HPLC Analysis of Manno-Oligosaccharides Derived from *Saccharomyces cerevisiae* Mannan Using an Amino Column or a Graphitized Carbon Column

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The chromatographic behavior of manno-oligosaccharides derived from *Saccharomyces cerevisiae* mannan on two kinds of HPLC columns, an aminopropyl-silica column or a graphitized carbon column (GCC), was investigated. The order of elution of manno-oligosaccharides on both columns with acetonitrile–water was almost the same, that is, the retention increased with increasing molecular size. However, the GCC made it possible to isolate completely two isomers of mannotrioses (M_3-1 and M_3-2) with different linkage positions. We reinvestigated the structures of mannobiose (M_2), M_3s, and mannotetraose (M_4) that were completely isolated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and NMR spectroscopy.

Key words HPLC; graphitized carbon column; manno-oligosaccharide; *Saccharomyces cerevisiae* mannan; NMR

The structure of *Saccharomyces cerevisiae* (commercial baker's yeast) mannan has been reported by many investigators.[2—8)] It is highly branched and contains 1→2, 1→3, and 1→6 linkages. Mannan has been proposed to have a "backbone" of 1→6-linked mannose units with 1→2- and 1→3-linked side chains that average about two sugar units in length.[6—8)] Stewart and Ballou,[8)] while not offering any particular ratio or order of units in the chain, have suggested that the mannan from *S. cerevisiae* has a structure like that depicted in Fig. 1. NMR spectra of mannans have been analyzed,[9—12)] and recently Vinogradov *et al.*[13)] have deduced the complete structure of mannan and assessed the ratio of different side chains on the basis of high-field NMR spectroscopy data.

Thus far, the deacetylated products of an acetolysate of yeast mannan have been separated by gel filtration, and the structures of each, along with the branch point of linkage, have been mainly established by methylation analysis. Although structural analyses of oligosaccharides of the polysaccharide mannan and mannoprotein using NMR spectroscopy have been performed,[9—13)] pure oligosaccharides could rarely be obtained.

We have chemically synthesized the branched cyclodextrins glucosyl-cyclodextrins[14,15)] and galactosyl-cyclodextrins[16)] and are now trying to synthesize branched cyclodextrins with mannose on the nonreducing ends in their side chains, because such compounds are expected to be useful as drug carriers in targeted drug delivery systems. To obtain the pure manno-oligosaccharides as side chains, we tried to isolate them from *S. cerevisiae* by isolating acetolysis fragments. Using an aminopropyl-silica column, the products were completely isolated as mannobiose (M_2), mannotrioses (M_3s), and mannotetraose (M_4). Also, using a graphitized carbon column (GCC), two isomers of M_3s, M_3-1 and M_3-2, were obtained. Their structures were elucidated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and NMR spectroscopy.

Experimental

HPLC Conditions HPLC analyses were performed with a Jasco 980-PU pump and a Shodex RI-71 monitor. The columns used were a Hibar LiChroCART NH_2 (250×4.0 mm i.d., Kanto Chemical) and a Hypercarb (5 μm, 100×4.6 mm i.d., Thermo Hypersil). For preparative HPLC, a TSKgel Amide-80 column (300×21.5 mm i.d., Tosoh) and a Hypercarb (5 μm, 100×10 mm i.d., Thermo Hypersil) were used. HPLC analyses at constant temperature were conducted using a column oven CA-202 (Flom).

MS MALDI-TOF-MS was performed in the positive-ion mode on a Vision 2000 reflector-type TOF instrument (Thermo Bioanalysis). The other conditions for measurements were as reported in a previous paper.[17)]

NMR ^{1}H- and ^{13}C-NMR spectra data were recorded for 6—10% solutions in D_2O at 50 °C with a Jeol GSX-500 spectrometer. Chemical shifts were expressed in ppm downfield from the signal of Me_4Si referenced to external 1,4-dioxane (67.40 ppm). The other conditions for ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H–^{13}C COSY measurements were as reported in a previous paper.[18)]

Isolation of Mannan Pressed baker's yeast (2.0 kg) was crumbled into 900 ml of water and 90 ml of 0.2 M citrate buffer solution (pH 7.0), and the mixture was autoclaved at 125 °C for 2 h. Crude mannan (30.0 g) was obtained and purified according to the method described by Peat *et al.*,[5)] with a yield of 18.8 g.

Acetolysis of Mannan and Isolation of Mannose Oligosaccharides Mannan 10.1 g was dissolved in a mixture of 52.5 ml of acetic acid–acetic anhydride–sulfuric acid (10 : 10 : 1 v/v), and the mixture was stirred at 38 °C for 23 h, then at room temperature for 40 h according to the method described by Peat *et al.*[5)] and Lee and Ballou.[6)] The progress of the reaction was monitored by TLC on Silica Gel 60 (Merck) (benzene–acetone 2 : 1). After neutralization with sodium hydrogen carbonate, the reaction products were extracted with chloroform. The chloroform layer was evaporated to dryness to yield 7.0 g of a mixture of sugar acetates. Most of the acetolysate was fractionated by centrifugal chromatography (Harrison Centrifugal Thin-Layer Chromatotron, Model 7924) with hexane–acetone, and then each fraction was deacetylated. M_2, M_3s, and M_4 were purified by HPLC on a TSKgel Amide-80 column (300×21.5 mm i.d.) (60 : 40 acetonitrile–water; flow rate, 4.0 ml/min; detector, Shodex RI-71) and two mannotrioses were isolated on a Hypercarb (100×10 mm i.d.) (0.5 : 99.5 acetonitrile–water; flow rate, 0.5 ml/min; detector, Shodex RI-71; temperature, 50 °C).

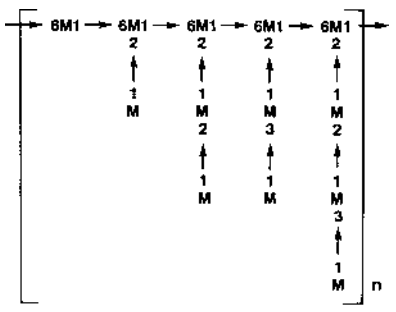

Fig. 1. Structure of Mannan from *S. cerevisiae* as Proposed by Stewart and Ballou[8)]

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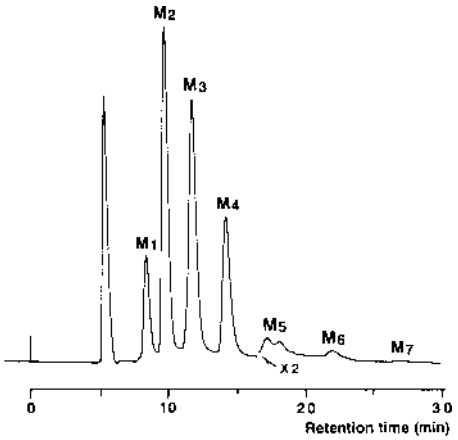

Fig. 2. Elution Profile of Mannose Oligosaccharides from *S. cerevisiae*

Chromatographic conditions: column, Hibar LiChroCART NH_2 (250×4.0 mm i.d.); eluent, 65 : 35 acetonitrile–water; flow rate, 0.5 ml/min; detector, Shodex RI-71.

Results and Discussion

A portion of the acetolysate obtained (100 mg) was deacetylated in the usual way, and then HPLC analyses of manno-oligosaccharides were performed with an amino-propyl-silica column and a GCC. The elution profiles are shown in Figs. 2 and 3, respectively. Figure 2 shows the elu-

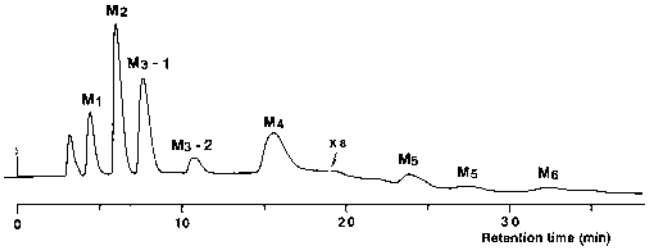

Fig. 3. Elution Profile of Mannose Oligosaccharides from *S. cerevisiae*

Chromatographic conditions: column, Hypercarb (100×4.6 mm i.d.); eluent, 1.0% acetonitrile aqueous solution containing 1.0 mM sodium hydroxide; flow rate, 0.5 ml/min; detector, Shodex RI-71; temperature, 20 °C.

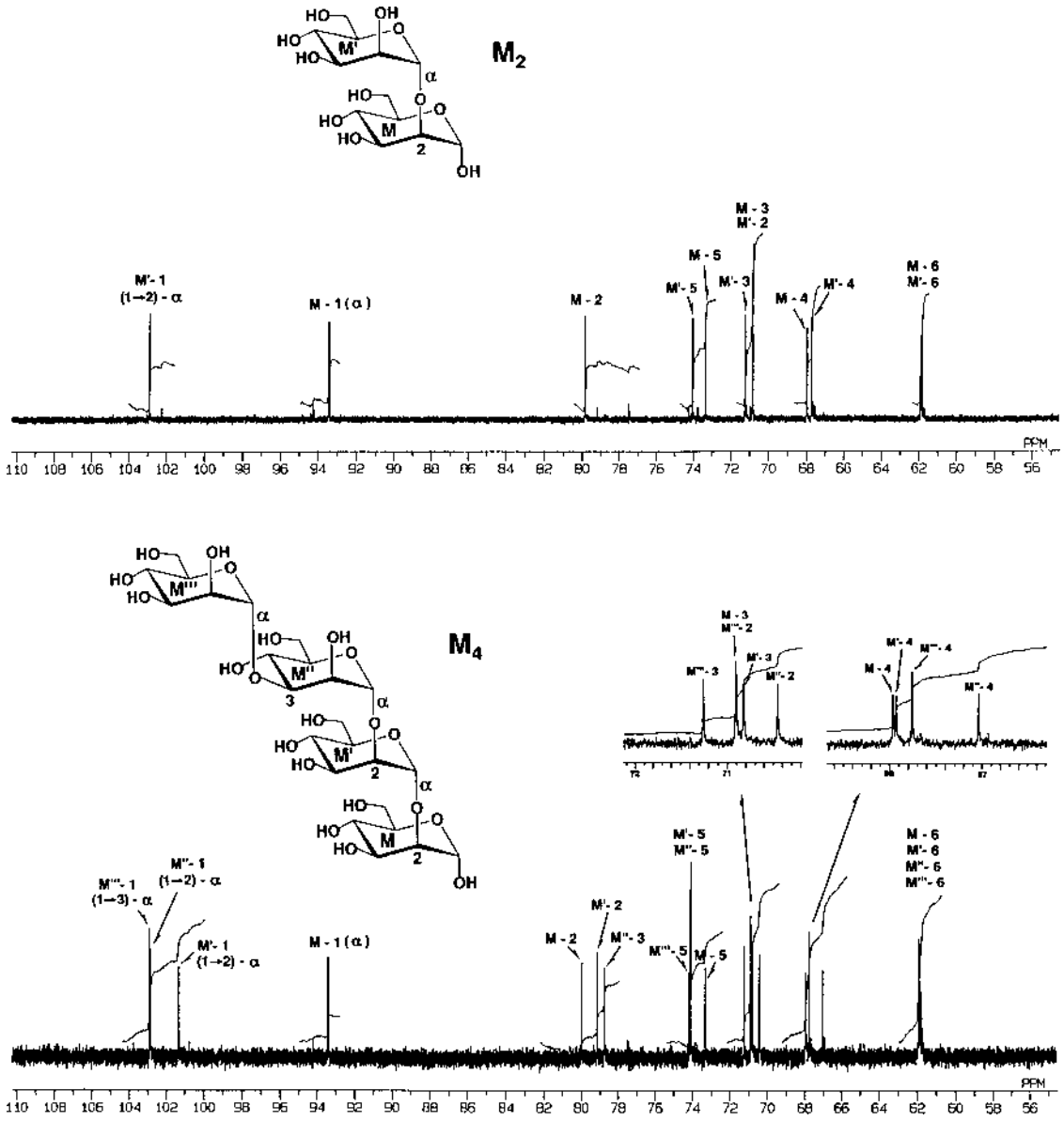

Fig. 4. ^{13}C-NMR Spectra of M_2 and M_4 Measured in D_2O at 125.65 MHz

tion profile of the product of acetolysis; the ratio of mannose (M_1) : M_2 : M_3s : M_4 : mannopentaose (M_5) : mannohexaose (M_6) was 14 : 44 : 44 : 38 : 2 : 1. In the analysis using this amino-propyl-silica column, the two M_3s (M_3-1 and M_3-2) were not separated. Retention on an amino column is similar to that on an anion-exchange resin, and the retention rate increases with increasing molecular size.[19)]

Figure 3 shows the elution profile of the same acetolysate on a GCC with 1 mM sodium hydroxide solution containing 1.0% acetonitrile. The ratio of M_1 : M_2 : M_3-1 : M_3-2 : M_4 : M_5 : M_6 was 36 : 92 : 79 : 21 : 83 : 2 : 1. Two M_3s (M_3-1 and M_3-2) were clearly separated, and the ratio of M_3-1 and M_3-2 was 3.7 : 1. Koizumi *et al.*[20)] reported the first use of this GCC for the separation of carbohydrate compounds. According to the manufacturer's description, the surface of the GCC packing is flat, which enables unique selectivity and the ability to resolve isomeric and closely related compounds. Retention on a GCC occurs mainly by an adsorption mechanism. In general, there is more retention of planar than non-planar molecules. For a series of saccharides with the same molecular configuration, the retention rate increases with increasing molecular size. The hydrophobicity of the GCC is greater than those of other reverse-phase materials. A column with a highly structured graphite surface offers chemical and physical stability to enable repeated use without loss of performance or reproducibility. The conditions

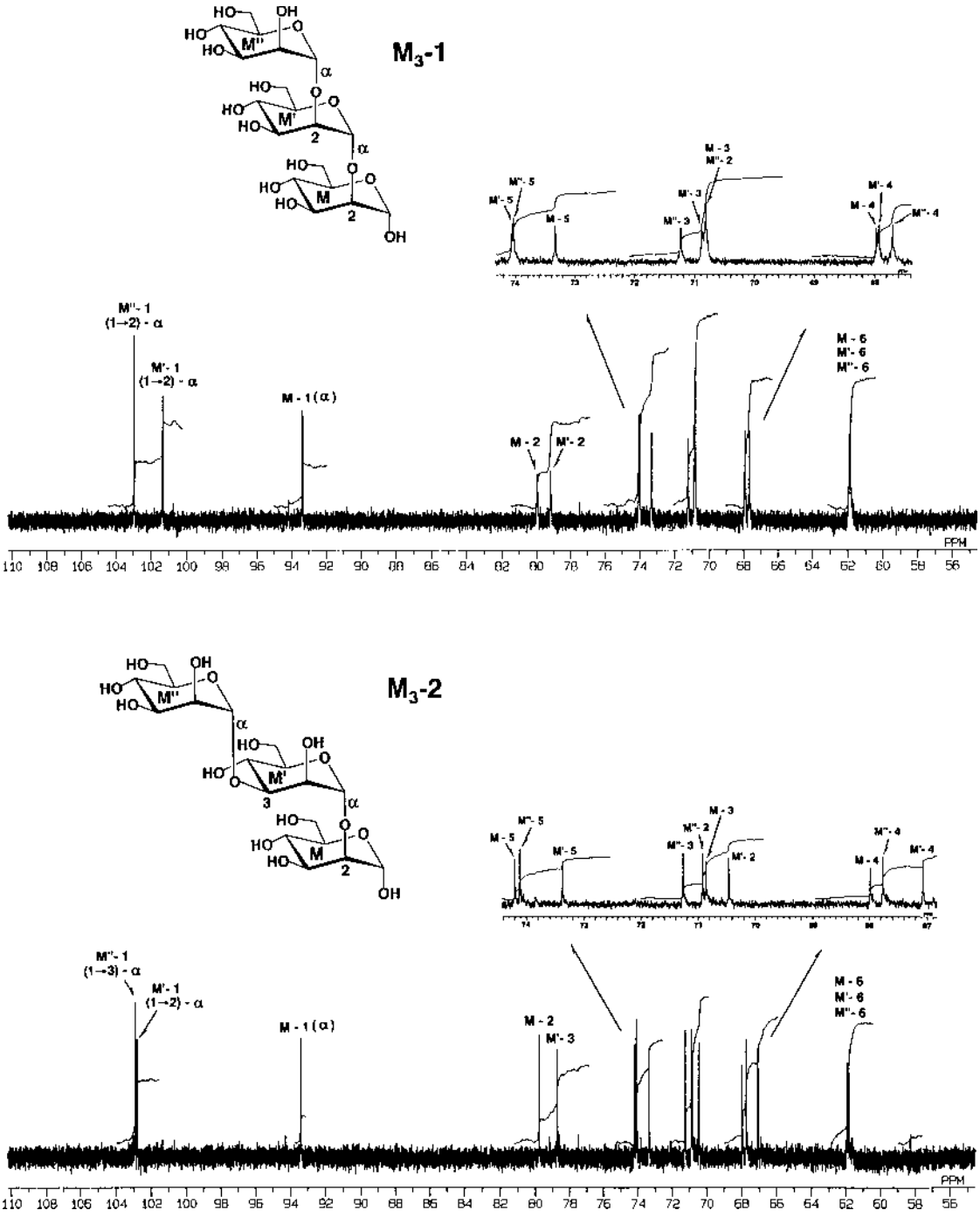

Fig. 5. ^{13}C-NMR Spectra of M_3-1 and M_3-2 Measured in D_2O at 125.65 MHz

used should be applicable to the analyses of high-mannose oligosaccharides in glycoproteins.

Most of the acetolysate was fractionated by centrifugal chromatography with hexane–acetone, and then each fraction was deacetylated. The main products, M_2, M_3s, and M_4, were isolated and purified by HPLC on a TSKgel Amide-80 column (300×21.5 mm i.d.) with 60 : 40 acetonitrile–water, and two M_3s were isolated on a Hypercarb (100×10 mm i.d.) with 0.5 : 99.5 acetonitrile–water. The molecular weights of M_2, M_3s, and M_4 were confirmed to be 365.4, 527.6, and 689.6, respectively, based on each molecular ion peak in the positive-ion mode $[M+Na]^+$ by TOF-MS. To analyze their structures in detail, the NMR analysis was performed using ^1H–^1H COSY and ^1H–^{13}C COSY measurements, and all carbons in the spectra of M_2, M_3-1, M_3-2, and M_4 were completely assigned. The assignments of C-6 signals were confirmed by distortionless enhancement by the polarization transfer (DEPT) method. Figures 4 and 5 show ^{13}C-NMR spectra of M_2 and M_4, and M_3-1 and M_3-2, respectively. The structures of M_2, M_3-1, M_3-2, and M_4 were identified as M (1α)→2M, M (1α)→2M (1α)→2M, M (1α)→3M (1α)→2M, and M (1α)→3M (1α)→2M (1α)→2M, respectively. These findings match the structure presented in Fig. 1.

Acknowledgment The authors thank Dr. A. Misaki (professor emeritus of Osaka City University) for helpful discussions on how to obtain manno-oligosaccharides. We also thank Professor M. Yamaki and her staff (Mukogawa Women's University) for recording and measuring the NMR spectra.

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