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Characterization of N- and O-linked glycosylation of recombinant human bile salt–stimulated lipase secreted by *Pichia pastoris*

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Recombinant human bile salt–stimulated lipase (hBSSL) was expressed in and secreted by *Pichia pastoris*, an organism exploited for the large-scale production of recombinant (glyco)proteins by bioprocessing technology. The 76.3-kDa glycoprotein was associated with 75–80 Man and a small amount of GlcNAc. hBSSL has one N-glycosylation site at Asn187, which was 38–40% occupied with a $Man_{10}GlcNAc_2$ structure defined previously in *Pichia* as the oligosaccharide-lipid form of $Man_9GlcNAc_2$ trimmed of the middle-arm terminal α1,2-Man and elongated with Manα1,2Manα1,6-disaccharide attached to the lower-arm core α1,3-Man (Trimble *et al.* [1991], *J. Biol. Chem.*, 266, 22807–22817). The C-terminal 192 residues of hBSSL contain 16 Pro-rich 11-amino-acid repeats, which include 32 Ser/Thr residues as potential O-glycosylation sites. Using hBSSL as a platform to study *Pichia*'s O-glycosylation capabilities, we found that nearly all of these sites were occupied by mannose-containing O-glycans, whose structures, after β-elimination and purification, were assigned by ^1H NMR and, in some cases, by linkage-specific exoglycosidases and methylation analysis. The most abundant O-glycan was α1,2-mannobiitol (55%), followed by α1,2-mannotriitol (16%) and mannitol (10%) and a lesser amount was α1,2-mannotetraitol. Unexpectedly, Man_5 and Man_6 O-glycans were present, which had the structure Manβ1,2Manβ1,2Manα1,2$(Manα1,2)_{1,2}$mannitol. Also a small amount of a phosphorylated Man_6 O-glycan was characterized by MALDI-TOF MS postsource decay analysis as having the reducing-end mannitol disubstituted with a glycosidically linked phosphorylated Man and an unbranched Man_4 polymer elongated from a different mannitol carbon. This is the first report of the synthesis of β-Man- and phosphate-containing O-linked constituents on glycoproteins synthesized by *P. pastoris*.

Key words: bile salt-stimulated lipase/glycan NMR N-glycosylation/O-glycosylation/*P. pastoris*

Introduction

Pancreatic human bile salt–stimulated lipase (hBSSL) works in concert with colipase-dependent pancreatic lipase to digest and assimilate triglycerides in adults of many species. Both enzymes are present in low levels in newborn humans, which leads to malabsorption of fats in some formula-fed neonates. Human milk triglycerides, which initially may account for 50% of a breast-fed infant's dietary energy supply, are efficiently utilized, even in preterm infants, due to the presence of hBSSL in the mother's milk (reviewed in Hernell and Bläckberg, 1994).

The hBSSL gene was cloned from a mammary gland cDNA library (Baba *et al.*, 1991; Nilsson *et al.*, 1990), and sequencing revealed the human form to be 742 aa in length, with the first 20 residues removed *in vivo* as a cleavable signal sequence. The catalytic domain, consisting of the first 530 residues, is highly conserved in nature, with an N-glycosylation site at Asn187. The C-terminal tail region in hBSSL, consisting of residues 531–722, contains 16 repeats of a proline-rich 11-residue sequence within residues 538–713. Different species have different numbers of the mucin-like C-tail repeat units. hBSSL is heavily glycosylated, and a number of studies have characterized both the N- and O-glycoforms of the human enzyme (Landberg *et al.*, 1997; Mechref *et al.*, 1999; Wang *et al.*, 1995), including natural variants (Strömqvist *et al.*, 1997) as well as changes in glycoforms during lactation (Landberg *et al.*, 2000).

Recombinant DNA constructs of human BSSL reveal that neither elimination of the N-glycosylation consensus sequence at Asn187 by an N187Q substitution nor removal of the heavily O-glycosylated tail region's 16 proline-rich 11-residue repeats negatively effects the expression, activation by bile salts, catalytic activity, or substrate specificity of the enzyme (Bläckberg *et al.*, 1995). The dispensability of either N- or O-glycosylation is confirmed by the expression of active full-length and C-terminal tail-free constructs in *Escherichia coli* (Hansson *et al.*, 1993). Thus the role of the extensive O-glycosylation is unproven, but the large C-terminal tail should be very hydrophilic and accessible (Wang *et al.*, 1995), factors that may relate to its retention in the intestinal track *in vivo*.

Efficient production of a recombinant form of hBSSL by *Pichia pastoris* with the enzymatic properties of the native protein for potential use as an infant formula supplement has been described (Sahasrabudhe *et al.*, 1998). *Pichia pastoris* has been used extensively by biopharma for the large-scale production of glycopharmaceuticals, but little is known about this organism's ability to synthesize other than short α1,2-linked mannose O-glycans (reviewed by

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Bretthauer and Castellino, 1999). Thus the abundance of this recombinant full-length hBSSL provided a platform to explore the capacity of *P. pastoris* to form O-glycans with potentially novel linkage arrangements. Like many other yeast, *Pichia* synthesizes α1,2Man$_{1-4}$ O-linked glycans. Unexpectedly, however, a portion of the α1,2-Man$_3$ and α1,2-Man$_4$ chains were found to be capped at the nonreducing end with a Manβ1,2Manβ1,2-disaccharide, and a small amount of a branched Man$_6$-phosphorylated O-glycan also was identified.

Results and Discussion

Characterization of purified hBSSL secreted by P. pastoris

The full-length hBSSL gene was cloned into vector pHILD4 to form pARC5799, which was used to transform *P. pastoris* for high-level expression and secretion of hBSSL (Sahasrubudhe *et al*., 1998). The hBSSL protein present in the fermentor broth was purified as described (Sahasrubudhe *et al*., 1998) and served as the starting material for the current studies on hBSSL glycosylation by *Pichia*.

Figure 1 shows the sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE) pattern of purified hBSSL stained with Coomassie blue. The full-length protein without its cleavable signal sequence has a predicted mass of 76.3 kDa (ENTREZ accession number P19835), and the protein migrates at about 102 kDa. Previous studies have shown that the *Pichia*-expressed hBSSL binds the lectin conacanavalin A, indicating the presence of Man. Acid hydrolysis and Dionex high-performance anion exchange chromatography (HPAEC) revealed Man as the only hexose present, with a small amount of GlcNAc associated with hBSSL, and phenol sulfuric acid analysis using Man as a standard revealed 75–80 Man/peptide (data not shown).

N-linked glycosylation of Pichia*-secreted hBSSL*

There is one potential N-glycosylation sequon at Asn187 in the hBSSL sequence (Baba *et al*., 1991), and the presence of GlcNAc by Dionex HPAEC suggested that the site was at least partially occupied. However, endo β-*N*-acetylglucosaminidase H (Endo H) treatment of the hBSSL had little effect on the glycoprotein's apparent migration (Figure 1). Nevertheless, solvent precipitation and methanol extraction (Verostek *et al*., 2000) of the Endo H–digested sample provided an N-glycan fraction, over 80% of which eluted as Man$_{10}$GlcNAc on a calibrated Bio-Gel P-4 column (Figure 2). This assignment was confirmed by the single matrix-assisted laser desorptin/ionization time-of-flight mass spectrometry (MALDI-TOF MS) signal at 1865 Da for the sodium adduct of Hex$_{10}$GlcNAc in the pooled fractions 127–133 (Figure 2, inset). Recovery of the Endo H–released N-glycans from three separate trials with this batch of purified hBSSL indicated an Asn187 site occupancy of 38–40%. Small amounts of Man$_{8,9,11,12}$GlcNAc were also present in the profile (Figure 2).

Interestingly, in a previous study on recombinant *Saccharomyces cerevisiae* invertase (Suc2p) expressed in *Pichia* (Trimble *et al*., 1991), the same range of Man$_{8-12}$ GlcNAc N-glycan sizes was found to be present, but the distribution comprised nearly equal amounts of Man$_{8,9}$GlcNAc, representing 75% of the N-glycans present, with Man$_{10}$GlcNAc constituting only 18% of the total. To assess whether the Man$_{10}$GlcNAc present on hBSSL had the same structure as that on Suc2p, we analyzed the pooled Man$_{10}$GlcNAc by high-field ^{1}H nuclear magnetic resonance (NMR) (data not shown). The hBSSL structure (Scheme 1), which includes residue numbers and their nominal anomeric proton chemical shifts (δ, ppm), is identical to the Suc2p Man$_{10}$GlcNAc pool characterized earlier (Trimble *et al*., 1991). This isomer is the Man$_8$GlcNAc core derived by trimming in the endoplasmic reticulum of three Glc and the middle-arm α1,2-Man from the Glc$_3$Man$_9$GlcNAc$_2$ precursor initially transferred to nascent proteins, followed by outer-chain mannan initiation with α1,6-Man residue **12** added by Och1p and capped with α1,2Man residue **13** in the Golgi apparatus (reviewed in Gemmill and Trimble, 1999a).

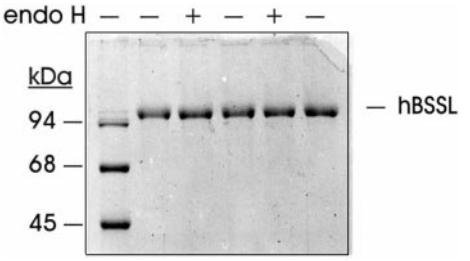


Fig. 1. SDS–PAGE of hBSSL secreted by *P. pastoris* before and after Endo H digestion. hBSSL N-glycans were hydrolyzed with endo H, 50 mU/ml, overnight at 30°C, and 0.5 μg aliquots of control and treated protein were run in alternate lanes on an 8% SDS–acrylamide gel. Molecular weight markers, in kDa, are identified by their masses in the lefthand gel lane.

O-glycosylation of hBSSL synthesized by Pichia

After Endo H digestion to remove N-glycans, the hBSSL O-glycans were β-eliminated in the presence of sodium borohydride, which converts the reducing-end Man to Man-ol. The pool of O-glycans, prepared as described in *Materials and methods*, was chromatographed on the calibrated Bio-Gel P-4 column with an included internal marker of ^{14}C-Man-ol eluting in peak 1 (Figure 3). The phenol–sulfuric acid assay profile revealed five additional peaks, which were collected as pools 2–6 by combining the fractions indicated by the bars over the peaks in Figure 3. These represented ManMan-ol through Man$_5$Man-ol, whose masses were confirmed by MALDI-TOF MS (Table I). The Man-ol in pool 1 was quantitated by ratio analysis of the ^{14}C in the profile with the recovery of Man-ol by Dionex HPAEC on an MA1 analytical column using an authentic Man-ol standard for calibration.

MALDI-TOF MS and Dionex HPAEC analysis of hBSSL O-glycans

Preliminary MALDI-TOF MS analysis of pools 2–6 revealed some cross-contamination in pools 4 and 5, so these were rechromatographed on Bio-Gel P-4 and the

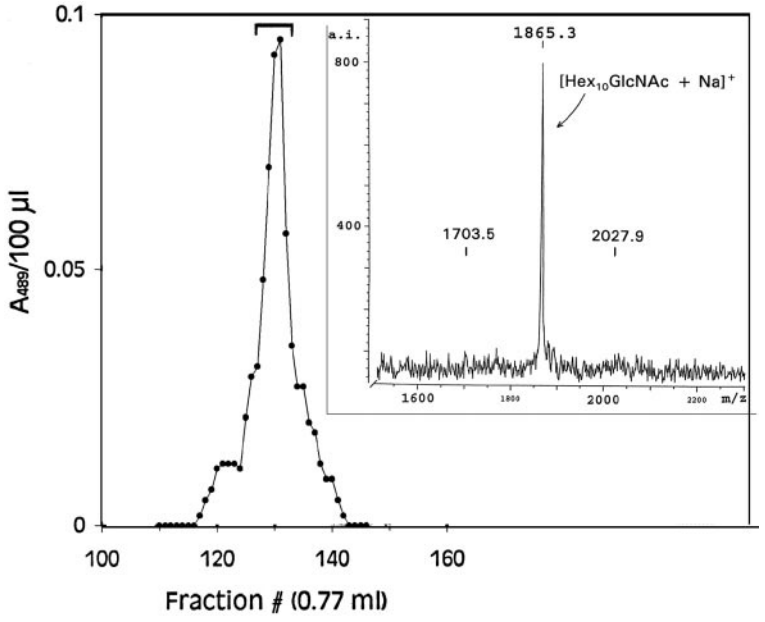
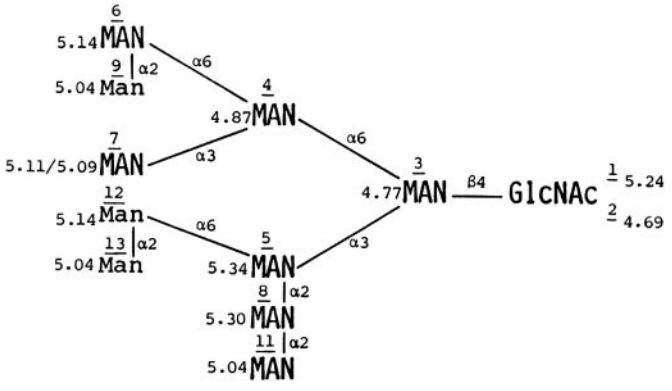


Fig. 2. Characterization of the N-glycans released from hBSSL by Endo H. The main panel shows the Bio-Gel P-4 column (1.6 × 95 cm) elution profile of the material solubilized on 50% CH_3OH extraction of the acetone-precipitated digest (see *Materials and methods* for additional details). Fractions 127–133 (top bracket) were pooled and ~10 pmol of glycan subjected to MALDI TOF MS analysis (inset).



Scheme 1. Structure of $Man_{10}GlcNac$ released from hBSSL by endoH.

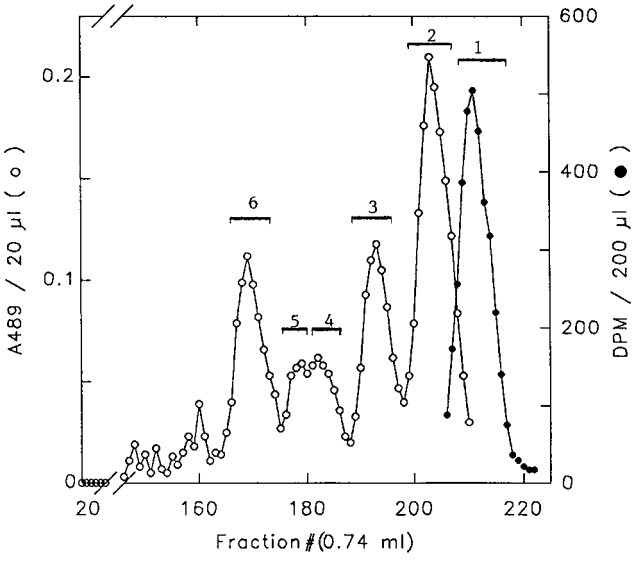


Fig. 3. Bio-Gel P-4 (1.6 × 95 cm) chromatography of the β-eliminated, reduced O-linked oligosaccharides from recombinant hBSSL secreted by *P. pastoris*. Reducing hexoses were assayed by the phenol–sulfuric acid method (open circles), and the monosaccharide alditol peak was quantitated by Dionex MA1 chromatography of the material coeluting with an authentic ^{14}C-mannitol internal standard (closed circles). Fractions constituting pools 1–6 are shown by the bars over the peaks.

central 85% of the phenol–sulfuric acid assay absorbance for each peak was pooled (data not shown). The final pools 2–6 were analyzed by MALDI-TOF MS, and all but pool 4 revealed a single molecular weight species representative of Man_xMan-ol, as summarized in Table I. The additional glycan in pool 4 is a phosphorylated Hex_5Hex-ol, which must have a compact solution structure to elute with a Hex_4Hex-ol species on gel permeation chromatography.

Each of the pools was analyzed by Dionex HPAEC on a PA1 analytical column; the resultant profiles are shown as a montage in Figure 4. Each pool has one main component with traces of species coeluting with the main components in adjacent peaks of the Bio-Gel P-4 column (Figure 3). The absence of a novel peak in the Dionex trace for pool 4 (Figure 4) suggests that the phosphorylated glycan in this pool (Table I) is a minor component.

On the basis of phenol–sulfuric acid assay absorbance values for three separate β-elimination experiments with this batch of hBSSL, the recovery of O-linked glycans in a typical Bio-Gel P-4 profile (Figure 3) was 74–92% of the starting material, depending on the amount of hBSSL treated, column pooling, and assay losses. With

Table I. Size and distribution of O-glycans on hBSSL secreted by *P. pastoris*

Pool no.[a]	Glycan size[b]	$M_r(Na^+)$ Measured[c]	$M_r(Na^+)$ Calculated	No. Chains/ hBSSL[d]	No. Man/ hBSSL
1	Man-ol	ND[e]		2–3	–3
2	ManMan-ol	366.8	366.4	17	34
3	Man_2Man-ol	529.1	528.6	5	15
4	Man_3Man-ol	691.4	690.8	1	4
	$Man_4(HPO_3Man)$Man-ol	1095.0	1095.2	ND	ND
5	Man_4Man-ol	853.4	853.0	1–2	5–10
6	Man_5Man-ol	1015.7	1015.2	1–2	6–12
				$\sum$ = 27–30	$\sum$ = 66–78

[a]Pools 1–6 as identified in P-4 profile in Figure 3.
[b]Man-ol quantitated by HPAEC, others by phenol–sulfuric acid assay.
[c]By MALDI-TOF MS in the positive ion mode.
[d]Average of three hBSSL preparations analyzed.
[e]Not determined.

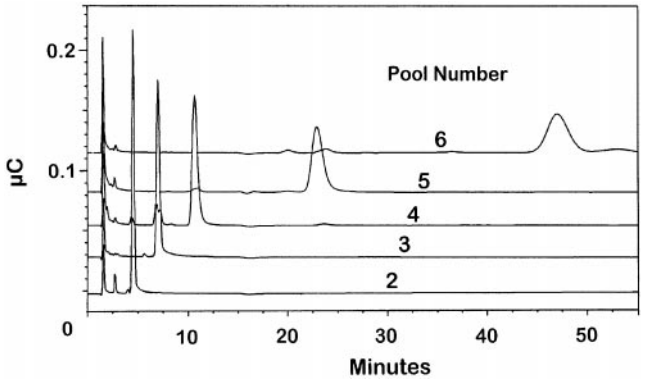


Fig. 4. Dionex HPAEC of alditol pools 2–6 from the Bio-Gel P-4 column profile in Figure 3. Approximately 5 nmol of each oligosaccharide were chromatographed isocratically on the Dionex PA1 analytical column in 16 mM NaOH. Detection was by pulsed amperometry (μC). Additional details are in *Materials and methods*.

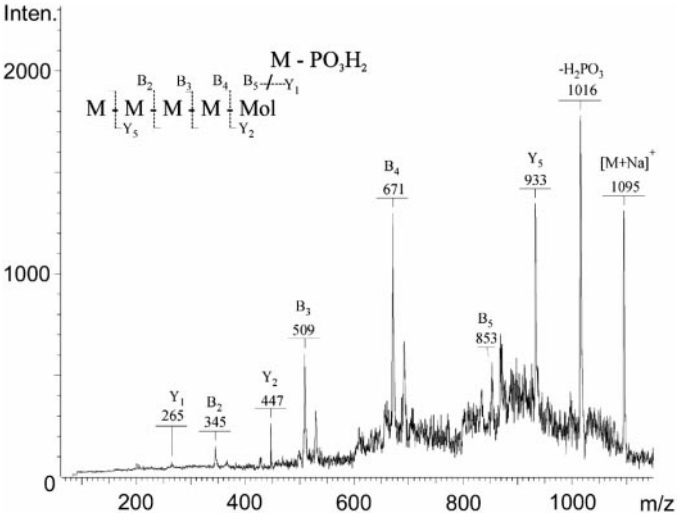


Fig. 5. PSD of $Man_4(ManH_2PO_3)$Man-ol present in pool 4. The sources of **B** and **Y** ion fragments from the proposed structure are noted on the figure as major molecular masses in kDa. A more comprehensive compilation of mass assignments found in the spectrum is summarized in Table II. Signals, in kDa, are identified in the figure.

the recoveries normalized, Table I summarizes the number of $Man_{1–5}$ Man-ol chains present on an "average" hBSSL molecule. Also included is the monosaccharide value assayed as Man-ol. Note that the distribution of chain sizes (Table I) is in agreement with the starting calculation of about 75 O-linked Man/hBSSL. The C-terminal proline-rich 11-amino-acid repeats in residues 538–713 have a total of 32 Ser and Thr residues as potential O-glycosylation sites. The assignment of 27–30 O-glycans/ hBSSL (Table I) means that 85–95% of these residues are glycosylated.

Postsource decay (PSD) MALDI-TOF MS analysis of pool 4 phosphorylated hexasaccharide alditol

The unusual phosphorylated O-glycan in pool 4 was present in such a small amount that no effort was made to isolate it from the major Man_3Man-ol component for ^{1}H NMR analysis. However, some structural information could be deduced from the PSD MALDI-TOF MS spectrum of the 1095-Da ion shown in Figure 5.

The relationship of parent/daughter ions is consistent with the structure included in Figure 5. Table II summarizes the measured and calculated masses of the fragments and their structures. A second, minor $Man_5(H_2PO_3Man)$-Man-ol isomer appears to be present, in which the PO_4 may be on the penultimate Man of the compound; this would account for the small signal at 428 Da for H_2PO_3-Man_2. Note that this fragment cannot be readily obtained

Table II. PSD ions formed on MALDI-TOF MS of the $Man_5(H_2PO_3)$Man-ol present in hBSSL O-glycan pool 4

m/z observed	*m/z* calculated	Identity
1095[a]	1095.2	$[Man_5(H_2PO_3)Man\text{-}ol]Na^+$
1015	1015.2	$[Man_5Man\text{-}ol]Na^+$
933	933.0	$[Man_4(H_2PO_3)Man\text{-}ol]Na^+$
853	853.0	$[Man_4Man\text{-}ol]Na^+$
833	832.8	$[Man_5]Na^+$
771	770.8	$[Man_3(H_2PO_3)Man\text{-}ol]Na^+$
692	690.8	$[Man_3Man\text{-}ol]Na^+$
671	670.8	$[Man_4]Na^+$
609	608.6	$[Man_2(H_2PO_3)Man\text{-}ol]Na^+$
530	528.6	$[Man_2Man\text{-}ol]Na^+$
509	508.6	$[Man_3]Na^+$
447	446.4	$[Man(H_2PO_3)Man\text{-}ol]Na$
428[b]	426.4	$[Man_2(H_2PO_3)]Na^+$
367	366.4	$[ManMan\text{-}ol]Na^+$
345	346.4	$[Man_2]Na^+$
265	264.2	$[Man(H_2PO_3)]Na$
205	204.2	$[Man\text{-}ol]Na^+$

[a]Parent ion.
[b]Assigned to a minor isomer.

from the major isomer shown in Figure 5, even by multiple fragmentations.

The major $Man_4(H_2PO_3Man)$Man-ol isomer would be expected to be composed solely of α/β1,2- and α1,6-linkages on the basis of the O-glycan structures in pools 2–6 determined by ^{1}H NMR, previous studies on *Pichia* glycans (Kobayashi *et al*., 1986), and this organism's resident glycosyltransferases (Verostek and Trimble, 1995). It is interesting to note that the secretion leader sequence of the *S. cerevisiae BAR1* protease gene product is heavily O-glycosylated and that many of the O-mannosyl chains bear terminal phosphate residues (Jars *et al*., 1995). This is in contrast to the Man phosphate in diester form found on *S. cerevisiae* N-glycans (Hernández *et al*., 1989).

Structure of the neutral O-glycans in pools 2–6

ManMan-ol and Man_2Man-ol. The most abundant O-linked oligosaccharides on recombinant hBSSL are the di- and trisaccharides, which represent nearly 60% and 20% of the total, respectively (Table I). Both the 1D and 2D double quantum filtered (DQF)–correlation spectroscopy (COSY) spectra (not shown) of pool 2 and 3 constituents were identical to spectra recently published for Manα1,2Man-ol and Manα1,2Manα1,2Man-ol β-eliminated and characterized from *Schizosaccharomyces pombe* glycoproteins (Gemmill and Trimble, 1999b). These O-glycans are also common to *Hansenula polymorpha* (Cohen and Ballou, 1980), *S. cerevisiae* (Ballou, 1990), and *Saccharomyces kluyveri* (Zhang and Ballou, 1981) glycoproteins. Both glycans were hydrolyzed to free Man and Man-ol in the expected ratios by *Aspergillus satoi* α1,2-mannosidase (not shown), in agreement with their proposed structures.

Man_3Man-ol. A 2D relayed rotating-frame Overhauser spectroscopy (ROESY) experiment (Cipollo *et al*., 2000) on the pool 4 O-glycan (Figure 6A) revealed three anomeric protons at chemical shifts of 5.057, 5.243, and 5.335 ppm. These correlated with C2-Hs at 4.091, 4.007, and 4.132 ppm, respectively, with $J_{1,2}$ coupling constants of ~1.5 Hz characteristic of α1,2-linked Man. The experiment (Figure 6A) shows the linkage of residues **d** → **c** → **b** through cross-peaks between **d**'s H1 (5.057 ppm) and **c**'s H1 (5.335 ppm) and **b**'s H1 (5.243 ppm), thus providing a proof of the $(Man\alpha1,2)_3$Man-ol assignment. Note the similarity of the anomeric proton chemical shifts for residues **d** and **c** in the pool 4 O-glycan with the α1,2-linked lower arm Man residues **11** and **8** on the N-linked Man_{10}GlcNAc in Scheme 1. A reduced α1,2-linked Man tetrasaccharide released from *Candida parapsilosis* cell wall mannan by acetolysis and chemically defined by methylation analysis provides an anomeric proton signal match (Funayama *et al*., 1983) to the chemical shift values recorded here for pool 4.

	d	**c**	**b**	**a**
	Manα1,2	Manα1,2	Manα1,2	Man-ol
C1-H(δ_{ppm})	5.057	5.335	5.243	

Interestingly, conversion of the reducing-end Man to Man-ol shifts the anomeric proton of the Man 2-O substituting the Man-ol (residue **b**) upfield by nearly 0.1 ppm (Funayama *et al*., 1983). A similar observation has been made by Shibata *et al*. (1995) on comparing reduced and nonreduced α1,2-linked mannan fragments isolated from *C. albicans* cell walls. The shift appears to result from increased shielding of this residue's anomeric center by the loss of the rigid ring structure of the reducing-end Man, on formation of the alditol.

Man_4Man-ol. The initial ^{1}H NMR spectrum of the pool 5 O-glycan included, in addition to nominal levels of α/β1,2-Man anomeric protons, a large signal at 4.904 ppm, characteristic of α1,6-linked Man. However, the C1-H/C2-H cross-peak and coupling constants to the putative ring C2-Hs for α1,6Man were inconsistent with these being glycan protons (Vliegenthart *et al*., 1983). Concerned that the sample was contaminated with a residual volatile organic compound from the column chromatography buffer, we rechromatographed the pool 5 glycan on Bio-Gel P-4, lyophilized the material and again exchanged it with D_2O for NMR spectroscopy. Because of the small amount of Hex_5-ol remaining (see Figure 3), ^{1}H NMR analysis was performed using a Nalorac microsample probe, which provided the 1D spectrum in Figure 6B. Note in this well-shimmed data set, the ~1.5 Hz $J_{1,2}$ splitting of the anomeric protons at ~5.1 and ~5.4 ppm, indicative of α-mannosides,

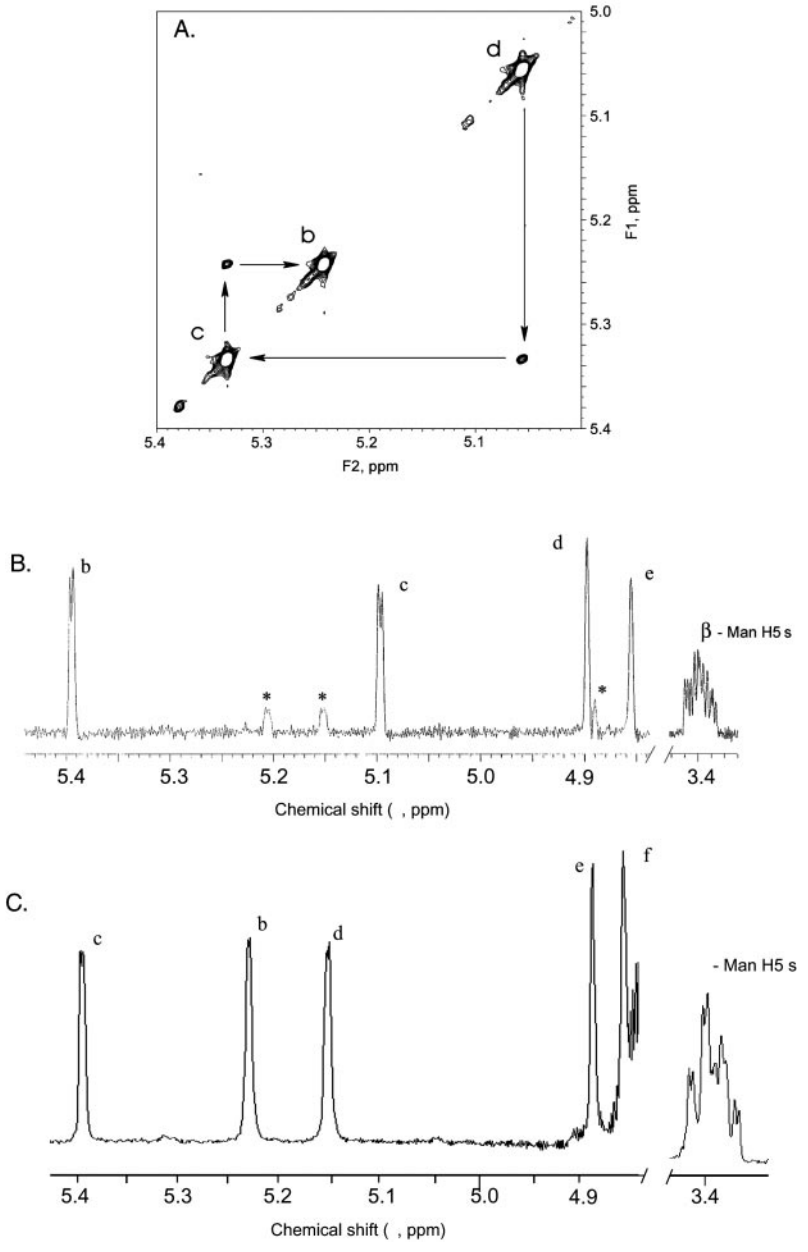


Fig. 6. ^{1}H NMR spectra at 500 MHz of pool 4–6 glycans. (**A**) 2D relayed ROESY spectrum of Man_3Man-ol (pool 4). (**B**) 1D spectrum of Man_4Man-ol (pool 5). (**C**) 1D spectrum of Man_5Man-ol (pool 6). Additional details are in *Materials and methods*.

and the lack of splitting of the anomeric protons at ~4.85 and ~4.9 ppm, characteristic of β-mannosides (Vliegenthart *et al.*, 1983). C2-H chemical shifts were determined from a separate 2D DQF COSY experiment (data not shown).

Thus the pool 5 O-glycan appears to be composed of two α- and two β-mannosides with the fifth residue a mannitol. Methylation followed by MS indicated that all residues were glycosidically 1,2-linked (data not shown). Studies on acetolysis fragments from *P. pastoris* strain 1FO 0948 cell-wall mannan (Kobayashi *et al.*, 1986) provided a minor Man_5 compound with anomeric ^{1}H signals for a glycan containing β1,2-linked mannoses that was identical to those recorded for the pool 5 O-glycan reported here. This in concert with the more comprehensive data generated for the more abundant pool 6 O-glycan suggests the pool 5 O-glycan has following structure:

	e	**d**	**c**	**b**	**a**
	Manβ1,2Manβ1,2Manα1,2Manα1,2Man-ol				
C1-H(δ,ppm)	4.855	4.897	5.097	5.392	--
C2-H(δ,ppm)	4.265	4.340	4.058	4.181	--

The minor signals in Figure 6B (∗) at 5.21, 5.15, and 4.88 ppm are from a residual amount of the pool 6 O-glycan in the sample and will be identified shortly.

Man_5Man-ol. The 1D ^{1}H NMR spectrum of the pool 6 O-glycan revealed five anomeric protons at 5.386, 5.225, 5.148, 4.883, and 4.850 ppm (Figure 6C). The first three resonances reveal $J_{1,2}$ coupling constants of ~1.2–1.5 Hz expected for α-mannosides and are reminiscent of the (Manα1,2)$_3$Man-ol anomeric protons seen in the pool 4 O-glycan (Figure 6A). The two upfield resonances show no splitting and duplicate the β-mannoside resonances seen in the pool 5 O-glycan (Figure 6B). A 2D DQF COSY spectrum provided the C1-H/C2-H cross-peaks for the pool 6 O-glycan listed later. Interestingly, Kobayashi and co-workers (1986) identified β1,2-Man-containing structures in acetolysis fragments from pathogenic *C. albicans* cell wall mannan; of particular interest among these was a pentasaccharide, Manβ1,2Manα1, 2Manα1,2-Manα1,2Manαβ (Kobayashi *et al*., 1989), which was used in more recent experiments as an acceptor in *C. albicans* extracts to assay for a nonreducing-end β1,2-mannosyltransferase (Suzuki *et al*., 1995). The product formed was a hexasaccharide shown by ^{1}H NMR at 45°C to be:

	f	**e**	**d**	**c**	**b**	**a**
	Manβ1,2Manβ1,2Manα1,2Manα1,2Manα1,2Manαβ					
C1-H(δ,ppm)	4.840	4.852	5.141	5.262	5.262	5.348(α)
C2-H(δ,ppm)	4.15	4.26	4.26	4.1+	4.1+	--

The pool 6 O-glycan reveals nearly identical $J_{1,2}$ cross-peaks, except that, due to residue **a**'s reduction to an alditol, residue **b**'s C1-H moves upfield to 5.225 ppm and residue c's C1-H moves downfield to 5.386 ppm:

	f	**e**	**d**	**c**	**b**	**a**
	Manβ1,2Manβ1,2Manα1,2Manα1,2Manα1,2Man-ol					
C1-H(δ,ppm)	4.850	4.883	5.148	5.386	5.225	--
C2-H(δ,ppm)	4.265	4.343	4.265	4.065	4.016	--

These representative shifts in both **b**'s and **c**'s anomerics on the reduction of Man to Man-ol have been documented previously (Funayama *et al*., 1983; Shibata *et al*., 1995). In keeping with the structure shown, β-mannosidase released two Man residues and provided a Man_3Man-ol tetrasaccharide alditol on Dionex PA1 chromatography. The intact hexositol was resistant to jackbean and *A. satoi* α-mannosidases (data not shown).

Conclusions

To date, only Man and α1,2-linked $Man_{2–4}$ polymers have been shown to constitute the O-linked glycans β-eliminated either from *Pichia* cell wall mannans or from recombinant kringle 1–4 domain (Duman *et al*., 1998) and mouse gelatinase B produced in *Pichia* (reviewed in Bretthauer and Castellino, 1999). The current study on recombinant hBSSL glycosylation have revealed the capability of *P. pastoris* to form a wider variety of O-linked glycan structures than previously known. Expectedly, the most abundant saccharides were α1,2-Man polymers of two and three residues, with a small percentage elongated to the α1,2-linked tetrasaccharide. Surprisingly, a portion of the tri- and tetrasaccharide were elongated further to pentaose and hexaose sizes by addition of a Manβ1,2Manβ1,2-cap (pools 5 and 6). Although these structures have been reported as minor components among the N-glycan side chains present in insoluble cell wall mannan from both *Candida* and *Pichia* sp. (Funayama *et al*., 1983; Kobayashi *et al*., 1986, 1989; Shibata *et al*., 1995), they have never been shown to be O-glycan constituents on *Pichia* sp. or any other fungal glycoproteins that we can document.

Extracts of *C. albicans* contain multiple β1,2-mannosyltransferases (Suzuki *et al*., 1995). Pathogenic *Candida* strains reveal few if any cell wall mannoproteins with only one β1,2-linked Man cap on the N-glycan side chains (Suzuki, 1997). In pathogenic *C. tropicalis* strains, ~15% of the side chains have two, whereas 5% have three nonreducing terminal β1,2-linked Man residues. This suggests that addition of the first nonreducing-end β1,2-linked Man by β1,2-mannosyltransferase I is rate limiting (Suzuki *et al*., 1995). *Pichia* does not appear to elongate the O-linked glycan β1,2-Man cap beyond two residues. It is unknown whether *Candida* sp. synthesize comparable α1,2-Man-containing O-glycans.

MALDI-TOF MS experiments revealed the presence—although in too small an amount for the glycosidic linkages to be established by NMR methods—of a phosphorylated O-glycan in pool 4 with a mass of 1095 Da. This glycan fragmented by PSD in a manner consistent with a H_2PO_3Man attached in diester linkage to the reducing-end Man-ol that was also substituted by a tetrasaccharide (Figure 5). Given the linkage structures of O-glycans defined here and elsewhere (Duman *et al*., 1998) and given the complement of mannosyltransferases present in *Pichia* (Verostek and Trimble, 1995), we speculate that the extended polymer is an α/β1,2-linked Man with PO_4Manα1,6-linked to the reducing-end Man-ol. ManPO_4 in diester linkage has been found α1,6-linked in N-glycan cores in *S. cerevisiae* mannan (Hernández *et al*., 1989) and in O-linked manno-oligosaccharides previously characterized on the Bar1p protease found in this organism (Jars *et al*., 1995).

β1,2-Man-linked mannose is immunogenic *in vivo*, and cell wall structures with β1,2-mannobiose caps described in this article constitute antigenic factor 6 in *C. albicans* and antigenic factor 9 in *Candida quilliermondii*, both of which are pathogenic in humans (reviewed in Suzuki, 1997). The capacity of *P. pastoris* to synthesize these structures, even in small amounts, should be of interest to academic researchers, but, more importantly, will have to be

considered in the context of the future application of this organism in industrial bioprocess design.

Materials and methods

Approximately 250 mg recombinant full-length hBSSL was purified from the culture broth of *P. pastoris* strain GS115 transformed with the BglII-digested plasmid pARC5799 (Sahasrabudhe *et al*., 1998). Bio-Gel P-4 (extra-fine mesh) was from Bio-Rad Laboratories (Hercules, CA) and $NaBH_4$, 99.8% D_2O, and 2,5-dihydroxybenzoic acid were from Sigma Chemical (St. Louis, MO). CIL (Cambridge, MA) supplied the 99.996% D_2O. NMR tubes (535 pp) were supplied by Wilmad Glass Works (Buena, NJ). *A. satoi* α1,2-mannosidase was from Glyco (formerly a product of Oxford Glycosystems) and Endo H (Trumbly *et al*., 1985) and jack bean α-mannosidase (Trimble *et al*., 1978) were prepared in our laboratory. *Helix pomatia* β-mannosidase was purchased from Seikagaku America (Ijamsville, MD). All solvents, sulfuric acid, and phenol were ACS reagent grade or better from commercial suppliers. Monosaccharide and monosaccharide alditol standards were obtained from Dionex (Sunnyvale, CA). [U-^{14}C]Man (279 μCi/umol) was purchased from Amersham Pharmacia Biotek (Piscataway, NJ). Dowex 50 H^+ (50–100 mesh) and Amberlite MB-3 mixed bed resin were from Fisher Scientific (Rochester, NY). SepPak C_{18} cartridges were supplied by Millipore (Bedford, MA).

N-linked oligosaccharides were hydrolyzed from SDS-denatured hBSSL with Endo H at 50 mU/ml overnight at 30°C (Trimble and Maley, 1984), and the released N-glycans were isolated by acetone precipitation followed by solubilization in 60% CH_3OH (Verostek *et al*., 2000). O-linked oligosaccharides were β-eliminated from Endo H–treated hBSSL by solubilization of the precipitated protein in 0.1 M NaOH in the presence of 1 M $NaBH_4$ (Gemmill and Trimble, 1999b). After overnight incubation at 45°C, residual $NaBH_4$ was destroyed by dropwise addition of glacial acetic acid. The mixture was passed over a 40-ml column of Dowex 50 H^+, and borate was removed from the eluate as methylborate by rotary evaporation three times from 1% acetic acid in methanol. The reduced O-glycan pool was solubilized in dH_2O; residual peptide and/or SDS was removed by passage through a 1 × 3 cm column of Amberlite MB-3 mixed bed resin prior to polishing by passage through a disposable C_{18} SepPak cartridge.

N- and O-linked glycans were concentrated by rotary evaporation to 1.5–2 ml and chromatographed on a 1.6 × 95 cm Bio-Gel P-4 (extra-fine mesh, lot 44671A) in 0.1 N acetic acid/1% butanol at 8.4 ml/h and room temperature. Fractions of 0.74–0.77 ml were collected and analyzed for neutral hexose by a modification (Byrd *et al*., 1982) of the phenol–sulfuric acid assay (Dubois *et al*., 1956).

MS of glycans was performed with a Bruker Reflex MALDI-TOF instrument. Sample spots of 25–50 pmol glycan were prepared in 2,5-dihydroxybenzoic acid matrix. Molecular weight data were obtained from the resultant average of 50–200 3-ns pulse shots from a 337 nm laser. A PSD spectrum of the pool 4 phosphorylated O-glycan was generated by isolation of a roughly 20 *m/z* window about the parent ion mass and recording the PSD product ions observed through 10 reflectron voltage reduction step spectra. These 10 spectra were then pasted together to form the final PSD spectrum.

The Endo H–released N-glycans and O-glycans in pools 2–6 were prepared for ^{1}H NMR spectroscopy in 99.996% D_2O at 1–2 mM final concentration in 0.5 cm NMR tubes as described (Ziegler *et al*., 1999). ^{1}H NMR 1D and 2D DQF COSY experiments were conducted on a Bruker Avance 500 MHz DRX spectrometer at 296 K as described (Cipollo and Trimble, 2000), and 2D relayed ROESY experiments to selectively detect 1,2-glycosidic linkages have been detailed (Cipollo *et al*., 2000). Some NMR spectra were analyzed using NMRPipe/NMRDraw software (Delaglio *et al*., 1995; available online at http://spin.niddk.nih.gov/bax/software/nmrpipe). After rechromatography of the pool 5 glycan (see *Discussion*), the remaining sample, exchanged with 99.996% D_2O, was examined in a Nalorac microprobe on the Varian 500 MHz spectrometer at the University of Colorado Health Sciences Center, and a portion was analyzed by methylation and MS fragmentation at the Boston University NIH-supported Mass Spectrometry Resource Center.

Analytical HPAEC of reduced O-glycan pools (Figure 4) was performed using a Dionex GP50 gradient pump system and ED-50 electrochemical detector on an 0.4 × 25 cm PA1 column employing PeakNet 6 software. The column was eluted isocratically with 16 mM NaOH at a flow rate of 1 ml/min. Man-ol was quantitated using an 0.4 × 25 cm MA1 column eluted at 0.4 ml/min with 0.26 M NaOH. At 29 min the NaOH was reduced to 0.15 M for 5 min prior to reequilibration in 0.26 M NaOH for 36 min.

Other methods

[^{14}C]Man-ol was prepared by reducing [U-^{14}C]Man (279 μCi/μmol) with 1 M $NaBH_4$ in 0.1 M $NaKB_3O_4$, pH 9.4, overnight at room temperature. Cations were removed after acidification with glacial CH_3COOH by passage through a Dowex 50 H^+ column. Borate was removed by rotary evaporation as described, and the [^{14}C]Man-ol was further purified by chromatoraphy on the Bio-Gel P-4 column described. Exoglycosidase digestions with jack bean α-mannosidase were conducted in 50 mM sodium acetate buffer, pH 4.5, 50 mM NaCl, and 0.1 mM zinc acetate; α1,2-mannosidase digestions were performed in 100 mM sodium acetate buffer, pH 5.0. β-Mannosidase assays were conducted in 10 mM sodium citrate/phosphate buffer, pH 5.0. After overnight hydrolysis at 30°C, ice-cold methanol was added to 60% final concentration, and after 1 h at −20°C, precipitated protein was removed by centrifugation. Supernatant fractions were flash-evaporated to dryness in the tip of a 5-ml screw-capped glass conical test tube. Samples were dissolved in 30 μl dH_2O, and a portion of each was subjected to Dionex PA1 chromatography as described. SDS–PAGE on 8% resolving gels was performed as described by Laemmli (1970).

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Abbreviations

COSY, correlation spectroscopy; DQF, double quantum filtered; Endo H, endo β-*N*-acetylglucosaminidase H; hBSSL, human bile salt–stimulated lipase; HPAEC, high-performance anion exchange chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PSD, post-source decay; ROESY, rotating-frame Overhauser spectroscopy; SDS, sodium dodecyl sulfate.

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