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Note

Isolation and characterization of non-labeled and ^{13}C-labeled mannans from *Pichia pastoris* yeast

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Abstract

Mannans from genetically modified *Pichia pastoris* yeast, used for overproduction of neural cell adhesion molecule protein, grown on normal media or on uniformly ^{13}C-labeled glucose and methanol, were isolated and characterized by high-field (750 MHz) NMR spectroscopy. Fully ^{13}C-labeled oligosaccharide fragments were prepared from mannans by acetolysis. According to the data obtained, the mannan is made up of a main chain of α-(1→6)-linked mannopyranosyl residues, substituted at O-2 with α-mannopyranosyl or a α-D-Man*p*-(1→2)-β-D-Man*p*-(1→2)-β-D-Man*p*-(1→2)-α-D-Man*p*- group, and with much lower content of substitution with β-D-Man*p*-(1→2)-α-D-Man*p*-. A fraction of these oligosaccharide side chains is again substituted with α-D-Glc*p* or α-D-Glc*p*NAc through a phosphodiester linkage to the 6 position of the first mannopyranosyl residue. Improved conditions of acetolysis, cleaving all α-(1→6) linkages, but not β-mannoside linkages, are proposed.

Keywords: Yeast; *Pichia pastoris*; Mannan; ^{13}C labeled

The yeast *Pichia pastoris* is widely used for the preparation of recombinant proteins, particularly isotopically labeled ones. Yeast synthesizes cell-wall mannoprotein (mannan), which can be obtained as a by-product of protein synthesis. Proteins, expressed in *P. pastoris*, are usually glycosylated with *Pichia*'s own oligomannoside structures, which can present a serious problem for the medical use of these proteins [1,2]. Detailed structural data on *Pichia* mannans may be important for the evaluation of the glycosylation of expressed products. ^{13}C-labeled mannans and their fragments can also be useful in the study of mannan-binding proteins and other mannose-recognizing biomolecules. Furthermore, NMR of ^{13}C-labeled oligo- and polysaccharides is advantageous for structural analysis taking advantage of the higher sensitivity in inverse experiments, such as HMBC, HMQC–TOCSY and ^{13}C–^{13}C TOCSY spectra, and allows the use of ^{13}C–^{13}C coupling constants for conformational analysis [3–8]. In the present work the isolation and structural characterization of the mannan from a recombinant *P. pastoris* strain with inserted plasmid encoding Ig module-2 of neural cell adhesion molecule (NCAM) and the preparation of its fragments are described.

Mannan was isolated from the culture fluid and from cells of *P. pastoris* strain His 4

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GS-115 (Invitrogen), grown in either ^{13}C-labeled or normal media. The yeast were transformed to produce the module-2 of NCAM protein [9]. About half of the total mannan was released into the culture medium. Chemical and spectroscopic data suggested that mannans extracted from culture fluid and from the cells had essentially the same structure. Monosaccharide analysis showed the presence of 55–60% of mannose, 4–6% of glucose, and 0.5–2% of glucosamine, the rest being mainly protein and salt. The combined mannan samples were separated by ion-exchange chromatography on DEAE TSK 650M gel into 'neutral' (eluted with void volume), intermediate, and acidic fractions (mannan **A**) in relative amounts of approximately 2:1:2. These fractions differed by the content of glucose and glucosamine, increasing with the retention on the DEAE column. NMR spectra of the DEAE fractions contained the signals of several mannose residues, as well as peaks of different intensity, corresponding to anomeric protons from phosphates of glucose and *N*-acetylglucosamine (Fig. 1, Tables 1 and 2). ^{31}P NMR spectra of all fractions contained a major signal at 0.8 ppm from a phosphodiester group, and two minor signals (about five times less intense) at 2.6 and 0.03 ppm. Mild acid hydrolysis of the intact mannan (mannan **A**) removed the residues of glucose and *N*-acetylglucosamine, but left phosphate groups intact, to produce mannan **B** preparation.

The structure of the mannan was determined using a series of one- and two-dimensional NMR spectra (1D ^{1}H, $^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$ TOCSY, $^{1}H-^{1}H$ NOESY, $^{1}H-^{13}C$ HSQC, $^{1}H-^{13}C$ HSQC–TOCSY, $^{1}H-^{13}C$ HSQC–NOESY, $^{1}H-^{13}C$ HMBC, 1D ^{31}P, $^{1}H-^{31}P$ HMQC, $^{13}C-^{13}C$ COSY). The experiments including ^{13}C in one or two dimensions were carried out on the fully ^{13}C-labeled samples. The overall structure of the polysaccharide for mannan **A** and **B**, as determined from NMR data, is indicated in Scheme 1. The only difference between mannan **A** and **B** is the lack of GlcP and GlcNAcP in mannan **B**. As the spectra of the two polysaccharides are very similar, only the data for mannan **A** are reported here (Tables 1 and 2). Several of the NMR signals could not be assigned, mainly due to the broad lines of the large polysaccharide, but also because of the similarity in the nature of the residues and the low amounts present of some residues.

Molar amounts of mono-, di-, and tetrasaccharide side chains are approximately 10:1:8.

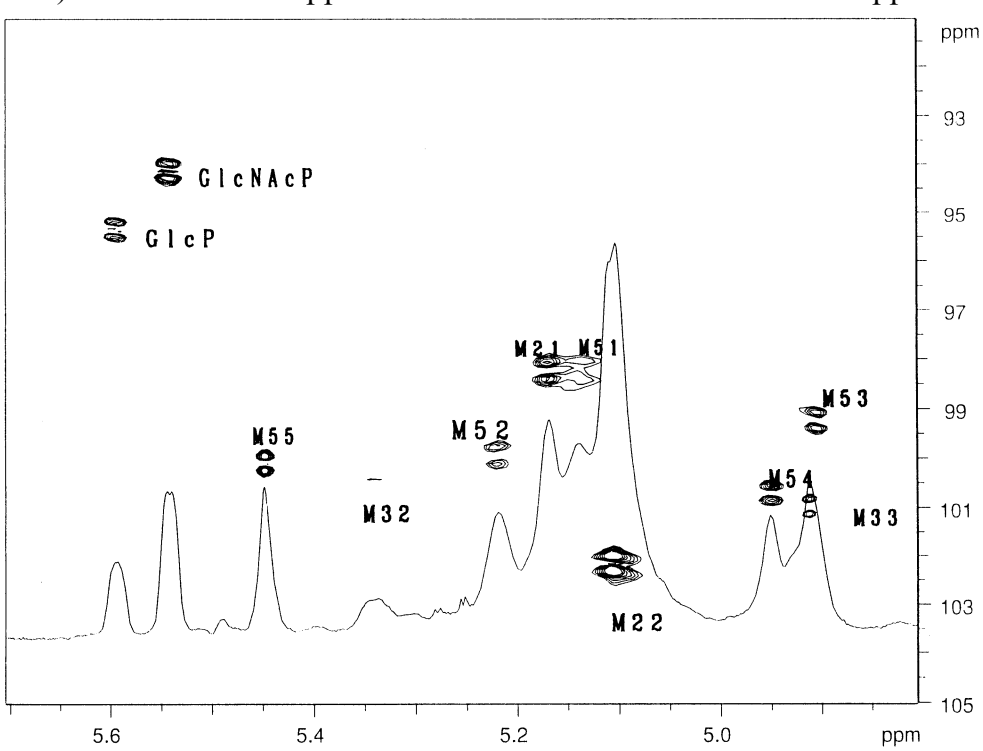


Fig. 1. Anomeric region of $^{1}H-^{13}C$ HSQC spectrum of ^{13}C-labeled mannan **A**.

Table 1
^{1}H NMR data [a] for the mannan **A** polysaccharide and pentasaccharide **1**

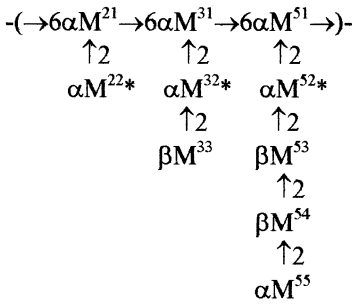
Substance	Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Mannan **A**	M^{21} [b]	5.17	4.09	3.99				
Mannan **A**	M^{51} [b]	5.13	4.10	3.99				
1	αM^{51}	5.42	4.01	4.00	3.74	3.84	3.92	3.83
1	βM^{51}	4.98	4.14	3.82	3.63	3.45		
Mannan **A**	M^{22}	5.10	4.13					
Mannan **A**	M^{32}	5.34	4.16					
Mannan **A**	M^{52}	5.22	4.33	3.92	3.70	3.81		
Mannan **A**	M^{x2}-6P-Glc [c]					3.89	4.25	4.19
Mannan **A**	M^{x2}-6P-GlcNAc [c]					3.89	4.22	4.15
1	M^{52} (αM^{51})	5.19	4.32	3.96	3.68	3.80	3.91	3.80
1	M^{52} (βM^{51})	5.29	4.35	4.04	3.73	4.12	3.86	3.82
Mannan **A**	M^{33}	4.91	4.21	3.67				
Mannan **A**	M^{53}	4.90	4.32	3.73	3.52	3.46	4.00	3.79
1	M^{53}	4.90	4.31	3.71	3.52	3.45	3.99	3.79
Mannan **A**	M^{54}	4.94	4.39	3.81	3.70	3.45	3.99	3.80
1	M^{54}	4.94	4.38	3.79	3.69	3.43	3.98	3.79
Mannan **A**	M^{55}	5.44	4.12	4.01	3.78	4.02	3.95	3.84
1	M^{55}	5.44	4.11	4.02	3.78	4.02	3.95	3.84
Mannan **A**	α-D-Glc*p*P	5.59	3.63	3.82	3.54	3.92		
Mannan **A**	α-D-Glc*p*NAcP	5.54	4.02	3.86	3.63	3.93	3.93	3.89

[a] The coupling constants are not reported, but when measurable these are in agreement with expected values.
[b] Separate assignment of M^{31} signals could not be obtained due to low intensity and overlap with M^{21} and M^{51}.
[c] M^{x2}-6P: can be in positions corresponding to either M^{22}, M^{32} or M^{52} and can be substituted with either α-D-Glc*p* or α-D-Glc*p*NAc.

Table 2
^{13}C NMR data for the mannan **A** polysaccharide and pentasaccharide **1**

Substance	Unit	C-1	C-2	C-3	C-4	C-5	C-6
Mannan **A**	M^{21} [a]	98.5	78.8	70.5			66.7 [b]
Mannan **A**	M^{51} [a]	98.5	78.8	70.5			65.8 [b]
1	αM^{51}	92.6	79.3	70.0	67.1	72.6	61.0
1	βM^{51}	93.5	78.3	73.5	66.8	76.8	61.1
Mannan **A**	M^{22}	102.3	70.2	70.2	66.9	73.3	61.1
Mannan **A**	M^{32}	100.8	78.5				
Mannan **A**	M^{52}	100.1	78.2	69.7	67.4	73.5	61.1
Mannan **A**	M^{x2}-6P [c]				66.0	72.1	64.6
1	M^{52} (αM^{51})	100.2	78.3	69.5	67.5	73.3	60.7
1	M^{52} (βM^{51})	99.4	78.3	69.3	67.3	73.0	60.4
Mannan **A**	M^{33}	101.2	70.4	73.0	66.9	76.4	61.2
Mannan **A**	M^{53}	99.5	78.2	72.1	67.7	77.0	61.2
1	M^{53}	99.4	78.3	72.0	67.6	76.7	61.2
Mannan **A**	M^{54}	100.9	73.9	74.7	66.9	77.0	61.1
1	M^{54}	100.8	73.9	74.2	66.8	77.0	61.1
Mannan **A**	M^{55}	100.2	70.2	70.3	66.5	72.9	60.9
1	M^{55}	100.2	70.0	70.2	66.3	72.9	61.0
Mannan **A**	α-D-Glc*p*P	95.4	71.6	72.8	69.4	73.0	60.6
Mannan **A**	α-D-Glc*p*NAcP	94.2	53.8	70.8	69.7	73.2	60.5

[a] Separate assignment of M^{31} signals could not be obtained due to low intensity and overlap with M^{21} and M^{51}.
[b] Signals might be interchanged.
[c] M^{x2}-6P: can be in positions corresponding to either M^{22}, M^{32} or M^{52} and can be substituted with either α-D-Glc*p* or α-D-Glc*p*NAc.



where M represents a D-mannopyranosyl residue.

* indicates the presence of the following substitution at O6

H, α-D-Glc*p*-P- or α-D-Glc*p*NAc-P

Scheme 1.

The identification of the linkage between the GlcP and GlcNAcP residues and the mannan was only possible using the full battery of experiments indicated above, as the phosphorylated units are present only about one per six units represented in the formula. The high apparent intensity of the GlcP and GlcNAcP signals in the spectra suggest a higher proportion of these residues in the mannan; however, this is due to narrower line width, probably as a result of higher mobility. The connection of Glc and GlcNAc through a phosphodiester linkage can clearly be assigned to the innermost residues of the oligosaccharide side chains, even though the mannose residues carrying the phosphate could only be partly assigned. The remaining signals (H-1–H-4 and C-1–C-3) for these residues are expected be identical to those of unphosphorylated residues [10]. The assignments rely on weak NOE correlations between H-1 in GlcP and GlcNAcP and H-6a and H-6b of the mannosyl residue and also that the same pairs of ^{1}H correlate with the same ^{31}P signal in a $^{1}H-^{31}P$ HMQC spectrum. The attachment of the phosphorylated mannosyl residue to the innermost positions can be assigned by NOE contact between the H-1 of M^{21} and M^{51} to H-5 (C-5 72.1 ppm) of M^{x2} in a HSQC–NOESY spectrum. This NOE is normally observed in α-D-Man*p*-(1→2)-α-D-Man*p*- structures [11].

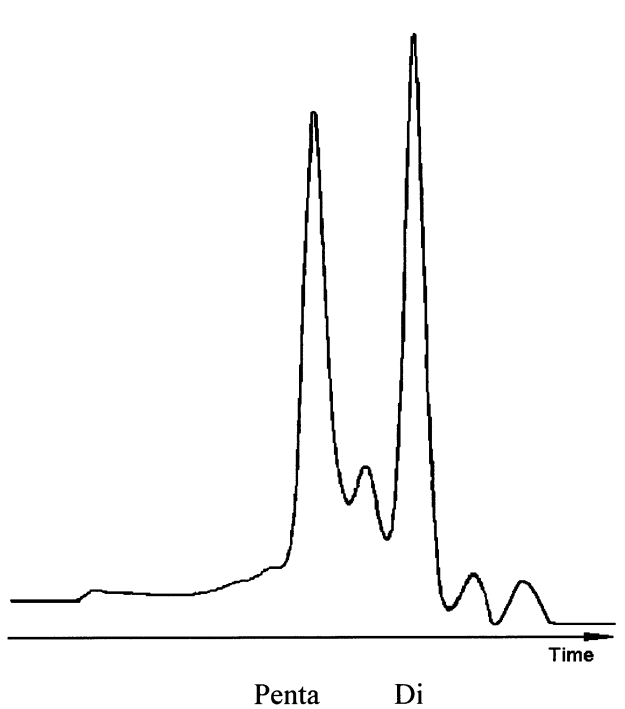


Fig. 2. Separation of the oligosaccharides obtained after acetolysis of *P. pastoris* mannan on TSK HW40 gel.

In order to prepare pure oligosaccharide fragments, the mannan was acetylated and treated with $Ac_2O–AcOH–H_2SO_4$ in the ratio of 20:20:1 at room temperature for 3 days. These conditions gave complete cleavage of all α-(1→6) linkages, but did not cleave the β linkages. Gel chromatography afforded two major products, a pentasaccharide **1** and a disaccharide **2** (see Fig. 2). Low amounts of other oligosaccharides proved that β linkages were not destroyed during the reaction. The MALDI mass spectrum of the ^{13}C-labeled pentasaccharide **1** contained only peaks at *m*/*z* 881.7 and 897.5 ($[M+Na]^+$ and $[M+K]^+$, respectively), indicating complete isotopic substitution in agreement with protein labeling [9]. The NMR data for **1** are reported in Tables 1 and 2. The ^{13}C NMR data for **2** are in agreement with previously published data [12] and are not reported here. Phosphorylated oligosaccharides in significant amount were not found among acetolysis products. Small amounts of free mannose released after acetolysis suggests that the mannan backbone is completely or nearly completely substituted with side chains.

α-D-Man*p*-(1→2)-β-D-Man*p*-(1→2)-β-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α,β-D-Man*p*

1

α-D-Man*p*-(1→2)-α,β-D-Man*p*

2

The structure of *P. pastoris* N-linked mannan from different strains and growth conditions, as well as the structure of oligomannoside O-chains of its proteins were reported previously [13–16]. All structures described so far include the pentasaccharide fragment **1** as the largest side chain. At the same time, smaller fragments are found in variable amount, which may reflect interstrain differences or a degree of undesired hydrolysis. The presence of phosphate substituents has not been previously reported, although an acidic fraction of a mannan has been reported [13].

1. Experimental

Cell growth and isolation of mannan.—The cDNA fragment encoding for Ig module-2 of NCAM was subcloned into pPIC9K plasmid (Invitrogen Corporation, San Diego, USA) and used for transformation of *P. pastoris* strain His 4 GS-115 (Invitrogen) [9]. The cells were grown on yeast normal base (Difco) without amino acids and with normal or uniformly ^{13}C-labeled glucose and methanol at 30 °C and pH < 4.9 for 36 h. The expression medium was subsequently concentrated 10 times by ultrafiltration. Mannan, released to culture medium, was isolated by gel filtration on Sephadex G-25 (Pharmacia). Cells were extracted by boiling in 50 mM Na-citrate buffer pH 7.5 for 1 h, followed by dialysis and chromatography on Sephadex G25 column, to give an additional amount of the mannan.

Preparation of mannan derivatives.—Mannan was treated with Proteinase K (50 °C, 3 h), dialyzed, chromatographed on DEAE TSK 6503228M column using a gradient of NaCl from 0 to 1 M and dialyzed to give the neutral, intermediate and acidic fractions. Hydrolysis of the acidic mannan (mannan A) with 2% AcOH (100°, 3 h), followed by separation of the products by gel chromatography on TSK HW40 column gave mannan **B**, glucose and *N*-acetylglucosamine.

Acetolysis.—Peracetylated mannan (100 mg) was dissolved completely in AcOH (4 mL), then Ac_2O (4 mL) was added and the mixture cooled in a freezer, H_2SO_4 (0.2 mL) was added with stirring and the mixture was kept for 3 days at room temperature. The mixture was diluted with water (100 mL), extracted with chloroform, the organic layer washed with $NaHCO_3$ and water. The product was deacetylated by NaOMe and separated on a TSK HW40 column to give pentasaccharide **1**, disaccharide **2** and a mixture of trisaccharide and tetrasaccharide.

NMR spectroscopy.—NMR spectra were recorded in 5 mm tubes at 50 °C in D_2O. $^{13}C-^{13}C$ COSY, ^{31}P–1D spectra and $^{31}P-^{1}H$ HSQC were recorded on a Varian Unity Inova 500 using standard pulse sequences. Standard experiments like DQCOSY, NOESY, TOCSY, HSQC and HSQC–TOCSY were recorded either on a Bruker DRX 600 or on a Varian Unity Inova 750 using standard pulse sequences. Specifically, the HSQC–NOESY being crucial for the assignment were recorded at the 750 MHz instrument. Chemical shifts are given relative to acetone (2.225 ppm for proton and 31.45 ppm for carbon).

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