Registry No. MO, 111-03-5; DOPC, 4235-95-4; DMPC, 18194-24-6; NaO, 143-19-1; OA, 112-80-1; MA, 544-63-8; DOG, 25637-84-7.

REFERENCES

Caffrey, M. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 159-186.
Charvolin, J., & Sadoc, J. F. (1988) *J. Phys. 49*, 521-526.
Clerc, M., Levelut, A. M., & Sadoc, J. F. (1991) *J. Phys.* (in press).
Das, S., & Rand, R. P. (1986) *Biochemistry 25*, 2882-2889.
Eriksson, P. O., Lindblom, G., & Arvidson, G. (1987) *J. Phys. Chem. 91*, 846-853.
Gulik, A., Luzzati, V., DeRosa, M., & Gambacorta, A. (1985) *J. Mol. Biol. 182*, 131-149.
Heimburg, T., Ryba, N. J. P., Würtz, U., & Marsh, D. (1990) *Biochim. Biophys. Acta 1025*, 77-81.
International Tables for X-ray Crystallography (1952) Kynoch Press, Birmingham, U.K.
Larsson, K. (1983) *Nature (London) 304*, 664.
Luzzati, V., Tardieu, A., & Taupin, D. (1972) *J. Mol. Biol. 64*, 269-286.
Luzzati, V., Mariani, P., & Delacroix, H. (1988) *Makromol. Chem., Macromol. Symp. 15*, 1-17.
Mariani, P., Luzzati, V., & Delacroix, H. (1988) *J. Mol. Biol. 204*, 165-189.
Mariani, P., Rivas, E., Luzzati, V., & Delacroix, H. (1990) *Biochemistry 29*, 6799-6810.
Rançon, Y., & Charvolin, J. (1987) *J. Phys. 48*, 1067-1073.
Seddon, J. M. (1990) *Biochemistry 29*, 7997-8002.
Seddon, J. M., Bartle, E. A., & Mingins, J. (1990a) *J. Phys. Condens. Matter 2*, SA285-SA290.
Seddon, J. M., Hogan, J. L., Warrender, N. A., & Pebay-Peyroula, E. (1990b) *Prog. Colloid Polym. Sci. 81*, 189-197.
Shyamsunder, E., Gruner, S. M., Tate, M. W., Turner, D. C., So, P. T. C., & Tilcock, C. P. S. (1988) *Biochemistry 27*, 2332-2336.
Tardieu, A. (1972) Thesis, Université Paris-Sud.

Structural Characterization of the *N*-Glycans of a Recombinant Hepatitis B Surface Antigen Derived from Yeast†

Charlotte C. Yu Ip,‡ William J. Miller,‡ and Dennis J. Kubek§

Department of Cellular and Molecular Biology and Biochemical Process Research and Development, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

Anne-Marie Strang and Herman van Halbeek

Complex Carbohydrate Research Center and Department of Biochemistry, The University of Georgia, Athens, Georgia 30602

Susan J. Piesecki and Jack A. Alhadeff*

Department of Chemistry, Division of Biochemical Sciences, Lehigh University, Mountaintop Campus, Bethlehem, Pennsylvania 18015

Received June 12, 1991; Revised Manuscript Received August 29, 1991

ABSTRACT: The *N*-glycans of purified recombinant middle surface protein (preS2+S) from hepatitis B virus, a candidate vaccine antigen expressed in a mnn9 mutant strain of *Saccharomyces cerevisiae*, have been characterized structurally. The glycans were released by *N*-glycanase treatment, isolated by size-exclusion chromatography on Sephadex G-50 and Bio-Gel P-4 columns, and analyzed by 500-MHz ^{1}H NMR spectroscopy and fast atom bombardment mass spectrometry. The mixture of oligosaccharides was fractionated by HPLC, the major subfractions were isolated, and their carbohydrate compositions were determined by high-pH anion-exchange chromatography with pulsed amperometric detection. The combined results suggest that high-mannose oligosaccharides account for all the *N*-glycans released from preS2+S: structures include $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ isomers in the ratios of 3:6:1. Approximately 80% of the oligosaccharides contain the C2,C6-branched trimannosyl structural element typical of yeast high-mannose oligosaccharides but not usually found in high-mannose oligosaccharides in animal glycoproteins.

Hepatitis B is a major disease chronically afflicting more than 300 million people worldwide (Blumberg & London, 1982; Stephenne, 1990). The disease is caused by a hepatotropic DNA virus designated hepatitis B virus (HBV)[1] (Chisari et al., 1989). Disease manifestations range from asymptomatic infection to chronic severe and progressive liver disease associated with high morbidity and mortality (Blumberg & London, 1982; Stephenne, 1990). Prolonged hepatitis B infection over many years can lead to cirrhosis and a significantly increased risk (200-fold) of developing hepatocellular carcinoma

† This investigation was supported in part by National Institutes of Health Grant P41-RR-05351 from the Division of Research Resources (to H.v.H.).

* Author to whom correspondence should be addressed.
‡ Department of Cellular and Molecular Biology, Merck Sharp & Dohme.
§ Biochemical Process Research and Development, Merck Sharp & Dohme.

[1] Abbreviations: DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; FABMS, fast atom bombardment mass spectrometry; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HPAEC, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; ORF, open reading frame; PAD, pulsed amperometric detection; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; WEFT, water elimination Fourier transform.

0006-2960/92/0431-285$03.00/0 © 1992 American Chemical Society

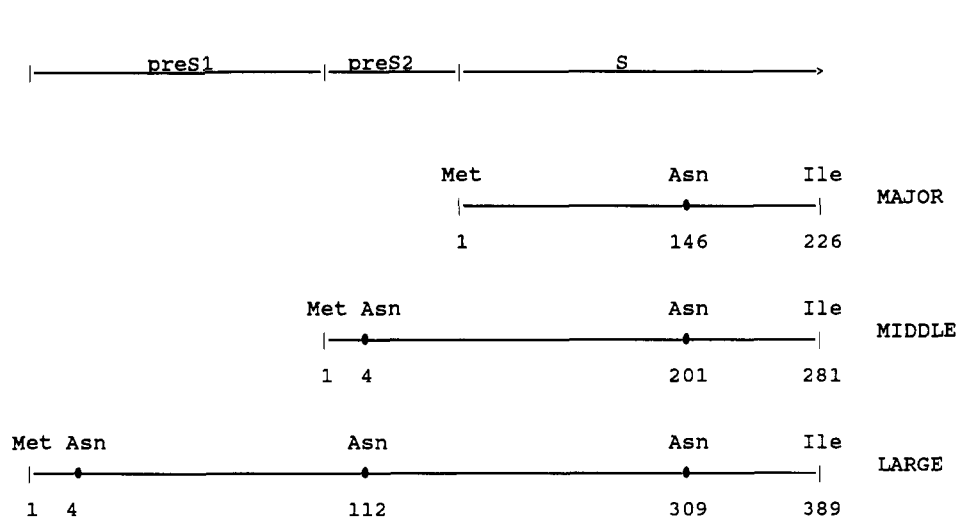


FIGURE 1: Diagram indicating the relationship of the major (S), middle (preS2+S), and large preS1+preS2+S) hepatitis B surface antigen proteins and the single open reading frame (ORF) from which they are derived.

(Beasley et al., 1981), a major cause of cancer death throughout the world. The control of HBV infection requires an effective vaccination program. First-generation vaccines were made from noninfectious hepatitis B surface antigen (HBsAg) particles extracted from the plasma of hepatitis B carriers (Francis, 1983). Since concern existed regarding a continuing supply of suitable carrier plasma to prepare the vaccine, more recent vaccines have been developed using recombinant DNA technology with expression of HBsAg proteins in eukaryotic hosts, primarily yeast (McAleer et al., 1984; Valenzuela et al., 1982; Miyanohara et al., 1983; Choo et al., 1985; Hitzman et al., 1983) and mammalian (Alexander et al., 1976; Moriarty et al., 1981; Dubois et al., 1980) cells. Yeast cells have proven to be the more widely accepted host system for productivity and regulatory reasons, and two yeast-derived vaccines (against the HBsAg S protein; see below) have been licensed in most countries worldwide (Ellis et al., 1988).

The HBV genome encodes several surface proteins which are products of the same open reading frame (ORF) (Peterson, 1987). The major protein, also called the S protein, is 226 amino acid residues long and, in glycosylated and nonglycosylated forms, comprises approximately 80% of the polypeptides in HBsAg particles. The middle protein (designated preS2+S) and "large" protein (designated preS1+preS2+S) contain varying NH_2-terminal extensions of the S protein and contain 281 and 389 amino acid residues, respectively (Peterson, 1987; Blum et al., 1989) (Figure 1). HBsAg generically refers to particles containing one or more of the polypeptides and is the key unit of immunogenicity. The preS2+S protein contains two asparagines in the appropriate sequon [Asn-X-Ser(Thr)] (Lehle & Bause, 1984) for N-glycosylation: Asn 4 and Asn 201 (the latter one corresponding to Asn 146 on the S protein) (Peterson, 1987; Blum et al., 1989). The complete amino acid sequences of the three polypeptides have been deduced from the cDNA, and more than 85% of the amino acid sequence of the S protein has been corroborated by mass spectrometric peptide mapping (Hemling et al., 1988). However, little is known about the structures of the carbohydrate moieties of these polypeptides. Structural information on the carbohydrate moieties is important for the development of recombinant vaccines primarily because there is evidence that carbohydrate moieties may influence the antigenicity of glycoproteins (Feizi, 1985). In addition, different host expression systems as well as different conditions for growing and harvesting cells can affect the glycosylation of cloned proteins quantitatively or qualitatively (Goochee & Monica, 1990).

Licensed yeast-derived hepatitis B vaccines consist of S polypeptides (Ellis et al., 1988). Yeast cells express these polypeptides which assemble into 20- to 22-nm lipoprotein particles similar to those isolated from human plasma (Stephenne, 1990; Peterson, 1987). Recently, yeast-derived preS2+S vaccines have been under evaluation as candidate hepatitis B vaccines with the hope of increased immunogenicity relative to S vaccines (Ellis et al., 1988). Studies on recombinant preS2+S proteins derived from yeast have suggested that the S protein domain is unglycosylated (Burnette et al., 1985) and that a high-mannose *N*-glycan is present on the preS2 portion of the middle protein (Langley et al., 1988). Since native N-linked glycans in wild-type yeast can have more than 100 Man residues (Ballou, 1986) which might compromise the immunogenicity of the preS2+S polypeptides, the preS2+S ORF was expressed in the mnn9 mutant of *Saccharomyces cerevisiae*, which is incapable of elaborating such hypermannosylated N-linked glycans (Tsai et al., 1984).

In the present investigation, the *N*-glycans of purified preS2+S derived from the mnn9 yeast mutant have been released by *N*-glycanase treatment and fractionated by size-exclusion chromatography (SEC) and high-performance liquid chromatography (HPLC). The structures of the *N*-glycans have been investigated by 500-MHz ^1H nuclear magnetic resonance (NMR) spectroscopy and fast atom bombardment mass spectrometry (FABMS). Compositional analysis of each of five HPLC-separated oligosaccharide fractions using high-pH anion-exchange chomatography with pulsed amperometric detection (HPAEC-PAD) gave results consistent with the structures proposed by NMR and FABMS analysis.

Experimental Procedures

Purification of PreS2+S Polypeptides. PreS2+S polypeptides were produced by expression in the *S. cerevisiae* mnn9 mutant (Tsai et al., 1984) and purified essentially as described previously (Ellis et al., 1988; Scolnick et al., 1984; Wampler et al., 1985). A 25% (w/v) slurry of recombinant yeast cells was lysed in a Stanstead press and clarified by phase separation in a mixture of poly(ethylene glycol) (PEG) 3350 and dextran T500 (Ellis et al., 1988). The antigen was adsorbed from the

upper PEG phase onto colloidal silica, eluted with hot borate buffer, and further purified by hydrophobic chromatography on hexyl-agarose with a reversed ammonium sulfate gradient followed by SEC on Superose 6 (Scolnick et al., 1984; Wampler et al., 1985). The product was treated with 3 M potassium thiocyanate (Ellis et al., 1988; Wampler et al., 1985) to effect disulfide cross-linking among the more than 1000 Cys residues in the ca. 100 preS2+S polypeptides in the purified 20-nm particle.

Digestion of PreS2+S with N-Glycanase. Five portions of purified preS2+S-containing particles (3 mg/portion) were incubated individually with 30 units of *N*-glycanase (Genzyme, Boston, MA) in 8.5 mL of 0.2 M sodium phosphate, pH 8.6, containing 0.36 mg of 1,10-*o*-phenanthroline (Genzyme) for 24 h at 37 °C. A control sample of preS2+S also was incubated under these conditions in the phosphate buffer without *N*-glycanase.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Electrophoresis was performed on preS2+S before and after *N*-glycanase digestion by the method of Laemmli (1970) using a precast 8–16% (w/v) acrylamide gradient gel from Novex (Encinitas, CA). The samples were reduced with dithiothreitol and 2% (w/v) SDS in boiling water for 10 min and electrophoresed for 1.5 h using the Novex mini-cell unit. Radiolabeled (^{14}C) molecular weight markers (0.5 μg/band) (Amersham, Arlington Heights, IL) included lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase *b* (92.5 kDa). One gel was silver stained (Morrissey, 1981), and a duplicate gel was electroblotted to a nitrocellulose membrane (Novex). The transfer was carried out using the MilliBlot-SDE electroblotting system (Millipore, Bedford, MA) according to the manufacturer's instruction. The antibodies used for immunoblotting were anti-p24, a polyclonal rabbit antibody raised against reduced and SDS-denatured HBsAg (Dennis-Sykes et al., 1985). The bound antibodies were detected with ^{125}I-labeled protein A (Amersham) and then autoradiographed for 12 h at −70 °C.

Concentration and Fractionation of PreS2+S Oligosaccharides. Each of the five *N*-glycanase-treated preS2+S samples (3 mg/sample) was concentrated individually on a Savant Speed Vac concentrator until the deglycosylated antigen began to precipitate out of solution (at approximately 4 mL). Each sample was centrifuged at 900*g* for 10 min at 22 °C, and the resulting supernatant fluids were subjected to SEC on a column (0.9 × 95 cm) of Sephadex G-50 (Pharmacia, Piscataway, NJ). The samples were applied and eluted with 50 mM pyridine–acetate buffer, pH 5.0, at an approximate flow rate of 30 mL/h at 22 °C, and 1.0-mL fractions were collected. Aliquots (50 μL) of these fractions were analyzed for protein (Lowry et al., 1951) and for carbohydrate by the resorcinol/H_2SO_4 method (Monsigny et al., 1988). A standard carbohydrate curve was determined over a range of 0.5–5.0 μg using a 5:1 molar mixture of Man/GlcNAc (Sigma, St. Louis, MO). The Sephadex G-50 column was calibrated with the following M_r standards: glycerol (92 Da), fucose (164 Da), stachyose (666 Da), bacitracin (1450 Da), melittin (2846 Da), and blue dextran (2 × 10^6 Da).

The carbohydrate-containing fractions from each of five runs on the Sephadex G-50 column were combined, lyophilized, and redissolved in 1.4 mL of 50 mM pyridine–acetate buffer, pH 5.0, and each individually was fractionated further on a column (0.9 × 150 cm) of Bio-Gel P-4 (Bio-Rad, Richmond, CA) resin at an approximate flow rate of 8.6 mL/h. Fractions (0.45 mL) were collected, and 100-μL aliquots were assayed for carbohydrate by the resorcinol/H_2SO_4 method (Monsigny et al., 1988). The P-4 column was calibrated with the following M_r standards: glycerol (92 Da), isomaltooctaose (1315 Da), melittin (2846 Da), and cytochrome *c* (12.5 kDa). The carbohydrate-containing fractions from the Bio-Gel P-4 columns were combined, lyophilized, reconstituted in 2.0 mL of Milli-Q H_2O, and relyophilized two more times. The final lyophilysates (containing 250 μg of carbohydrate) was dissolved in 2.0 mL of Milli-Q H_2O and divided into two portions: 1.2 mL (150 μg) for 500-MHz ^1H NMR and FABMS analyses; 0.8 mL (100 μg) for HPLC fractionation and compositional analysis by HPAEC-PAD (see below).

500-MHz ^1H NMR Spectroscopy. The oligosaccharides released from preS2+S by *N*-glycanase and purified by SEC on Sephadex G-50 and Bio-Gel P-4 were analyzed by ^1H NMR spectroscopy at 500 MHz. The oligosaccharide sample (150 μg) was dissolved repeated in ^2H_2O (99.8 and the 99.96 atom % ^2H, respectively; Aldrich, Milwaukee, WI) at room temperature and p^2H 6, with intermediate lyophilization. Prior to ^1H NMR spectroscopic analysis, the sample was redissolved in 0.5 mL of ^2H_2O (99.99 atom % ^2H; Cambridge Isotope Laboratories, Cambridge, MA) and transferred into a 5-mm NMR tube (535-PP; Wilmad, Buena, NJ). 500-MHz ^1H NMR spectroscopy was performed on a Bruker AM-500 spectrometer interfaced with an Aspect-3000 computer. The residual HO^2H resonance was suppressed with a water elimination Fourier transform (WEFT) pulse sequence that included a composite nonselective π-pulse (Haasnoot, 1983; Nilsen et al., 1991). The probe temperature was kept at 27 °C. Further experimental details have been described (Argade et al., 1988; Green et al., 1988; Spellman et al., 1989). Chemical shifts (δ) are expressed in parts per million downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), measured by reference to internal acetone (δ 2.225 in ^2H_2O at 27 °C) with an accuracy of 0.002 ppm.

Fast Atom Bombardment Mass Spectrometry (FABMS). A portion (40 μg) of the preS2+S oligosaccharides subjected to NMR analysis was reduced with $NaBH_4$ as described (Argade et al., 1988) and then per-O-acetylated using trifluoroacetic anhydride and acetic acid according to the procedure of Dell and Tiller (1986). The products were analyzed by FABMS on a VG ZAB-SE mass spectrometer. The sample was dissolved in methanol and mixed with thioglycerol and sodium acetate on the probe tip of the mass spectrometer. FAB was performed with 6-keV xenon atoms (10-mA emission current), and spectra were recorded in the positive-ion mode. Data were acquired over a mass range of 100–3650 amu [compare Nilsen et al. (1991)], with 1:1000 resolution (by the 10% overlap definition).

HPLC Separation of PreS2+S Oligosaccharides. A portion of the mixture of preS2+S oligosaccharides (100 μg) (isolated by Sephadex G-50 and Bio-Gel P-4 chromatography) was separated into individual oligosaccharide fractions using an HPLC system consisting of a Perkin-Elmer 410 LCBio pump, LC-235 diode array detector, SS100 autosampler, and solvent environment chamber. Oligosaccharides were fractionated on a Waters Carbohydrate Analysis column (4.6 × 250 mm) (Millipore) using isocratic elution with 43% H_2O/57% acetonitrile (HPLC grade, Fisher Scientific, Malvern, PA) at a flow rate of 1.2 mL/min. Detection of the oligosaccharides was by UV absorption at 200 nm.

Monosaccharide Compositional Analysis of Individual Oligosaccharide Fractions. Each of the HPLC-separated oligosaccharide fractions was individually hydrolyzed in

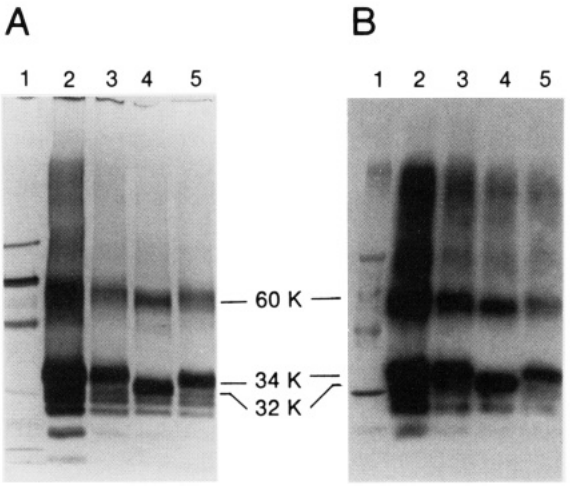


FIGURE 2: SDS–PAGE (panel A) and immunoblot (panel B) analyses of duplicate samples of preS2+S before and after *N*-glycanase treatment. Detection on panel A was with silver staining and on panel B (following transfer onto nitrocellulose) was with anti-p24 polyclonal antibodies, ^{125}I-labeled protein A, and autoradiography. (Lane 1) M_r markers, 0.5 μg/protein: lysozyme (14.4 kDa); soybean trypsin inhibitor (21.5 kDa); carbonic anhydrase (31 kDa); ovalbumin (45 kDa); bovine serum albumin (66 kDa); and phosphorylase *b* (92.5 kDa). (Lane 2) 4 μg of untreated preS2+S. (Lane 3) 1 μg of untreated preS2+S. (Lane 4) 1 μg of preS2+S treated with *N*-glycanase for 24 h at 37 °C. (Lane 5) 1 μg of preS2+S treated with sodium phosphate buffer (pH 8.6) for 24 h at 37 °C.

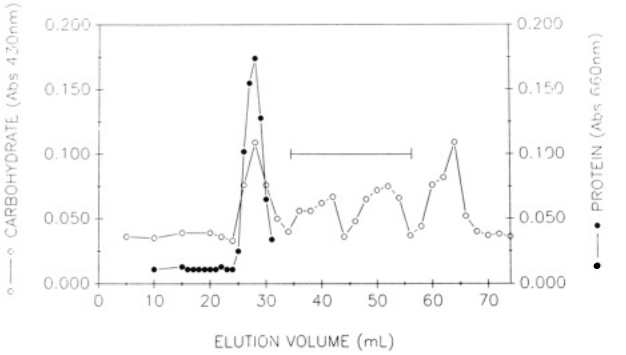


FIGURE 3: Elution profile from a Sephadex G-50 column (0.9 × 95 cm) of oligosaccharides derived from *N*-glycanase treatment of preS2+S. Fractions indicated by the bar represent pooled carbohydrate-positive material.

Reacti-Vials (Pierce, Rockford, IL) in 2 M trifluoroacetic acid (TFA) (Pierce) at 100 °C for 5 h (Hardy et al., 1988). TFA was removed from the hydrolysate by a stream of N_2, the sample was reconstituted in 100 μL of Milli-Q H_2O, and monosaccharides were analyzed using a system consisting of a Dionex BioLC gradient pump module (Dionex, Sunneyvale, CA) PAD, post-column delivery reservoir, and Spectra Physics 8880 autosampler. The Dionex eluent degas module was employed to sparge and pressurize the eluents with He. NaOH eluents were prepared by dilution of a 50% NaOH solution (Fisher Scientific) with degassed Milli-Q H_2O. Samples were injected using the autosampler equipped with a 200-μL sample loop and a Tefzel rotor seal to withstand the alkalinity of the eluents.

Monosaccharides were separated on a column (4.6 × 250 mm) of Dionex Carbopac PA1 pellicular anion-exchange resin, equipped with a Dionex AG-6 guard column, using a flow rate of 1 mL/min at 22 °C. Isocratic elution was carried out with 10 mM NaOH for 30 min with postcolumn delivery of 500 mM NaOH at a flow rate of 0.3 mL/min. The column was regenerated after each run by a 500 mM NaOH wash for 20 min followed by a 25-min equilibration with 10 mM NaOH. Detection of the separated monosaccharides was by PAD using a gold working electrode. The error limits for monosaccharide determinations were approximately ±5%. The pulsing potentials and durations used for monosaccharide analysis were as follows: $E_1 = 0.05$ V ($t_1 = 480$ ms), $E_2 = 0.60$ V ($t_2 = 300$ ms), $E_3 = -0.60$ V ($t_3 = 240$ ms). The response time of the PAD was set to 3 s. The resulting chromatographic data were analyzed using Nelson Turbochrom 2700 software. The chromatograms were plotted using a Hewlett Packard colorpro plotter.

RESULTS

The protein purity of preS2+S and the effectiveness of carbohydrate removal by *N*-glycanase were assessed by SDS–PAGE. Figure 2 depicts the silver-stained (panel A) and immunoblotted (panel B) results of SDS–PAGE before and after *N*-glycanase digestion. The major 34-kDa preS2+S polypeptide was reduced to 32 kDa with the apparent removal of the oligosaccharides from a single N-glycosylation site (lanes 3–5). Minor bands in the 16–30-kDa region represent the products of proteolytic cleavage of preS2+S as evidenced by amino acid sequence data (unpublished observations). Dimers and higher aggregates of preS2+S (Peterson, 1987) appear in the regions of 60 and 90 kDa. All bands appearing in the silver-stained gel (panel A) are immunoblot-reactive with anti-p24 antibody (Dennis-Sykes et al., 1985) (panel B). Densitometric scans of the results shown in panels A and B of Figure 2 (data not shown) indicate that the preS2+S (including the proteolytic fragments) is greater than 98% pure and the N-linked glycans of preS2+S appear to have an M_r of approximately 2 kDa. Densitometric scanning also suggests that approximately 95% of the N-linked carbohydrate was removed by *N*-glycanase treatment of preS2+S.

The *N*-glycanase-treated preS2+S was chromatographed on a column of Sephadex G-50 to separate the deglycosylated protein, any remaining glycosylated protein, and *N*-glycanase from the released intact oligosaccharides. The G-50 profile in Figure 3, which depicts typical results for 3.0 mg of *N*-glycanase-digested preS2+S, indicates a peak centered around 27 mL which is primarily protein with some carbohydrate, two intermediate M_r carbohydrate-positive broad peaks centered at 40 and 52 mL, respectively (accounting for approximately 66% recovery of the carbohydrate expected from 95% deglycosylation of preS2+S), and a low M_r carbohydrate-positive peak for glycerol (which is present in the purchased *N*-glycanase preparation) centered at 63 mL. The carbohydrate materials representing the approximate M_r's of oligosaccharides, designated by the bar in Figure 3 (34–56 mL), were combined, lyophilized, resuspended in 1.4 mL of pyridine–acetate buffer, pH 5.0, and chromatographed on the Bio-Gel P-4 column. The P-4 profile in Figure 4, representative of five runs, indicates one major carbohydrate-positive region (designated by the bar) from 50 to 57 mL which was combined and lyophilized. The recovery of carbohydrate from the P-4 column fractionation was approximately 45%. It is not understood why the P-4 column gave only one carbohydrate-positive peak while the G-50 column gave two such peaks, but these findings were consistent in the five P-4 and five G-50 fractionations. The five post-Bio-Gel P-4 samples were combined and found to contain a total of 250 μg of carbohydrate (Monsigny et al., 1988). This mixture of oligosaccharides, which represents an overall recovery of approximately 30%, was used for NMR and FABMS analyses as well as for HPLC fractionation and monosaccharide compositional analysis by HPAEC-PAD.

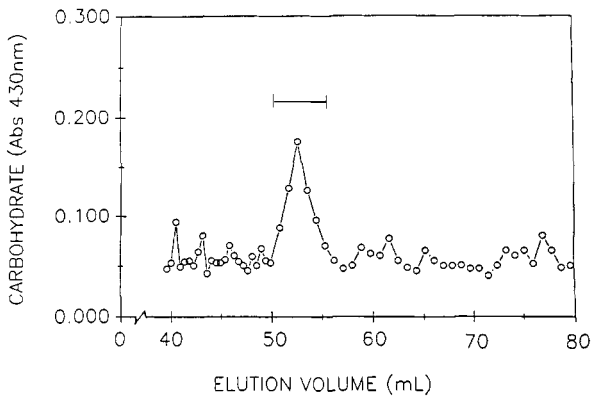


FIGURE 4: Elution profile from a Bio-Gel P-4 column (0.9 × 150 cm) of post-Sephadex G-50 preS2+S oligosaccharides. Fractions indicated by the bar represent pooled carbohydrate-positive material.

The ^{1}H NMR spectrum (Figure 5) of the oligosaccharides and the chemical shifts of the structural reporter groups (Table I) provided evidence for a mixture of high-mannose oligosaccharides differing in the number of mannosyl residues. No NMR evidence was found for the presence of *N*-acetyllactosamine- or hybrid-type *N*-glycans. The α-anomeric region ($4.8 < \delta < 5.5$ ppm) shows nine signals in nonintegral intensity ratios (see inset in Figure 5). Comparison of these data with the ^{1}H NMR spectra of other high-mannose oligosaccharides (Nilsen et al., 1991; Spellman et al., 1989; Vliegenthart et al., 1983; Trimble & Atkinson, 1986; Hernandez et al., 1989) indicates that the majority (~78%) of the high-mannose oligosaccharides contain a C2,C6-disubstituted $\alpha(1\rightarrow3)$mannosyl residue in the core, a structural element that is typical for yeast glycoprotein carbohydrates (Tsai et al., 1984; Hernandez et al., 1989) (see Table II, structures M7-I, M8-I, -III, and -IV, and M9-I, -II, and -III).

All components in the mixture appear to have an intact (reducing) *N*,*N*′-diacetylchitobiose unit, as usual for *N*-glycanase-released oligosaccharides, as evidenced by the presence in the ^{1}H NMR spectrum of the H-1 signals of free reducing GlcNAc-**1** at δ 5.192 and 4.70 (in ratio 3:1) for the α and β anomer of the oligosaccharides, respectively, the H-1 signal of GlcNAc-**2** at δ 4.602, in conjunction with the appearance of two NAc methyl signals at δ 2.038 (GlcNAc-**1**) and 2.065 (GlcNAc-**2**) (see Tables I and II for structures and numbering system). In addition, the two trimannosyl branch points common to high-mannose oligosaccharides were found intact in each component of the mixture as evidenced by signals for Man-**4** H-1 and Man-**4**′ H-1 at positions (δ 5.338 and 4.873) characteristic for substitution of Man-**4** at C2 (by Man-**C**) and disubstitution of Man-**4**′ at C3 and C6 (by Man-**A** and Man-**B**), respectively. The intensity ratio of the signals at δ 5.338 and 4.873 was 1:1; their intensities were set to 100% (see inset to Figure 5), to enable expression of the intensities of the other anomeric signals in a fraction of the molecules in the mixture. The error margins in the integrated intensities of the anomeric signals listed beneath the ^{1}H NMR spectrum are ±5%. Residue Man-**A** appeared terminal in virtually all (>97%) oligosaccharides in the mixture, on the basis of the absence of an H-1 signal at δ 5.40. The reason for the doubling of the Man-**A** H-1 signal [δ 5.104 (~30%) and 5.095 (~70%); combined intensity 100%; see Figure 5] will be explained below.

Heterogeneity was observed with respect to the substitution of Man-**C** by Man-$\mathbf{D_1}$ in $\alpha(1\rightarrow2)$-linkage, substitution of Man-**B** by Man-$\mathbf{D_3}$ in $\alpha(1\rightarrow2)$-linkage, extension of Man-**4** by Man-**E** in $\alpha(1\rightarrow6)$-linkage, and substitution of Man-**E**, in turn, by Man-$\mathbf{D_4}$ in $\alpha(1\rightarrow2)$-linkage. The signal at δ 5.301 is typical for H-1 for a Man-**C** residue which is substituted at C2 with Man-$\mathbf{D_1}$. The relative intensity of the signal at δ

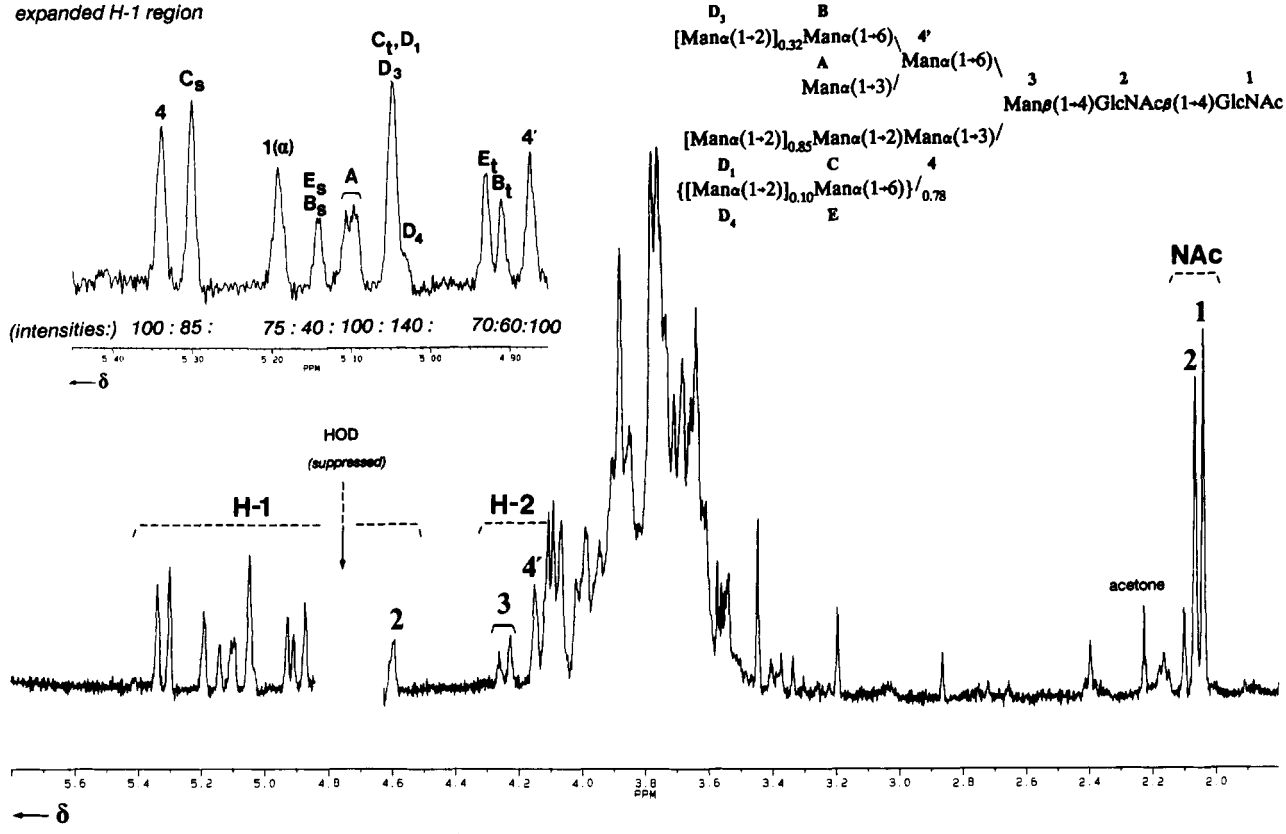


FIGURE 5: 500-MHz ^{1}H NMR spectrum (^2H_2O, p^{2}H 6, 27 °C) of the mixture of oligosaccharides derived from preS2+S. Inset: Anomeric region ($4.8 < \delta < 5.5$ ppm). The bold numbers in the spectrum refer to the corresponding monosaccharide residues in the structure. The subscripts s and t stand for substituted and terminal, respectively.

Table I: ^{1}H Chemical Shifts of Structural Reporter Groups of Constituting Monosaccharides for Oligosaccharides Released from Recombinant preS2+S by *N*-Glycanase and for Some Pertinent Reference Oligosaccharides

		chemical shift[b] (ppm) in[c]						
		preS2+S[d]		allergen *Art*vII from pollen[e]		WT yeast invertase		*Saccharomyces cerevisiae* mnn1mnn9 mutant[f]
		$Man_{7-9}GlcNAc_2$		$Man_6GlcNAc_2$	$Man_9GlcNAc_2$	$Man_9GlcNAc$		$Man_{10}GlcNAc$
reporter group	residue[a]	nonextended	extended[g]					
H-1	GlcNAc-**1**α[h]	5.192	5.192	5.189	5.189			
	GlcNAc-**1**β[h]	4.70	4.70	4.698	4.698			
	GlcNAc-**2**α[i]					5.249	5.243	5.243
	GlcNAc-**2**β	4.602	4.602	4.597	4.597	nd[j]	4.178	4.723
	Man-**3**	4.77	4.77	4.765	4.765	4.782	4.778	4.778
	Man-**4**	5.338	5.338^{D_1,E}	5.340	5.340	5.347	5.343	5.343
	Man-**C**	5.048	5.301^{D_1}	5.046	5.301	5.304	5.297	5.293
	Man-D_1		5.048		5.046	5.050	5.053	5.053
	Man-**4**′	4.873	4.873	4.870	4.870	4.874	4.875	4.878
	Man-**A**	5.104	5.095^{E}	5.093	5.093	5.085(α)[k]	5.093(α)[k]	5.093(α)[k]
						5.115(β)	5.118(β)[k]	5.118(β)[k]
	Man-D_2				5.046			
	Man-**B**	4.910	5.141^{D_3}	4.909	5.142	5.147	5.133	5.133
	Man-D_3		5.048		5.046	5.042	5.043	5.043
	Man-**E**	4.931	5.141^{D_4}			4.932	4.933	5.133
	Man-D_4		5.042					5.053(α)[k]
								5.063(β)[k]
H-2	Man-**3**	4.226	4.261^{E}	4.230	4.230	4.166(α)[k]	4.17	nd
						4.158(β)[k]		
	Man-**4**	4.106	4.106	nd	nd	4.089	4.10	nd
	Man-**C**	4.067	4.092^{D_1}	nd	nd	4.117	nd	nd
	Man-D_1		4.067	nd	nd	4.074	nd	nd
	Man-**4**′	4.149	4.149	4.143	4.143	4.150	4.15	nd
	Man-**A**	4.067	4.067	nd	nd	4.053		nd
	Man-D_2				nd			
	Man-**B**	3.988	4.018^{D_3}	nd	nd	4.027	nd	nd
	Man-D_3		4.067		nd	4.074	nd	nd
	Man-**E**	3.988	4.018^{D_4}			3.991	nd	nd
	Man-D_4		4.067					nd
NAc	GlcNAc-**1**	2.038	2.038	2.039	2.039		nd	nd
	GlcNAc-**2**	2.065	2.065	2.064	2.064	2.045[j]	nd	nd

[a] For numbering and lettering of monosaccharide residues, see Figure 5. [b] Data were acquired at 500 MHz for neutral solutions of the compounds in ^2H_2O at 27 °C, except for the *S. cerevisiae* mnn1mnn9 oligosaccharides. Those data were acquired at 40 °C (Hernandez et al., 1989), and converted from the reported values by adding 0.008 ppm, to compensate for the differences in the chemical shift values used for the internal standard [δ acetone was taken to be 2.217 ppm by Hernandez et al. (1989)]. [c] Structures are schematically illustrated in the column headings by using the number-and-letter representation for the constituting monosaccharides (see Figure 5). The data for reference compound $Man_9GlcNAc$ from wild-type yeast invertase have not been published before (H. van Halbeek, unpublished results); they may, however, be compared to those published by Trimble and Atkinson (1986) and Hernandez et al. (1989). [d] This study. [e] From Nilsen et al. (1991). [f] From Hernandez et al. (1989). [g] The boldface superscripts in this column indicate the residue(s) responsible for the observed effect in chemical shift compared to in the nonextended column. [h] In oligosaccharides ending in the reducing chitobiose unit, H-1 of GlcNAc-**1** is the only structural reporter group whose chemical shift shows a significant anomerization effect (see text). [i] In oligosaccharides ending in the reducing chitobiose unit, GlcNAc-**2** is β-linked (to GlcNAc-**1**). However, GlcNAc-**2** is the reducing residue in the endo H released oligosaccharides in the last three columns. Therefore, its structural reporter groups experience the anomerization effect. [j] nd, value could not be determined. In this case, the H-1 signal of GlcNAc-**2** in the β-anomer is hidden under the residual HO^2H peak at 27 °C. [k] In oligosaccharides ending in reducing GlcNAc-**2**, Man-**A** H-1, Man-D_4 H1 (Hernandez et al., 1989), and Man-**3** H-2 show profound anomerization effects [see also Vliegenthart et al. (1983)].

5.301 being 85% indicates that ~15% of the molecules in the mixture are lacking the Man-D_1 residue. The presence of the signal at δ 4.910 (relative intensity 60–65%) suggests that ~65% of the molecules in the mixture contain Man-**B** in terminal position. Consequently, the remaining 35% of the molecules in the mixture have Man-**B** substituted at C2 by Man-D_3; the H-1 signal of substituted Man-**B** contributes to the intensity of the resonance observed at δ 5.141. The presence of Man-**E** in the terminal position in a portion of the molecules in the mixture was inferred from the presence of an H-1 signal at δ 4.931 (Trimble & Atkinson, 1986; Hernandez et al., 1989). The location of the $\alpha(1\rightarrow6)$-linked Man residue designated **E**, typical for high-mannose structures in yeast glycoproteins, to Man-**4** (rather than to Man-**B**) is based on mass spectrometric studies of similar oligosaccharides (only differing from the oligosaccharides released from hepatitis B surface antigen in the absence of GlcNAc-**1**) (see Table I) (Hernandez et al., 1989). The relative intensity of the signal at δ 4.931 suggests that ~70% of the molecules in the mixture contain Man-**E** as a terminal extension of Man-**4**. The relative intensity of the signal at δ 5.141 (40%) suggests that an additional 5–10% of the molecules in the mixture have Man-**E** substituted by an $\alpha(1\rightarrow2)$-linked mannosyl residue, denoted Man-D_4. The presence of Man-**E** is known not to affect the chemical shift of Man-**4** H-1 or that of Man-**C** H-1. However, it apparently does have a minor effect on the chemical shift

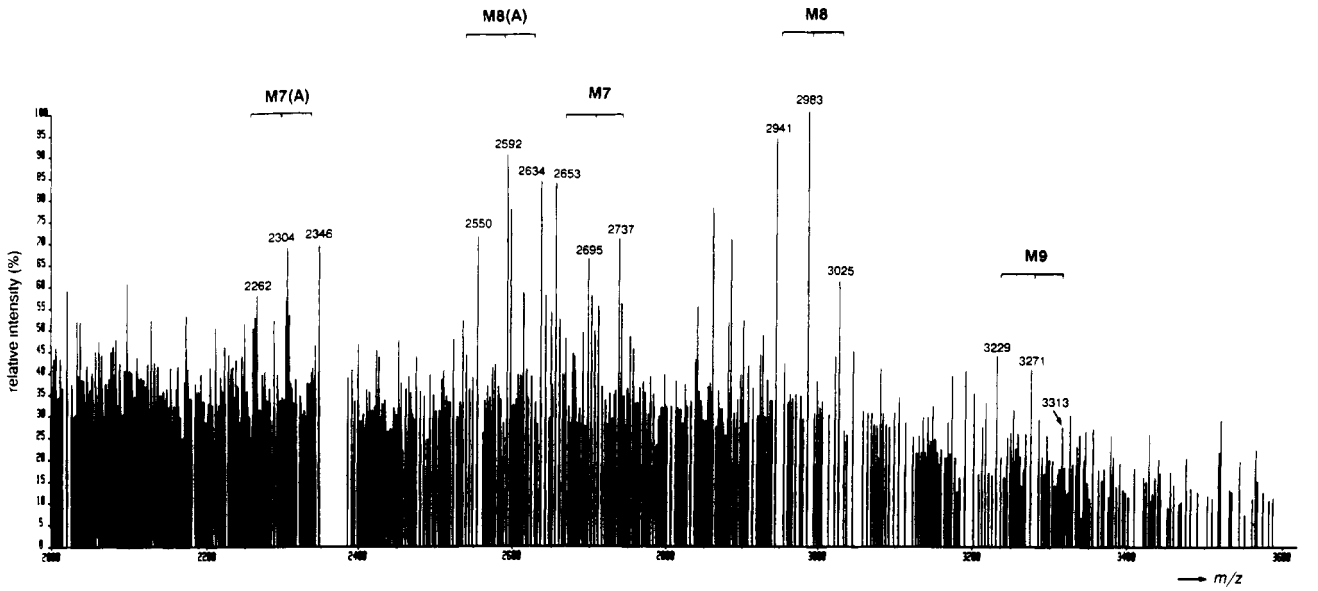


FIGURE 6: Relevant portion ($2000 < m/z < 3620$) of the FAB mass spectrum of the per-O-acetylated, reduced oligosaccharides derived from preS2+S. The numbers in the spectrum are the nominal masses of the corresponding fragment ions. M7, M8, and M9 denote the ion clusters corresponding to $[M + H]^+$, $[M + H]$ minus ketene$]^+$ and $[M + H]$ minus 2× ketene$]^+$ for $Man_7GlcNAc$–GlcNAc-ol, $Man_8GlcNAc$–GlcNAc-ol, and $Man_9GlcNAc$–GlcNAc-ol, respectively. M7(A) and M8(A) denote the A-fragments (loss of GlcNAc-ol) from M7 and M8, respectively.

of H-1 of Man-A: the latter signal shifts from δ 5.104 to 5.095 (Table I). The H-2 signal of Man-3 had been observed before (Trimble & Atkinson, 1986; Hernandez et al., 1989), for oligosaccharides ending in GlcNAc-2, to undergo an upfield shift due to the presence of Man-E. We observed two signals for H-2 of Man-3, the downfield one of which (δ 4.261) was assigned to the structures that lack Man-E, the other one (δ 4.226) to the structures that contain Man-E, on the basis of their relative intensities (25% and 75%, respectively).

The comprehensive structure of the proposed high-mannose oligosaccharides released from preS2+S, including the relative abundance of each of the four residues not present in all of the compounds in the mixture, is given on the top right portion of Figure 5. The intensity ratios of the H-1 signals in the ^1H NMR spectrum of the oligosaccharides are consistent with the presence of up to 12 different components occurring in the mixture, namely, the basic $Man_6GlcNAc_2$ structure, three $Man_7GlcNAc_2$ isomers, four $Man_8GlcNAc_2$ isomers, three $Man_9GlcNAc_2$ isomers, and a $Man_{10}GlcNAc_2$ compound (see Table II).

To verify the molecular mass of the oligosaccharides present in the mixture, a portion (40 μg) of the oligosaccharides was reduced by $NaBH_4$, desalted, O-acetylated, and analyzed by FABMS. Isotopomeric ions of high-mass ion clusters were not resolved under the low-resolution conditions used. Therefore, the VG software was used to determine the centroid m/z values corresponding to the average mass of the ion clusters. The centroid m/z values were converted readily into the nominal masses of the isotopomers containing only ^{12}C, ^1H, ^{14}N, and ^{16}O atoms using the CARBOMASS software developed by W. S. York at the CCRC (Athens, GA) (Kiefer et al., 1990). The FAB mass spectrum (Figure 6) showed clusters of peaks at the m/z values 2737 $[M + H]^+$, 2695 $[M + H$ minus ketene$]^+$, and 2653 $[M + H$ minus ketene$]^+$, and corresponding A-type fragments (loss of GlcNAc-ol from the reduced end of the oligosaccharide) at m/z 2346, 2304, and 2262; this set of six peaks suggested the presence of $Man_7GlcNAc$–GlcNAc-ol. The analogous series of peaks observed at m/z values 3025, 2983, and 2941 with the A-type fragments at m/z 2634, 2592, and 2550, suggested the presence of $Man_8GlcNAc$–GlcNAc-ol, in the sample. The evidence for a $Man_9GlcNAc$–GlcNAc-ol species in the sample was inferred from the observation of (low-intensity) peaks at m/z 3313, 3271, and 3229; the corresponding A-type fragment ions were not observed. No pseudomolecular ion clusters were observed at 2449 and 3601, thereby making it unlikely that $Man_6GlcNAc$–GlcNAc-ol and $Man_{10}GlcNAc$–GlcNAc-ol, respectively, occur in relative abundance >2%. Thus, 10 oligosaccharide structures remain to be considered as likely candidates for the preS2+S-derived components (Table II).

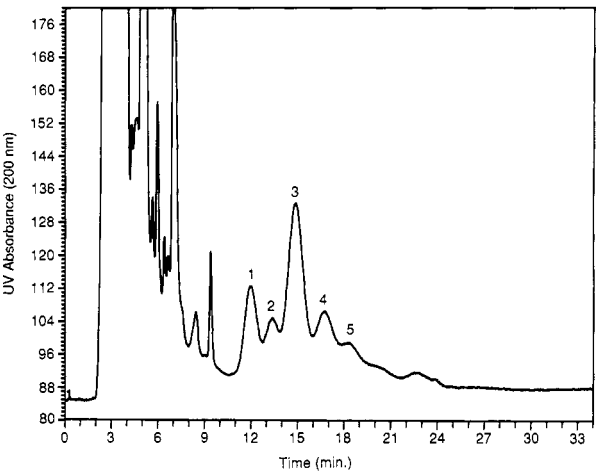


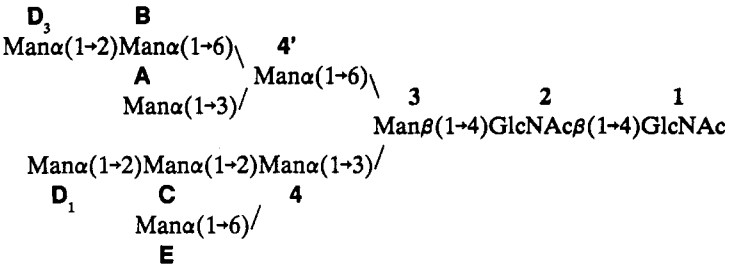
FIGURE 7: HPLC elution profile of preS2+S oligosaccharides from a Water's carbohydrate analysis column using isocratic elution with acetonitrile/H_2O (57:43).

The mixture of oligosaccharides from preS2+S was fractionated by HPLC on a Waters carbohydrate analysis column (Figure 7). Five major peaks (eluting at 11–19 min) were identified, collected, hydrolyzed, and analyzed for monosaccharide composition by HPAEC-PAD. The peaks eluting at 2–10 min are solvent artifacts as evidenced by the fact that they are seen when only solvent is injected into the HPLC. Potential peaks centered around 20, 22.5, and 24 min contained insufficient amounts of material for compositional analysis. The five major peaks from the carbohydrate analysis column (Figure 7) each contained only GlcN and Man, providing further evidence that all N-linked oligosaccharides of preS2+S

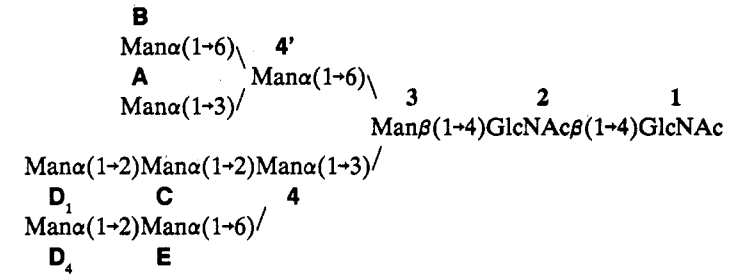
Table II: Structures Proposed[a] for the Asn-Linked Oligosaccharides Isolated from Yeast-Derived Recombinant preS2+2

Structure	Estimated Relative Abundance

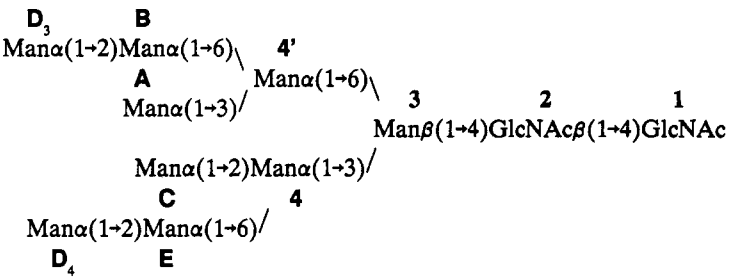
$Man_9GlcNAc_2$ isomer I (M9-I)



$Man_9GlcNAc_2$ isomer II (M9-II)

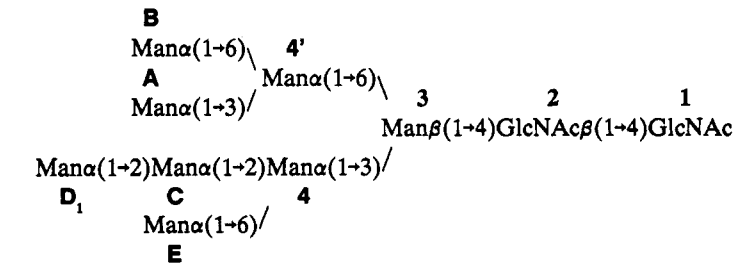


$Man_9GlcNAc_2$ isomer III (M9-III)



M9-I + M9-II + M9-III = 10%

$Man_8GlcNAc_2$ isomer I (M8-I)



$Man_8GlcNAc_2$ isomer II (M8-II)

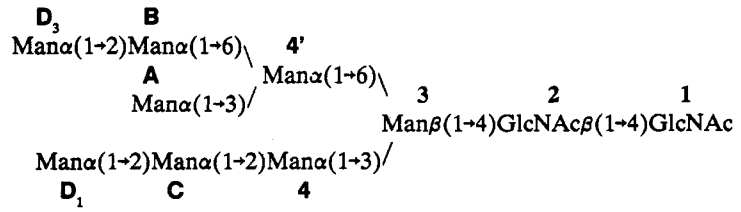
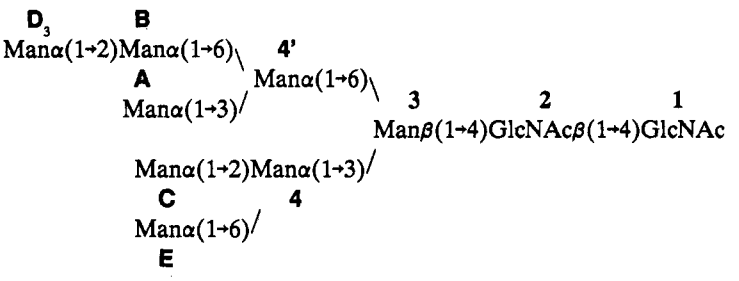
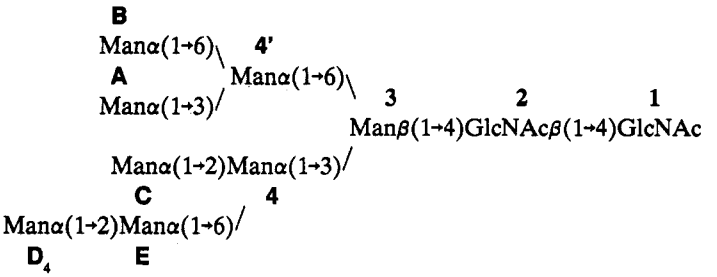
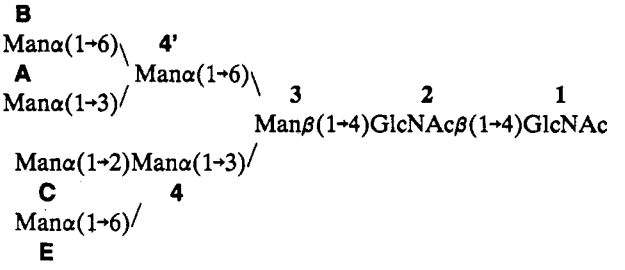
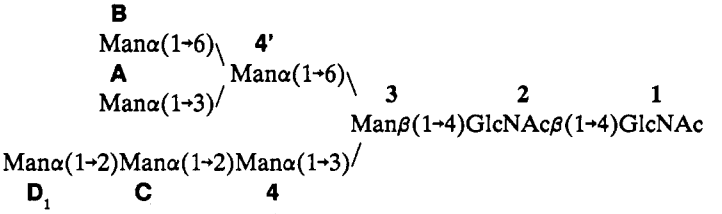
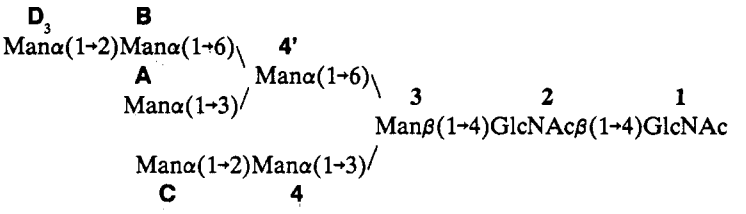


Table II (Continued)

Structure	Estimated Relative Abundance
$Man_8GlcNAc_2$ isomer III (M8-III) 	
$Man_8GlcNAc_2$ isomer IV (M8-IV) 	*M8-I + M8-II + M8-III + M8-IV* = 62%
$Man_7GlcNAc_2$ isomer I (M7-I) 	
$Man_7GlcNAc_2$ isomer II (M7-II) 	
$Man_7GlcNAc_2$ isomer III (M7-III) 	*M7-I + M7-II + M7-III* = 28%

[a] The data presented provide evidence that the oligosaccharides released from preS2+S are exclusively high-mannose structures, with compositions $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ in the ratio of 28:62:10. The detailed primary structures in this table are tentative, in part on the basis of the assumption that the structures assemble according to the known set of biosynthetic pathways for yeast glycoproteins. The combination of techniques we used for the structural characterization of this complicated mixture of oligosaccharides does not rule out entirely the presence of minor amounts of unexpected isomers of the structures listed above, of $Man_6GlcNAc_2$ and/or $Man_{10}GlcNAc_2$ or of novel saccharide linkages.

Table III: Relative Amounts of Five Major Oligosaccharide Peaks Separated by HPLC (See Figure 7) and Their Monosaccharide Composition as Determined by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

peak no.	retention time (min)	relative amount (area %)		molar ratio[a] (GlcNAc:Man)	integral molar ratio (GlcNAc:Man)
1	11.5–12.8	17	28	1.10:3.93	2:7
2	13.1–14.0	11		0.35:1.21	2:7
3	14.3–16.4	42	62	0.55:2.27	2:8
4	16.6–18.0	20		0.58:2.43	2:8
5	18.1–19.0	10		0.32:1.47	2:9

[a] The error limits for the monosaccharide determinations were approximately ±5%.

are high-mannose structures. The relative amounts of the five oligosaccharide fractions separated by HPLC and their molar ratios of GlcN (GlcNAc prior to hydrolysis) to Man are summarized in Table III and suggest the presence of at least two isomers of $Man_7GlcNAc_2$, at least two isomers of $Man_8GlcNAc_2$, and at least one $Man_9GlcNAc_2$ with relative total abundances of 28:62:10, respectively. Each of the five oligosaccharide fractions could contain more than one isomeric species which were not separable under the HPLC conditions employed. The compositional data are consistent with the NMR and FABMS results.

Discussion

Studies on HBsAg proteins derived from human plasma have provided direct evidence for diantennary *N*-acetyllactosamine-type *N*-glycans with and without sialic acid residues (Hanaoka et al., 1986; Gillece-Castro et al., 1987), and circumstantial evidence has been provided from glycosidase digestions (endo H and neuraminidase) for a mannose-rich *N*-glycan and, surprisingly, terminal sialic acid residues (Stibbe & Gerlich, 1982). Peterson et al. (1982) also have provided evidence from radiolabel incorporation studies (and selective removal of the label) that sialic acid is present in the carbohydrate moiety of the S protein of HBsAg derived from human plasma.

As indicated previously, studies on recombinant preS2+S proteins derived from yeast have suggested that the S domain is not glycosylated (Burnette et al., 1985) and the preS2 domain contains a single high-mannose *N*-glycan (Langley et al., 1988). In the latter studies, all of the carbohydrate could not be removed by endo H or endo F treatments, and therefore the investigators speculated that *O*-glycans might be present and linked to serine and/or threonine residues on the preS2 region (Langley et al., 1988). Itoh et al. (1986) also have provided evidence that yeast-derived preS2+S protein contains both N-linked and O-linked glycans by removing carbohydrate using endo H digestion and mild alkali treatment, respectively. However, there is increasing evidence that some N-linked glycans can be released by mild alkali treatment (Argade et al., 1989; Debray et al., 1984); thus, there is no definitive evidence to date for O-glycosylation in preS2+S proteins. Our preS2+S is presumed to contain carbohydrate only on Asn 4 since amino acid sequencing from the N-terminus indicated that the fourth amino acid was blocked. In addition, an engineered point mutant form of preS2+S with Asn 4 converted to Gln 4 contained no carbohydrate and a significantly lower M_r on SDS–PAGE (unpublished data).

In the present investigation we have analyzed the structures of the *N*-glycans derived from preS2+S, which was expressed in the mnn9 mutant of *S. cerevisiae* as a candidate vaccine antigen. High-field ^1H NMR spectroscopy suggested that only high-mannose oligosaccharides were present including $Man_7GlcNAc_2$ isomers, $Man_8GlcNAc_2$ isomers, and $Man_9GlcNAc_2$ isomers (see Table II for tentative structures). The FABMS data are consistent with the results obtained by NMR spectroscopy in demonstrating the presence of three species that are different in molecular mass, i.e., $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$, and helping to rule out the occurrence of $Man_6GlcNAc_2$ and $Man_{10}GlcNAc_2$ species. The relatively low intensity of the peaks indicative for $Man_9GlcNAc_2$ suggests that the latter isomeric species are not a major component of the sample, as was indicated from the NMR data. However, care has to be taken when FABMS data is being interpreted in a quantitative fashion. The relative amounts of each of the tentatively proposed structures in Table II could not be calculated precisely. However, integration of the appropriate peaks in the HPLC chromatogram used to fractionate the mixture of oligosaccharides (Figure 7) provided the ratios for total $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ species. The structural similarity of the oligosaccharides and the small amount of sample available possibly prevented the complete HPLC separation of each of the potential species present. Each of the major HPLC peaks was isolated and hydrolyzed by TFA, and the relative amounts of the constituent monosaccharides were determined by HPAEC-PAD (Table III). Each peak contained only Man and GlcN, presumably GlcNAc prior to hydrolysis (as evidenced by the chemical shifts of the GlcNAc *N*-acetyl reporter groups given in Table I). The molar ratios of Man and GlcNAc for the five oligosaccharide fractions (Table III) are consistent with the structures proposed from the NMR and FABMS data. Therefore, the preS2+S oligosaccharides appear to include a mixture of high-mannose structures composed of two GlcNAc residues and 7–9 Man residues (Table II). The majority (~80%) of the oligosaccharides contain the 2,6-branched trimannosyl structural element typical of yeast high-mannose oligosaccharides (Hernandez et al., 1989), but not usually found in high-mannose oligosaccharides in animal glycoproteins. It remains to be seen whether yeast-derived glycosylated preS2+S is a better (more immunogenic) antigen than yeast-derived S polypeptides for the production of hepatitis B vaccines.

Acknowledgments

We thank Mr. William S. York (CCRC, Athens, GA) for expert assistance with the FABMS; Peter J. Kniskern, Arpi Hagopian, and Loren Schultz for the molecular biology contributions; D. Eugene Wampler for antigen purification; and Ronald W. Ellis for helpful discussions. We acknowledge Mrs. Fern Stozing for excellent secretarial help.

References

Alexander, J. J., Bey, E. M., Geddes, E. W., & Lecatsus, G. S. (1976) *S. Afr. Med. J. 50*, 2124–2128.

Argade, S., Hopfer, R. L., Strang, A.-M., Van Halbeek, H., & Alhadeff, J. A. (1988) *Arch. Biochem. Biophys. 266*, 227–247.

Argade, S., Daves, G. D., Jr., Van Halbeek, H., & Alhadeff, J. A. (1989) *Glycoconjugate J. 6*, 45–56.

Ballou, C. E. (1986) *Adv. Microb. Physiol. 14*, 93–158.

Beasley, R. P., Lin, C.-C., Hwang, L. Y., & Chen, C.-S. (1981) *Lancet*, 1129–1133.

Blum, H. E., Gerok, W., & Vyas, G. N. (1989) *Trends Genet. 5*, 154–158.

Blumberg, B. S., & London, W. T. (1982) *Cancer 50*, 2657–2665.

Burnette, W. N., Samal, B., Fieschko, J. C., Langley, K. E., & Bitter, G. (1985) in *Hepatitis Viruses and Hepatocellular Carcinoma* (Nishioka, K., Ed.) pp 143–149, Academic Press, Tokyo, Japan.

Chisari, F. V., Ferrari, C., & Mondelli, U. (1989) *Microb. Pathog. 6*, 311–325.

Choo, K.-B., Wu, S.-M., Lee, H.-H., & Lo, S. J. (1985) *Biochem. Biophys. Res. Commun. 131*, 160–166.

Debray, H., Strecker, G., & Montreuil, J. (1984) *Biochem. Soc. Trans. 12*, 611–612.

Dell, A., & Tiller, P. R. (1986) *Biochem. Biophys. Res. Commun. 135*, 1126–1134.

Dennis-Sykes, C. A., Miller, W. J., & McAleer, W. J. (1985) *J. Biol. Stand. 13*, 309–314.

Dubois, M. F., Pourcel, C., Rousset, S., Chany, C., & Tiollais, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4549–4553.

Ellis, R. W., Kniskern, P. J., Hagopian, A., Schultz, L. D., Montgomery, D. L., Maigetter, R. Z., Wampler, D. E., Emini, E. A., Wolanski, B., McAleer, W. J., Hurni, W. M., & Miller, W. J. (1988) in *Viral Hepatitis and Liver Disease* (Zuckerman, A. J., Ed.) pp 1079–1086, Alan R. Liss, Inc., New York.

Feizi, T. (1985) *Cancer Surveys 4*, 245–269.

Francis, D. P. (1983) *Infect. Dis. Rev. 5*, 322–335.

Gillece-Castro, B. L., Fisher, S. J., Tarentino, A. L., Peterson, D. L., & Burlingame, A. L. (1987) *Arch. Biochem. Biophys. 256*, 194–201.

Goochee, C. F., & Monica, T. (1990) *Bio/Technology 8*, 421–427.

Green, E. D., Adelt, G., Baenziger, J. U., Wilson, S., & Van Halbeek, H. (1988) *J. Biol. Chem. 263*, 18253–18268.

Haasnoot, C. A. G. (1983) *J. Magn. Reson. 52*, 153–158.

Hanaoka, K., Takasaki, S., Kobata, A., Miyamoto, H., Nakamura, T., & Mayumi, M. (1986) *J. Biochem. 99*, 1273–1276.

Hardy, M. R., Townsend, R. R., & Lee, Y. C. (1988) *Anal. Biochem. 170*, 54–62.

Hemling, M. E., Carr, S. A., Capian, C., & Petre, J. (1988) *Biochemistry 27*, 699–705.

Hernandez, L. M., Ballou, L., Alvarado, E., Gillece-Castro, B. L., Burlingame, A. L., & Ballou, C. E. (1989) *J. Biol. Chem. 264*, 11849–11856.

Hitzman, R. A., Chen, C. Y., Hagie, F. E., Patzer, E. J., Liu, C.-C., Estell, D. A., Miller, J. V., Yaffe, A., Kleid, D. G., Levinson, A. D., & Oppermann, H. (1983) *Nucleic Acids Res. 11*, 2745–2763.

Itoh, Y., Hayakawa, T., & Fujisawa, Y. (1986) *Biochem. Biophys. Res. Commun. 138*, 268–274.

Kiefer, L. L., York, W. S., Albersheim, P., & Darvill, A. G. (1990) *Carbohydr. Res. 197*, 139–158.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Langley, K. E., Egan, K. M., Barendt, J. M., Parker, C. G., & Bitter, G. A. (1988) *Gene 67*, 229–245.

Lehle, L., & Bause, E. (1984) *Biochim. Biophys. Acta 799*, 246–251.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McAleer, W. J., Buynak, E. B., Maigetter, R. Z., Wampler, D. E., Miller, W. J., & Hilleman, M. R. (1984) *Nature 307*, 178–180.

Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N., & Matsubara, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1–5.

Monsigny, M., Petit, C., & Roche, A. (1988) *Anal. Biochem. 175*, 525–530.

Moriarty, A. M., Hoyer, B. H., Shih, J. W.-K., Gerin, J. L., & Hamer, D. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2606–2610.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Nilsen, B. M., Sletten, K., Paulsen, B. S., O'Neill, M., & Van Halbeek, H. (1991) *J. Biol. Chem. 266*, 2660–2668.

Peterson, D. L. (1987) *BioEssays 6*, 258–262.

Peterson, D. L., Nath, N., & Gavilanes, F. (1982) *J. Biol. Chem. 257*, 10414–10420.

Scolnick, E. M., McLean, A. A., West, D. J., McAleer, W. J., Miller, W. J., & Buynak, E. B. (1984) *J. Am. Med. Assoc. 251*, 2812–2815.

Spellman, M. W., Basa, L. J., Leonard, C. K., Chakel, J., O'Connor, J. V., Wilson, S., & Van Halbeek, H. (1989) *J. Biol. Chem. 264*, 14100–14111.

Stephenne, S. (1990) *Vaccine 8* (Suppl.), S69–S73.

Stibbe, W., & Gerlich, W. H. (1982) *Virology 123*, 436–442.

Trimble, R. B., & Atkinson, P. H. (1986) *J. Biol. Chem. 261*, 9815–9824.

Tsai, P.-K., Frevert, J., & Ballou, C. E. (1984) *J. Biol. Chem. 259*, 3805–3811.

Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G., & Hall, B. D. (1982) *Nature 298*, 347–350.

Vliegenthart, J. F. G., Dorland, L., & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.

Wampler, D. E., Lehman, E. D., Boger, J., McAleer, W. J., & Scolnick, E. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6830–6834.