Oligosaccharide Structures of the Major Exoglucanase Secreted by *Saccharomyces cerevisiae*†

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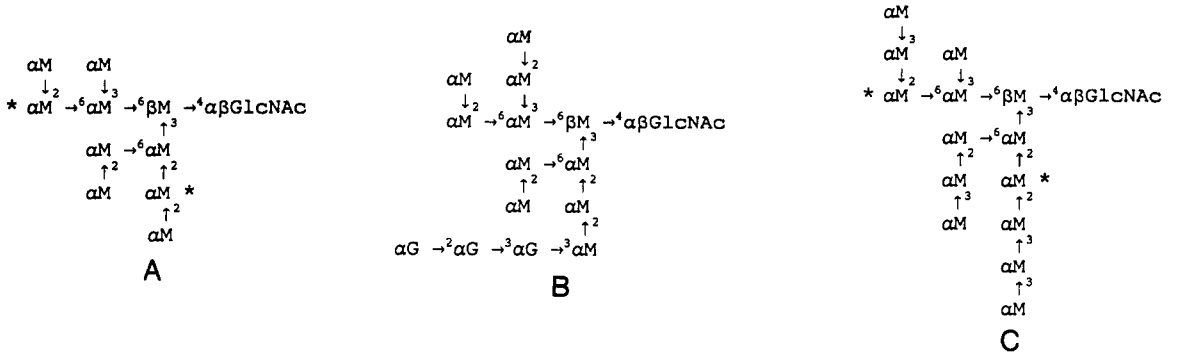
ABSTRACT: We have determined the structures of the N-linked carbohydrate chains, released by endo H, of exoglucanase II that are secreted by wild-type *Saccharomyces cerevisiae* and by the *mnn1 mnn9* and *mnn1* glycosylation mutants. The *mnn9* mutation does not significantly affect N-linked oligosaccharides of exoglucanase II since we found almost identical structures in both mutant strains consisting of a slightly enlarged core with the basic structure shown in A (where M = mannose). Most of the molecules (77%) were phosphorylated on one of the starred mannoses (34%) or on both (43%) with a diesterified (αM → P →) or monoesterified phosphate group. In addition, some of the molecules apparently escape normal processing and retain the α-(1→2)-linked mannose (italicized) and/or the three glucoses that are characteristic of the lipid-linked precursor (structure B). In the wild type, we found the same basic structure but more



than 90% of the molecules were modified with one to four α-(1→3)-linked mannoses, which were absent in the strains bearing the *mnn1* mutation (structure C). The proportion of acidic components was similar to that found in the mutants (78%), although, in this case, the monophosphorylated forms were more abundant (50%) than the diphosphorylated ones (28%). Most of the phosphate groups (69%) were diesterified by a disaccharide (αM → $^3\alpha$M → P →) instead of the single mannose found when the *mnn1* mutation was present. In both *mnn1* and wild type 10–15% of the oligosaccharides had an extra α-(1→6)-linked mannose in the outer chain, a structure described in the recently isolated *vrg1* mutant [Ballou, L., Hitzeman, R. A., Lewis, M. S., & Ballou, C. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3209–3212].

N-Linked protein glycosylation in *Saccharomyces cerevisiae* starts by the *en block* transfer of Glc_3-Man_9-$GlcNAc_2$ from pyrophosphoryldolichol to asparagine residues in the protein. The three glucoses and one of the mannoses are removed in the endoplasmic reticulum to generate the processing intermediate Man_8-$GlcNAc_2$ (Byrd et al., 1982; Kukuruzinska et al., 1987). From this intermediate, *S. cerevisiae* builds up two types of polymannose oligosaccharides: one consisting of a slightly enlarged core with Man_{9-14}-$GlcNAc_2$ found in the vacuolar protease carboxypeptidase Y (CPY)[1] (Ballou et al., 1990), on some sites in periplasmic invertase (Trimble et al., 1983), and in the cell wall mannoproteins of the glycosylation mutants *mnn9* (Tsai et al., 1984b) and *vrg6* (Ballou et al., 1991); and another type with a long and highly branched outer chain consisting of $Man_{>100}$-$GlcNAc_2$, characteristic of cell wall mannoproteins of wild-type cells (Ballou, 1990) and some sites on periplasmic invertase. These two patterns suggest the existence of two glycosylation pathways (Ballou et al., 1990). Figure 1 shows the structure of these oligosaccharides as well as the defects presented by some glycosylation mutants related to this study.

S. cerevisiae exoglucanases are enzymes capable of releasing glucose from the nonreducing end of (1→3)-β-glucans and, to a lesser extent, from the (1→6)-β-linked polymer. In addition, they show hydrolytic activity against the synthetic derivative *p*-nitrophenyl β-D-glucopyranoside. Exoglucanase activity secreted by exponentially growing *S. cerevisiae* cells can be fractionated by ion-exchange chromatography into two forms called exoglucanase I and exoglucanase II. Exoglucanase I has a lower electrophoretic mobility and accounts

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[1] Abbreviations: CPY, carboxypeptidase Y; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; endo H, endo-β-*N*-acetylglucosaminidase H; G, glucose; H-1, anomeric proton; HPLC, high-performance liquid chromatography; M, mannose; *mnn*, yeast mutant defective in protein glycosylation; *p*-NPG, *p*-nitrophenyl β-D-glucopyranoside.

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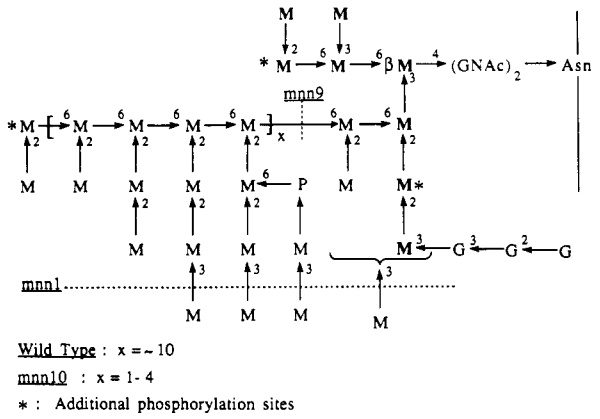


FIGURE 1: *S. cerevisiae* N-linked carbohydrate structure. The core region of the oligosaccharide is shown in block letters, and the dotted lines indicate the defects presented by the *mnn* mutants. The carboxypeptidase Y oligosaccharides are similar to those from the *mnn9* mutant. The glucose units (G) are only present in the *gls1* mutant, while the *vrg1* mutant synthesizes oligosaccharides with structure similar to *mnn9* but with one additional unbranched α-(1→6)-linked mannose in the outer chain (see structure XII).

for 10–15% of the secreted activity, while exoglucanase II acounts for the remaining 85–90% (Ramírez et al., 1989a). Preliminary studies on the carbohydrate portions (Ramírez et al., 1989b) have shown that exoglucanase II has two short N-linked chains of about 2500 Da, while exoglucanase I seems to arise by elongation of at least one of the carbohydrate chains present in exoglucanase II. When deglycosylated by endo H, the two enzymes appear identical in terms of electrophoretic mobility, immunological cross-reactivity, amino acid composition, and amino-terminal sequence (48 amino acids) (Ramírez et al., 1989a, 1990). In addition, both enzymes are coded by the same structural gene (EXG1), which has been cloned (Nebreda et al., 1986) and sequenced (Vázquez de Aldana et al., 1991); a mutation in EXG1 prevents secretion of both exoglucanases (Nebreda et al., 1987). The predicted polypeptide is 448 amino acids long and has two potential N-glycosylation sites at positions 165–167 (Asn-Asn-Ser) and 325–327 (Asn-Glu-Ser) (Vázquez de Aldana et al., 1991). Thus, native exoglucanases I and II must arise by heterogeneous glycosylation of a single gene product.

In the present work, we have characterized the N-linked oligosaccharides from exoglucanase II of wild-type *S. cerevisiae* and of the *mnn1* and *mnn1 mnn9* glycosylation mutants. In wild-type cells we found structures identical to those previously reported for the vacuolar enzyme CPY (Ballou et al., 1990). The strains with the *mnn1* mutation lacked the terminal α-(1→3)-linked mannoses as expected (Raschke et al., 1973), whereas the *mnn9* mutation did not affect the oligosaccharide structure. Thus, the exoglucanase II apparently follows the same glycosylation pathway as the vacuolar protease CPY, which is the sole or predominant pathway expressed in the *mnn9* mutant (Ballou et al., 1990). The exoglucanase system in *S. cerevisiae* therefore provides a good model for study of the factors that determine the glycosylation pattern followed by a protein in terms of modification of the polymannose outer chain.

EXPERIMENTAL PROCEDURES

Materials and Methods. Wild-type *S. cerevisiae X2180* and the mutant strains *mnn1* and *mnn1 mnn9* were used. The mutants were kindly supplied by Lun Ballou, University of California, Berkeley, CA, and have been described previously (Raschke et al., 1973; Tsai et al., 1984b; Ballou, 1990). The strains were grown in a synthetic liquid medium (Olivero et al., 1985) buffered at pH 5.2 with 0.45 M sodium citrate/sodium phosphate and containing 4% glucose. The cultures were started with a heavy inoculum (A_{600} ~ 0.05) from a YEPD plate (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and incubated at 28 °C in a rotary shaker until the late exponential phase of growth was reached (usually 24 h).

Exo-(1→2)-α-D-mannosidase was prepared from *Aspergillus phoenicis* (Ichishima et al., 1981), and endo-β-*N*-acetylglucosaminidase H (endo H) (Tarentino et al., 1978) was from ICN Biochemicals (Cleveland, OH). DEAE-Bio-Gel, Bio-Gel P-4 (–400 mesh), and AG 50W-X8 were obtained from Bio-Rad, QAE-Sephadex and Sephacryl S-200 were from Pharmacia-LKB, and 100% ^2H_2O was from Aldrich. Protein was determined according to the Lowry method (Lowry et al., 1951) and carbohydrate by the phenol–sulfuric acid method (Dubois et al., 1956). Mild acid hydrolysis was performed by boiling the samples for 30 min in 0.01 N HCl.

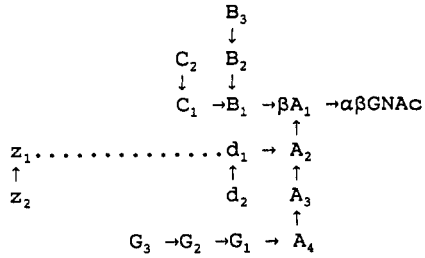
^{1}H NMR (Cohen & Ballou, 1980; Vliegenthart et al., 1983; Derome, 1987) was done at 40 °C on a 500-MHz spectrophotometer in the Department of Chemistry, University of California, Berkeley, CA. Samples were resuspended in ^2H_2O, and chemical shifts are referred to internal acetone at δ 2.217 relative to DSS (Cohen & Ballou, 1980; Vliegenthart et al., 1983; Hernández et al., 1989a).

Cell wall mannoproteins were solubilized by autoclaving whole cells in sodium citrate buffer at pH 7 and purified by the cetavlon method (Tsai et al., 1984a; Ballou et al., 1989). HPLC was carried out on a Bio-Gel TSK DEAE-5-PW column (7.5 × 75 mn) from Bio-Rad. Oligosaccharides were fractionated on a Dionex BioLC carbohydrate system equipped with a pulsed amperometric detector. A CarboPac PA1 column (4 × 250 mm) was used under the conditions described previously (Ballou et al., 1990; Hernández et al., 1990).

Purification of Exoglucanases. Secreted exoglucanases were purified as described previously (Ramírez et al., 1989a). The culture supernatant was concentrated on a Pellicon cassette system from Millipore (exclusion limit, 10 000 Da) and dialyzed against 25 mM sodium acetate buffer, pH 5.2. For small volumes an Amicon cell, fitted with a PM10 membrane, or a Centricon 10 microconcentrator from Amicon Corp. was used. The concentrate was applied to a DEAE-Bio-Gel column (2.5 × 20 cm) equilibrated with the same buffer and eluted with 500 mL of a continuous gradient up to 0.4 M NaCl. Exoglucanases I and II were collected separately, concentrated, and dialyzed against 0.2 M sodium acetate buffer, pH 5.2, and the enzyme II was rechromatographed on a Sephacryl S-200 column (2.5 × 120 cm) equilibrated and eluted with the same buffer. The purity of the exoglucanase II was checked by SDS–PAGE. In some cases, the solution containing the pure enzymes was dialyzed against water and lyophilized. To assay exoglucanase activity, 0.5 mL of the enzyme solution (properly diluted) in acetate buffer, pH 5.2, was incubated at 30 °C with 0.1 mL of 40 mM *p*-NPG. The reaction was stopped by addition of 1 mL of saturated Na_2CO_3 and the released *p*-nitrophenol was read at 410 nm (Tingle & Halvorson, 1971). One unit of enzymatic activity is defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from *p*-NPG per hour under the above conditions.

Enzymatic Digestions. For digestion with endo H, about 50 mg of pure lyophilized exoglucanase II or mannoprotein was resuspended with sonication in 0.5 mL of 50 mM sodium citrate buffer, pH 5.5, containing 0.02% sodium azide and 50 milliunits of endo H and incubated at 37 °C for 72 h. The reaction was stopped by heating at 100 °C for 3 min and centrifuged. The precipitate contained most of the protein,

Scheme I



while the released oligosaccharides remained in solution. After 24 and 48 h of incubation, 25 more milliunits of endo H was added (Hernández et al., 1989b). Oligosaccharides were digested with exo-(1→2)-α-D-mannosidase as in Ballou et al. (1990).

Convention for Identifying Mannose Units in Yeast Oligosaccharides. In Scheme I, G stands for glucose and the rest of the letters stand for mannose units in which the horizontal bonds represent 1→6 linkages, with the exception of the linkage A_1→GlcNAc, which is 1→4, and the linkages between the glucoses, which are 1→2 and 1→3. The vertical bonds represent 1→2 and 1→3 linkages. Unless indicated, the monosaccharides are in the α configuration.

Units derived from the lipid-linked oligosaccharide precursor are shown in upper case letters, and those units added during processing are in lower case letters. The dotted line indicates an extension of the α-(1→6)-linked outer chain, but when the outer chain is extended, d_2 is not added and an equivalent α-(1→2)-linked mannose (z_2) is added. Mannoses C_1, A_3, and z_1 are sites of phosphorylation (Hernández et al., 1989b).

Most of the units in this structure are easy to identify by the characteristic chemical shifts of their anomeric protons (H-1) (Cohen & Ballou, 1980; Hernández et al., 1989b; Alvarado et al., 1990; Tsai et al., 1984b). In a neutral oligosaccharide, the shifts are as follows: A_1, δ 4.77; A_2, δ 5.32; A_3, δ 5.29; A_4, δ 5.03; B_1, δ 4.87; B_2, δ 5.379 (δ 5.08–5.11 when unsubstituted); B_3, δ 5.05; C_1, δ 5.125; C_2, δ 5.04; d_1, δ 5.125; d_2, δ 5.04; G_1, δ 5.27; G_2, δ 5.52; G_3, δ 5.17. When the outer chain is extended, the signals for z_1 and z_2 replace those for d_1 and d_2. When the oligosaccharide is phosphorylated in C_1, no significant change is observed in the spectrum if the phosphate group is not diesterified, but if the diesterifying mannose is present, a new doublet appears at δ 5.43–5.45. When the phosphorylation occurs in A_3 and the phosphate group is diesterified, the signal for the αM →P is present at δ 5.43–5.45 and significant changes are seen in the signals for the mannoses A_1, A_2 and A_3: A_1, δ 4.80; A_2, δ 5.31, and A_3, δ 5.28. If the phosphate group is not diesterified, the doublet at δ 5.43–5.45 is not present and the signals for A_1, A_2, and A_3 change to A_1, δ 4.81; A_2, δ 5.35; and A_3, δ 5.24. The changes observed in the spectra of the monophosphates in the positions C_1 and A_3 are also valid for the diphosphorylated forms in the same phosphorylation sites. In strains not bearing the *mnn1* mutation, some of the arms in the core and the outer chain are capped with α-(1→3)-linked mannoses (a_5, c_3, d_3, ...) that resonate at δ 5.12–5.14, and the mannose linked to the phosphate may also be substituted at position 3, in which case the doublet at δ 5.43–5.45 shift upfield to δ 5.407–5.418.

RESULTS

Structure of the N-Linked Oligosaccharides of Exoglucanase II from the mnn1 mnn9 Mutant. The enzyme was purified from 105 L of culture medium (49 000 units) harvested in the late exponential phase of growth. The clear supernatant of the endo H digest was loaded in a Bio-Gel P-4 column

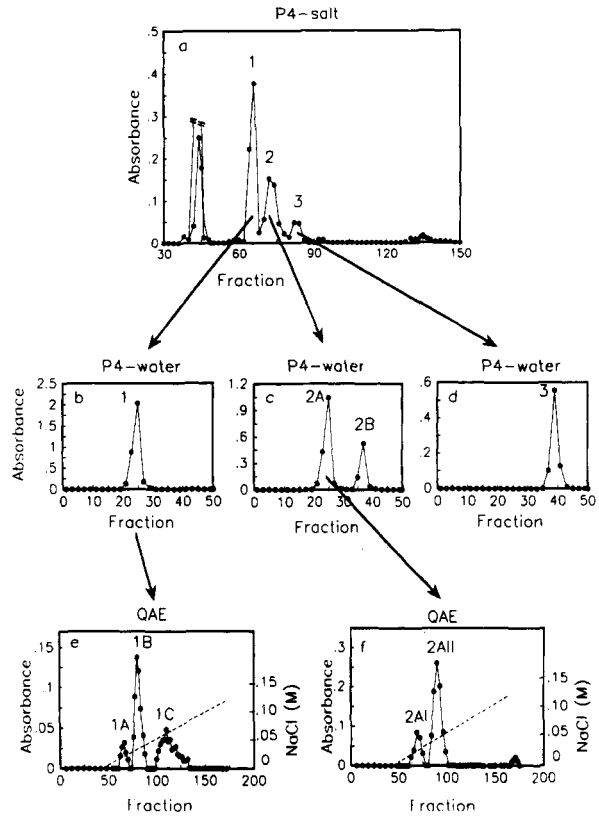


FIGURE 2: Fractionation of exoglucanase II endo H-released oligosaccharides from the *mnn1 mnn9* mutant. In all panels the carbohydrate (●) was monitored by the phenol–sulfuric acid method at 490 nm, protein (—) at 280 nm, and concentration of NaCl (- - -) by conductivity. The endo H digest of exoglucanase II was loaded in a Bio-Gel P-4 column (1.5 × 170 cm) eluted with 0.1 M ammonium acetate (panel a). As indicated by the arrows, each of the three peaks containing the endo H-released oligosaccharides was rerun in a Bio-Gel P-4 column (1 × 30 cm) by elution with water (panels b–d). The phosphorylated oligosaccharides (peaks 1 and 2A) were fractionated by ion-exchange chromatography in a QAE-Sephadex column (1.5 × 9.5 cm) equilibrated with 2 mM Tris. The samples were eluted with 150 mL of a continuous NaCl gradient up to 125 mM (panels e and f).

equilibrated and eluted with 0.1 M ammonium acetate to separate the remaining protein from the endo H-released oligosaccharides (Figure 2a). The peak in the void volume included the protein portion of exoglucanase II as well as a variable amount of undigested carbohydrate which represented around 20% of the total. The possibility that the excluded carbohydrate could be very long oligosaccharide chains from contaminant exoglucanase I must be discarded because, in addition to the high electrophoretic homogeneity of the enzyme preparation, the *mnn1 mnn9* does not secrete detectable amounts of exoglucanase I. Peaks 1–3, which correspond to the endo H-released oligosaccharides, were collected separately, desalted by passing through Dowex (AG 50W–X8), and lyophilized. The lyophilized powder was resuspended in water and rechromatographed in a P-4 column equilibrated and eluted with water. In this chromatography, peaks 1 and 3 eluted again as single peaks (Figure 2b,d), while peak 2 was separated into two fractions (Figure 2c), one of them in the void volume of the column and the other one slightly included. The ratio of carbohydrate in these two samples was approximately 7:3, and we will refer to them as 2A and 2B, respectively.

As the following ^{1}H NMR analysis will establish, all of the oligosaccharides had the basic structure previously proposed for the carbohydrate of *S. cerevisiae mnn1 mnn9* mannoproteins: M_{10-11}-GlcNAc (Tsai et al., 1984b; Hernández et

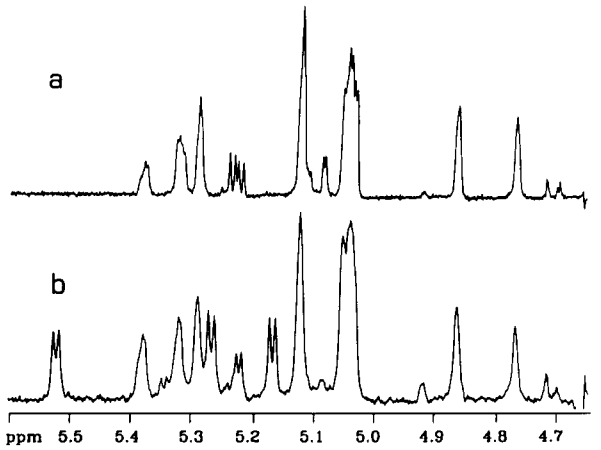
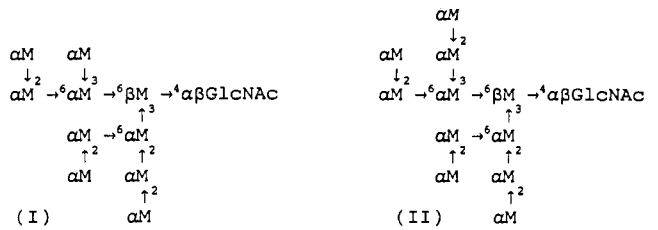


FIGURE 3: H-1 NMR spectra of exoglucanase II neutral oligosaccharides from *mnn1 mnn9*. (a) Sample from peak 3 (Figure 2d); (b) sample from peak 2B (Figure 2c).

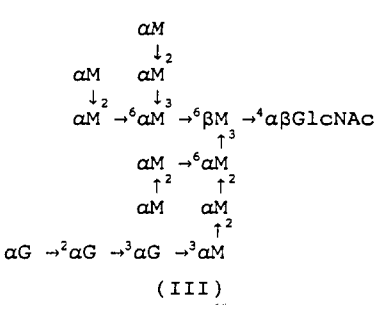
al., 1989a), some of them being phosphorylated at the mannoses A3, C1, or both (Hernández et al., 1989b). In addition, some of the molecules retained the three glucoses characteristic of the lipid-linked precursor that participate in early steps of the N-glycosylation pathway.

The samples 2B and 3, from the gel filtration columns, were neutral oligosaccharides, as shown by the H-1 NMR spectra in Figure 3. The spectrum of sample 3 (Figure 3a) shows the presence of the two neutral forms, $M_{10}GNAc$ and $M_{11}GNAc$, previously found in the cell wall mannoproteins of this mutant (Hernández et al., 1989b) in a ratio of 1:1 and with structures I and II. The presence of molecules with 11



mannoses is supported by the signal at δ 5.379 for the mannose B_2 that shifted downfield (from δ 5.08–5.11) as a consequence of the substitution in position 2 by mannose B_3 (italicized). The small upfield shift of the α-glucosamine signal in $M_{11}GlcNAc$ (δ 5.24 → δ 5.22) also corroborated the presence of mannose B_3 (Hernández et al., 1989b).

Sample 2B (spectrum in Figure 3b) was an almost pure $G_3Man_{11}GlcNAc$ with structure III. The signals at δ 5.27,



5.52, and 5.17 in Figure 3b correspond to glucoses G_1, G_2, and G_3, respectively (Tsai et al., 1984b), while the integral of the signal at δ 5.379 supports the presence of the mannose B_3 (italicized) in more than 90% of the molecules.

Sample 2A from the above gel filtration column (Figure 2c) was lyophilized, resuspended the 2 mM Tris, and loaded

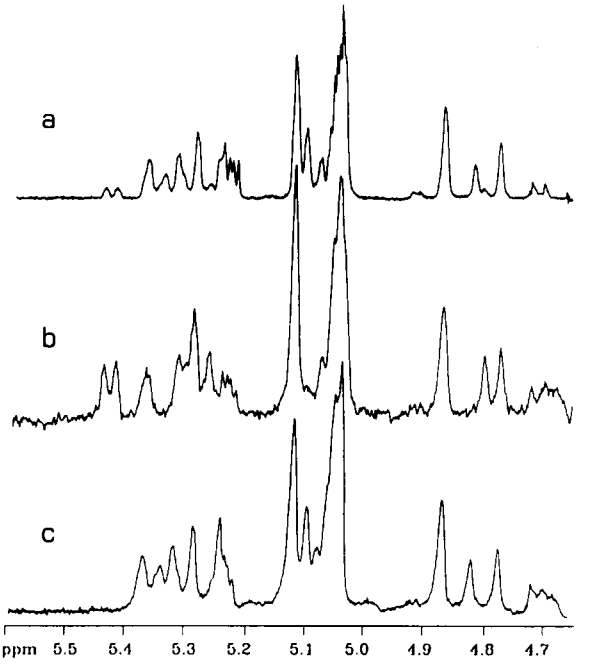
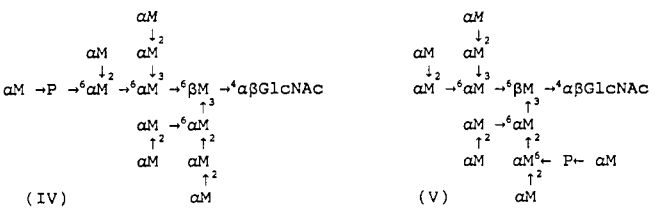


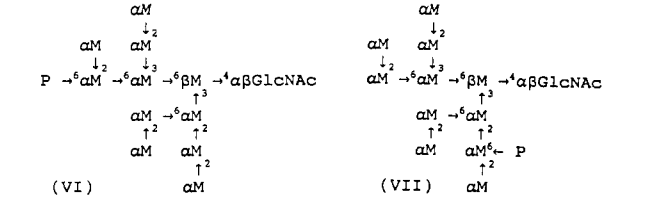
FIGURE 4: H-1 NMR spectra of exoglucanase II acidic oligosaccharides from *mnn1 mnn9*. (a) Sample from peak 2A (Figure 2c); (b) monophosphate diester from peak 2AI (Figure 2f); (c) monophosphate monoester from peak 2AII (Figure 2f).

in a QAE-Sephadex column. Then, it was eluted with a continuous NaCl gradient up to 125 mM, and two peaks were separated (Figure 2f) in the positions expected for molecules with one (peak 2AI) and two (peak 2AII) negative charges (Hernández et al., 1989b). The H-1 NMR spectrum of peak 2AI is shown in Figure 4b. The integral of the doublet at δ 5.43–5.45 clearly indicates the presence of one $\alpha M \rightarrow P$ group per molecule, which fully agrees with the elution position in QAE-Sephadex. The signals for A_1 at δ 4.77 and 4.80 indicate the presence of oligosaccharides with structrures IV and V in



an approximated molar ratio of 1:1. In this case, the italized mannose (B_3) appeared in 60% of the molecules as deduced from the integral of the signal at δ 5.379.

The two negative charges in peak 2AII of Figure 2f could be explained by the presence of one monoesterified or two diesterified phosphate groups. The H-1 NMR spectrum in Figure 4c shows the absence of diesterifying mannoses ($\alpha M \rightarrow P$) because it lacks the doublet at δ 5.43–5.45. In addition, the chemical shifts of the signals for the mannoses A_1 at δ 4.77 and 4.82, A_2 at δ 5.31 and 5.35, and A_3 at δ 5.24 and 5.28 suggest an equimolecular mixture of monoesterified monophosphates at both phosphorylation sites (C_1 and A_3) with structures VI and VII. For the same reasons as in the previous

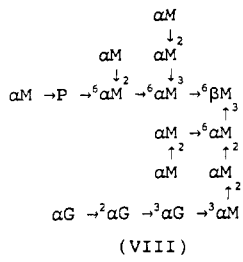


spectra, the mannose B_3 (italicized) appeared in about 60% of the oligosaccharides.

The spectrum in Figure 4a corresponds to peak 2A from gel filtration before fractionation in the ion-exchange column. It shows signals that agree with a mixture of the structures present in spectra of Figure 4b,c in an approximated ratio of 1:6.

The H-1 NMR spectrum of sample 1 showed that it was a mixture of phosphorylated oligosaccharides (Figure 5a). The major peaks in the spectrum agree with a diphosphate diester, while the small signal at δ 4.77 corresponds to the mannose A_1 in the oligosaccharide monophosphorylated in C_1. In addition, the signal at δ 5.37 indicates the presence of the mannose B_3, the signal at δ 5.35 is present in molecules with a monoesterified phosphate in A_3, and, finally, the doublets at δ 5.17 and 5.52 correspond to glucoses G_3 and G_2 (see above). The signal for G_1, at δ 5.27, is hidden by other signals in the spectrum.

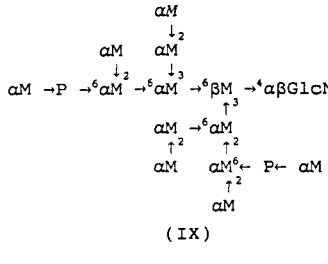
The fractionation of this sample by ion-exchange chromatography on QAE-Sephadex allowed us to separate three peaks (Figure 2e) eluting at the positions expected for molecules with one, two, and three negative charges (Hernández et al., 1989b). The H-1 NMR spectrum of peak 1A (Figure 5b) is characteristic of a diesterified monophosphate in the mannose C_1, retaining the three glucoses, having structure VIII.



(VIII)

In this case the italicized mannose (B_3) is present in about 60% of the molecules.

The ^{1}H NMR analysis of peak 1B in Figure 2e gave the spectrum shown in Figure 5c, which corresponds to an almost pure diphosphate diester having structure IX.



(IX)

The italicized mannose (B_3) is present in about 40% of the molecules as deduced from the integral of the signal at δ 5.37.

The spectrum in the Figure 5d corresponds to peak 1C in Figure 2e and is characteristic of an equimolecular mixture of diphosphates having one diesterified and one monoesterified phosphate group in any of the phosphorylation positions as indicated by the chemical shift for the anomeric proton of mannose A_1 at δ 4.80 and 4.81 in a 1:1 molar ratio. The signals for A_2 at δ 5.35 and A_3 at δ 5.24 also indicate the presence of monoesters in A_3; in addition, the integral of the doublet at δ 5.43–5.45 suggests the presence of only one αM→P group per molecule. From the spectrum, we propose for these molecules the structures X and XI.

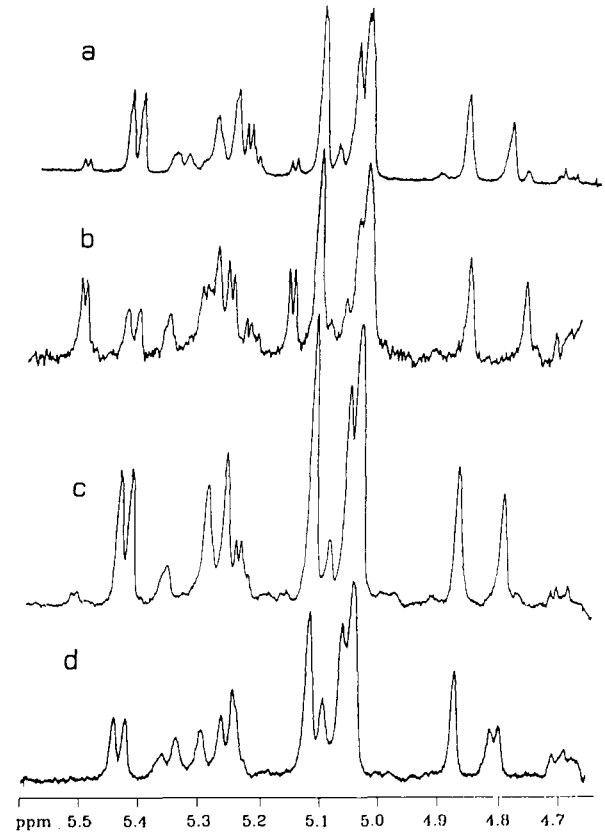
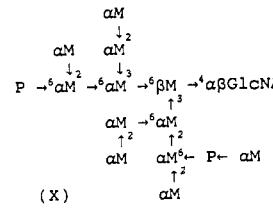
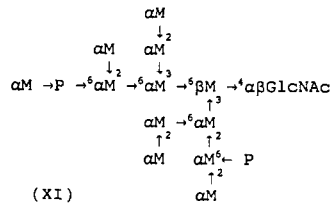


FIGURE 5: H-1 NMR spectra of exoglucanase II acidic oligosaccharides from *mnn1 mnn9*. (a) Sample from peak 1 (Figure 2b); (b) glucose containing monophosphate diester from peak 1A (Figure 2e); (c) diphosphate diester from peak 1B (Figure 2e); (d) diphosphate monoester/diester from peak 1C (Figure 2e).



(X)



(XI)

For the same reasons as in previous structures, the italicized mannoses seem to be present in about 30% of the molecules.

The structures shown above for the N-linked carbohydrate of *mnn1 mnn9* exoglucanase II appeared in the following proportions: I, 6%, II, 6%, III, 11%, IV, 2.1%; V, 2.1%; VI, 12%; VII, 12%; VIII, 6%; IX, 30%; X, 6.4%; XI, 6.4%.

The Glucoses Are Also Present in Some N-Linked Oligosaccharides of the mnn1 mnn9 Bulk Mannoprotein. As we pointed out above, the main difference between the structures found in the N-linked oligosaccharides of exoglucanase II and those previously shown for the bulk mannoprotein of this mutant (Tsai et al., 1984a; Hernández et al., 1989a) was the presence in the former of molecules retaining the three glucoses characteristic of the lipid-linked precursor (structures III and VIII). Two reasons could account for that apparent discrepancy: first, the incomplete processing of exoglucanase II oligosaccharides could be a specific feature of the enzyme secretory pathway or, second, it could be a general misfunction of the glycosylation apparatus due to the growth conditions used. Note that, to facilitate the purification of the enzymes, we have used a synthetic medium with glucose as the sole carbon and energy source, in contrast with the complex YEPD medium used in the above-mentioned study of the mannoprotein. To discern between these two hypotheses, we extracted some mannoproteins from *mnn1 mnn9* cells grown

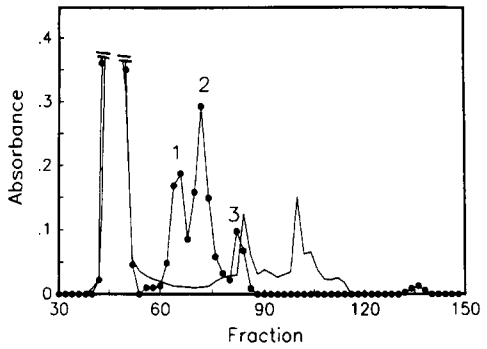


FIGURE 6: Fractionation of the endo H digest of *mnn1 mnn9* mannoprotein by gel filtration. The sample was loaded in a Bio-Gel P-4 column (1.5 × 170 cm) and eluted with 0.1 M ammonium acetate. The absorbances were read at 280 nm for protein (—) and at 490 nm for carbohydrate (●) as in Figure 2.

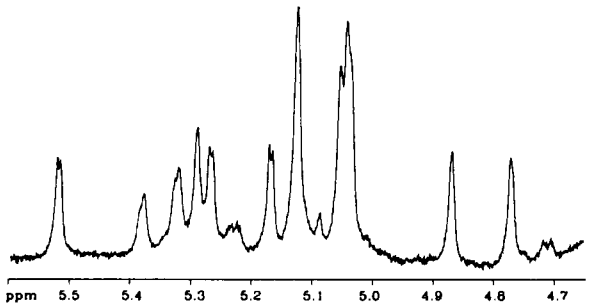


FIGURE 7: H-1 NMR spectra of glucose containing neutral oligosaccharides from *mnn1 mnn9* mannoprotein.

in the same conditions used to purified the enzymes and analyzed the N-linked oligosaccharides.

Mannoproteins were exhaustively digested with endo H and loaded in the Bio-Gel P-4 column equilibrated and eluted with 0.1 M ammonium acetate (Figure 6). Most of the protein came out in the void volume of the column together with a large peak of carbohydrate including the O-linked oligosaccharides. The endo H-released molecules were fractionated into three peaks eluting in the same positions as those found for the exoglucanase II (compare to Figure 2a), although in different proportions. Each one of the peaks was subjected to the same fractionation shown in Figure 2 for peaks 1–3 of Figure 2a, and the results were essentially the same (not shown). We only show here the H-1 NMR spectrum of the corresponding 2B peak (Figure 7), which was almost identical to that shown in Figure 3b. The most abundant molecule has structure III (see above), although there seems to be a low proportion of contaminant molecules lacking the mannose B_3. This result favors the hypothesis that the presence of partially unprocessed oligosaccharides in exoglucanase II may be due to the growth conditions used in our experiments.

Structure of the N-Linked Oligosaccharides of Exoglucanase II from the mnn1 Mutant. The enzyme was purified from 90 L of culture supernatant of the *mnn1* mutant to compare the structure of its carbohydrate chains with that shown above for the *mnn1 mnn9*. The lyophilized enzyme was digested by endo H, and the reaction mixture was chromatographed in the Bio-Gel P-4/salt column (Figure 8). The same three peaks found in Figures 2a and 6 also appeared here, which suggests that the same structures are present. Each of the peaks was run in the Bio-Gel P-4/water column (not shown), and the H-1 NMR spectra of the corresponding peaks 1 and 2A (see Figure 2b,c) are shown in Figure 9. The spectrum of peak 1 (Figure 9a) is very similar to that shown in Figure 5a for oligosaccharides from the *mnn1 mnn9* mutant. The signals agree with the presence of molecules with structures

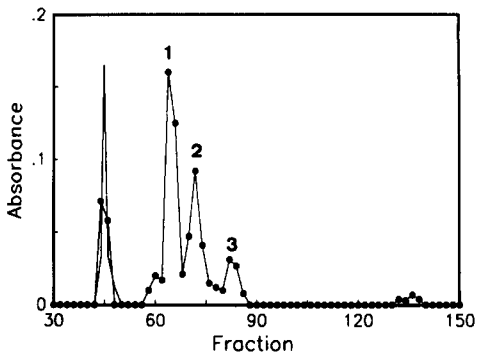


FIGURE 8: Fractionation of the endo H digest of *mnn1* exoglucanase II by gel filtration. Purified exoglucanase II from culture fluids of the *mnn1* mutant was digested with endo H and fractionated in the Bio-Gel P-4 column (1.5 × 170 cm) by elution with 0.1 M ammonium acetate as in Figure 2a. The absorbances were read at 280 nm for protein (—) and at 480 nm for carbohydrate (●) as in Figure 2.

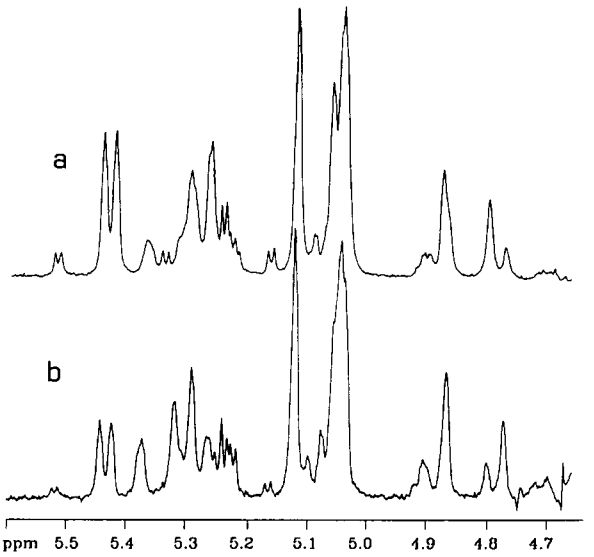
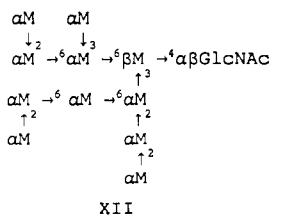


FIGURE 9: H-1 NMR spectra of exoglucanase II acidic oligosaccharides from *mnn1*. Endo H-released exoglucanase II oligosaccharides of *mnn1* were fractionated as shown in Figure 2 for the *mnn1 mnn9* mutant. (a) Spectrum of the correspondent peak 1; (b) spectrum of the correspondent peak 2A (see text).

VIII–XI, in almost the same proportions as in the *mnn1 mnn9* mutant. The spectrum of peak 2A (Figure 9b) is equivalent to that shown in Figure 4a, and it suggests the presence of monophosphorylated molecules mainly with structures IV and V. In this case, the integral of the signal at δ 5.43–5.45 is very close to 1, indicating that the monoesterified forms VI and VII are present only in trace amounts. It has been shown that the monoesterified forms found in yeast mannoproteins seem to be an artifact formed during the manipulation of the samples (Hernández et al., 1989b). That could be an explanation for the different proportions of such forms found in the two mutant strains. Perhaps the most significant difference between the spectra shown in Figure 9 and their equivalents for the *mnn1 mnn9* (Figures 5c and 4a,b) is the presence in the former of a small signal at δ 4.906, which suggests the presence of an extra α-(1→6)-linked mannose in the short outer chain of ~15% of the oligosaccharides. Such structure (XII) is characteristic of the recently isolated *vrg1* mutant (Ballou et al., 1991). Note that the signal is quite broad in Figure 9a, probably as a consequence of the two mannosyl phosphate groups attached to the oligosaccharide. Some of the molecules with structure XII could be extended with mannose B_3 and/or the glucoses G_1, G_2, and G_3.

The neutral forms present in peaks 3 and 2B were also identical to those found in the *mnn1 mnn9* mutant. Peak 3



XII

included molecules with structures I and II, while structure III was present in peak 2B (not shown). In all cases, 10–15% of the molecules contained the extra α-(1→6)-linked mannose shown above (XII).

Structure of the N-Linked Oligosaccharides of Exoglucanase II from Wild Type. The enzyme was purified from 95 L of culture supernatant, and the endo H digest was loaded in the Bio-Gel P-4 column equilibrated and eluted with 0.1 M ammonium acetate. In this case (Figure 10), most of the endo H-released oligosaccharides eluted as a broad peak in a position slightly ahead of peak 1 in Figures 2a, 6, and 8, indicating an average molecular weight a little higher and additional heterogeneity in the molecules. As we will show, both the heterogeneity and the increase in the average molecular weight are due to the presence of α-(1→3)-linked mannoses that were absent in the strains bearing the *mnn1* mutation. The fractions 52–76 from Figure 10 were collected together, lyophilized, and fractionated in a QAE-Sephadex column to separate the neutral oligosaccharides from the mono- and diphosphorylated ones. Figure 11 shows the results of the ion-exchange chromatography. The neutral molecules (peak 1) were not retained in the column and represented 22% of the total carbohydrate, while the mono- and diphosphorylated forms (peaks 2 and 3, respectively) were eluted with the gradient and included 50 and 28% of the molecules.

As expected, all three peaks were a heterogeneous mixture of several different structures as shown by the H-1 NMR spectra in Figure 12. The signal at δ 4.906 supports the presence of *vrg1*-like structures which are more abundant in the neutral fraction (Figure 12a). In the spectrum of the diphosphate (Figure 12c), the signal is smaller and broader as it was in Figure 9a.

The main difference between the spectra shown before for the *mnn1 mnn9* and *mnn1* mutants and those in Figure 12 is the presence, in the latter, of additional signals in the region δ 5.12–5.14, an indication of the presence of terminal α-(1→3)-linked mannoses prevented by the *mnn1* mutation. The signal at δ 5.43–5.45 changed to δ 5.407–5.418 in about 90% of the monophosphates (Figure 12b) and in 50% of the diphosphates, which indicates that 69% of the phosphate groups were diesterified with α-(1→3)-mannobiose instead of a single mannose. The diesterifying disaccharide was also detected by anion-exchange chromatography with pulsed amperometric detection after milk acid hydrolysis of the phosphorylated fraction (Alvarado et al., 1990) (results not shown). The synthesis of the α-(1→3)-mannobiosyl group is also prevented by the *mnn1* mutation.

Due to the limited amount of neutral carbohydrate and its heterogeneity deduced from the H-1 NMR spectrum, it was not feasible to try a fractionation of the sample and further analysis of the resulting individual peaks; instead, we tried an analysis similar to that previously done with the neutral oligosaccharides from CPY (Ballou et al., 1990) by using ion-exchange chromatography with pulsed amperometric detection and digestion with specific exo-(1→2)-α-mannosidase. Figure 13a shows the HPLC pattern of the intact oligosaccharides. The capital letters A–E represent peaks that coelute with oligosaccharides present in the CPY which

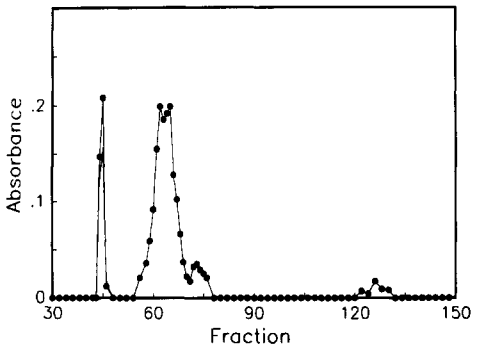


FIGURE 10: Fractionation of endo H-released oligosaccharides of exoglucanase II from wild type. Purified exoglucanase II was digested with endo H and fractionated in a Bio-Gel P-4 column (1.5 × 170 cm) by elution with 0.1 M ammonium acetate. The absorbances were read at 280 nm for protein (—) and at 490 nm for carbohydrate (●) as in Figure 2.

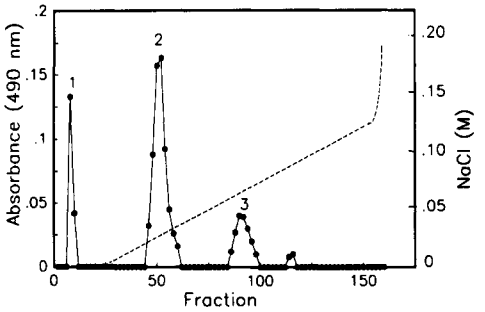
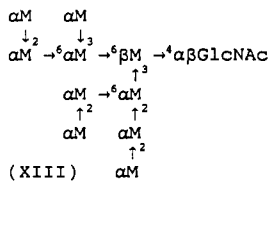
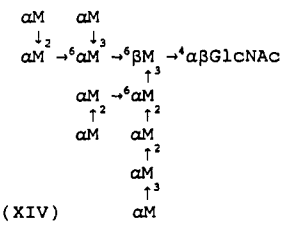


FIGURE 11: Fractionation of the endo H-released oligosaccharides of exoglucanase II from wild type. Fractions 52–76 from Figure 10 were desalted, lyophilized, and chromatographed in QAE-Sephadex (1.5 × 9.5 cm) as in Figure 2e,f. The fractions were assayed for carbohydrate at 490 nm (●) and for NaCl by conductivity (- - -).

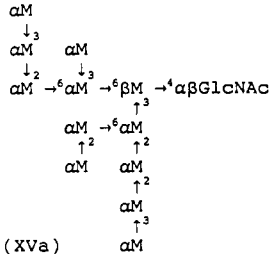
had the following structures (Ballou et al., 1990): A, structure XIII; B, structure XIV; C, structure XVa or XVb; D, structure XVI; E, structure XVII.



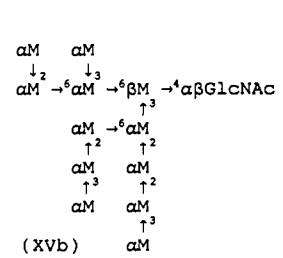
(XIII)



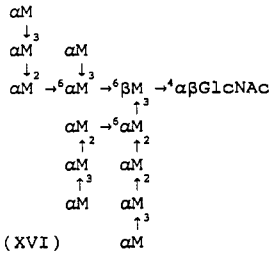
(XIV)



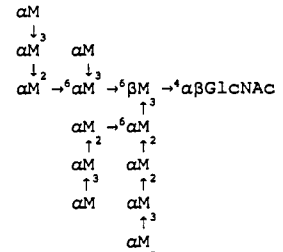
(XVa)



(XVb)



(XVI)



(XVII)

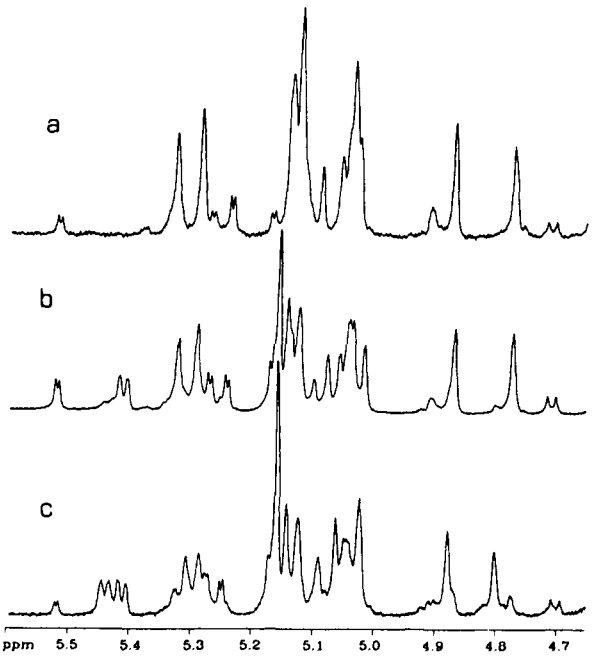


FIGURE 12: H-1 NMR spectra of exoglucanase II oligosaccharides from wild type. (a) Neutral oligosaccharides from peak 1 in Figure 11; (b) acidic oligosaccharides from peak 2 in Figure 11; (c) acidic oligosaccharides from peak 3 in Figure 11.

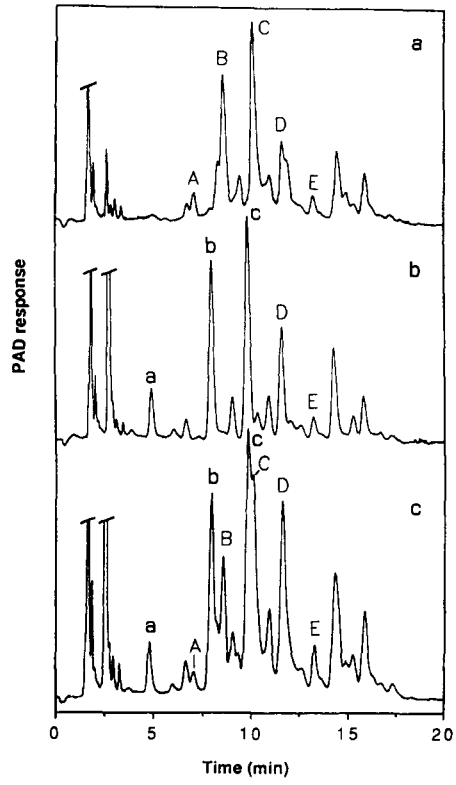


FIGURE 13: Ion-exchange chromatography of oligosaccharides from peak 1 in Figure 11. The peaks in upper case letters are intact oligosaccharides, and those in lower case letters are products of digestion with exo-(1→2)-α-D-mannosidase. (a) Undigested mixture; (b) digested mixture; (c) mixture of undigested and digested oligosaccharides (for structures, see text). The eluent was 100 mM NaOH with a gradient from 50 to 130 mM sodium acetate in 25 min. PAD, pulsed amperometric detector.

The identity of exoglucanase neutral oligosaccharides with those from CPY was confirmed by the changes in retention times after digestion of the sample with exo-(1→2)-α-mannosidase (Figure 13b). Digested peak A eluted in a position that agreed with the loss of its four α-(1→2)-linked mannoses present in structure XIII (peak a). In addition, peak a coeluted with authentic M_6-GlcNAc obtained by exhaustive exo-(1→2)-α-mannosidase digestion of a molecule with structure XIII from another source (Hernández et al., 1989a) (not shown). Peak b eluted 0.64 min before its parental peak B, which also agreed with a loss of the two accessible α-(1→2)-linked mannoses present in structure XIV. For the same reason, the decrease of 0.35 min in retention time of peak c with respect to peak C accounts for the hydrolysis of a single α-(1→2)-linked mannose accessible in structure XVa or XVb. Peaks D and E were not digested by the mannosidase, which supports structures XVI and XVII, respectively, for them. Also, due to the differences in retention time (about 0.3 min), the shoulder on the left side of peak B must correspond to molecules with structure XIV but having one less α-(1→2)-linked mannose (C_2 or d_2), while the shoulder on the right side of peak D must have structure XVI with an additional α-(1→2)-linked mannose (most probably B_3). In both cases, the digestion products are the same as those from B and D, respectively. Note that it has been proposed previously (Hernández et al., 1990) that the change in retention time produced by an α-(1→3)-linked mannose is larger than that produced for an α-(1→2)-linked one, probably due to the presence in the former molecules of an extra free 2-hydroxyl group which is known to be the most acidic in a hexose (Hardy & Townsend, 1989).

The rest of the peaks in the chromatogram of Figure 13a must account for the minor peaks in the spectrum of Figure 12a; they should include molecules with some of the structures shown above but having the glucoses G_1, G_2, and G_3 and/or the extra α-(1→6)-linked mannose present in *vrg1* oligosaccharides (Ballou et al., 1991). However, due to the low proportion in which they appear and the lack of appropriate standards, we are not able to make structural assignments to each of them. In the chromatogram of Figure 13c we injected a mixture of intact and α-(1→2)-mannosidase-digested oligosaccharides.

DISCUSSION

In the present study we have determined the structure of the N-linked oligosaccharides of the major exoglucanase secreted by *S. cerevisiae* (exoglucanase II). In contrast to other extracellular mannoproteins so far described (i.e., cell wall mannoproteins, invertase), which have long and highly branched outer chains in most of the glycosylation sites, exoglucanase II has two short N-linked carbohydrate chains consisting mainly of an "inner core" with a single branch of the outer chain attached to mannose A_2, thus having a structure similar to that found in the vacuolar enzyme CPY (Ballou et al., 1990) or in the cell wall mannoproteins of the *mnn9* mutant (Tsai et al., 1984b; Hernández et al., 1989a). The glycosylation mutants *mnn1* and *mnn1 mnn9* showed almost identical structures in these carbohydrate chains, which indicates that the *mnn9* mutation does not affect the oligosaccharide structure of exoglucanase II. It has been proposed (Ballou et al., 1990) that the *mnn9* defect leads to a block in the normal glycosylation pathway of cell wall mannoproteins, redirecting them to the pathway followed by the vacuolar protease CPY. Apparently, exoglucanase II follows this pathway even in wild type. It is interesting that some exoglucanase molecules (10–15%) apparently escape this pathway and become glycosylated like cell wall mannoproteins with a long and branched outer chain in at least one of the two N-linked oligosaccharides. As expected, these heavily glycosylated molecules are not secreted by the *mnn9* mutant and have been referred to as exoglucanase I (Ramírez et al., 1990). The oligosaccharides are quite

heterogeneous in size, and the structure is currently being studied in our laboratory.[2]

The proportion of phosphorylated oligosaccharides in exoglucanase II (78%) is similar to that found in CPY (Ballou et al., 1990) (70%) as well as the proportion of the mono- and diphosphorylated forms (50 and 28% for exoglucanase and 44 and 26% for CPY, respectively). In the presence of the *mnn1* mutation, the proportion of diphosphorylated forms in exoglucanase II rises to 43%, although the total phosphorylated forms remain the same (77%).

Some of the neutral and monophosphorylated forms of exoglucanase II oligosaccharides retained the α-linked glucoses characteristic of the lipid-linked precursor. This was not a special feature of the exoglucanase secretory pathway since, when we prepared some mannoproteins from *mnn1 mnn9* cells growing in the same conditions, we also found some neutral and monophosphorylated forms retaining the three glucoses. These reults suggest that the growth conditions affect the processing of the carbohydrate moiety of glycoproteins. Since we did not find any forms having only two glucose units, a low effectiveness of glucosidase I might be responsible for the presence of these unprocessed structures (Esmon et al., 1984).

The presence of the mannose B_3 in a high proportion of the molecules (54%) in *mnn1 mnn9* also indicates an effect on processing by the α-(1→2)-mannosidase. When the cells were grown in a complex medium, the proportion of such forms did not exceed 30% (Hernández et al., 1989b). In both cases, the presence of the mannose B_3 failed to prevent further processing of the oligosaccharides as suggested before (Byrd et al., 1982). In wild-type oligosaccharides, only trace amounts of mannose B_3 were observed (see Figure 12), which may indicate that the *mnn* mutant strains are also affected in some way in endoplasmic reticulum function, as has been suggested for the *vrg1* mutant (Ballou et al., 1991). However, the removal of the glucoses, which also occurs in the endoplasmic reticulum (Esmon et al., 1984), is equally affected in both wild type and *mnn* mutants. The possibility of mannose B_3 being added later during processing seems unlikely because the addition of an α-(1→2)-linked mannose to a terminal α-(1→3)-linked one has not been observed in the outer chain, and it is difficult to understand how the growth conditions used in our experiments could activate such supposed enzymatic activity.

In summary, the N-linked oligosaccharides of the exoglucanase protein portion in wild-type *S. cerevisiae* have structures characteristic of mannoproteins synthesized in several glycosylation mutants previously described: *vrg1* (Ballou et al., 1991), *mnn9* (Tsai et al., 1984b), *mnn10* (Ballou et al., 1989), and *gls1* (Esmon et al., 1984). In addition, some molecules bear an extra α-(1→2)-linked mannose, and some others have a fully elongated outer chain. A plausible explanation for this heterogeneity could be the existence of a linear, multistep glycosylation pathway. Only the cell wall mannoproteins from wild type will go through all the steps while, in the mutants, the mannoproteins will bypass one or several such steps. The defects leading to unprocessed forms in the endoplasmic reticulum could be the malfunction of the processing enzymes glucosidase I and α-(1→2)-mannosidase as shown for the *gls1* mutant (Esmon et al., 1984), although transport defects that alter the time glycoproteins are in contact with the processing enzymes must also be considered.

[2] L. M. Hernández, I. Olivero, and G. Larriba, manuscript in preparation.

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REFERENCES

Alvarado, E., Ballou, L., Hernández, L. M., & Ballou, C. E. (1990) *Biochemistry 29*, 2471–2482.
Ballou, C. E. (1990) *Methods Enzymol. 185*, 440–4701.
Ballou, L., Alvarado, E., Tsai, P.-K., Dell, A., & Ballou, C. E. (1989) *J. Biol. Chem. 264*, 11857–11864.
Ballou, L., Hernández, L. M., Alvarado, E., & Ballou, C. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3368–3372.
Ballou, L., Hitzeman, R. A., Lewis, M. S., & Ballou, C. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3209–3212.
Byrd, J. C., Tarentino, A. L., Maley, F., Atkinson, P. H., & Trimble, R. B. (1982) *J. Biol. Chem. 257*, 14657–14666.
Cohen, R. E., & Ballou, C. E. (1980) *Biochemistry 19*, 4345–4358.
Derome, A. E. (1987) *Modern NMR Techniques for Chemistry Research*, pp 183–244, Pergamon Press, New York.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.
Esmon, B., Esmon, P. C., & Scheckman, R. (1984) *J. Biol. Chem. 259*, 10322–10327.
Hardy, M. R., & Townsend, R. R. (1989) *Carbohydr. Res. 188*, 1–7.
Hernández, L. M., Ballou, L., Alvarado, E., Gillece-Castro, B. L., Burlingame, A. L., & Ballou, C. E. (1989a) *J. Biol. Chem. 264*, 11849–11856.
Hernández, L. M., Ballou, L., Alvarado, E., Tsai, P.-K., & Ballou, C. E. (1989b) *J. Biol. Chem. 264*, 13648–13659.
Hernández, L. M., Ballou, L., & Ballou, C. E. (1990) *Carbohydr. Res. 203*, 1–11.
Ichishima, E., Arai, M., Shigematsu, Y., Kumagai, H., & Sumida-Tanaka, R. (1981) *Biochim. Biophys. Acta 658*, 45–53.
Kukuruzinska, M. A., Bergh, M. L. E., & Jackson, B. J. (1987) *Annu. Rev. Biochem. 57*, 915–944.
Lowry, O. H., Rosebrough, N. H., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Nebreda, A. R., Villa, T. G., Villanueva, J. R., & del Rey, F. (1986) *Gene 47*, 245–259.
Nebreda, A. R., Vázquez, C. R., Villa, T. G., Villanueva, J. R., & del Rey, F. (1987) *FEBS Lett. 220*, 27–30.
Olivero, I., Hernández, L. M., & Larriba, G. (1985) *Arch. Microbiol. 143*, 143–146.
Ramírez, M., Hernández, L. M., & Larriba, G. (1989a) *Arch. Microbiol. 151*, 391–398.
Ramírez, M., Muñoz, M. D., & Larriba, G. (1989b) *Biochim. Biophys. Acta 990*, 206–210.
Ramírez, M., Muñoz, M. D., Basco, R. D., Giménez-Gallego, G., Hernández, L. M. & Larriba, G. (1990) *FEMS Microbiol. Lett. 71*, 43–48.
Raschke, W. C., Kern, K. A., Antalis, C., & Ballou, C. E. (1973) *J. Biol. Chem. 248*, 4660–4666.
Tarentino, A. L., Trimble, R. B., & Maley, F. (1978) *Methods Enzymol. 40*, 574–580.
Tingle, M. A., & Halvorson, H. O. (1971) *Biochim. Biophys. Acta 250*, 165–171.
Trimble, R. B., Maley, F., & Chu, K. (1983) *J. Biol. Chem. 258*, 2562–2567.
Tsai, P.-K., Ballou, L., Esmon, B., Schekman, R., & Ballou, C. E. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6340–6343.
Tsai, P.-K., Frevert, J., & Ballou, C. E. (1984b) *J. Biol. Chem. 259*, 3805–3811.
Vázquez de Aldana, C. R., Correa, J., San Segundo, P., Bueno, A., Nebreda, A. R., Méndez, E., & del Rey, F. (1991) *Gene 97*, 173–182.
Vliegenthart, J. F. J., Dorland, L., & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.