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The oligosaccharides in a recombinant hepatitis B virus surface antigen (HBsAg) carrying the pre-S2 region derived from yeast

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The *N*- and *O*-linked oligosaccharides in a yeast-derived HBsAg M protein (pre-S2 + S) were analyzed. Two major structures of the *N*-linked oligosaccharides bound to residue Asn[4] were determined to be high-mannose type oligosaccharides, $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$, by two-dimensional sugar mapping of the corresponding pyridylamino oligosaccharides. Peptide mapping of the M protein, sequence analysis of the glycopeptides after β-elimination under reducing conditions and sugar-composition analysis revealed that the *O*-linked oligosaccharides were composed solely of mannose and bound to residue Ser[5], Thr[6], Thr[7], Ser[27], Ser[28], Ser[29] and Thr[31] in the pre-S2 region.

Hepatitis B vaccine; Pre-S2; *N*- and *O*-linked oligosaccharides

1. INTRODUCTION

Infection with hepatitis B virus (HBV) is a worldwide health problem which is especially serious in Southeast Asia, the Middle East and Africa. To date various hepatitis B (HB) vaccines have been developed using a variety of immunogens: HBV surface antigen (HBsAg) small particles from healthy carriers (first-generation HB vaccine), yeast-derived HBsAg S protein particles (second-generation HB vaccine) [1–4] and yeast or mammalian cell-derived HBsAg M protein (pre-S2 + S) particles (third-generation HB vaccine) [5–9].

We have already reported the purification and characterization of recombinant yeast-derived modified M protein particles (HBsAg M-P31c). M-P31c was found to be composed mainly of two major glycoproteins, GP37 and GP34. Protein chemical analyses of these glycoproteins revealed that (1) GP37 and GP34 have the same polypeptide backbone, P31, (2) GP37 has an *N*-linked oligosaccharide bound to Asn[4] and an additional *O*-linked oligosaccharide(s) bound to the pre-S2 region of P31, and (3) GP34 has an *N*-linked oligosaccharide bound to Asn[4] [7]. However, the structures of these oligosaccharides and the binding sites of *O*-linked oligosaccharides have until now remained unresolved.

Two-dimensional sugar mapping of the pyridylamino (PA)-oligosaccharides revealed that Asn[4] has a high-mannose type oligosaccharide, either $Man_7GlcNAc_2$ or $Man_8GlcNAc_2$. In this report, we describe the structures of the two major *N*-linked oligosaccharides and the binding sites of the *O*-linked oligosaccharides.

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2. MATERIALS AND METHODS

2.1. *Materials*

HBsAg M-P31c particles were prepared from recombinant yeast according to the method described in [7]. TPCK-trypsin was purchased from Millipore, pepsin from Sigma and endo-β-*N*-acetylglycosaminidase H (endo H) from Seikagaku Kogyo Co., Ltd. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd.

2.2. *Preparation of PA-oligosaccharides*

Oligosaccharides released from HBsAg M-P31c by hydrazinolysis were *N*-acetylated with acetic anhydride and coupled with 2-aminopyridine by the method of Hase et al. [10]. The reaction mixture was extracted three times with ethyl acetate, and fractions containing PA-oligosaccharides were obtained by Asahipak GS-220 column (Asahi Chemical Industry Co., Ltd.) chromatography.

2.3. *Structure elucidation of PA-oligosaccharides*

Structure elucidation of PA-oligosaccharides was performed according to the method of Tomiya et al. [11]. In brief, PA-oligosaccharides were purified using a TSK-gel amide-80 column (amide silica column; 0.46 × 25 cm; Tosoh), and peak fractions were further analyzed using a Shimpak CLC-ODS column (ODS silica column; 0.6 × 15 cm). PA-oligosaccharides were detected by fluorescence using excitation and emission wavelength of 320 and 400 nm, respectively. The glucose units were determined by comparing the elution positions of each PA-oligosaccharide on both amide silica and ODS silica columns with those of standard PA-glucose oligomers and were plotted on a two-dimensional sugar map. The structures of the oligosaccharides were estimated with aid of the sugar map described in [11].

2.4. *Preparation of standard PA-glucose oligomers*

Dextran (100 mg) was dissolved in 1 ml of 0.1 N HCl and hydrolyzed in vacuo at 100°C for 4 h. The resulting mixture of glucose oligomers was subjected to coupling with 2-aminopyridine.

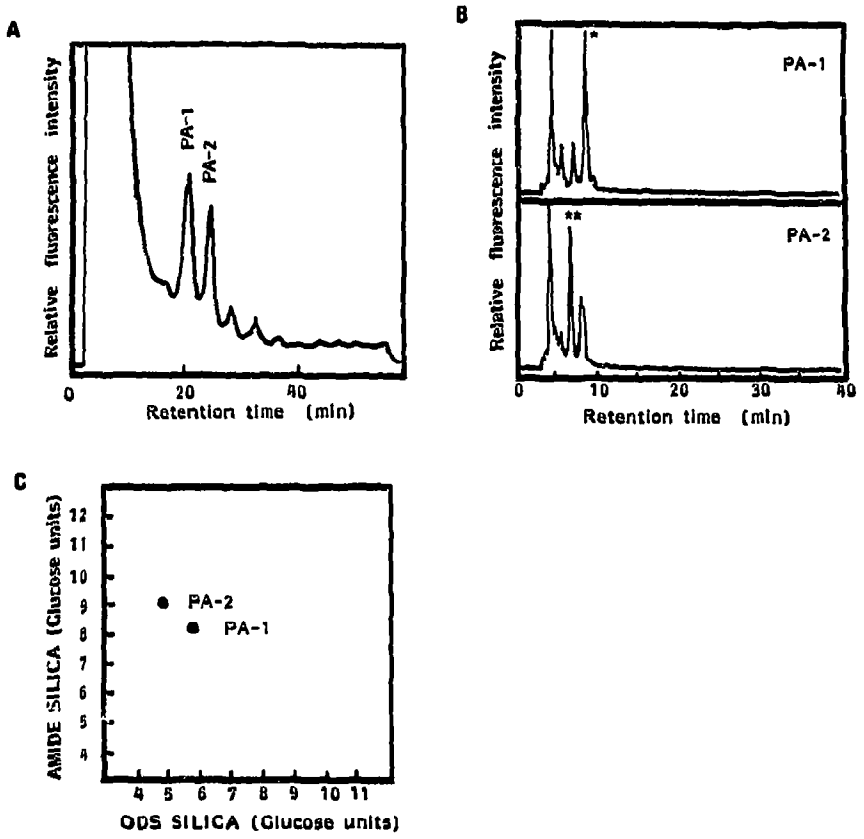

Fig. 1. Structure determination of *N*-linked oligosaccharides. (A) Amide silica chromatography of PA-oligosaccharides. Preparation of PA-oligosaccharides is described in section 2. PA-oligosaccharides were loaded onto a TSK-gel amide-80 column previously equilibrated with a mixture of 3% CH_3COOH-triethylamine buffer (pH 7.3) and CH_3CN (35:65, v/v). PA-oligosaccharides were eluted by increasing the ratio of 3% CH_3COOH-triethylamine buffer (pH 7.3) to CH_3CN from 35:65 to 50:50. Fractions for two peaks, designated PA-1 and PA-2, were collected for further analysis. (B) ODS silica chromatography of PA-1 and PA-2. PA-1 and PA-2 were loaded onto a Shimpak CLC-ODS column previously equilibrated with 10 mM sodium phosphate buffer (pH 3.8), 0.1% 1-butanol. The PA-oligosaccharides were eluted by using a gradient of 1-butanol concentration from 0.1% to 0.25%. * and ** are peaks used for determining glucose units. (C) Two-dimensional sugar map of PA-1 and PA-2. The glucose units were determined by comparing the elution positions of PA-1 and PA-2 on both amide silica and ODS silica columns with those of standard PA-glucose oligomers. The glucose units of PA-1 and PA-2 are plotted on a two-dimensional sugar map according to the method of Tomiya et al. [11].

2.5. *Sugar composition analysis*

PA-oligosaccharides were hydrolyzed in vacuo in 4 M trifluoroacetic acid (TFA) at 100°C for 4 h. Monosaccharides released were analyzed using the BioLC system (Dionex) equipped with a CarboPak PA-1 column.

2.6. *Preparation of glycopeptide, Met^1-Arg^{16}*

HBsAg M-P31c (6.1 mg) was incubated with endo H at an enzyme to substrate ratio (E/S) of 1:1000 (w/w) at 37°C for 20 h to remove the *N*-linked oligosaccharides and was digested further with TPCK-trypsin at an E/S ratio of 1:1000 (w/w) at 37°C for an additional 5 h. Met^1-Arg^{16} was purified by HPLC using a Nucleosil 5C18 column (0.4 × 30 cm; Macherey-Nagel Company).

2.7. *Preparation of glycopeptide, Gly^{19}-Glu^{51}*

HBsAg M-P31c (30 mg) was digested with TPCK-trypsin at an E/S ratio of 1:250 (w/w) at 37°C for 2 h. TPCK-trypsin and small molecular-weight tryptic peptides were removed by Sephacryl S-300 column chromatography. The thus purified des-(Met^1-Arg^{18}) M-P31c was dialyzed against PBS, and the pH was adjusted to 2.5. Des-(Met^1-Arg^{18}) M-P31c was digested with pepsin at an E/S ratio of 1:50 (w/w) at 37°C for 3 h. The reaction was stopped by adjusting the pH to 7.7, and Gly^{19}-Glu^{51} was purified by affinity chromatography using a Con A Sepharose (Vb = 15 ml; Pharmacia) column and HPLC using a YMC-packed A324-ODS column (YMC Co., Ltd).

2.8. *β-Elimination of O-linked oligosaccharides under reducing conditions*

β-Elimination of the *O*-linked oligosaccharides of Met^1-Arg^{16} and Gly^{19}-Glu^{51} was performed under reducing conditions according to the methods described in [12] and [13]. Each glycopeptide (20 nmol) was dissolved in 0.8 ml of 0.1 M NaOH, 0.3 M $NaBH_4$, and 1.7 mg of Palladium black was added. The reaction was allowed to proceed at room temperature for 19 h under a N_2 atmosphere and was stopped by adding 0.4 ml of 1 M AcOH. Palladium black was removed by centrifugation, and deglycosylated peptide was purified by reverse-phase HPLC.

2.9. *Protein chemical analyses*

The N-terminal amino acid sequence was determined with a gas-phase protein sequencer model 470A coupled with a model 120A analyzer (Applied Biosystems, Inc.). Amino acid composition was determined with a PICO-TAG amino acid analysis system (Millipore) after hydrolysis of the sample.

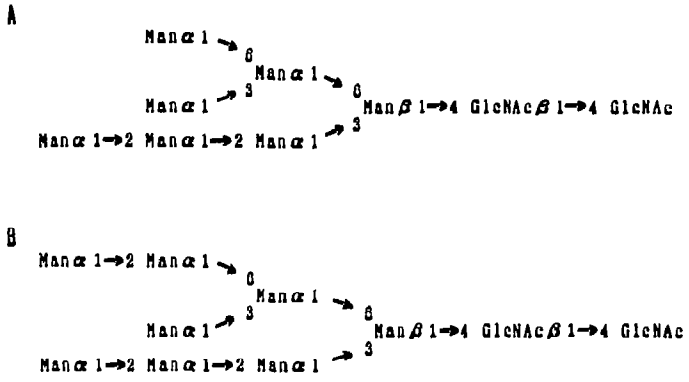

Fig. 2. Structures of *N*-linked oligosaccharides of HBsAg M-P31c. (A) PA-1; (B) PA-2.

3. RESULTS AND DISCUSSION

3.1. *Structure elucidation of* N-*linked oligosaccharides*

We previously reported that an *N*-linked oligosaccharide was bound to residue Asn^4 of the polypeptide backbone P31 based on peptide mapping, glycosidase digestion and suger composition analysis [7]. To elucidate the structure of the *N*-linked oligosaccharide, M-P31c was degraded by hydrazinolysis, and the oligosaccharides released were *N*-acetylated and modified by 2-aminopyridine according to the method of Hase et al. [10]. Fig. 1A shows the elution profile of PA-oligosaccharides from an amide silica column. The major PA-oligosaccharides, PA-1 and PA-2, were collected separately and subjected to ODS silica column chromatography (Fig. 1B). The glucose units of PA-1 and PA-2 for both amide silica and ODS silica columns were determined using a PA-glucose oligomer mixture as the standard and were plotted on a two-dimensional sugar map (Fig. 1C). The structures of PA-1 and PA-2 were estimated to be those shown in Fig. 2A and Fig. 2B, respectively, with the aid of the sugar map described in [11]. PA-1 and PA-2 are typical high-mannose type oligosaccharides. Outer chains composed of a number of mannose residues are often observed in glycoproteins of yeast origin. The oligosaccharides in M-P31c do not have such outer chains which might hinder the vaccine's ability to elicit anti-S and anti-pre-S2 antibodies.

Sugar composition of these oligosaccharides determined after hydrolysis of PA-1 and PA-2 at 100°C for 4 h in 4 M TFA indicated that the mannose/glucosamine ratios for PA-1 and PA-2 were 7.0:1.1 and 8.0:0.95, respectively. This is consistent with the results described above. (GlcNAc at the reducing end was converted into a PA-derivative by pyridylamination and was not detected in this assay.)

3.2. *Determination of* O-*linked oligosaccharide binding sites*

HBsAg M-P31c contains *O*-linked oligosaccharides in the pre-S2 region as well as *N*-linked oligosaccharides

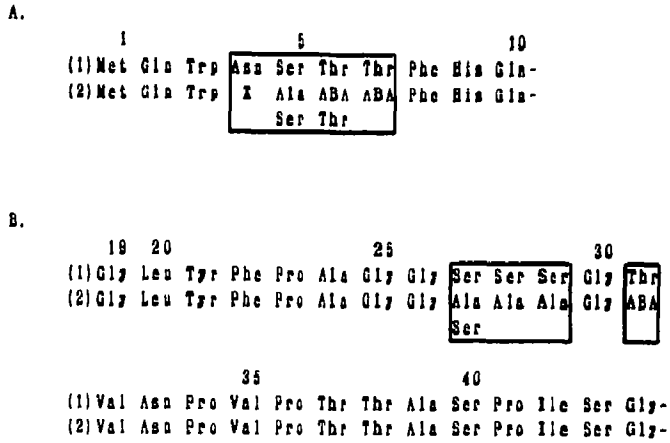

Fig. 3. N-terminal acid sequences of glycopeptides, Met^1-Arg^{16} (A) and Gly^{19}-Glu^{51} (B). (1) Putative amino acid sequence derived from the DNA sequence; (2) Amino acid sequence obtained after β-elimination. X, unidentified; ABA, 2-aminobutyric acid. Glycosylation sites are indicated by the boxes. Ser^5 and Thr^6 for A and Ser^{27} for B were also identified upon sequencing after β-elimination.

as evidenced by peptide mapping [7]. To precisely determine the *O*-linked oligosaccharide binding sites, we tried to isolate glycopeptides having *O*-linked oligosaccharides. M-P31c was treated with endo H to remove *N*-linked oligosaccharides, and Met^1-Arg^{16} was purified from the digest by reverse-phase HPLC. Alternatively, M-P31c was first digested with trypsin then with pepsin, and Gly^{19}-Glu^{51} was purified from the digest by Con A affinity column chromatography and reverse-phase HPLC. N-terminal amino acid sequence analysis after β-elimination of these glycopeptides gave PTH-Ala at residue numbers 5, 27, 28 and 29 and PTH-2-aminobutyric acid (ABA) at 6, 7 and 31 (Fig. 3). These results clearly indicate that the *O*-linked oligosaccharides were bound to Ser^5, Thr^6, Ser^{27}, Ser^{28}, Ser^{29} and Thr^{31}, because a Ser residue with an *O*-linked oligosaccharide is converted into Ala, and Thr into ABA, upon β-elimination under reducing conditions. We also observed PTH-Ser at residues 5 and 27 and PTH-Thr at residue 6 after β-elimination (Fig. 3). This suggests that there is heterogeneity with regard to the *O*-glycosylation sites. Sugar composition analysis revealed that the *O*-linked oligosaccharides were composed of only mannose.

Yu Ip et al. recently reported the structures of *N*-linked oligosaccharides in a recombinant M protein produced by the mnn9 strain of *Saccharomyces cerevisiae* [15]. They found that the major structures of the *N*-linked oligosaccharides were $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$, similar to our observations. However, they did not investigate the *O*-linked oligosaccharides in their M protein.

In conclusion, M-P31c has an *N*-linked oligosaccharide, either $Man_7GlcNAc_2$ or $Man_8GlcNAc_2$, bound to residue Asn^4 and *O*-linked oligosaccharides bound variably to Ser^5-Thr^7 and Ser^{27}-Thr^{31}. These oligosaccharides do not have any effect on the ability of the

HBsAg M-P31c vaccine to elicit anti-S and anti-pre-S2 antibodies in experimental animals (Kobayashi et al., to be published elsewhere). Structural analysis of the *O*-linked oligosaccharides remains to be elucidated.

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