MICROHETEROGENEITY OF THE INNER CORE REGION OF YEAST MANNO-PROTEIN

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SUMMARY: The inner core linkage region fragment from *Saccharomyces cerevisiae* mannan has been fractionated into 6 components and their structures have been analyzed. They form a family of homologous oligosaccharides ($Man_{12}GNAc$ to $Man_{17}GNAc$) with 6 or 7 mannose units in α1→6 linkage attached to N-acetylglucosamine by a β1→4 linkage, and with different amounts of side chain mannose units attached by α1→2 and α1→3 linkage.

Yeast mannan is a covalently-linked polysaccharide-protein complex (manno-protein) (1-4). Two kinds of linkages between the carbohydrate and protein components have been characterized. Some of the carbohydrate is attached to hydroxyamino acids by O-glycosidic bonds, but the majority is linked as polysaccharide chains to asparagine units by way of di-N-acetylchitobiose (Fig. 1).

The polysaccharide units linked to asparagine have two genetically differentiated regions, an inner core near the point of attachment to the protein and an outer chain extending from the inner core (2). The structure of the inner core of the manno-protein has been partially characterized, and the results show that it possesses microheterogeneity. We have now fractionated the inner core preparation and studied the individual components. Although they are all closely related to each other, they differ in size, in mannose content, and in the linkages between the mannose units.

EXPERIMENTAL

Yeast strains--Saccharomyces cerevisiae X2180-1A-5 (*mnn2* mutant) was obtained from the laboratory collection (5) and *S. cerevisiae* LB-34-B (*mnn1,2* double mutant) was provided by D. L. Ballou (6). The yeasts were grown in 1% yeast extract, 2% Bacto-peptone and 2% D-glucose, and mannan was isolated by citrate buffer extraction combined with Fehling's precipitation (7).

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Materials--Bio-Gel P-4 (-400 mesh) and Dowex AG50 were from Bio-Rad, and $NaBT_4$ (180 Ci/mole) was from New England Nuclear. Standard partially methylated sugars were samples characterized previously (2).

General methods--Carbohydrate was determined by the phenol-sulfuric acid method. Descending paper chromatography was carried out on Whatman No. 1 filter paper using the following solvents (in volume ratio): A, ethyl acetate-pyridine-water (8:2:1); B, ethyl acetate-pyridine-water-acetic acid (5:5:3:1). Sugars and sugar alcohols were detected on paper chromatograms with a $AgNO_3$-NaOH dip reagent. Paper chromatograms were scanned for radioactivity by cutting a 2 cm wide strip into 1 cm horizontal bands which were counted in 10 ml of Bray's solution in a Packard Tricarb liquid scintillation counter. Combined chromatography-mass spectrometry was carried out on a Varian Aerograph 1400 gas chromatograph coupled to a DuPont 21-491 mass spectrometer. Partially methylated alditol acetates were chromatographed on a glass column (1/8 inch x 4 feet) of 3% OV-210 on Supelcoport at 180°.

Isolation of the inner core fragment from cultural filtrate of Alkaligenes sp. TN-31 grown with S. cerevisiae X2180-1A-5 mannan--The organism was grown as before on 0.2% *S. cerevisiae* X2180-1A-5 mannan (2), and 10 liters of cultural filtrate was concentrated to a small volume. An aliquot (10 ml, about 1,500 mg of carbohydrate) was applied to a Bio-Gel P-4 column (4 x 200 cm). The mixture of fragments (1,350 mg) from the first gel filtration was passed through a Dowex 50(H^+) column (2 x 50 cm) to remove salts and amino acids. The decationized material (1,090 mg) was applied to a second Bio-Gel P-4 column (4 x 200 cm) and separated partially into six components. Each component peak was pooled separately and applied to a third Bio-Gel P-4 column (2 x 200 cm), which yielded the individual components free from each other. The purified components (1-2 mg each) were hydrolyzed in 0.5 ml of 2 N HCl for 3 hr at 100° in a sealed tube, and the hydrolysates were chromatographed on Whatman No. 1 paper with solvent A for 48 hr at 25°.

Determination of sugar ratios and molecular weights--1. *Proton magnetic resonance method:* The samples (30-50 mg) were lyophylized twice from 99.5% D_2O, dissolved in 0.5 ml of the same solvent, and the spectra were measured with a Varian A-60 spectrometer. All showed a signal at 3.5-4 ppm for the sugar ring protons and one at 2.0 ppm for the acetyl methyl group (8) of N-acetylglucosamine (2). The ratio of N-acetylglucosamine to mannose was estimated from integration of these two PMR signals.

2. *Reducing end assay:* The reducing power of each component (1 mg) was assayed (9) using mannose, α1→6-mannobiose and α1→6-mannohexaose as standards. The values, compared to the total carbohydrate by the phenol-sulfuric acid assay, gave the desired ratios.

3. *Gel filtration:* The degree of polymerization was calculated from the elution position on a Bio-Gel P-4 column (2 x 200 cm) that was calibrated with α1→6-mannooligosaccharides (Man-Man_{20}) (2). N-Acetylglucosamine was counted as 2 mannose units (10,11).

Immunological precipitation reaction--The reactions were carried out by the method of Ballou (12) with 25 μl of *S. cerevisiae* X2180-1A (wild-type) antiserum (5).

Methylation analysis--Methylation was done according to Hakomori (13,2). The methylated product was treated with 90% formic acid for 2 hr at 100°, and the formolysate was hydrolyzed with 0.3 N HCl for 6 hr at 100°. The mixture of methylated sugars was reduced with $NaBH_4$, after which the borate was removed and the alditol acetates prepared for gas chromatography. Methylation was judged to be complete by the absence of monomethyl-pentaacetyl derivatives and by a constant ratio of tetramethyl to dimethyl derivatives (about 1:1).

Acetylation and acetolysis--The inner core component (about 2 mg) was acetylated with pyridine and acetic anhydride, and the acetylated samples were acetolyzed in 2 ml of a mixture of glacial acetic acid-acetic anhydride-sulfuric acid (10:10:1) for 12 hr at 40° (14). The recovered acetolysis products were deacetylated, and the deacetylated products were reduced with $NaBT_4$. The reduced acetolysis products were chromatographed on Whatman No. 1 paper in solvent B for 17 hr at 25°, and the chromatogram was then analyzed for radioactivity.

RESULTS AND DISCUSSION

In earlier studies concerning the attachment between the carbohydrate and protein parts of yeast mannan (1,2), we isolated a fragment corresponding to the polysaccharide protein linkage region. It was composed of about 12 mannose units with a single N-acetylglucosamine at the reducing end. The mannose formed an α1→6-linked backbone with short α1→2- and α1→3-linked side chains. This linkage fragment was produced by the combined action of a bacterial endo-α1→6-D-mannanase and an endo-N-acetyl-β-D-glucosaminidase on the mutant mannan from *S. cerevisiae* X2180-1A-5, but its characterization was incomplete owing to its microheterogeneity. We have now fractionated this material into several components and have elucidated the basis of the heterogeneity.

The enzymes secreted by *Alkaligenes* sp. TN-31 degrade the manno-protein to produce 3 kinds of fragments (Fig. 2). FI is eluted in the void volume,

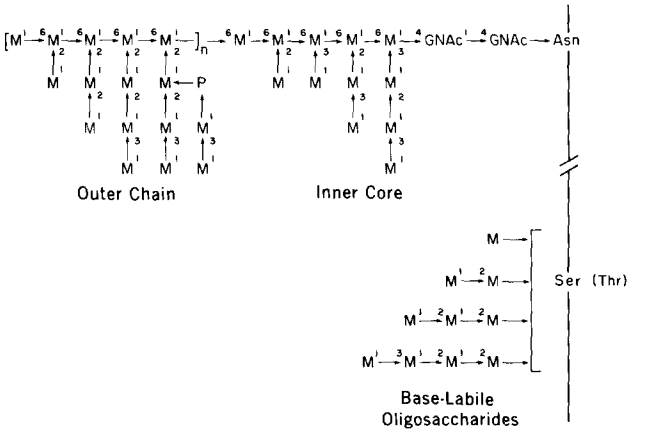

Fig. 1. The structure of *Saccharomyces cerevisiae* X2180 wild-type mannoprotein showing the oligosaccharides attached to hydroxyamino acid, the inner core and the outer chain. All anomeric linkages have the α-configuration except for the trisaccharide unit βMan(1→4)βGNAc(1→4)GNAc attached to asparagine.

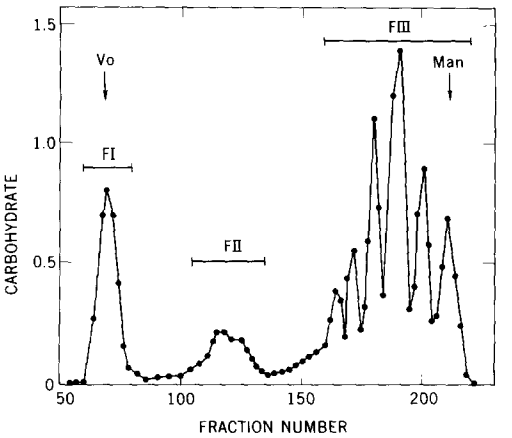

Fig. 2. Fractionation on a Bio-Gel P-4 (-400 mesh) column of the products from enzymic digestion of *S. cerevisiae* X2180-1A-5 (*mnn2* mutant) mannan. FI is a resistant glycopeptide fragment containing FII-like units, FII is the inner core mixture of branched oligosaccharides with about 12 mannose units and one N-acetylglucosamine unit, and FIII is a mixture of homologous α1→6-mannooligosaccharides with a degree of polymerization 1 to 6.

FII is of intermediate size and corresponds to the inner core fragment, whereas FIII is a mixture of α1→6-mannooligosaccharides (2). After removing salts and amino acids on a Dowex 50(H^+) column, the FII fragment was fractionated on

a Bio-Gel P-4 column (Fig. 3). The filtration was repeated until each component appeared homogeneous, and patterns of the six isolated components (FII-A to FII-F) are shown in Fig. 4.

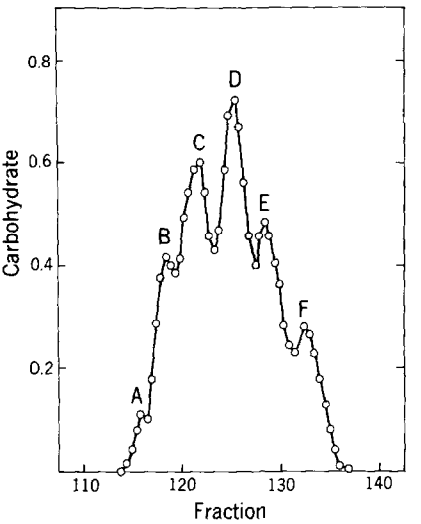

Fig. 3. Fractionation of FII from Fig. 2 on a Bio-Gel P-4 column.

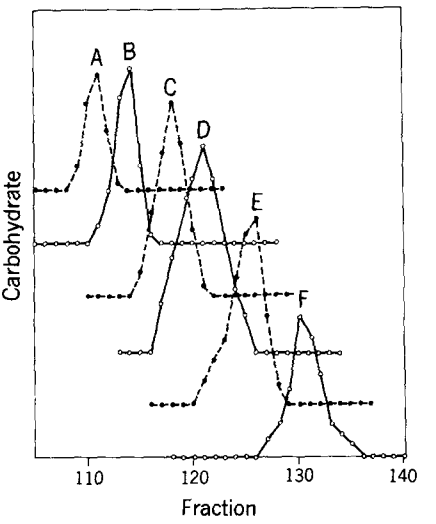

Fig. 4. Rechromatography of the inner core components from Fig. 3 on a Bio-Gel P-4 column.

Some properties of the individual inner core components are listed in Table I. The sugars are predominately in α-configuration as shown by a high positive rotation and by their enzymatic hydrolysis by the exo-α-D-mannanase (2). The molecular sizes range from $Man_{12}GNAc$ to $Man_{17}GNAc$, although the reducing sugar determinations gave slightly higher values.

The unfractionated inner core fragment from *S. cerevisiae* X2180-1A-5 mannan gives a strong precipitation reaction with *S. cerevisiae* X2180-1A antiserum (2). Thus, the material must have a branched structure with two or more terminal α1→3-linked mannose units in order to cross-link the antibody. Considerable variation was observed in the reactions of individual FII components with the antiserum (Fig. 5). FII-A, the largest component from *S. cerevisiae*

TABLE I

Properties of the inner core components

Component	Yield (mg)	$[\alpha]_{546}$	Ratio of Man to GNAc[a]	Total hexose/reducing end[b]	Polymer size[c]	Proposed structure
FII-A	14	+117	17.2	18.9	18	$Man_{17}GNAc$
FII-B	66	+122	16.7	18.0	17	$Man_{16}GNAc$
FII-C	120	+119	15.1	17.3	16	$Man_{15}GNAc$
FII-D	174	+118	14.3	16.7	15	$Man_{14}GNAc$
FII-E	87	+111	12.9	15.6	14	$Man_{13}GNAc$
FII-F	45	+113	12.2	14.7	13	$Man_{12}GNAc$

[a]Determined by proton magnetic resonance.

[b]These values are about one hexose unit larger than expected from the other data.

[c]Determined by gel filtration on a column calibrated with mannooligosaccharides. The original data gave apparent sizes one hexose unit larger, but correction has been made for the fact that N-acetylglucosamine has the retention volume of a disaccharide.

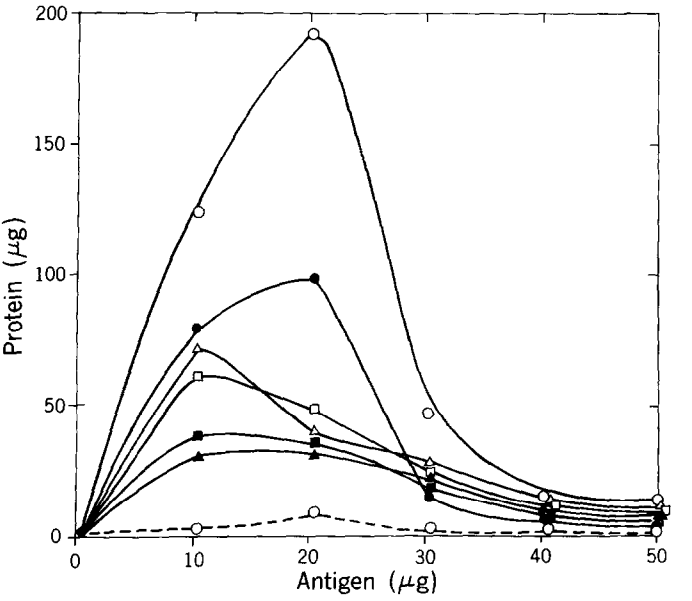

Fig. 5. Precipitin curves for *S. cerevisiae* X2180-1A antiserum with the inner core components from *S. cerevisiae* X2180-1A-5 mannan: FII-A (0—0), FII-B (●—●), FII-C (□—□), FII-D (■—■), FII-E (Δ—Δ), and FII-F (▲—▲). The inner core fragment from *S. cerevisiae* A2180-LB34-B (*mnn1,2* mutant) is represented by (0---0).

X2180-1A, gave the strongest precipitation suggestive of two or more terminal α1→3 linkages, whereas the inner core fragment from *S. cerevisiae* X2180-LB34-B (*mnn1,2* mutant) did not react with X2180-1A (wild-type) serum. This indicates that the mutant lacking the α1→3-mannosyltransferase activity (*mnn1*) lacks terminal α1→3-linked mannose units in the inner core of the mannan as well as in the outer chain, which supports the conclusion that the *mnn1* mutation is pleiotropic (2).

A typical methylation pattern is shown in Fig. 6, and the results in Table II show that all of the components have 2,3,4,6-tetra-, 2,4,6-tri-, 3,4,6-tri-, 2,3,4-tri-, 3,4-di- and 2,4-di-O-methyl mannitol. Thus, all are highly branched and the side chains are attached by both 1→2 and 1→3 linkages. The direct attachment by 1→3 linkages to the backbone is observed only in the inner core (2). We previously established the existence of the 1→6-linked backbone and

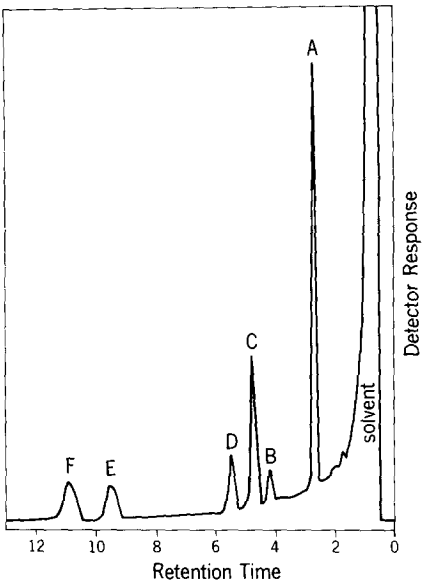

Fig. 6. Typical gas chromatographic pattern of the partially methylated mannitol acetates from an FII fragment. The peaks, identified by retention time and mass spectra, correspond to (A) 2,3,4,6-tetra-, (B) 2,4,6-tri-, (C) 3,4,6-tri-, (D) 2,3,4-tri-, (E) 2,4-di-, (F) 3,4-di-O-methylmannitol acetate.

TABLE II

Methylation analysis of the inner core components[a]

Component	Tetra-O-methyl	Tri-O-methyl			Di-O-methyl	
	2,3,4,6-	2,4,6-	3,4,6-	2,3,4-	2,4-	3,4-
FII-A	1 (1)	0.40 (0.33)	0.48 (0.5)	0.19 (0.17)	0.45 (0.33)	0.47 (0.5
FII-B	1 (1)	0.13 (0.17)	0.48 (0.5)	0.21 (0.17)	0.35 (0.33)	0.40 (0.5
FII-C	1 (1)	0.51 (0.4)	0.49 (0.4)	0.43 (0.4)	0.38 (0.4	0.42 (0.4
FII-D	1 (1)	0.25 (0.2)	0.51 (0.6)	0.18 (0.2)	0.43 (0.4)	0.44 (0.4
FII-E	1 (1)	0.24 (0.2)	0.52 (0.4)	0.21 (0.2)	0.42 (0.4)	0.44 (0.4
FII-F	1 (1)	0.12 (0)	0.50 (0.5)	0.23 (0.25)	0.31 (0.5)	0.48 (0.5

[a]All results are normalized to the tetra-O-methyl peak, and those in parentheses are calculated from the structures in Fig. 7.

TABLE III

Molar ratios of acetolysis fragments[a]

Component	Mannitol	Mannobiitol	Mannotriitol	Mannotetraitol
FII-A	1	1.1	0.6	1.2
FII-B	1	0.6	0.8	0.6
FII-C	1	0.5	1.1	0.4
FII-D	1	0.3	0.7	0.6
FII-E	1	0.5	0.6	0.5
FII-F	1	0.2	0.2	0

[a]Based on mannitol. Man_3GNAc and Man_4GNAc were not determined.

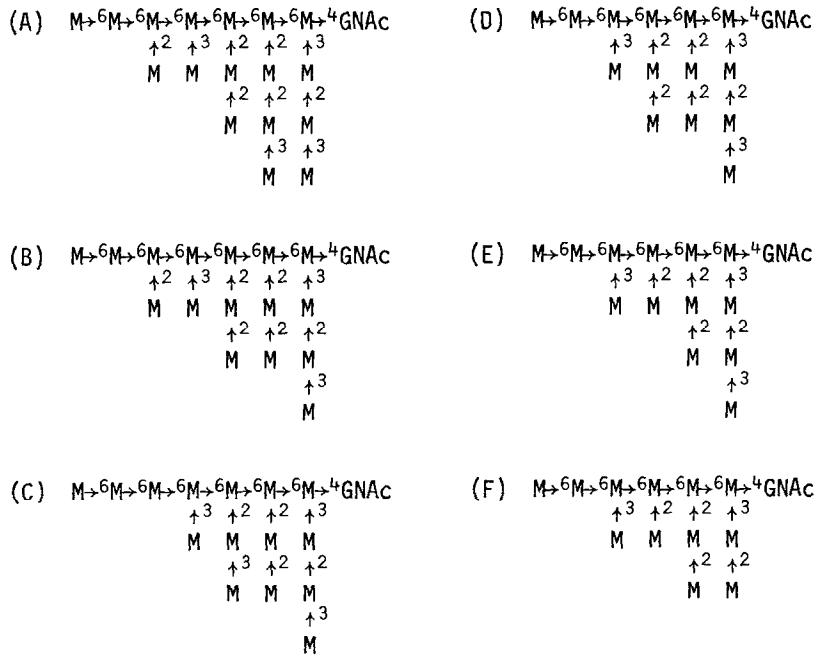

Fig. 7. Proposed structures of the inner core components from *S. cerevisiae* mannan. The order of side chains has not been established, except that if a mannotetraose unit is present it is attached directly to the N-acetylglucosamine.

the 1→4 linkage to the N-acetylglucosamine at the reducing end (2).

Partial acetolysis cleaves the 1→6 linkages preferentially (15) and gives fragments representing the side chains of the inner core components (Table III). As expected from the immunochemical results, the FII-A fragment contained the largest amount of the longer side chains which are often terminated by α1→3 linkages, whereas the FII-F fragment lacked the tetrasaccharide side chain. Although all of the data are not perfectly consistent, the results in this paper together with those reported earlier (2) indicate that the inner core fragments from *S. cerevisiae* X2180-1A-5 mannan have structures similar to those presented in Fig. 7.

The origin of the heterogeneity of the inner core is uncertain. Because the preparation is derived from the bulk cell wall manno-protein, it could be a hint that there are many species of mannan with slightly different structures. Alternatively, it could be an artifact of the isolation if other mannosidases were active during digestion of the mannan preparation. Further studies are underway to resolve these uncertainties.

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