Effects of Mannoprotein Mutations on Saccharomyces cerevisiae Core Oligosaccharide Structure

Robert E. Cohen‡, Wei-je Zhang§, and Clinton E. Ballou¶

From the Department of Biochemistry, University of California, Berkeley, California 94720

By the combined actions of an endo-α1 → 6-mannanase and an endo-β-N-acetylglucosaminidase, the core oligosaccharides can be released from Saccharomyces cerevisiae X2180 mnn2 mannoproteins. The effects of various mannoprotein mutations were evaluated by structural comparison of these core oligosaccharides with those prepared from double mutant strains with the genotypes mnn1 mnn2, mnn3 mnn4, mnn2 mnn4, and mnn2 mnn5. The results indicate that only the mnn1 lesion has a major effect on the mannoprotein core structure. Whereas the mnn2 mannoprotein yields a core composed of 6 fragments that differ in size from each other by single mannose units, only the two smallest species predominate in the mnn1 mnn2 preparation. This change is correlated with a loss of terminal α1 → 3-mannosyl residues, an effect of the mnn1 lesion that is found also in the polysaccharide outer chain and hydroxyamino acid-linked mannooligosaccharides. The mnn3 and mnn5 mutations also had slight effects on the core size, but clear differences in linkage composition were not apparent. The results suggest that core oligosaccharides have an average composition of Man₃GlcNAc, whereas Man₄GlcNAc is the major oligosaccharide in strains containing the mnn1 defect. These values are 2 to 3 sugars less than those estimated previously (Nakajima, T., and Ballou, C. E. (1975) Biochem. Biophys. Res. Commun. 66, 870-879). Detailed analysis of the major core oligosaccharide from the mnn2 mutant revealed that the two mannoses in α1 → 3 linkage to the backbone were adjacent to each other and that the oligosaccharide is nearly identical with one isolated from Chinese hamster ovary cell membranes (Li, E., and Kornfeld, S. (1979) J. Biol. Chem. 254, 1600-1605). This finding provides strong evidence for the evolutionary conservation of this structural feature of the high mannose core oligosaccharides.

Yeasts contain a variety of extracellular mannoproteins with up to 90% by weight of mannose (1, 2). Most are associated with the cell wall or periplasm, and the linkages between the carbohydrate and protein components are of two types (3, 4). Short mannooligosaccharides are attached O-glycosidically to serine and threonine, whereas long polysaccharide chains, constituting 90% of the carbohydrate, are linked to asparagine residues via di-N-acetyllactosamine. In Saccharomyces cerevisiae X2180, the polysaccharide consists of α1 → 6 backbone of about 75 mannoses to which are attached mono- and di- trimannoside units in α 1 → 2 and α1 → 3 linkages. Some of the side chains are substituted with mannosylphosphate and mannobiosylphosphate units.

Several mannoprotein mutants with altered carbohydrate structure have been isolated (5-7). One of these, S. cerevisiae X2180 mnn2, lacks side chains on most of the α1 → 6-poly-mannose backbone (5), although a branched core fragment at the carbohydrate-protein linkage region is unaffected (4). This core fragment, recovered from the culture filtrate of a Bacillus circulans strain grown on the mnn2 mannoprotein (8), can be resolved into at least six homologous oligosaccharides. In this study we have obtained similar fragments using a purified endo-α1 → 6-mannanase and endo-β-N-acetylglucosaminidase. Because similar oligosaccharides have been derived from both mnn2 and wild type yeast invertases (9), the differentiation of the mannoprotein polysaccharide into "core" and "outer chain" regions is not an artifact of the mnn2 defect.

The S. cerevisiae mnn1, mnn3, mnn4, and mnn5 mutations are known to affect the mannoprotein outer chain, whereas mnn1 and mnn3 alter the hydroxyamino acid-linked carbohydrate as well (3, 5, 6). Most of the effects appear to result from the loss of specific mannosyltransferase activities (5, 6, 10, 11). In this study we have isolated and compared core oligosaccharides from the various mutant mannoproteins. Only the mnn1 mutation alters the core dramatically, giving smaller fragments that lack most terminal α1 → 3-mannosyl units. The mnn3 and mnn5 mutations have pleiotropic effects that may result from changes in cellular concentrations of mannosyl donors. Our results indicate that the average wild type core fragment size is Man₃GlcNAc, which is reduced to Man₄GlcNAc by the mnn1 lesion. The core of the mnn1 strain is identical with that reported for a mammalian high mannose oligosaccharide (12).

EXPERIMENTAL PROCEDURES

Materials—Bio-Gel P-2 (200-400 mesh), Bio-Gel P-4 (~400 mesh), Bio-Gel P-100 (200-400 mesh), Chelex 100, and DEAE-cellulose (Cellex D) were from Bio-Rad, and Sephadex G-75 was from Pharmacia Fine Chemicals. Acetonitrile (U. V. grade) was from Burdick and Jackson Laboratories, Inc., and deuterated solvents were from Bio-Rad and Aldrich. Ovalbumin glycopeptides AC-E (Man₃GlcNAcAsn), AC-D (Man₃GlcNAc₆Asn), and AC-C (Man₃GlcNAc₆Asn) were gifts from Dr. Chris Reading of this laboratory; the structures of these compounds, which correspond to GP-V, GP-IV, and GP-II in theomenclature of Tan et al., have been reported (13, 14). The N[6-H] dansyl¹ ovalbumin glycopeptides were also prepared by Dr. Reading. β1 → 3-linked mannitetraose, a Man₃ → 3oMan₁ → 2oMan₁ → 2Man, and a Man₃ → 3oMan₁ → 2oMan₁ → 3βMan₁ → 4GlcNAc

The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
were prepared by partial acetylation of ivory nut (Phytelephas macrocarpa) mannan (15), *S. cerevisiae* X2180 cell wall mannoprotein (16), and *S. cerevisiae mnna*2 mutant mannoprotein core fragments (4), respectively. α1→6-Linked mannotetraose was made by endo-α1→6-mannanase digestion of the mnna2 mutant mannoprotein (17). These oligosaccharides were labelled by reduction with sodium borohydride and by comparison to the retention times of *S. cerevisiae mnna*2 mutant mannoprotein core fragments (4), carpa) mannan (15), jima et al. (17), omitting the hydroxylapatite step and using an chromatograph equipped with a flame ionization detector and coupled differential refractometer.

These oligosaccharides were labeled by reduction with sodium borohydride dip reagent (26) and radioactivity with a Packard model 7201 radiochromatogram scanner. The oligosaccharide products from Smith degradation were chromatographed in ethyl acetate/pyridine/water (5:5:3, v/v) (Solvent B), and the labeled components were located by cutting the paper strip into 1-cm segments and determining radioactivity by scintillation counting in Bray’s solution.

Oligosaccharides were methylated (27), and the permethylated mixtures that contained 0.25 M sodium citrate, pH 6.5. One unit of enzyme produces 1 μmol of danyal Aan-GlcNAc per min at 37 °C under the standard assay conditions.

**Enzyme and Assays**—Endo-α1→6-p-mannanase and endo-β-N-acetyl-d-glucosaminidase were purified from the culture filtrate of *B. circulans* sp. TN31 (ATCC 29201). The organism was grown with aeration at 30 °C in the medium described (17) except that the organism was grown with aeration at 30 °C in the medium described (17) except that the content of *S. cerevisiae mnna*2 mannoprotein was increased from 0.1 to 0.35%. Endo-α1→6-p-mannanase was purified according to Nakajima et al. (17), omitting the hydroxylapatite step and using an Amicon hollow fiber system with an H1P10 cartridge for the initial concentration of the enzyme. The enzyme had a specific activity of 87 units/mg of protein (17), and it was free of other mannosidases.

The purification and properties of the endo-β-N-acetylglucosaminidase from *B. circulans* are described in “Results.” The activity was assayed using [H3]asparyl-Ac-C glycopeptide (21) in 24 reaction mixtures that contained 0.25 M sodium citrate, pH 6.5. One unit of enzyme produces 1 μmol of danyal Aan-GlcNAc per min at 37 °C under the standard assay conditions.

**General Methods**—Carbohydrate was determined by the phenol-sulfuric acid method (22) and reducing sugar by the Nelson-Somogyi (23) or the Park-Johnson (24) procedure; p-nassaccharides were used as the standard. Protein was determined by the method of Lowry et al. (25) with bovine serum albumin as a standard. β-Elimination of mannoproteins was performed as described (3).

Descending paper chromatography was done on Whatman No. 1 filter paper using ethyl acetate/pyridine/water (5:3:2) (Solvent A) for monosaccharides. Sugars were detected with the silver nitrate/sodium hydroxide dip reagent (29) and radioactivity with a Packard model 7201 radiochromatogram scanner. The oligosaccharide products from Smith degradation were chromatographed in ethyl acetate/pyridine/acetic acid/water (5:5:3, v/v) (Solvent B), and the labeled components were located by cutting the paper strip into 1-cm segments and determining radioactivity by scintillation counting in Bray’s solution.

Oligosaccharides were methylated (27), and the permethylated samples were formylized in 90% formic acid at 100 °C for 2 h and then hydrogenated in 0.3 M HCl at 100 °C for 6 h. The products were reduced and acetylated (28), and the partially methylated aldol acetates were analyzed using a Varian Aerograph model 1400 gas chromatograph equipped with a flame ionization detector and coupled to a DuPont model 21-491 mass spectrometer. A glass column (1/8 inch X 4 feet), packed with 3% OV-210 on Supelcoport, was used at 180 or 210 °C with He as the carrier gas. Peaks were identified by mass spectrometry (29) and by comparison to the retention times of standards. Partial acetylation (30) and Smith degradation (31) of oligosaccharides followed published procedures.

Mannoprotein core oligosaccharides were separated on a Waters Associates, Inc., model ALC/GPC 201 high performance liquid chromatograph using a μBondapak/carbohydrate column (0.40 × 30 mm) and acetonitrile/water in a ratio (v/v) between 55:45 and 60:40, depending upon the efficiency of the column. A flow rate of 3.5 or 4.5 ml/min was used, and the effluent was monitored with a Waters differential refractometer.

**NMR Spectroscopy**—1H-NMR spectra were obtained at 180 and 250 MHz on instruments equipped with superconducting magnets and Nicolet 1180 computers operated in the Fourier transform mode. The experimental parameters and methods for sample preparation have been described (18). Chemical shifts are expressed relative to sodium 3-(trimethylsilyl)-propanesulfonate and are referenced indirectly with an internal acetone standard (δ = 2.217 ppm at 40.0 °C). Spectra were determined in D2O at 40.0 °C.

### RESULTS

**Purification of Endo-β-N-Acetylglucosaminidase**—When grown on *S. cerevisiae* X2180 mnna2 mannoprotein, *B. circulans* strains TN31 secretes an endo-α1→6-mannanase and an endo-β-N-acetylglucosaminidase into the medium (4). Both enzymes are present in the 30 to 65% ammonium sulfate fraction, the two activities can be separated by DEAE-cellulose chromatography, and purification of the endomannanase has been reported (17). Table I summarizes the purification of the endo-β-N-acetylglucosaminidase. The activity reached a maximum value in a 36-h culture, when the cells were centrifuged for 30 min at 12,900 × g, and the supernatant liquid was concentrated and dialyzed against 0.05 M potassium phosphate, pH 7.0, with an Amicon hollow fiber concentrator using an H1P10 cartridge. Ammonium sulfate was added to 30% of saturation at 4 °C and, after 4 h, the mixture was centrifuged (30 min at 13,700 × g), and the supernatant solution was

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture filtrate</td>
<td>18,880</td>
<td>22,76</td>
<td>0.0017</td>
<td>100</td>
</tr>
<tr>
<td>2. Hollow fiber concentrate</td>
<td>835</td>
<td>20.73</td>
<td>0.0039</td>
<td>91</td>
</tr>
<tr>
<td>3. Ammonium sulfate fraction</td>
<td>189</td>
<td>9.66</td>
<td>0.0344</td>
<td>42</td>
</tr>
<tr>
<td>4. DEAE-cellulose (1st)</td>
<td>170</td>
<td>7.53</td>
<td>0.050</td>
<td>33</td>
</tr>
<tr>
<td>5. DEAE-cellulose (2nd)</td>
<td>149</td>
<td>5.93</td>
<td>0.023</td>
<td>26</td>
</tr>
<tr>
<td>6. Sephadex G-75</td>
<td>83</td>
<td>5.59</td>
<td>0.0369</td>
<td>82</td>
</tr>
</tbody>
</table>
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adjusted with ammonium sulfate to 65% of saturation. After 18 h, the mixture was centrifuged for 30 min at 12,000 × g, and the precipitate was dissolved in a minimum of 0.05 M, pH 7.0, potassium phosphate and dialyzed against the same buffer. This material was chromatographed on DEAE-cellulose (Fig. 1), and the fractions containing the endo-N-acetylglucosaminidase were lyophilized, dissolved in 5 mM sodium citrate, pH 6.5, and dialyzed against the same buffer. The enzyme recovered from the first DEAE-cellulose column (Table I, step 4) was lyophilized, dialyzed against 0.005 M sodium citrate, pH 6.5, and applied to a DEAE-cellulose column (2 × 25 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of 0.005 to 0.05 M sodium citrate, pH 6.5, and 5-ml fractions were collected. The enzyme activity appeared in fractions 40–60, which were combined and lyophilized, and the material was dissolved in 3 ml of water and applied to a Sephadex G-75 column (2.5 × 90 cm) which was eluted with 0.05 M sodium citrate, pH 6.5, in 4-ml fractions. The activity was eluted as a single peak just after the void volume (fractions 35–50). On Bio-Gel P-100, the enzyme coeluted with bovine serum albumin, indicating an approximate size of 6 × 10^6 daltons. The purified and lyophilized endo-N-acetylglucosaminidase retained full activity when redissolved in water, although repeated freezing and thawing gradually inactivated it. In 0.05 M sodium citrate at pH 6.5, the enzyme was stable at 37 °C for 100 h and was 50% inactivated after 2 h at 50 °C. It had a broad pH optimum between 5.5 and 7.5.

The B. circulans enzyme, assayed by the release of reducing sugar, degraded ovalbumin glycopeptides AC-C, AC-D, and AC-E, and the mannose-rich glycopeptides from a human IgM invertase or cell wall mannoprotein. The purified enzyme had N-acetylglucosaminidase with IgM or S. cerevisiae wild type invertase and the mannose-rich glycopeptides from a human IgM (18). In contrast, IgM complex glycopeptides were unaffected. Carbohydrate was not released from intact glycoproteins, with the exception of the S. cerevisiae mnn2 mutant mannoprotein. The absence of contaminating mannosidases was confirmed by failure of the endoglucosaminidase to attack H-reduced mannooligosaccharides listed under “Materials.”

Preparation of Core Oligosaccharides—Core oligosaccharides were prepared from mannoproteins as outlined in Fig. 2. After incubation of the mannoproteins with endomannanase to remove the outer chain, the products were chromatographed on a Bio-Gel P-2 column to separate mannose and small α1 → 6-linked manno-oligosaccharides. The recovered glycoprotein was then treated with endo-N-acetylglucosaminidase to release the core fragments, which were isolated by gel filtration (Fig. 3).

The endoglycosidase digestion products from the mnn2 mutant and double mutant mannoproteins all showed similar gel filtration patterns, except for the mnn1 mnn2 preparation. The latter core fragments were eluted as if they were smaller than the oligosaccharides from the other strains (Fig. 3), and this result was confirmed by other criteria (see below). Endomannanase digestion of all mannoproteins released approximately 30% of the carbohydrate as small oligosaccharides, and endo-N-acetylglucosaminidase treatment of the residual glycoprotein yielded 30% of the remaining carbohydrate as core oligosaccharides. Most of the mannose remaining on the protein could be removed by β-elimination, indicating that these units were O-glycosidically linked to hydroxyxamino acid residues. Incubation of the β-eliminated mannoprotein with endo-N-acetylglucosaminidase resulted in little or no further

Fig. 3. Isolation of mannoprotein core oligosaccharides. Mannoprotein (250 mg) was digested at 50 °C under a drop of toluene with 12.4 units of endo-α1 → 6-mannanase in 3 ml of 0.1 M potassium citrate, pH 6.0, containing 50 μM CaCl2. After 70 h, an additional 12.4 units of enzyme was added, and, at 128 h, the reaction was stopped by heating at 100 °C for 5 min. The mixture was applied to a Bio-Gel P-2 column (2 × 90 cm) and eluted with water; 4-ml fractions were collected. The residual glycoprotein, which was eluted in the void volume, was pooled and lyophilized. This material was incubated with 4 milliunits of endo-β-N-acetylglucosaminidase at 37 °C in 2.7 ml of 0.05 M sodium citrate, pH 6.5, under toluene. After 53 h, 4 milliunits more of enzyme were added, and the reaction was stopped at 96 h by heating for 5 min at 100 °C. The mixture was applied to a Bio-Gel P-4 column (2.5 × 100 cm) and eluted with water; 5-ml fractions were collected. The core-sized fragments, indicated by the bar, were combined and lyophilized to yield approximately 10 mg from each preparation. A is mnn2, B is mnn1 mnn2, and C is mnn2 mnn3 mannoprotein. Note that the mnn1 mnn2 core fragments in B were eluted later than those in A and C; the mnn2 mnn4 and mnn2 mnn5 preparations gave patterns similar to A.

Fig. 2. Scheme for the preparation of core oligosaccharides from S. cerevisiae X2180 mnn2 mutant mannoproteins. A, the mnn1 mnn2 mannoprotein; B, the product of endomannanase digestion; C, the product of subsequent endoglucosaminidase digestion.
release of core fragments (data not shown). Thus, a portion of
the carbohydrate remains linked to the residual protein, and
the reason for its resistance to endoglucanomimidase digestion
and β-elimination is unknown.

High Pressure Liquid Chromatography of Core Oligosaccharides—The core oligosaccharide preparation obtained from the culture filtrate of B. circulans TN31 grown on mnn2 cell wall mannoprotein is heterogeneous and contains 6 fragments of different size (8). Whereas these fragments are poorly resolved by gel filtration (8), improved separation is possible with high pressure liquid chromatography (9). Chromatographic patterns for the core oligosaccharides from mnn2, mnn2 mnn3, and mnn1 mnn2 mannoproteins are shown in Fig. 4, and the size heterogeneity is obvious. Compared with mnn2 (Fig. 4C), the mnn2 mnn3 core oligosaccharides (Fig. 4B) show less of fragments d and e and more of b and c. This shift is more pronounced for the mnn1 mnn2 preparation (Fig. 4A) in which fragments a and b account for most of the carbohydrate. In contrast, the mnn2 mnn3 core is enriched in the larger oligosaccharides d, e, and f, whereas the mnn2 mnn4 preparation is nearly identical with that of the mnn2 mutant (data not shown). The mixture of mnn2 core oligosaccharides produced by sequential treatment with both endoglucosidases is similar to the material recovered from the B. circulans culture filtrate, although the relative amounts of components c and d are reversed. Studies on the mnn1 mnn2 core described below suggest that some of the heterogeneity observed probably reflects differences in the completeness of endomannanase digestion.

Methylation Analysis of Core Oligosaccharides—Linkage compositions determined by methylation (Table II) suggest that the mnn2 core produced by sequential enzymatic degradation of mannoprotein is very similar to that from the B. circulans culture filtrate (4). Calculated on the basis of 4 terminal mannoses, the data indicate an average size of Man1;GlcNAc for mnn2 fragments, a result supported by the H-1 NMR integrations given below. The presence of a single GlcNAc at the reducing end was established previously (4).

All of the core samples contain both 2,6- and 3,6-di-O-substituted mannoses in a 1:2 molar ratio, characteristic of this portion of the mannoprotein (4). Only the mnn1 mnn2

![Fig. 4. Fractionation of mannoprotein core oligosaccharides by high pressure liquid chromatography.](http://www.jbc.org/)

| TABLE II |
|——|
| **Methylation analyses of S. cerevisiae mannoprotein core preparations** |
| **Core preparation** | **Molar ratios of linkages** | **Mannoses per oligosaccharide** |
|——|——|——|
| **Strain** | **Genotype** | **Man1** | **→** | **3Man1** | **→** | **2Man1** | **→** | **6Man1** | **→** | **5Man1** | **→** | **2Man** | **→** | **4GlcNAc** |
| X2180-1A-5 | (mnn2) | 4.00 | 0.96 | 2.1 | 1.3 | 2.1 | 1.04 | 0.76 | 11.5 |
| LB34-B | (mnn1 mnn2) | 4.00 | 0.07 | 2.1 | 0.5 | 2.0 | 1.0 | 9.5 |
| LB57-1D | (mnn2 mnn3) | 4.00 | 0.50 | 2.1 | 0.7 | 1.9 | 1.1 | 10.4 |
| LB13D-5D | (mnn3 mnn4) | 4.00 | 0.50 | 2.0 | 1.25 | 2.1 | 1.2 | 11.2 |
| LB68-7A | (mnn2 mnn5) | 4.00 | 1.06 | 2.3 | 1.15 | 1.9 | 1.3 | 11.7 |

* Values are normalized to terminal mannose, assuming 4 end groups. The variable amounts of the → 6Man1 → linkage probably reflects differences in the completeness of endomannanase digestion.
* Before fractionation into the homologs listed on Table III.
* This sugar was present but not quantitated.

![TABLE III]

| **TABLE III** |
|——|
| **'H-NMR assignments for reduced core oligosaccharide anomeric protons** |
| **Signal** | **Chemical shift** | **Integration** | **Linkage assignment** |
|——|——|——|——|
| A | 5.34 | 0.95 | 1.02 | → 2aMan → 3 |
| B | 5.29 | 1.10 | 1.06 | → 2aMan → 2 |
| C | 5.13 | 3.24 | 1.97 | → 3aMan → 3, → 3aMan → 3, → 3aMan → 6, → 6aMan → 6 |
| D | 5.04 | 2.03 | 2.25 | → 3aMan → 2 |
| E | 4.91 | 0.98 | 1.67 | → 3aMan → 6, → 6aMan → 6 |
| F | 4.87 | 0.92 | 0.81 | → 6aMan → 6 |
| G | 4.77 | 1.00 | 1.00 | → 6aMan → 4 |

* The signals are identified as in Fig. 6.
* Relative to signal G (the β-mannosyl anomeric proton).
* From Fig. 5.
* According to Reference 18.
* Some preparations of the mnn2 core show about 4 protons in this signal.
core shows a reduced amount of 1 → 3-linked mannose, and because the extent of branching is unaffected, the change indicates that the side chains in this oligosaccharide are shorter than the others.

**1H-NMR Spectroscopy of Core Oligosaccharides**—The anomeric proton resonances of mannosyloligosaccharides are characteristic of the linkages and sequences of the sugars (16, 18, 32, 33). This methodology is particularly useful for analyzing oligosaccharide mixtures, because the H-1 signals can be assigned to specific sugars that can be quantitated by integration.

The spectral data for the reduced mnn1 mnn2 and mnn1 mnn2 core oligosaccharides are given in Table III. The major difference between the two spectra is in resonance C, which has decreased from slightly over 3 protons in the mnn2 core spectrum to about 2 for mnn1 mnn2. This signal could correspond to any of the 4 α-mannosyl units listed in Table III at δ 5.13. We can eliminate one of these structures, Man → 6, because previous studies show that the endomannanae used to generate the core is unable to remove a terminal α1 → 6-linked mannose from a structure (6) such as

\[
\alpha\text{Man} \rightarrow 6\alpha\text{Man} \rightarrow 6.
\]

A change in the amount of → 6αMan → 6 can also be eliminated because the methylation results (Table II) show that the core preparations all have equal proportions of 2,6-di-O-substituted mannose.

By normalizing the NMR peak integrations, an average size of Man\textsubscript{1}GlcNAc is obtained for the mnn2 core and Man\textsubscript{1}GlcNAc for mnn1 mnn2. Core units of this size could contain at most two 3,6-di-O-substituted mannoses, and the spectra show H-1 resonances at δ 4.77 and δ 4.87, each equiva-

\[C \]lent to 1 proton, that correspond to these units. From the methylation data, core units of this size could contain only a single 2,6-di-O-substituted mannose, from which we conclude that only one of the H-1 resonances at peak C can be due to such a unit. Therefore, the change in peak C must be due to a loss of αMan → 3 or → 3 αMan → 3 units from the mnn1 mnn2 core, a conclusion that agrees with the changes that occur in other parts of the mnn1 mannan (5).

**Fine Structure of Two mnn1 mnn2 Core Oligosaccharides**—The mnn1 mnn2 core oligosaccharide is a mixture of two homologs that differ by one mannose unit (Fig. 5). The NMR spectrum for the smallest fragment is shown in Fig. 6, and a proposed structure is shown in the inset figure based on the integrations listed in Table III. Mannoprotein core oligosaccharides contain βMan → 4GlcNAc at their reducing ends (4), and a terminal α1 → 6-mannosyl residue must remain from the endomannanae digestion used for their isolation (6).

From this information and the NMR results, the structure in Fig. 6 is postulated for this molecule. That the larger homolog in Fig. 5 is derived from this compound by addition of an αMan → 6 unit to the backbone is supported by the integrations in Table III. The sizes of these oligosaccharides, Man\textsubscript{1}GlcNAc and Man\textsubscript{0}GlcNAc, are consistent with the estimates from methylation and gel filtration.

In confirmation of this structure, Smith degradation of the borotritide-reduced mnn1 mnn2 oligosaccharide yielded a radioactive fragment with the chromatographic properties expected for Man\textsubscript{1}XylNAc\textsubscript{2} (Fig. 7), which supports the assignment of the second 1 → 3-linked mannose unit to a position adjacent to the β-linked mannose in the backbone. Fig. 8 shows the acetolysis pattern for the mnn1 mnn2 core oligosaccharide, and the two fragments of greatest importance are those labeled peak B and F. The structure of peak F was derived from its H-1 NMR spectrum (Fig. 9), which agrees with that of a pentasaccharide αMan → 2αMan → 2αMan →

**References**


**Fig. 5.** Fractionation of mnn1 mnn2 core oligosaccharide.

The material prepared as in Fig. 3 was fractionated on a Bio-Gel P-4 column (2 x 200 cm) by elution with water in 12.5-m1 fractions. The elution was monitored for carbohydrate (○) and protein (●). Fractions A and B correspond to core oligosaccharides with 9 and 10 mannoses, respectively. Residual glycoprotein is in fraction C.

**Fig. 6.** Anomeric proton NMR spectra of the reduced major mnn1 mnn2 core oligosaccharide. The relative intensities are listed in Table III, and the signals are assigned in the inset figure. A minor component with one more mannose showed an enhanced intensity of signal E which is consistent with an additional α1 → 6-linked mannose unit in the backbone. Signal H is a spinning side band and I is the water peak.
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FIG. 7. Smith degradation of mann1 mann2 core oligosaccharide. The oligosaccharide (2.6 x 10^6 cpm), labeled by reduction with sodium borohydride, was oxidized in 0.1 M NaO4 at pH 4.0 for 2 days in the dark at 4 °C (8) or for 7 days at 37 °C (C). Ethylene glycol was added to consume the excess periodate, followed by which it was lyophilized, treated with a mixed bed resin, and chromographed in Solvent A.

FIG. 8. Separation of mann1 mann2 core oligosaccharide acetylation fragments. Ten mg of the oligosaccharide was acetylated in a 1:1 mixture of pyridine and acetic anhydride for 6 h at 105 °C, and the acetylated product was recovered by evaporation of the reagents under a stream of N2 gas. The product was acetylated for 8 h at 40 °C in a 10:1:0.1 mixture of acetic acid, acetic anhydride, and sulfuric acid (2 ml). The reaction was stopped by adding pyridine, and the acetylated fragments were extracted into dichloromethane, which was washed with water, dilute HCl, dilute boric acid, and water to remove the acid and salts. The solution was evaporated to dryness, and the products were deacetylated in dry methanol with sodium methoxide before fractionation on a Bio-Gel P-4 column (210 cm) by elution with water. The ratio of mannose to glucosamine was peak F, 4.2; peak E, 6.9; peak D, 15; peak C, 27. Peaks A and B did not contain glucosamine. The probable identities and molar ratios of the peaks are: A, mannose (1.17); B, mannotriose (2.0); C, mannobiose (0.13) plus ManGlcNAc (0.02); D, mannotetraose (0.13) plus ManGlcNAc (0.04); E, ManGlcNAc (0.15); and F, ManGlcNAc (0.5).

FIG. 9. Anomeric proton NMR spectrum of ManGlcNAc produced by partial acetylation of mann1 mann2 core oligosaccharide. The spectrum shows the borohydride-reduced peak F from Fig. 8. The relative intensities are A (1.17), B (1.00), C (1.05), and D (0.74). Only the intensity of D is slightly less than expected for the structure in the inset. F is the water signal, and the E signals are spinning side bands.

FIG. 10. Anomeric proton NMR spectrum of the mannotriose component produced by partial acetylation of mann1 mann2 core oligosaccharide. The spectrum shows peak B from Fig. 8, and it agrees with a mixture of 1 part aMan → 2αMan and 0.75 part of aMan → 3βMan. Signals A and C correspond, respectively, to the reducing a-anomer and the nonreducing end of the 1→2-isomer, while signal B corresponds to both the reducing a-anomer and the nonreducing end of the 1→3-isomer. Signal D is the small amount of β-anomer of both disaccharides, and E is the water peak. The structure in this figure predicts equimolar amounts of these two disaccharides. The reduced yield of the 1→3-linked isomer is expected, however, because of the fact that it is less stable to the acetylation conditions than the 1→2-isomer (30).

3βMan → 4GlcNAc. A mannose:glucosamine ratio of 4.2 supports this assignment. Finally, peak B represents the disaccharide component produced by acetylation, and the H-1 NMR spectrum (Fig. 10) is consistent with it being a mixture of aMan → 2Man and cMan → 3Man in a 1:0.75 ratio. The structure in Fig. 6 predicts a 1:1 mixture of these two fragments, and the low yield of the 1→3-linked isomer is expected because it is acetylated 10 times faster than the 1→2-isomer (30). This degradation of the 1→3-isomer leads, in turn, to a greater amount of mannose than predicted. Nonspecific acetylation of fragment F also reduces its yield to about 50% of the predicted amount and produces the fragments of intermediate size (peaks C, D, and E) that contain mannose and small...
amounts of glucosamine. Acetolysis patterns for well charac-
terized ovalbumin oligosaccharides show the same side re-
actions and poor quantitation (14). Peak E is the only other one
that contains a significant amount of glucosamine, and if it is
assumed that this in part represents a fragment with the structure \( a\text{Man} \rightarrow 6\text{Man} \rightarrow \text{Man} \rightarrow 4\text{GlcNAc} \), the \( mnn1 \)
\( mnn2 \) core could contain a small amount of the positional
isomer
\[
a\text{Man} \rightarrow 6\text{Man} \rightarrow 6\text{Man} \rightarrow 4\text{GlcNAc} \\
\downarrow  \quad \downarrow \\
\text{Man} \quad \text{Man}
\]
which would have a similar \( H-1 \) NMR spectrum as the isomer
in Fig. 6.

**DISCUSSION**

The yeast core oligosaccharides examined in this study were
prepared by the sequential digestion of \( S.\ cervisiae \) \( mnn2 \)
cell wall mannoproteins with an endo-\( a\) \( 6\)-mannanase and an
endo-\( N\)-acytethylglucosaminidase. The endoglucosaminidase
was purified 500-fold from the \( B.\ circulans \) culture filtrate
and was free of mannosidase activity. Its specificity resembles
that of endo-\( N\)-acytethylglucosaminidase \( H \) from \( S.\ piricus \)
\( (21) \), although the latter is more effective in the
release of carbohydrate from intact glycoproteins (34). The
greater size of the \( B.\ circulans \) enzyme \( (60,000 \text{ versus } 27,000 \)
daltons for endo-\( N\)-acytethylglucosaminidase \( H \) may contribute to
this difference in specificity.

To evaluate effects of various \( mnn \) mutations on the yeast
core structure, double mutant strains containing the \( mnn2 \)
lesion were compared. Use of the \( mnn2 \) background is neces-
sary to allow removal of the mannoprotein outer chain by
endo-\( a\) \( 6\)-mannanase digestion. Only the \( mnn1 \) mutation
substantially altered the core structure, the average size being
reduced 1 to 2 sugars due mostly to a loss of terminal \( a\) \( 3\)-
linked mannosides. This result is consistent with the observa-
tion that the \( mnn1 \) strains also lack the terminal \( a\) \( 5\)-mannosyl
residues in the outer chain (5) and on serine- and threonine-
linked oligosaccharides (3). The \( mnn1 \) phenotype is also corre-
lated with the absence of an \( a\) \( 1\)-mannosyltransferase
activity in cell extracts (10).

The precise effects of the \( mnn3 \) and \( mnn5 \) mutations on the
mannoprotein core are unclear. Slight size differences were
noted by chromatography, but the fragments had similar
linkage compositions and sequences. It has been suggested
that a decrease in the GDP-mannose pool could explain the
\( mnn3 \) phenotype that leads to a general shortening of the side
chains of both the outer chain and hydroxyaxidon acid-linked
carbohydrate (6).

The \( mnn4 \) mutation prevents the addition of mannosyl-
phosphate units to the mannoprotein side chains (5), so we
would not expect it to alter the size of the core. Phosphate
has not been detected in the \( mnn2 \) mannoprotein core, but in
mutants lacking most or all of the outer chain the core may
be phosphorylated (7). Inclusion of the \( mnn4 \) lesion could
alter the core structure of such strains.

Because of its smaller size and reduced heterogeneity, we have
analyzed the \( mnn1 \) \( mnn2 \) core in greater detail to clarify
some previously undefined features of yeast core oligosaccha-
ride structure. Smith degradation established that the two
mannoses in \( a\) \( 1\) \( 3\) linkage to the backbone are adjacent to
each other as they are in mammalian glycoproteins (35).
Methylation and partial acetolysis studies, as well as the \( H-1\)
NMR analysis, support the following structure for the \( mnn1 \)
\( mnn2 \) core, which is strikingly similar to a fragment found on
human IgM (18, 36) and to the dolichol-linked core oligosac-
charide precursor of mammalian tissues (37-41).

\[
a\text{Man} \rightarrow 6\text{Man} \rightarrow 6\text{Man} \rightarrow 6\text{Man} \rightarrow 4\text{GlcNAc} \\
\downarrow \quad \downarrow \quad \downarrow \quad \downarrow \\
\text{Man} \quad \text{Man} \quad \text{Man} \quad \text{Man} \\
\downarrow \quad \downarrow \quad \downarrow \\
\text{Man} \quad \text{Man} \\
\downarrow \quad \downarrow \\
\text{Man}
\]

The similarity becomes even more apparent if one assumes
that the terminal \( a\) \( 1\)-linked mannose in the \( mnn1 \) \( mnn2 \) core is a part of the outer chain that was not removed by
endomannanase digestion. If this is so, then the only process-
ning required to produce the yeast core from a mammalian-like
precursor would be removal of the glucose units and a single
\( a\) \( 1\)-linked mannosate unit (42, 43). This speculation implies
that the terminal \( a\) \( 1\)-linked mannosides found in the \( mnn2 \)
core are added to the oligosaccharide after its transfer to
protein, and the reports (44, 45) that the size of the lipid-
linked oligosaccharide precursor in yeast is not affected by the
\( mnn1 \) mutation are consistent with this idea. Some caution
should be observed, however, until a complete linkage analysis
on the yeast oligosaccharide precursor becomes available.

The \( mnn2 \) core oligosaccharides were first isolated from the
culture filtrate of \( B.\ circulans \) grown on \( S.\ cervisiae \) \( mnn2 \)
mannoprotein (4). These preparations were heterogeneous in
size (8), and it was considered possible that this was due to
the action of other mannosidases in the medium. Methylation
and \( H-1\) NMR spectroscopy of the core oligosaccharides pro-
duced by purified endoglycosidases gave similar results, how-
ever, and only a slight difference in size distribution was
apparent on high pressure liquid chromatography. The \( mnn2 \)
core oligosaccharides were previously estimated to range in
size from \( \text{Man}_{2}\text{GlcNAc} \) to \( \text{Man}_{4}\text{GlcNAc} \) (8). Our results now
suggest the average composition \( \text{Man}_{3}\text{GlcNAc} \), approxi-
ately 3 sugars fewer than previously estimated, although we
find a similar distribution of 4 to 6 fragments that differ by
single mannosate units.

The heterogeneity in the \( mnn2 \) core fragments appears to
have two origins. In part, it is introduced by the endomann-
anse, which leaves either one or two \( a\) \( 1\)-linked mannosate
units from the outer chain attached to the core. Part of the
heterogeneity, however, appears to represent a real feature of
the mannoprotein structure and results from a variable
amount of terminal \( a\) \( 1\)-linked mannosate. This conclusion
is supported by the dramatic effect of the \( mnn1 \) mutation in
reducing the heterogeneity such that the core fragments ob-
tained from this mutant mannoprotein differ only in the
amount of \( a\) \( 1\)-linked mannosate. Microheterogeneity of
mannose-rich core oligosaccharides is observed in mammalian
glycoproteins (35) and in yeast carboxypeptidase \( Y \) (46) where
fragments of different size and isomeric structure are found
attached to the same protein site.

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