Structure of Oligosaccharides on Saccharomyces SUC2 Invertase Secreted by the Methylotrophic Yeast Pichia pastoris

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Saccharomyces SUC2 invertase, secreted by the methylotrophic yeast Pichia pastoris and purified to homogeneity from the growth medium by DE-52 chromatography, appeared on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a diffuse ladder of species at 85–90 kDa, while the secreted Saccharomyces form migrated as a broad band from 100 to 150 kDa. Endo-β-N-acetylglucosaminidase H released the Pichia invertase carbohydrate generating a 60-kDa protein with residual Asn-linked GlcNAcs and oligosaccharides separated on Bio-Gel P-4 into Manα,1GlcNAc. Nearly 75% of the oligosaccharides were equally distributed between Manα,1GlcNAc, while 17% were Manβ,2GlcNAc and 8% were Manα,1GlcNAc. Oligosaccharide pools were analyzed for homogeneity by high-pH anion-exchange chromatography, and structures were assigned using 500 MHz one- and two-dimensional 1H NMR spectroscopy. Pichia Manα,2GlcNAc was the same isomer as found in Saccharomyces, which arises by removing the α1,2-linked terminal mannose from the middle arm of the lipid-oligosaccharide Manα,1GlcNAc (Byrd, J. C., Tarentino, A. L., Maley, F., Atkinson, P. H., and Trimble, R. B. (1982) J. Biol. Chem. 257, 14657–14666). The Manα,2GlcNAc pool was 5% lipid-oligosaccharide precursor and 95% Manα,2GlcNAc isomer with a terminal α1,6-linked mannose on the lower-arm α1,3-core-linked residue (Hernández, L. M., Ballou, L., Alvarado, E., Gillece-Castro, B. L., Burlingame, A. L., and Ballou, C. E. (1989) J. Biol. Chem. 264, 11849–11856). An α1,2-linked mannose on the new α1,6-linked branch in Manα,2GlcNAc provided 90% of the Manα,2GlcNAc, which is the structure on Saccharomyces invertase (Trimble, R. B., and Atkinson, P. H. (1986) J. Biol. Chem. 261, 9815–9824). A minor Manα,1GlcNAc (12%) and the principal Manα,2GlcNAc (82%) were the major Manα,2GlcNAc with novel α1,2-linked mannosides on the preexisting α1,2-linked ter-

mini. Although Pichia glycans did not have terminal α1,3-linked mannosides as found on Saccharomyces core oligosaccharides, over 60% of the structures were isomeric configurations unique to lower eukaryotes.

Pichia pastoris is a methylotrophic yeast, which has been exploited for the high level expression of heterologous proteins by ligation to the methanol-inducible alcohol oxidase (AOX1) promoter of gene sequences of interest (1, 2). As part of a study to determine the characteristics of heterologous glycoprotein secretion from yeast, a vector (pGS102) consisting of the Saccharomyces SUC2 invertase coding sequence coupled to the AOX1 promoter was constructed and used to transfect Pichia (3). On induction with methanol, invertase was secreted by the transformed cells over a period of 100 h to a level of 2–3 g/liter.

The invertase secreted by wild-type Saccharomyces strains consists of 140-kDa subunits which are in excess of 50% by weight glycans (4, 5). The carbohydrate is distributed as families of asparagine-linked Manα,1GlcNAc2 and Manβ,2GlcNAc2 oligosaccharides attached to 13 of 14 -Asn-X-Thr/Ser- sequences in the protein (6, 7). In contrast to Saccharomyces, SUC2 invertase secreted by Pichia is a more homogeneous product consisting of ~85-kDa subunits, which by indirect methods appear to be associated with 8–10 oligosaccharides of the size Manα,4GlcNAc (8). Incorporation of [3H]Man by Pichia revealed that over 85% of the invertase label was in short oligosaccharides, nearly 70% of which were Manα,2GlcNAc (8).

The presence of short oligosaccharides on Pichia invertase suggested that glycan processing may be different in this organism from that in Saccharomyces. Accordingly, Pichia invertase, isolated from the medium of methanol-induced cells, was treated with endo H1; the individual Manα,1GlcNAc oligosaccharide pools were purified by gel filtration on Bio-Gel P-4. High pH anion-exchange chromatography (HPAEC) was used to determine the number of isomers in each size pool (10, 11), while one- and two-dimensional 1H NMR spectroscopy at 500 MHz was employed to assign structures. Manα,1GlcNAc and the major component of Manα,2GlcNAc were identical to the structures found previously on Saccharomyces invertase. The remaining Manα,2GlcNAc and Manα,1GlcNAc isomers differed from those in Saccharomyces

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1 The abbreviations used are: endo H, endo-β-N-acetylglucosaminidase H (EC 3.2.1.96); PAG, polyacrylamide gel electrophoresis; HPAEC, high-pH anion-exchange chromatography; PAD, pulsed amperometric detection; AMMS, anionic micromembrane suppressor; SDS, sodium dodecyl sulfate.
(9), in that no terminal α1,3-linked mannoside was present. Rather, the novel Pichia invertase Man$_{0,11}$GlcNAc oligosaccharides were the Saccharomyces forms of Man$_{0,14}$GlcNAc elongated by addition of α1,2-linked mannosides to preexisting terminal α1,2-linked residues.

**EXPERIMENTAL PROCEDURES**

**Materials**

P. pastoris strain GS115 (his4) transformed with the SUC2 expression vector pGS102 was used for the production of invertase (3). Invertase was also induced in Saccharomyces strain MBBY-21Aa (sec18-1,tryp8,leu2-3,112), provided by Dr. R. Schekman (University of California, Berkeley), which was transformed with SUC2 multicopy plasmid pRB88, from Dr. M. Carlson (Columbia University). endo H was the cloned, protease-free enzyme described previously by this laboratory (12). Resins were obtained from the following suppliers: DE-52 microgranular cellulose, Whatman, Inc.; Sephadex G-25 and protein-A-Sepharose, Pharmacia LKB Biotechnology Inc.; and Bio-Gel P-4 (400 mesh), Bio-Rad Laboratories. Immobilon polyvinylidene difluoride membranes for Western blotting and Cls phosphatase-conjugated anti-rabbit IgG was from Promega Biotech.

Invertase was also induced in Saccharomyces strain MBY-21Aa (sec18-1, trp289, leu2-3,112), which was transformed with a plasmid expressing invertase (13), and the IgG fraction was purified by protein A-Sepharose chromatography as directed by the manufacturer. Alkaline phosphatase-conjugated anti-rabbit IgG was from Promega Biotech and used according to the provided protocol. NMR tubes (0.5 cm, Cat. number 5366p) were from Willmack Glass, and 99.86% $^3$H$_2$O was obtained from Sigma, while 99.96% $^3$H$_2$O was from Cambridge Isotopes Laboratory. For HPAGE with PAD sodium hydroxide (50%) solution in water and sodium acetate were from Fisher and Fluka, respectively. SDS-PAGE chemicals were obtained from Bio-Rad.

**Methods**

**Purification of Pichia SUC2 Invertase**—Approximately 250 ml of shake-culture broth from methanol-induced, transformed Pichia cells containing 385,000 units of activity (100 mg of invertase protein) was dialyzed against several changes of 10 mM sodium phosphate buffer, pH 6.5, and loaded onto a 20-cm column of DE-52 microgranular cellulose equilibrated in the same buffer. Invertase was eluted with a linear 800-ml gradient of 0-0.3 M NaCl in 10 mM sodium phosphate, pH 6.5, at a flow rate of 40 ml/h, and 3-ml fractions were collected at 4 °C. Approximately 95% of the activity eluted in a sharp peak at 40 mM NaCl.

The DE-52 peak was homogeneous with respect to invertase protein (4000 IU/mg) but contained a large excess of phosphomannan not covalently associated with the enzyme. The invertase and phosphomannan were coprecipitated with two volumes of cold acetone at −20 °C. The precipitate was centrifuged for 15 min at 5000 × g and 4 °C, and the supernatant was discarded. The pellet, containing invertase and mannan, was washed with 50 ml of cold 10% trichloracetic acid, which solubilized the mannan but not the invertase. The invertase precipitate was centrifuged as above, dissolved in a minimum volume of 50 mM NaOH (∼5 ml), and immediately dialyzed at 4 °C against 1 liter of sodium acetate buffer, pH 5.5. The final recovery of invertase was 93 mg (93%) which, was covalently associated with 75-80 mannose residues/60-kDa subunit, determined with the phenol sulfuric assay using mannan as a standard (14).

SDS-PAGE was performed on 12%, 0.5-mm-thick slab gels, and SDS-PAGE was performed on 12%, 0.5-mm-thick slab gels, and three cycles of chromatography on a 1.6-× 96-cm Bio-Gel P-4 (400 mesh) column exactly as described (9). Prior to the final chromatography step on Bio-Gel P-4, which is shown in Fig. 2, the oligosaccharide pools were passed through a disposable C$_8$-RP Sep-Pak cartridge to remove any residual peptide material.

**HPACE of Monosaccharides and Oligosaccharides**—Analyses were performed using HPACE with PAD essentially as described (10). The chromatograph consisted of a Dionex GPM-II pump a PAD-II detector, an Eluent Degas Module, a post-detector anionic micro-membrane suppressor unit (AMMS), and an AutoRegen pump and cartridge. The pulse potentials for the PAD-II were $E_1 = 0.05$ V, $E_2 = 480$ ms (range 2, position 5); $E_3 = 0.60$ V, $E_4 = 120$ ms (position 2); and $E_5 = -0.60$ V, $E_6 = 60$ ms (position 1). The time constant was set to 3 s. The Dionex Eluent Degas Module was used to sparge and pressurize the eluents with helium. The system was controlled and data was collected using Dionex A1450 software. Sample injection was with a Spectra-Physics SP8880 autosampler equipped with a 200-μl sample loop. The Dionex injection valve was fitted with a Tefzel rotor seal to withstand the alkalinity of the eluents.

Oligosaccharide alditols of the Man$_{0,11}$GlcNAc Bio-Gel P-4 fractions were prepared (9) using nonradioactive NaBH$_4$, and oligosaccharides were separated using CarboPac PA100 columns (4 × 250 mm). Eluent 2 was 200 mM sodium acetate, eluent 3 was water, and eluent 4 was 1 M NaOH (Eluent 1 was not used for this application). Eluent 2 was filtered through a 0.2-μm nylon membrane before use. The water for all eluents was glass-distilled using a Corning Mega Pure system and collected directly into a glass reservoir. Eluent 4 was prepared by a suitable dilution of a 50% NaOH solution (19.3 M) with water. The flow rate was 0.5 ml per min. For the preparative isolation of oligosaccharides, the post-detector AMMS was used to remove sodium ions from the eluent. The counter-current flow (from the AutoRegan pump) was 30 ml per min. Under these conditions, up to 250 mM sodium was removed. Samples were collected using an ISCO FOXY II fraction collector.

Electrochemical response foci of the sized oligosaccharides were determined relative to a glassy carbon working electrode. The concentration of the oligosaccharides was based on mannoside, which was determined using HPACE with PAD after hydrolysis with 2 N trifluoroacetic acid for 4 h at 100 °C (16). Monosaccharides were analyzed with a CarboPac PA100...
column (4 x 250 mm). The eluents for monosaccharide analysis were water (Eluent 1) and 200 mM sodium hydroxide (Eluent 2). Mono-
saccharides were eluted isocratically at 16 mM NaOH. After 20 min
the column was eluted for 10 min with eluent 2, followed by return
to initial conditions in 2 min. The time between injections was kept
constant at 50 min in order to minimize retention time drift (<1 min).

\[ ^1H \text{ NMR Spectroscopy} - \text{Samples were flash evaporated to dryness and exchanged three times by flash evaporation from 1 ml of 99.96\%} \ H_2O. \text{They were lyophilized from 1 ml of 99.996\%} \ D_2O \text{and dissolved in 0.7 ml of 99.996\%} \ H_2O \text{containing equimolar acetone as an internal}
\text{chemical shift marker (3 = 2.225 ppm relative to 4,4-dimethyl-4-
silapentane sulfonate). Samples were flame-sealed in 0.5-cm NMR}
tubes, and two-dimensional 500-MHz \(^1H\) NMR spectra were recorded at 296 K at the Albert Einstein College of Medicine NMR facility as
described (17). The two-dimensional spectra were from phase-sensitive

\[ \text{correlation spectroscopy acquired by the method of States et al.} \ (15). \text{Data were analyzed on a Varian VXR4000 work station and on a}
\text{Silicon Graphics Iris using Hare Research, Inc., software. Reso-
nance intensities were integrated by cutting out and weighing peaks from}
\text{expansions of the anomeric and C2-H regions of one-dimen-

sional spectra. The anomeric proton of the core p1,4-linked mannose}

(residue 3) was obscured by the residual HOH peak. However, residue

3's C2-H resonance is found at 4.241 ppm in Man\textsubscript{9}GlcNAc, at 4.154

ppm in Man\textsubscript{8}GlcNAc along with the C2-H of residue 4 and isolated at

4.158 ppm in Man\textsubscript{7}GlcNAc. By integration of the C1- and C2-H peaks,

residues 3 and 4 were confirmed to be present at 1 mol in all oligosaccharides studied.

RESULTS AND DISCUSSION

\[ P. \text{pastoris} \text{transformed with pGS102 secreted a low-molecu-

lar-weight form of SUC2 invertase into the medium as the}

principal protein, which was purified to homogeneity by a single DE-52 chromatography step (see "Experimental Pro-
cedures"). Nearly 95% of the enzyme was recovered, which

exhibited a specific activity of over 4,000 IU/mg protein, the

expected value for the homogeneous product (13). However,

further characterization of the diaлизed DE-52 pool revealed

the presence of 3–4-fold more carbohydrate than would be

expected for an 85-kDa glycoprotein with 20% carbohydrate by

weight (3, 8). Much of the carbohydrate was found to be soluble phosphomannan that could be removed from the DE-

52 invertase glycoprotein after coprecipitation with acetone

followed by selective solubilization with 10% trichloroacetic

acid. Fig. 1 shows that the final \textit{Pichia} invertase preparation

appears on SDS-PAGE as a ladder of species of about the

same size as the endoplasmic reticulum form of invertase

made by the \textit{Saccharomyces cerevisiae} strain at 37 °C, which has

9–11 Man\textsubscript{1}GlcNAc core oligosaccharides/subunit (9, 19).

In order to determine the size distribution and structure of the oligosaccharides associated with \textit{Pichia} invertase, the

protein was deglycosylated with endo H. Fig. 1 shows that

endo H treatment of both the \textit{Pichia}-derived and \textit{Saccharo-
myces cerevisiae} strains (37 °C) invertases generated the expected 60-kDa form of the protein, which retains only the asparagine-proxi-

mal GlcNAc residues (6, 7). Chromatography of the released

oligosaccharides indicated that over 90% of the neutral hexoses/subunit was in species of the size Man\textsubscript{9}GlcNAc (Fig. 2), while 10% or

less was found in oligosaccharides eluting in the void

volume of the Bio-Gel P-4 column (not shown). Upon esti-

mating the size of the voided glycans to be Man\textsubscript{9}GlcNAc (5), it was calculated that less than 3% of the oligosaccharides

associated with this preparation of \textit{Pichia} invertase were larger than Man\textsubscript{1}GlcNAc. Given the high level of mannan in the

starting material, it is probable that the large glycans

were carried through the purification as a contaminant and

were not covalently associated with the invertase. There was

no phosphate associated with the Man\textsubscript{9}GlcNAc oligosaccharides from \textit{Pichia} invertase.

The distribution of oligosaccharides on the 14 potential

glycosylation sites on invertase (7) ranges from 8 to 11 oligo-
saccharides per subunit in wild type and sec18 (37 °C) forms.

Analysis of the purified \textit{Pichia} invertase provided a value of

74 mannoses per subunit (see "Experimental Procedures")

associated with the Man\textsubscript{1}GlcNAc oligosaccharides (Fig. 2).

The distribution of these chain lengths on an "average" sub-

unit was estimated by dividing the total mannosae recovered

in each peak (Fig. 2) by the chain length, which provides the

molar ratio of species present (Table 1). Normalizing the

molar ratio of lengths to 74 mannosae yields the number of

mannoses in each size class, which on division by the chain

length gives the average number of each species/subunit

(Table 1). The distribution on the \textit{Pichia} enzyme of 6 to 10

oligosaccharides with a range of chain lengths (Man\textsubscript{9}–11) dimin-

ishes the resolution of the individual isoforms in compar-

ison with sec18 (37 °C) invertase which is associated with 8 to

11 Man\textsubscript{1}GlcNAc chains (Fig. 1).

To determine whether the \textit{Pichia} Man\textsubscript{1}GlcNAc had the same or different structures as those found previously on

\textit{Saccharomyces} glycoproteins (9, 20, 21), the separated oligo-
saccharide pools (Fig. 2) were subjected to one- and two-
dimensional 500-MHz \(^1H\) NMR spectroscopy. Fig. 3 shows the anomeric and partial C2-H proton regions of the spectra

of Man\textsubscript{9}GlcNAc. Tables 3–6 in the miniprint section sum-

marize the integration of proton intensities and their appor-

tionment to specific glycosidic linkages in Man\textsubscript{9}GlcNAc, respectively. See Figs. 5 and 7 for relevant parts of the two-
dimensional phase-sensitive COSY spectra. The distribution of oligosaccharide isomers in each pool was also assessed by

HPAEC, which is capable of separating high-mannose-branching

isomers within a given glycan size class (10, 11). A compila-

tion of the profiles for reduced Man\textsubscript{9}GlcNAc-ol is shown in Fig. 4, and Table 7 provides the retention times and

electrochemical response factors for the Man\textsubscript{9}GlcNAc-ol species. Table 2 summarizes the structures of oligosaccharides

deducted in this study. All structures contained the Man\textsubscript{9}

GlcNAc core (structure I), and include resonance identifica-
tion numbers and linkage assignments to aid in cross-refer-

encing residues in the figures and tables. The mannose resi-

dues of interest in the subsequent Man\textsubscript{10}GlcNAc structures

are boxed for clarity.

\textit{Man\textsubscript{9}GlcNAc}—The spectrum (Fig. 3A) and integration of reso-
nances (Table 3) reveal \textit{Pichia} Man\textsubscript{9}GlcNAc (Table 2, structure I) to be identical to \textit{Saccharomyces} invertase (20) and

whole-cell glycoprotein Man\textsubscript{9}GlcNAc species.\(^3\) Of the three possible isomers that can be generated by trimming a

\[ ^3 \text{Trimble, R. B., and Atkinson, P. H. (1992) Glycobiology, in press.} \]
single α1,2-linked mannose from the precursor lipid-oligosaccharide form of Man₉GlcNAc (residues 9, 10, or 11 in structure IIb, Table 2) only this one, which lacks the middle-arm α1,2-linked terminal residue 10, has been found in all fungal glycoproteins examined to date. It is generated in *Saccharomyces* by the action of an endoplasmic reticulum processing mannosidase (20), which recently has been purified and shown to generate only this isomer of Man₉GlcNAc in vitro (22, 23). HPAEC efficiently separates the possible Man₉GlcNAc branch isomers (10), and a single species (Fig. 4A) was found using HPAEC with PAD which eluted at 21.4 min (Table 7). This species coeluted with the endoplasmic reticulum α-mannosidase trimming product from *Saccharomyces* invertase

(data not shown) (20). Thus, *Pichia* would appear to utilize the same early processing pathway enzymes that are present in *Saccharomyces*.

Man₉GlcNAc—The Pichia invertase Man₉GlcNAc (Fig. 3B and Table 4) provides a spectrum which is also identical to that found for *Saccharomyces* invertase Man₉GlcNAc (9, 24). This compound (structure IIa, Table 2) is the Man₉GlcNAc structure to which an α1,6-linked mannose (residue 12) has been added to the α1,3-linked mannose of the lower-arm (the α1,3-branch) residue 5. This configuration was originally assigned in *Saccharomyces* man mutant oligosaccharides (24) and now has been confirmed to be the isomeric form associated with wild type *Saccharomyces* and *Pichia* glycoproteins.²

The small signal at 5.403 ppm indicates the presence of the precursor form of Man₉GlcNAc (20) from which mannose residue 10 was not completely removed during processing (structure IIb, Table 2). By integration of the NMR spectrum this isomer represents about 5% of the Man₉ pool (Table 4, isomer IIb). HPAEC with PAD of the *Pichia* Man₉ revealed two peaks (Fig. 4B). One comprised 4% of the total electrochemical response (Table 7), which eluted where the lipid-oligosaccharide Man₉ isomer elutes (RT = 26.1 min), while the remaining 95% eluted as a more-retained symmetrical peak (RT = 30.4 min) coincident with the *Saccharomyces* Invertase Man₉GlcNAc-ol (10).

The response factors for all species were found to be similar (2.3–2.6 relative to glucose external standard); therefore, peak area is directly related to molar proportion. Studies with monosaccharides indicate that the 2-OH of mannose are the major ionized groups during HPAEC of high mannose oligo-

⁴Kaur, S., Townsend, R. R., Liang, W., Trimble, R. B., and Burlingame, A. L., manuscript in preparation.
Oligosaccharide Structures on Pichia SUC2 Invertase

**TABLE 2**

| Structure and abundance of oligosaccharides on Pichia SUC2 invertase |
|---------------------------------------------------------------|
| 1-Man, GlcNAc | II-Man, GlcNAc | III-Man, GlcNAc | IV-Man, GlcNAc |
| ![Structures](image1) | ![Structures](image2) | ![Structures](image3) | ![Structures](image4) |
| 100 % | a-95 % | a-80 % | a-82 % |

**TABLE 7**

| Size and peak numbers | Retention time | Percent response | Response factor |
|-----------------------|----------------|-----------------|----------------|
| Man$_{10}$GlcNAc-ol   | 21.4           | 100             | 2.4            |
| Man$_{10}$GlcNAc-ol   | 26.1           | 4               | 2.6            |
| Man$_{10}$GlcNAc-ol   | 30.4           | 96              |                |
| Man$_{10}$GlcNAc-ol   | 33.5           | 72              | 2.3            |
| Man$_{10}$GlcNAc-ol   | 34.2           | 18              |                |
| Man$_{10}$GlcNAc-ol   | 37             | 10              |                |
| Man$_{10}$GlcNAc-ol   | 37.2           | 73              | 2.3            |
| Man$_{10}$GlcNAc-ol   | 38.4           | 5               |                |
| Man$_{10}$GlcNAc-ol   | 40.9           | 16              |                |
| Man$_{10}$GlcNAc-ol   | 45.3           | 6               |                |

*Corresponds to peak numbers in Fig. 4.
* Determined using the gradient conditions described in the legend of Fig. 4.
* Based on the signal area of 250 pmol of Glc from consecutive chromatographies.

Saccharide alditols (25). Thus, the increased retention time (approximately 4 min) of the yeast Man$_{10}$GlcNAc oligosaccharide (Table 2, structure IIa) compared to precursor Man$_{9}$GlcNAc (Table 2, structure IIb) may be explained by the net increase of one free 2-OH. Contributions of spatial factors (26), inter- and intraresidue hydrogen bonding, and cooperative interactions (27) to HPAEC retention times also have been discussed.

Man$_{10}$GlcNAc—On the basis of the spectrum (Fig. 3C) and its integration (Table 5), the principal Pichia invertase Man$_{10}$GlcNAc is the species found previously on Saccharomyces invertase (9). This is the glycoprotein form of Man$_{10}$GlcNAc in which a1,2-linked mannose 13 has been attached to the α1,6-linked residue 12 (structure IIIa, Table 2). By integration (Table 5, structure IIIa) this species is at least 80% of the Man$_{10}$ isomers in the pool. The small residual signals at 4.931 and 4.914 ppm suggest the presence of mannose in α1,6-linkage to 2-O-substituted or 2-O-unsubstituted mannose, respectively (28). The sum of resonance intensities at 5.149, 4.931, and 4.914 ppm is slightly in excess of 2 mol suggesting that all Man$_{10}$ isomers have both α1,6-linked residues 6 and 12, plus additional α1,6-linked mannose on some species. Unlike the Man$_{9}$ pool, there is no signal at 5.403 ppm in the Man$_{10}$GlcNAc pool to indicate the presence of any α1,2-linked substitution of residue 7. The total contribution of terminal α1,2-linked mannose (residues 9, 11, 13)
caused an -0.013 ppm of upfield shift in 5's C1-H relative to thyroglobulin Man9 isomers (20) and several IgM isomers which had the sequence

\[ \text{residue 9, as this would also provide two} \]

protons.

that Fig. 3C also shows a small peak at 5.127 ppm, which result from a through-space shielding of 5's C1-H anomeric center by the conformational proximity of residue 11's ring protons.

By analogy, a similar upfield shift should occur to residue 6's C1-H if an α1,2-linked residue was added terminally to the upper-arm internal α1,2-linked mannose residue 8. In Pichia Man9GlcNAc this resonance integrated as 1.13 mol (Table 5) indicating additional internal α1,2-linked mannose beyond that provided by residue 8. This additional intensity would result upon terminal α1,2-linked mannose substitution of a pre-existing terminal α1,2-linked residue, such as to 9 or 11 in structure IIa (Table 2).

Evidence to support a new 2-O-substituted α1,2-linked residue is found in the two-dimensional projections of the ManloGlcNAc spectrum (Fig. 5C). Additional multiplets are seen in the J1,2 cross-peaks at C1-H/C2-H = 5.304/4.113 ppm of ManloGlcNAc compared to this region in the Man9GlcNAc spectrum (Fig. 5B), which indicates internal α1,2-linked mannose beyond the 1 mol provided by residue 8. In the J1,2 cross-peak region, this partial residue is found at C2-H/C3-H = 4.113/3.955 ppm (Fig. 5G) and is clearly resolved from the J1,2 of residue 8. Note that the C3-H of residue 8 in ManloGlcNAc is found at 3.955 ppm (Fig. 5E) and shifts upfield to 3.920 ppm on addition of the α1,6-linked residue 12 to form ManloGlcNAc (Fig. 5F). The appearance of a partial C3-H resonance at 3.955 in Manlo (Fig. 5G) suggests that the 2-O-substituted α1,2-linked mannose is spatially removed from the oligosaccharide core which could result by terminal addition of α1,2-linked mannose residue 14 to either 11 or 9.

Previous NMR studies, which examined the three possible thyroglobulin Man8 isomers (20) and several IgM Man8 isomers (28), showed that the terminal α1,2-linked mannose 11 in oligosaccharides with the sequence

\[ (\text{Man1o})_{2} \text{Man1o} \rightarrow \]

caused an -0.013 ppm of upfield shift in 5's C1-H relative to isomers which had the sequence

\[ (\text{Man1o})_{2} \rightarrow \]

From the work of Cohen and Ballou (28), this shift appears to result from a through-space shielding of 5's C1-H anomic center by the conformational proximity of residue 11's ring protons.

On the basis of the NMR data (Fig. 3C) and the chemical shift database (20, 28), residue 14's attachment is assigned to residue 9 rather than 11.

The small residual signal at 4.914 ppm, indicative of α1,6-linked mannose in polymer form or to 2-O-unsubstituted mannose, can be accommodated by addition of another α1,6-linked residue 15 to extend residue 12 (Table 2, structure IIIc and Table 5). This isomer accounts for the excess of α1,6-linked mannose over the 2 mol contributed by residues 6 and 12 (Table 5). Verification that the minor resonance peaks at 4.931 and 4.914 ppm in the Manlo one-dimensional spectrum (Fig. 3C) were due to α1,6-linked mannose was provided by
their $J_{1,2}$ cross-peaks in a two-dimensional expansion of the COSY spectrum (not shown).

Three peaks were found after HPAEC with PAD of the Man$_{10}$GlcNAc oligosaccharide pool (Fig. 4C and Table 7). A major peak ($R_T = 33.5$ min) comprised 72% of the electrochemical response. A shoulder on the major peak ($R_T = 34.2$ min) and the most retained peak ($R_T = 37$ min) gave 18 and 10% of the response, respectively. The proportion, as well as the number of species, were in agreement with the Man$_{10}$GlcNAc isomers, which were deduced from the NMR spectra (Tables 2 and 5). Based on the published retention-time trends discussed above (11, 26), structure IIIc (Table 2) was assigned to the most retained peak ($R_T = 37$ min) in the Man$_{10}$GlcNAc pool.

The Man$_6$GlcNAc fractions from invertase of Saccharomyces were compared by HPAEC to those expressed in Pichia. Fig. 6A shows the major oligosaccharide species from Pichia and Saccharomyces co-eluted. Fig. 6, B and C, shows that the trailing shoulder of the major peak is not present in the Man$_6$GlcNAc fraction of Saccharomyces invertase oligosaccharides. However, the last eluting peak, assigned to the α1,6-extended Man$_6$GlcNAc oligosaccharide, co-eluted in both cases.

Man$_{10}$GlcNAc—This oligosaccharide pool represented a very small fraction of total Pichia invertase carbohydrate (Table 1), nevertheless the one-dimensional NMR spectrum revealed unique features (Fig. 3D) not seen for Saccharomyces invertase Man$_{10}$GlcNAc (9, 21). The first was the presence of over 1.8 mol of intensity at 5.305 ppm (Table 6), where α1,2-linked 2-O-substituted mannose appears (28). Saccharomyces Man$_{10}$GlcNAc preparations studied to date (9, 21) reveal 1 mol of 2-O-substituted α1,2-linked mannose (residue 8 in structure I, Table 2). The excess intensity at 5.305 ppm in Pichia Man$_{10}$GlcNAc indicates that most species have an additional α1,2-linked mannose in series, which means that one or more of the α1,2-linked terminal residues 9, 11, or 13 are 2-O-substituted.

Examination of the $J_{1,2}$ cross-peaks (Fig. 5D) reveals two overlapping multiplets at C1-H/C2-H = 5.305/4.112 ppm, which confirms the presence of additional 2-O-substituted α1,2-linked mannose beyond the signal contributed by residue 8 (compare this region in Man$_{10}$, Fig. 5, A–C). This is shown more clearly in the $J_{2,3}$ cross-peaks where the C2-H/C3-H = 4.112/3.955 ppm of the new resonance is completely separated from the C2-H/C3-H of residue 8 (Fig. 5H), and at a greater intensity than seen in Man$_{10}$GlcNAc (Fig. 5G). The data best fit the major Man$_{10}$GlcNAc being the Saccharomyces form of Man$_{10}$GlcNAc with an additional α1,2-linked terminal residue (structure IVa, Tables 2 and 6).

The arguments used in assigning α1,2-linked terminal residue 14 to upper-arm residue 9 in Man$_{10}$GlcNAc (Table 5, isomer IIIb), also apply to the major Man$_{10}$GlcNAc species. Thus, residue 14 appears to be attached to residues 9 and/or 13 rather than 11. The 1D spectrum of Man$_{10}$GlcNAc (Fig. 3D) provided 1 mol of intensity at 5.150 ppm and 0.9 mol shifted upfield to 5.130 ppm for α1,6-linked 2-O-substituted mannose. As discussed above (20, 28), this upfield shift appears to result from a through-space interaction of residue 14’s ring protons with the anomeric center of residue 6 (or 12), causing a shielding effect. Although mannoses 14 and 6 (or 12) are separated by two residues in the primary sequence (Table 2, structures IIIb and IVa), the conformation of this trisaccharide, based on a space-filling model, confirms their proximity (not shown).

The two-dimensional COSY NMR data provide evidence that both residues 9 and 13 are substituted. The $J_{1,2}$ cross-peaks in Fig. 7 show that the intensity of residue 6 at C1-H/C2-H = 5.144/4.026 ppm and 12 at C1-H/C2-H = 5.149/4.012 ppm in Man$_{10}$GlcNAc are both diminished with the appearance of a new cross-peak for 2-O-substituted α1,6-linked mannose appears at C1-H/C2-H = 5.126/4.011 ppm. This is also seen in the $J_{1,2}$ region by comparison of Man$_{10}$ and Man$_{11}$ (Fig. 5, G and H), where 6 and 12 are clearly separated in Man$_{10}$ at C2-H/C3-H = 4.026/3.955 ppm and C2-H/C3-H = 4.012/3.906 ppm, respectively, and in Man$_{11}$ where the intensity of both 6 and 12 are diminished with the appearance of a new $J_{1,2}$ cross-peak at C2-H/C3-H = 4.011/3.950 ppm. Evidence against a major substitution of residue 11 by residue by 14 is provided by the absence of an apparent through-space effect on residue 8’s anomeric center two mannoses away, which would be expected for this configuration as discussed above for Man$_{10}$ isomers (20, 28).

HPAEC of Man$_{10}$GlcNAc provided evidence for four components, the major one of which represented about 78% of the total (Fig. 4D and Table 7, Man$_{10}$GlcNAc peaks 1 and 2). Inspection of the peaks in Fig. 4D reveals skewing when compared to peaks for the Man$_{10}$ species, suggesting isomeric heterogeneity. Reprocessing of the integrated data for Fig. 4D showed the major peak ($R_T = 37.2$ min) to consist of two components in about a 3:2 ratio, which would correspond to the 9 and 13 substituted isomers. Assigning which is the major species will be done by mass spectrometry of the isolated
isomers, as one will generate a minus 5 Hex form while the other will generate a minus 4 Hex form upon fragmentation. The minor (5%) isomer on the backside of the main peak (Fig. 4D) may represent a small amount of a third possible variation where lower arm mannose 11 is terminally 2-O-substituted (Table 2, structure IVa). Chemical shift changes expected on residue 9 for such a minor component may not be apparent in the Man_{11} NMR spectrum.

The α1,2-linked terminal mannose region (5.038-5.063 ppm) for Man_{11}GlcNAc integrates to about 2.9 mol indicating that about 10% of the species lack 3 terminal α1,2-linked residues (Table 6). Note that there is a small α1,6-linked terminal mannose signal at 4.926 ppm and another at 4.914 ppm for 2-O-unsubstituted α1,6-linked mannose. The sum of all α1,6-linked mannoses is 2.2 mol, indicating that 20% of Man_{11} species have α1,6-linked mannose in addition to residues 6 and 12. From this information it can be deduced that one component of the Man_{11}GlcNAc pool is the yeast Man_{9}GlcNAc isomer IIIc (Tables 2 and 5) with additional α1,2-linked terminal mannose attached either to residue 12 or 15 (Tables 2 and 6, isomers IVb and IVc). These two isomers comprise about 11% of the total and appear to correspond to the two-component peak eluting at 40.9 min, which represents 16% of the integrated area on HPAEC (Fig. 4D, inset).

The final, minor component of the Man_{11} pool represented about 6% of the integrated area of HPAEC chromatograms and eluted at 45.3 min (Fig. 4D, Table 7). This isomer appears to be Man_{9}GlcNAc isomer IIC with an α1,2-linked terminal residue 14 attached to upper-arm residue 9 (Table 2, structure IVd). This isomer accounts for the excess α1,2-linked 2-O-substituted mannose over that provided by Man_{9}GlcNAc isomers IVa, as well as most of the α1,6-linked 2-O-unsubstituted mannose whose signal appears at 4.914 ppm (Fig. 3D and Table 5). The long retention time of isomer IVd is attributed to the absence of α1,2-substitution of the Man_{10}6Man_{10}6-chain on the lower arm (11).

The second notable difference in Saccharomyces and Pichia Man_{11}GlcNAc oligosaccharides is the absence of signals for α1,3-linked terminal mannose in the latter (9, 21, 28). Although it can be difficult to distinguish α1,3-linked mannose in one-dimensional spectra because the C1-H signal at 5.144 ppm coincides with 2-O-substituted α1,6-linked mannose (28), the C2-H of 3-O-substituted α1,2-linked mannose shifts markedly from the envelope region at 4.085 to 4.224 ppm, where it is easily integrated (9, 28). None of the Pichia oligosaccharides examined in this study reveal any C2-H signal at 4.224 ppm indicative of 3-O-substituted, α1,2-linked mannose (see Fig. 3).

Absence of α1,3-linkage isomers was also supported by HPAEC of Man_{9}GlcNAc from P. pastoris and S. cerevisiae invertase. The major Saccharomyces Man_{9}GlcNAc was found to contain one α1,3-linked Man, attached to residue 11 as shown in Scheme 1 (9, 21). Residues designated by upper-case letters are those added on elongation of the trimmed Man_{9}GlcNAc core after leaving the ER (9, 19). Analytical HPAEC of Saccharomyces Man_{9}GlcNAc is shown in Fig. 4A, while Man_{9}GlcNAc from Pichia is shown in B for comparison. A 1:1 admixture of Man_{9}GlcNAc from the two sources is shown in C. The major Man_{9}GlcNAc from Saccharomyces did not coelute with any of the four Man_{11}GlcNAc isomers from P. pastoris (Fig. 4C). Thus, HPAEC provides a clear separation of Pichia branch isomers of Man_{11}GlcNAc with various α1,2-substitutions (Table 2, IVa-d) from the major Saccharomyces Man_{11}GlcNAc with terminal α1,3-linked mannose (Scheme 1).

In conclusion, Pichia yeast synthesize and secrete high levels of heterologous Saccharomyces SUC2 invertase, which on gels resembles in size the endoplasmic reticulum form of the enzyme synthesized at 37 °C in sec18 mutants (Fig. 1). On Bio-Gel P-4 (Fig. 2), endo H-released oligosaccharides are mostly Man_{9}GlcNAc with lesser amounts of Man_{10,11}GlcNAc.
proposed metabolic interrelationship of the oligosaccharide novel structures, which are the Mang,loGlcNAc isomers with component of ManloGlcNAc and most of ManllGlcNAc are which demonstrate the ability of HPAEC to separate not only processed MangGlcNAc linkage isomers (Fig. 4B and Table 2, structures IIa and IIb), ManoGlcNAc linkage isomers IIIa and IIId from IIIc (Fig. 4C and Table 2), and the unique elution position of Man11GlcNAc from Saccharomyces (Scheme 1), which contains an α1,3-linked Man in contrast to the same size oligosaccharides from Pichia with α1,2-linked termini (Fig. 8). Under the conditions described, linkage isomers were separated by at least 4 min. Branch isomers (no change in substitution) were more difficult to resolve and appeared as shoulders and asymmetrical peaks (Fig. 4D), but the indicated heterogeneity was completely consistent with structural assignments made by NMR analysis.

An unexpected finding in this study was that Pichia invertase oligosaccharides do not undergo terminal addition of α1,3-linked mannose during processing of the core ManGlcNAc to larger species. It is not currently known whether Pichia can add α1,3-linked terminal residues to any cellular glycans, but this is under investigation. Although the ManGlcNAc pool revealed a minor contaminant of the lipid-oligosaccharide form of Manα (structure IIb, Tables 2 and 4), the one- and two-dimensional NMR spectra of larger species did not reveal signals from residual glucose residues. Therefore, if glucose is initially present on Pichia lipid-oligosaccharide, as is found in other fungal species (29, 30), it is efficiently removed during processing.

As a final note, it has been suggested that because Pichia glycans are shorter than Saccharomyces forms (8), they may be more “mammal-like” than “yeast-like.” The structural studies reported here show that, although 75% of the oligosaccharides are ManαGlcNAc, 95% of the ManβGlcNAc and all of the Manα,1GlcNAc are isomers uniquely found on fungal glycoproteins. From a biotechnological perspective, it is not known at present the extent to which the Pichia Manα,1GlcNAc species would be antigenic in mammals, but studies addressing this question also are in progress.

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Oligosaccharide Structures on Pichia SUC2 Invertase

SUPPLEMENTAL MATERIAL

FOR

STRUCTURE OF OLIGOSACCHARIDES ON SACCHAROMYCES SUC2 INVERTASE SECRETED BY THE METHYLOTROPIC YEAST, PICHIA PASTORIS

by

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Fig. 2 Bio-Gel P-4 profiles of endo H-released Pichia SUC2 invertase oligosaccharides. The 1.6 x 84.5 cm column was eluted at a flow rate of 8 ml/h and the 0.75 ml fractions were assayed for neutral hexose by the phenol-sulfuric acid method. Panel A, Man,GlcNAc plus Man,GlcNAc. Panel B, Man,GlcNAc plus Man,GlcNAc. Each run had Man,GlcNAc (=) as an internal marker. Horizontal bars indicate the fractions pooled for 1H NMR spectroscopy and HPAEC with PAD.

Fig. 6 HPAEC with PAD of Man,GlcNAc from Pichia and Saccharomyces invertase. Man,GlcNAc Bio-Gel P-4 fraction from Saccharomyces (9) was purified using HPAEC with in-line micro-membrane deashing as described in the Experimental Procedures and eluted in 0.75 ml fractions. The purified oligosaccharide (approximately 2 mol of starting material) was dissolved in 1000 µl of water and 10 µl was chromatographed in Panel A as described in the legend to Fig. 6. Approximately 125 pmol of Man,GlcNAc from Pichia invertase was analyzed in Panel B, and the results of a 1:1 admixture of the two is shown in Panel C.

Table 3. Man,GlcNAc peak integrals and assignment to structures

| Residue No. | Name | α- or β- | Intensity (Integrated) | Distribution in Telzym 1 | Assigned |
|-------------|------|---------|----------------------|-------------------------|----------|
| 5           | Man  | β        | 5.344                | 0.98                    | 1.0      |
| 8           | Man  | α        | 5.304                | 0.98                    | 1.0      |
| 10          | Man  | α        | 5.347                | 0.98                    | 1.0      |
| 12          | Man  | β        | 5.144                | 1.02                    | 1.0      |
| 13          | Man  | β        | 5.102                | 0.98                    | 1.0      |
| 15          | Man  | β        | 5.073                | 0.98                    | 1.0      |

Total Man IC1 + C2: 7.943
Total: 8.00

Abundance: 100% 100%

* Structures are presented in Table 2.
### Table 6. Oligosaccharide Structures on Pichia SUC2 Invertase

| Residue  | Name | Acceptor | Benzoate | Distribution of % Benzoate in Residue | Intensity Assigned |
|----------|------|----------|----------|--------------------------------------|-------------------|
| Total    |      |          |          |                                      |                   |
| 34       |      |          |          |                                      |                   |
| 35       |      |          |          |                                      |                   |
| 26       |      |          |          |                                      |                   |
| 25       |      |          |          |                                      |                   |
| 2          |      |          |          |                                      |                   |
| 4        |      |          |          |                                      |                   |
| 3        |      |          |          |                                      |                   |

### Table 7. Analysis of Oligosaccharide Structures

| Residue  | Name | Acceptor | Benzoate | Distribution of % Benzoate in Residue | Intensity Assigned |
|----------|------|----------|----------|--------------------------------------|-------------------|
| Total    |      |          |          |                                      |                   |
| 34       |      |          |          |                                      |                   |
| 35       |      |          |          |                                      |                   |
| 26       |      |          |          |                                      |                   |
| 25       |      |          |          |                                      |                   |
| 2        |      |          |          |                                      |                   |
| 4        |      |          |          |                                      |                   |
| 3        |      |          |          |                                      |                   |

* Structures are illustrated in Figure 2.