Carbohydrate Research, 231 (1992) 317–323
Elsevier Science Publishers B.V., Amsterdam

Note

Mild acetolysis and NMR studies of the D-mannan of *Saccharomyces cerevisiae* X2180-1A wild-type strain

Hidemitsu Kobayashi, Nobuyuki Shibata, Masahiko Watanabe, Mariko Komido, Nobutaka Hashimoto, Kanehiko Hisamichi [1] and Shigeo Suzuki

Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Sendai Aoba-ku, Miyagi 981 (Japan)

(Received October 2nd, 1991; accepted January 3rd, 1992)

It has been found[1,2] that, of the α-(1 → 2), β-(1 → 2), and α-(1 → 6) linkages in the mannan of *Pichia pastoris* strain, preferential cleavage of the last linkage occurs on acetolysis with 100:100:1 Ac_2O–AcOH–H_2SO_4 for 36 h at 40°. Subsequently, the usefulness of this mild method of acetolysis for investigating β-(1 → 2)-linkage-containing yeast D-mannans, such as phospho-D-mannan–protein complexes of *Citeromyces matritensis*[3], *Candida albicans* serotype A[4,5], *Candida glabrata*[6], and *Candida stellatoidea* Type II[7] strains, was reported. The results demonstrated that each D-mannan possessed a long backbone of (1 → 6)-linked α-D-mannopyranose residues with many side chains attached by α-(1 → 2) linkages as in the D-mannan of bakers' yeast, *Saccharomyces cerevisiae* X2180-1A wild-type strain (1A strain)[8]. Stewart et al.[9] reported that brief acetolysis of the D-mannan (1A) of the 1A strain with 10:10:1 Ac_2O–AcOH–H_2SO_4 for 1.25 h at 40° gave two isomeric D-mannohexaoses (**4** and **5**), each consisting of a D-mannotetraose (**3**) and a D-mannobiose (**1**) unit connected by an α-(1 → 6) linkage, and an isomer (**6**) composed of two D-mannotriose (**2**) units. The ratios of the structures **4**–**6** were 1.7:0.6:1. We now report the application of the mild-acetolysis technique[1] in order to investigate the branching frequency of 1A.

Fig. 1A shows the elution profile on Bio-Gel P-2 of the mild-acetolysis products of 1A, namely, D-manno-oligosaccharides of dp 2–4 and 6 (M_2–M_4 and M_6, respectively) and a phosphate-containing component (V_0).

^{1}H-NMR analyses of M_2–M_4 indicated the structures **1**–**3**, respectively (Fig. 2, D, C, and B; Table I). Methylation analysis of M_6 revealed one (1 → 6) linkage that originated from the backbone moiety. 2,4,6-Tri-*O*-methyl-1,3,5-tri-*O*-acetyl,

[1] First Department of Medicinal Chemistry.

Correspondence to: Dr. S. Suzuki, Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Sendai Aoba-Ku, Miyagi 981, Japan.

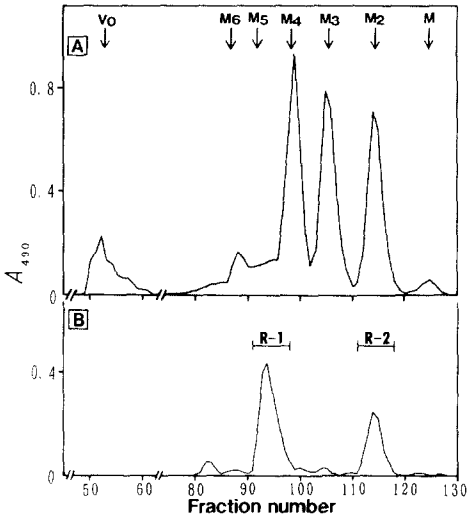

Fig. 1. Gel filtration on a column (2.5 × 100 cm) of Bio-Gel P-2 (elution with H_2O) of the products of mild acetolysis of 1A with 100:100:1 Ac_2O–AcOH–H_2SO_4 (A) and M_6-ol with 10:10:1 Ac_2O–AcOH–H_2SO_4 (B): M and M_2–M_6 indicate D-mannose and D-manno-oligosaccharides of dp 2–6; V_o refers to the void volume.

3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl, 3,4-di-*O*-methyl-1,2,5,6-tetra-*O*-acetyl, and 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl derivatives of mannitol were formed from M_6 in the molar ratios 0.48:1.01:0.42:1.00. Reduction of M_6 with $NaBH_4$ gave

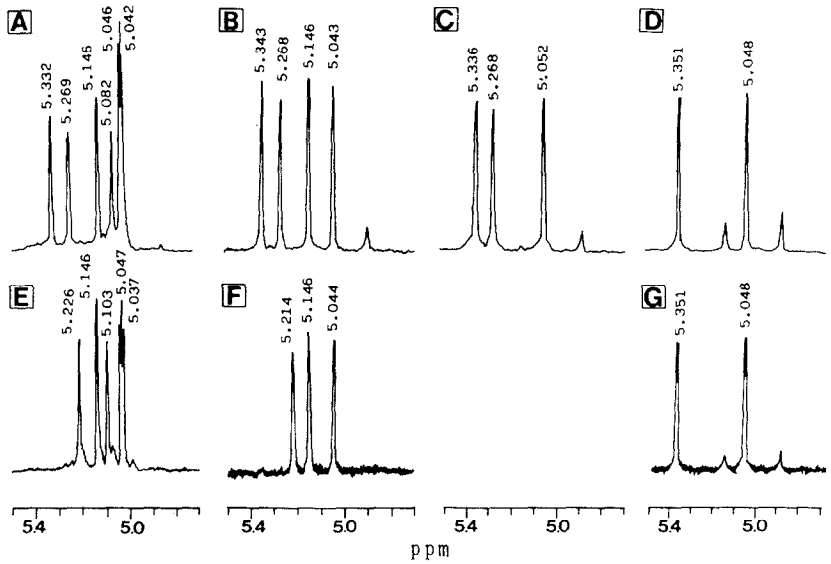

Fig. 2. ^{1}H-NMR spectra [D_2O, 70°, internal acetone (2.217 ppm)] of D-manno-oligosaccharides (see Fig. 1): (A) M_6; (B) M_4; (C) M_3; (D) M_2; (E) M_6-ol; (F) R-1; and (G) R-2.

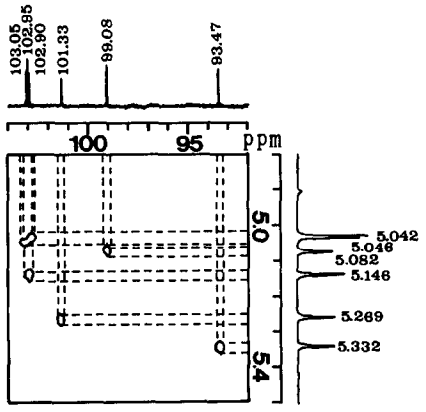

Fig. 3. $^{1}H,^{13}C$ COSY spectrum [D_2O, 55°, internal acetone (2.217 ppm) and CD_3OD (49.00 ppm)] of M_6.

M_6-ol, the ^{1}H-NMR spectrum of which (Fig. 2E) contained five resonances at 5.226, 5.146, 5.103, 5.047, and 5.037 ppm, corresponding to H-1 of D-manno-pyranose residues. Fig. 1B shows the elution profile of the products obtained from M_6-ol after conventional acetolysis. Two major oligosaccharides, R-1 and R-2, were obtained. The retention time of the latter was the same as that of M_2 described above and it was identified as **1** by ^{1}H-NMR spectroscopy (Fig. 2G). The ^{1}H-NMR spectrum of R-1 (Fig. 2F) was in agreement with the structure of the reduced tetraose **8**. Therefore, M_6 and M_6-ol were identified as **4** and **7**, respectively. $^{1}H,^{13}C$ COSY spectrometry of M_2–M_4 and M_6 (structures **1–4**, respectively) resulted in the ^{13}C assignments shown in Table I. The assignments of the C-1 signals for **1–3** were identical to those in the literature[10–12]. However, the assign-

TABLE I

^{1}H and ^{13}C Chemical shifts of D-manno-oligosaccharides (α anomer) obtained from 1A by acetolysis followed by *O*-deacetylation

Structure	Chemical shift (ppm)							
	H-1				C-1			
	Residue				Residue			
	l'''	*l''*	*l'* *l''''*	*l* *l''''*	*l'''*	*l''*	*l'* *l''''*	*l* *l''''*
1			5.048	5.351			102.95	93.47
2		5.052	5.268	5.336		102.97	101.39	93.44
3	5.146	5.043	5.268	5.343	102.95	102.90	101.38	93.89
4	5.146	5.042	5.269	5.332	102.95	102.90	101.33	93.47
			5.046	5.082			103.05	99.08
7	5.146	5.047	5.226					
			5.037	5.103				
8	5.146	5.044	5.214					

ment of the ^{13}C-NMR spectrum for M_6 (**4**) has not been reported hitherto. Fig. 3 shows ^{1}H,^{13}C COSY spectra of the regions for H-1 and C-1 resonances of M_6.

α-D-Man*p*-(1 → 2)-{α-D-Man*p*-(1 → 2)}$_n$-D-Man

1 $n = 0$
2 $n = 1$

α-D-Man*p*-(1 → 3)-{α-D-Man*p*-(1 → 2)}$_2$-D-Man

3

α-D-Man*p*-(1 → 3)-{α-D-Man*p*-(1 → 2)}$_2$-D-Man
6
↑
1
α-D-Man*p*-(1 → 2)-α-D-Man*p*

4

α-D-Man*p*-(1 → 2)-D-Man
6
↑
1
α-D-Man*p*-(1 → 3)-α-{D-Man*p*-(1 → 2)-α}$_2$-D-Man*p*

5

α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 2)-D-Man
6
↑
1
α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*

6

α-D-Man*p*-(1 → 3)-{α-D-Man*p*-(1 → 2)}$_2$-D-Man-ol
6
↑
1
α-D-Man*p*-(1 → 2)-α-D-Man*p*

7

α-D-Man*p*-(1 → 3)-{α-D-Man*p*-(1 → 2)}$_2$-D-Man-ol

8

The proportion of D-mannose produced by mild acetolysis of 1A (Fig. 1A) was small, suggesting that 1A had a highly branched structure. Nakajima and Ballou[8] showed that the unsubstituted core of 1A comprised (1 → 6)-linked α-D-mannopyranose residues because conventional acetolysis[13] gave a high yield of D-mannose. The branching frequency (93.1%) of 1A, calculated from the peak areas of

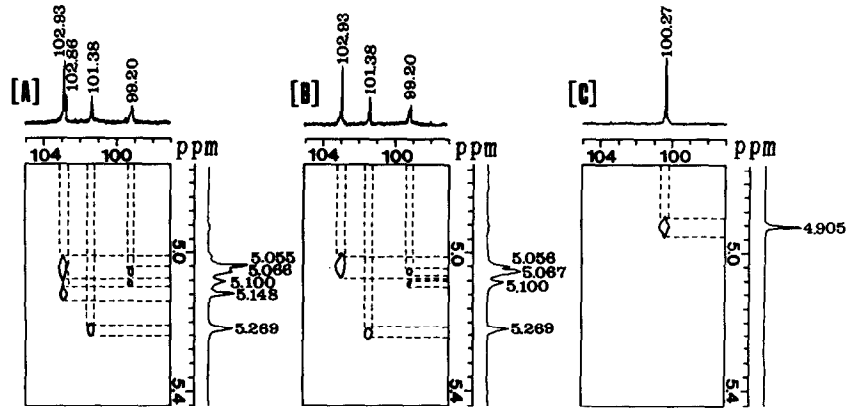

Fig. 4. $^{1}H,^{13}C$ COSY spectra (conditions as in Fig. 3) of D-mannans: (A) 1A; (B) D1; and (C) A5.

D-manno-oligosaccharides obtained by mild acetolysis, was supported by the ^{1}H, ^{13}C COSY spectra of the D-mannans of three *S. cerevisiae* strains. Thus, comparison of the H-1 and C-1 resonances of 1A (Fig. 4A) with those (Figs. 4B and C, respectively) of the D-mannans of *S. cerevisiae* 4484-24-D1 (D1) and X2180-1A-5 (A5) mutant strains indicated that D1 lacked (1 → 3)-linked α-D-mannopyranosyl units in the non-reducing terminal site, and that A5 lacked both (1 → 3)- and (1 → 2)-linked α-D-mannopyranosyl units corresponding to all branches[14]. The chemical shift data (H-1, 5.148 ppm; C-1, 102.86 ppm) corresponding to a (1 → 3)-linked α-D-mannopyranose unit in the non-reducing site[15] were not detected in the spectra of D1 and A5. Also, lack of the appropriate resonances in the spectrum of A5 reflects the loss of the side chain. Instead of these resonances, there was an increase of intensity of the resonances (H-1, 4.905 ppm; C-1, 100.27 ppm) corresponding to (1 → 6)-linked α-D-mannopyranose unit(s) of the unsubstituted core moiety[16]. These findings suggest that 1A and D1 did not contain unsubstituted (1 → 6)-linked α-D-mannopyranose residues in their core moeities.

Thus, the results of mild acetolysis and NMR spectroscopy indicated 1A to have a highly branched structure, and the D-mannohexaose (**4**) containing an α-(1 → 6)-linkage was isolated after mild acetolysis of 1A. These findings suggest that this procedure may be useful in the analysis of the structures of other yeast D-mannans.

EXPERIMENTAL

Materials.—The *S. cerevisiae* X2180-1A, 4484-24D-1, and X2180-1A-5 strains (1A, D1, and A5 strains, respectively) were kindly supplied by Drs. C.E. Ballou and T. Nakajima. Bio-Gel P-2 (−400 mesh), fractionation range 100–1800 Da, was purchased from Bio-Rad Laboratories (Richmond, CA, USA).

Cultivation of S. cerevisiae *strains and preparation of the* D-*mannans.*—The procedure of Shibata *et al.*[17] was used. The D-mannans (1A, D1, and A5, respectively) were obtained in yields (dry-weight basis) of 7.9, 6.8, and 6.5%, respectively. 1A, D1, and A5 contained, respectively, 98.0, 98.5, and 99.3% of carbohydrate; 0.77, 0.07, and 0.08% of phosphate; and had $[\alpha]_D^{20}$ values of +80°, +67°, and +76° (*c* 1.0, H_2O).

Mild acetolysis of 1A.—This procedure was as described by Kobayashi et al.[1], using 100:100:1 Ac_2O–AcOH–H_2SO_4 at 40° for 36 h. After *O*-deacetylation, the mixture of oligosaccharides was fractionated on Bio-Gel P-2.

Calculation of the branching frequency (X) of 1A.—*X* was calculated from the formula:

$$X(\%) = [(B/342) + (C/504) + (D/666)] \times 100/(A/180) + (B/342) + (C/504) + (D/666)$$

where *A*–*D* represent the peak areas of D-mannose (M), D-mannobiose (M_2), D-mannotriose (M_3), and D-mannotetraose (M_4) in the gel-filtration profile of the acetolysis products of A1 (Fig. 1A), and the numbers, 180, 342, 504, and 666, indicate the mol wts of D-mannose and M_2–M_4, respectively.

Reduction[8] *of hexaose* (M_6) *obtained from Fr. 1A by mild acetolysis.*—Aqueous 0.2% $NaBH_4$ was used at room temperature for 16 h. The excess of $NaBH_4$ was destroyed with Amberlite IR-120 (H^+) resin, the resin was collected and washed throughly with water, and the filtrate and washings were combined and concentrated in vacuo. The residual boric acid was removed by evaporation of MeOH (5 × 5 mL) to leave M_6-ol.

Acetolysis of M_6*-ol.*—The procedure of Kobayashi et al.[1] was used, which is a modification of the method of Kocourek and Ballou[10], with 10:10:1 Ac_2O–AcOH–H_2SO_4 for 13 h at 40°. After *O*-deacetylation, the mixture of oligosaccharides was fractionated on Bio-Gel P-2.

NMR spectroscopy.—^{1}H-NMR spectra [internal acetone, (2.217 ppm)] of D-mannans and D-manno-oligosaccharides were measured as described by Gorin and Spencer[18] with a Jeol JNM-GSX 400 spectrometer on solutions (10 and 3 mg/0.7 mL, respectively) in D_2O at 70°. The ^{13}C-NMR spectra [internal CD_3OD (49.00 ppm)] were measured with the same spectrometer, as described by Kobayashi et al.[19], on solutions (25 and 15 mg/0.7 mL, respectively) of D-mannans and D-manno-oligosaccharides in D_2O at 55°. ^{1}H,^{13}C COSY spectra were also recorded[20] for solutions in D_2O at 55°.

Methylation analysis.—Each D-manno-oligosaccharide (2 mg) was methylated by the Hakomori method[21], and the products were converted into a mixture of *O*-acetyl-*O*-methyl-D-mannitols as described by Lindberg[22].

Other methods.—Carbohydrate was determined by the phenol–H_2SO_4 method[23], using D-mannose as the standard, and phosphate was determined by the method of Ames and Dubin[24], using KH_2PO_4 as the standard. $[\alpha]_D^{20}$ values were determined with a JAS DIP-360 digital polarimeter on aqueous solutions that had

been stored for 3 h. GLC of *O*-acetyl-*O*-methylmannitols was conducted on a glass column (5 mm × 150 cm) containing 3% OV-210 on Supelcoport (100–200 mesh) at 185°, using N_2 as the carrier gas at 20 mL/min. Conversion of the peak areas into molar ratios of the sugar derivatives was made by means of a Shimadzu Chromatopac-E1A microcomputer.

REFERENCES

1 H. Kobayashi, N. Shibata, and S. Suzuki, *Arch. Biochem. Biophys.*, 245 (1986) 494–503.
2 H. Kobayashi, N. Shibata, T. Yonezu, and S. Suzuki, *Chem. Pharm. Bull.*, 36 (1988) 3168–3172.
3 H. Kobayashi, N. Shibata, T. Yonezu, and S. Suzuki, *Arch. Biochem. Biophys.*, 256 (1987) 381–396.
4 H. Kobayashi, N. Shibata, H. Mitobe, Y. Ohkubo, and S. Suzuki, *Arch. Biochem. Biophys.*, 272 (1989) 364–375.
5 H. Kobayashi, N. Shibata, T. Osaka, Y. Miyagawa, Y. Ohkubo, and S. Suzuki, *Phytochemistry*, 31 (1992) in press.
6 H. Kobayashi, H. Mitobe, K. Takahashi, T. Yamamoto, N. Shibata, and S. Suzuki, *Arch. Biochem. Biophys.*, 294 (1992) in press.
7 H. Kobayashi, M. Takaku, Y. Nishidate, S. Takahashi, M. Takikawa, N. Shibata, and S. Suzuki, *Carbohydr. Res.*, 231 (1992) 105–116.
8 T. Nakajima and C.E. Ballou, *J. Biol. Chem.*, 249 (1974) 7685–7694.
9 T.S. Stewart, P.B. Mendershausen, and C.E. Ballou, *Biochemistry*, 7 (1968) 1843–1854.
10 P.A.J. Gorin, *Can. J. Chem.*, 51 (1973) 2375–2383.
11 T. Ogawa and H. Yamamoto, *Carbohydr. Res.*, 104 (1982) 271–283.
12 A. Allerhand and E. Berman, *J. Am. Chem. Soc.*, 106 (1984) 2400–2412.
13 J. Kocourek and C.E. Ballou, *J. Bacteriol.*, 100 (1969) 1175–1181.
14 C.E. Ballou, K.A. Kern, and W.C. Raschke, *J. Biol. Chem.*, 248 (1973) 4667–4673.
15 H. Kobayashi, T. Kojimahara, K. Takahashi, T. Takikawa, S. Takahashi, N. Shibata, Y. Okawa, and S. Suzuki, *Carbohydr. Res.*, 214 (1991) 131–145.
16 M. Tojo, N. Shibata, Y. Ban, and S. Suzuki, *Carbohydr. Res.*, 199 (1990) 215–226.
17 N. Shibata, K. Mizugami, K. Takano, and S. Suzuki, *J. Bacteriol.*, 156 (1983) 552–558.
18 P.A.J. Gorin and J.F.T. Spencer, *Adv. Appl. Microbiol.*, 13 (1970) 25–89.
19 H. Kobayashi, N. Shibata, M. Nakada, S. Chaki, K. Mizugami, Y. Ohkubo, and S. Suzuki, *Arch. Biochem. Biophys.*, 278 (1990) 195–204.
20 H. Kobayashi, P. Giummelly, S. Takahashi, M. Ishida, J. Sato, M. Takaku, Y. Nishidate, N. Shibata, Y. Okawa, and S. Suzuki, *Biochem. Biophys. Res. Commun.*, 175 (1991) 1003–1009.
21 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
22 B. Lindberg, *Methods Enzymol.*, 28 (1972) 178–195.
23 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
24 B.N. Ames and D.T. Dubin, *J. Biol. Chem.*, 235 (1960) 769–775.