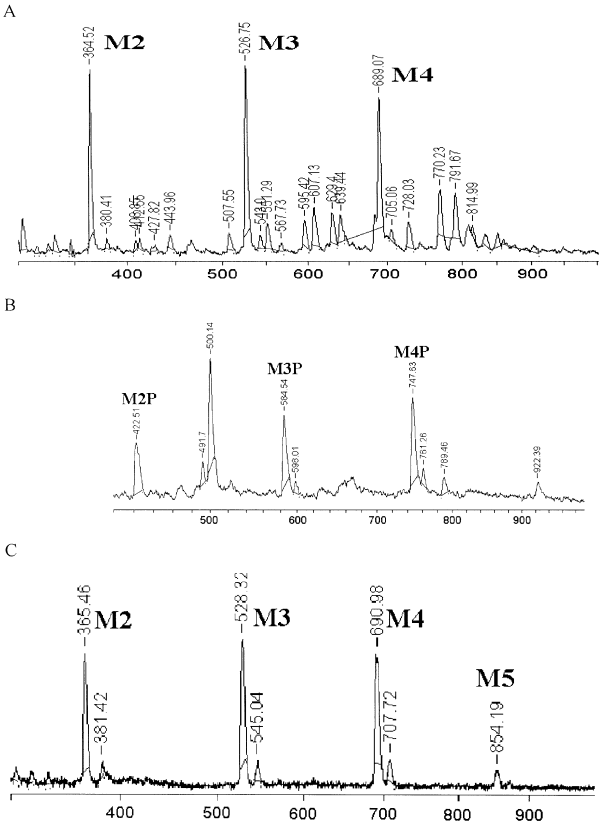


Fig. 4. MALDI mass spectra of the deacetylated products of the acetolysis of *S. cerevisiae* mannan. A: mannan-1, whole mixture, positive mode; B: mannan-1, whole mixture, negative mode; C: mannan-1, isolated neutral oligosaccharides, positive mode.

Bruker AMX-600 or Varian INOVA UNITY 750 spectrometers in D_2O at 70 °C using standard software. ^{31}P spectra and one-dimensional ^{31}P-^{1}H HMQC spectra were recorded on a Bruker DRX-250 spectrometer. Chemical shifts are given relative to acetone (δ_H 2.225 ppm, δ_C 31.45 ppm).

Acknowledgements

The mannan sample was a gift from Professor J. Jensenius, Aarhus University, Denmark. The 750 MHz data were obtained at the Danish National NMR Instrument Centre for Biological Macromolecules.

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