

Structure of an Allergenic Pentasaccharitol, Gp-1 β -b6, Isolated from a Sea Squirt Antigen, Gi-rep, as a Minimum Structural Unit Responsible for Its Allergenicity¹

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An allergenic pentasaccharitol, Gp-1 β -b6, was isolated as a minimum structural unit responsible for the allergic reaction in skin of patients with sea squirt allergy from a saccharitol fraction, Gp-1 β -b, that had been liberated by β -elimination from a glycopeptide in a Pronase digest of a sea squirt antigen, Gi-rep. Methylation/GC-MS and FAB-MS analyses indicated the sugar sequence of Gp-1 β -b6 to be GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ (GalNAc1 $\rightarrow$)3,4GlcNAc1 $\rightarrow$ 3GalNAc-ol. To analyze the structure in more detail, Gp-1 β -b6 was labeled with *p*-aminobenzoic acid ethyl ester (ABEE), i.e., the reducing terminal 3-*O*-substituted GalNAc-ol of the saccharitol was oxidized to 2-*O*-substituted L-ThrNAc with equimolar periodate, and the resultant aldehyde was labeled with ABEE by reductive amination. The ABEE-labeled Gp-1 β -b6 was subjected to sequential exoglycosidase digestion with β -*N*-acetylhexosaminidase, α -*N*-acetylgalactosaminidase, and α -fucosidase, and the digests were chromatographed on an HPLC column of TSK gel Amide 80. From the results of the HPLC, methylation/GC-MS, and FAB-MS analyses of the digests of the labeled substrate, the structure of Gp-1 β -b6 was determined to be GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ 3(GalNAc β 1 $\rightarrow$ 4)GlcNAc β 1 $\rightarrow$ 3GalNAc-ol. Enzymatic elimination of either the non-reducing terminal β -GalNAc or the non-reducing terminal α -GalNAc led to inactivation of the allergenic pentasaccharitol. Accordingly, it is possible that the allergenic saccharitol contains two disaccharide units as the allergy-specific epitopes, one GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ and the other GlcNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$.

A sea squirt antigen termed Gi-rep is an acidic glycoprotein capable of inducing a remarkable erythema in skin of patients with sea squirt allergy on subcutaneous injection at a dose of less than 1 μ g (1-3). Previously, a Pronase digest of Gi-rep was divided into large (Gp-1) and relatively small (Gp-2) size glycopeptide fractions, both of which were still allergenically active as examined by means of the skin reaction.

After β -elimination treatment, Gp-1 gave an *N*-acetyl-galactosaminitol-containing saccharitol (Gp-1 β -b) fraction and a relatively large size glycopeptide (Gp-1 β -a) fraction, while Gp-2 still remained as a glycopeptide substance (Gp-2 β). Thus, it was suggested that Gp-1 contained both *O*- and *N*-linked oligosaccharides and Gp-2 had only *N*-linked oligosaccharide (4).

All of the glycopeptide and oligosaccharitol preparations

were still allergenic as examined by means of the skin reaction, but allergenicity in all these preparations was eliminated by periodate oxidation (4). These findings suggested that these allergenic preparations contain a certain common epitope(s) responsible for the allergenicity in their carbohydrate structures.

In the previous study (5), the carbohydrate structure of the allergenically active glycopeptide, Gp-2, was analyzed in relation to the allergenicity, and two types of non-reducing terminal *N*-acetylgalactosaminyl disaccharide units, GalNAc α 1 $\rightarrow$ 2Fuc1 $\rightarrow$ and GalNAc α 1 $\rightarrow$ (3/4)HexNAc1 $\rightarrow$, were found as possible epitopes specific to sea squirt allergy.

Recently, the carbohydrate structure of another sea squirt antigen, H-antigen, of a substantially large molecular size ($M_r > 10^6$) was analyzed, and three types of non-reducing terminal disaccharide units, GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$, GalNAc1 $\rightarrow$ (3/4)GalNAc1 $\rightarrow$, and GalNAc1 $\rightarrow$ (3/4)GalNAc1 $\rightarrow$, were also characterized as major components of the allergy-specific epitopes residing in its *O*-glycosidically linked carbohydrate chains (6).

The present study deals with the structure of an allergenic pentasaccharitol, Gp-1 β -b6, isolated from the oligosaccharitol fraction, Gp-1 β -b, as a minimum structural unit responsible for the allergenicity specific to sea squirt allergy.

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Abbreviations: FAB, fast atom bombardment; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GalNAc-ol, *N*-acetyl-galactosaminitol; β -HexNAcase, β -*N*-acetylhexosaminidase; α -GalNAcase, α -*N*-acetylgalactosaminidase; Fucase, α -fucosidase; TFA, trifluoroacetic acid; L-ThrNAc, 3-acetamido-3-deoxy-L-threonine; L-LyxNAc, 4-acetamido-4-deoxy-L-threonine; ABEE, *p*-aminobenzoic acid ethyl ester.

MATERIALS AND METHODS

Allergenic Oligosaccharitol Fraction, Gp-1 β -b—The oligosaccharitol fraction, Gp-1 β -b, was prepared in the previous study (4) as a fraction containing various oligosaccharitols that had been O-glycosidically linked to and liberated from a large glycopeptide, Gp-1, in a Pronase digest of a sea squirt antigen termed Gi-rep by β -elimination. Gp-1 β -b was still comparable to Gp-1 and Gi-rep in allergenicity as examined by means of the skin reaction test on patients with sea squirt allergy (4). Chemically, Gp-1 β -b contained 271 μ mol/g of galactosaminitol, 333 μ mol/g of galactosamine, 499 μ mol/g of glucosamine, 212 μ mol/g of fucose, 136 μ mol/g of galactose, and smaller amounts of glucose and xylose (4).

Allergenicity—Allergenicities of samples were evaluated by the skin test method *in vivo* against patients with sea squirt allergy as previously described (1-5).

Liquid Chromatography—Gel chromatographic fractionation of a mixture of saccharitols was carried out on a set of two columns (2.0 $\times$ 100 cm each) packed with Bio-Gel P-4 (400 mesh, Bio-Rad, Richmond) connected in series with deionized water at a flow rate of 7 ml/h at 55°C under monitoring with a RI-8 differential refractometer (Toso). A saccharitol fraction was further purified on an HPLC column of YMC-Pac AL-312 ODS (0.6 $\times$ 15 cm, YMC, Kyoto) with deionized water at a flow rate of 1 ml/min under monitoring with the refractometer.

p-Aminobenzoic acid ethyl ester derivatives of saccharitols were purified on a Sep-Pak C₁₈ cartridge (Waters, Milford) with water/acetonitrile (9:1 by volume), and the purified derivatives were analyzed on an HPLC column of TSK gel Amide 80 (0.4 $\times$ 25 cm, Toso) with acetonitrile/water (7:3 by volume) at a flow rate of 0.5 ml/min under monitoring with a UV-8 spectrometer (Toso).

Sugar Composition—Sugar compositions were determined by the alditol acetate GLC method on a 20K gas chromatograph apparatus (JEOL, Tokyo). Oligosaccharitols (~50 μ g) were hydrolyzed in a sealed tube with 0.5 ml of 4 N TFA at 100°C for 4 h. The hydrolyzate was evaporated, reduced with NaBH₄, and acetylated according to the procedure of Sawardek *et al.* (7). Alditol acetates of neutral sugars were analyzed on a glass column (0.2 $\times$ 200 cm) packed with 3% ECNSS-M on Gaschrom Q (80/100 mesh) at 180°C. Alditol acetates of amino sugars were analyzed on a glass column (0.2 $\times$ 100 cm) packed with 2% OV-17 on Gaschrom Q (80/100 mesh) at 200°C.

Methylation Analysis—Samples were permethylated by the method of Hakomori *et al.* (8, 9). The permethylated samples were hydrolyzed in 4 N TFA at 100°C for 4 h, reduced with NaBH₄, and acetylated as described previously (6). The permethylated samples were analyzed by GLC-mass spectrometry (GC-MS) on a DX-300 mass spectrometric analyzer (JEOL) using a glass column (1.6 mm $\times$ 100 cm) packed with 2% OV-17 on Gaschrom Q (80/100 mesh) at increasing (2°C/min) temperature from 150 to 240°C (10, 11). Mass spectra were recorded at an ionization potential of 70 eV (12).

FAB-MS Analysis—Fast atom bombardment-MS (FAB-MS) analysis was carried out on a JMS HX-100 JMA-5100 mass spectrometer (JEOL) under the same conditions as in the preceding paper (6). An aqueous

solution of a saccharitol or a methanolic solution of a permethylated sample was mixed with glycerol and *o*-thioglycerol-1 N HCl on a stainless steel target, and the sample was bombarded with a xenon atom beam accelerated at a potential of 7 keV for analysis in the positive or negative ion mode (13).

Exoglycosidases—Exoglycosidases (Seikagaku Kogyo, Tokyo) used were β -N-acetylhexosaminidase (β -HexNAcase) from jack bean, α -N-acetylgalactosaminidase (α -GalNAcase) from *Acremonium* sp., and α -fucosidase (α -Fucase) from *Charonia lampas*.

¹H-Nuclear Magnetic Resonance Spectrometry (NMR)—NMR analysis was carried out on an Fx-500s FT NMR apparatus (500 MHz, JEOL) equipped with a data recorder operating in the Fourier-transform mode at 20°C. Samples were repeatedly freeze-dried from a solution in 99.98% D₂O to exchange hydrogen in the samples with deuterium before analysis (14).

Other Materials—Dextran (Nakarai, Kyoto) was hydrolyzed in 0.1 N HCl at 100°C for 4 h and used as a calibration standard in gel chromatography on a Bio-Gel P-4 column. A mixture of N-acetylgalactosamine oligomers (GalNAc α 1-4)_nGalNAc ($n=0-4$) was kindly given by Higeta Soy Sauce (Choshi) and a mixture of chitooligosaccharides was purchased from Hayashibara (Okayama). These oligosaccharides were reduced to the corresponding alcohols with NaBH₄ as reference saccharitols. *p*-Aminobenzoic acid ethyl ester (Katayama Kagaku, Osaka) and other reagents were of analytical grade.

RESULTS AND DISCUSSION

Isolation of Oligosaccharitols from Gp-1 β -b—A 240 mg portion of the previously isolated oligosaccharitol fraction, Gp-1 β -b, from the saccharides O-glycosidically linked to Gi-rep (4) was chromatographed into 15 subfractions.

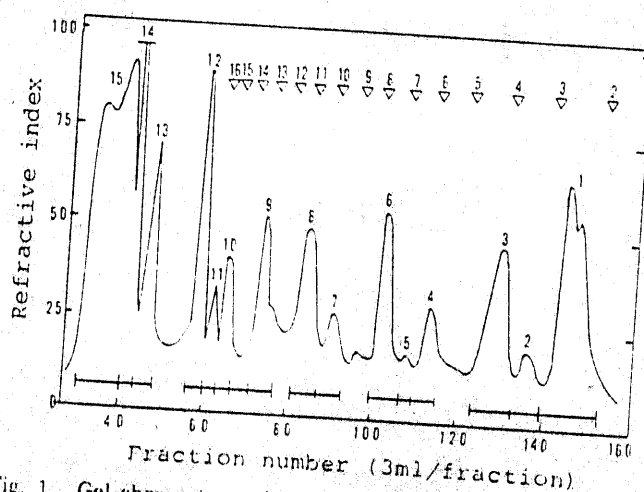


Fig. 1. Gel chromatographic fractionation of Gp-1 β -b. A 240 mg portion of an oligosaccharitol preparation, Gp-1 β -b, which had been liberated from a Pronase digest of Gi-rep by β -elimination (4), was chromatographed on a set of two columns (2.0 $\times$ 100 cm each) of Bio-Gel P-4 at 55°C with deionized water at a flow rate of 7 ml/h under monitoring with a differential refractometer at 256 nm. Marks ∇ show the elution positions of glucose oligomers in a dextran hydrolyzate as calibration standards; the numbers on the marks correspond to the degrees of polymerization of the respective components.

TABLE I. Yields, molecular weights, and sugar compositions of subfractions from Gp-1 β -b.

Subfraction	Yield (mg)	Molecular weight ^a	Sugar content (μ mol/mg)			
			Fuc	Gal	GlcNAc	GalNAc
Gp-1 β -b1	17.8	223	2.93	n.d.	n.d.	1.66
		369				
Gp-1 β -b2	1.7	223	0.36	0.67	2.48	0.42
Gp-1 β -b3	7.5	426	0.79	2.53	0.43	0.86
		531				
Gp-1 β -b4	10.1	629	0.98	1.56	1.06	0.97
		734				
Gp-1 β -b5	1.7	775	0.74	1.32	1.16	0.98
		936				
Gp-1 β -b6	8.0	978	0.92	0.12	1.44	2.00
Gp-1 β -b7	5.6	1237	0.48	0.54	1.27	0.75
Gp-1 β -b8	10.0	1035	0.63	n.d.	2.26	1.54
		1181				
		1327				
Gp-1 β -b9	6.8	1369	0.36	0.59	1.62	1.16
Gp-1 β -b10	6.6	1384	0.47	0.48	1.72	1.22
Gp-1 β -b11	5.8	1505	0.77	0.92	1.62	1.24
Gp-1 β -b12	0.4	1667	0.79	0.54	1.46	0.96
Gp-1 β -b13	0.6	n.d.	0.95	0.62	0.84	0.47
Gp-1 β -b14	8.8	465*	1.11	0.51	0.96	1.30
		611*				
		709*				
		855*				
		1017*				
Gp-1 β -b15	143	1058*	0.93	0.79	0.86	0.54
		1115*				
		1261*				
		1464*				
		1611*				
		1814*				

^aMolecular weights were estimated from the m/z values of pseudomolecular ions detected in FAB-MS analysis of the respective subfractions. The m/z values for Gp-1 β -b6-b12 were measured by FAB-MS analysis in the positive ion mode, while those for Gp-1 β -b14 and Gp-1 β -b15 were measured in the negative ion mode (*). No pseudomolecular ion from Gp-1 β -b13 was detected (n.d.) in either positive or negative FAB-MS analyses.

TABLE II. Allergenicity of subfractions from Gp-1 β -b. Allergenicity was examined by direct skin test against three of twelve patients (a-l) with sea squirt allergy by injecting 2 μ g each of three or four saccharide samples in 20 μ l of 0.9% NaCl, and 1 μ g of Gi-rep as a reference. When erythema, appearing after 15 min, was 0 to 10, 10 to 20, 20 to 30, 30 to 40, or >40 mm in diameter, the symbol -, $\pm$, +, #, or ## was assigned, respectively; no symbol means no skin test was done.

Subfraction	Skin reaction detected on patient											
	a	b	c	d	e	f	g	h	i	j	k	l
Gp-1 β -b1	-	-	-									
Gp-1 β -b2	-	-	-									
Gp-1 β -b3	-	-	-									
Gp-1 β -b4	-	-	-									
Gp-1 β -b5				-	-	-						
Gp-1 β -b6				+	-	-						
Gp-1 β -b7				-	-	-						
Gp-1 β -b8				-	-	-						
Gp-1 β -b9				-	-	-						
Gp-1 β -b10							+	-	-			
Gp-1 β -b11							-	-	-			
Gp-1 β -b12							-	-	-			
Gp-1 β -b13										+	-	-
Gp-1 β -b14										-	-	-
Gp-1 β -b15										-	-	-
Gi-rep as reference	-	+	-	-	-	-	-	-	-	-	-	-

Gp-1 β -b1-b15, on a column of Bio-Gel P-4 with deionized water under monitoring with a differential refractometer (Fig. 1). Table I shows the sugar compositions of the subfractions and the molecular weights estimated from the m/z values of their pseudomolecular ions detected in FAB-MS analysis.

Out of the 15 subfractions, 10, Gp-1 β -b6-b15, were found to be allergenically active, while the other 5, Gp-1 β -b1-b5, were inactive as examined by means of the skin reaction test (Table II). As Gp-1 β -b6 was the smallest in molecular size among the 10 allergenic subfractions, this subfraction was expected to be a minimum structure responsible for the skin reaction specific to the allergy. Thus, Gp-1 β -b6 was chromatographed on an HPLC column of YMC-Pack AL-312 ODS with deionized water for further purification, and the carbohydrate was mostly recovered in the fractions covering an asymmetric elution peak (Fig. 2A).

Since all of the standard chitooligosaccharitols also gave essentially the same asymmetric elution peaks (Fig. 2B) as that in Fig. 2A, the HPLC-purified Gp-1 β -b6 was suggested to be substantially pure. Composition analysis showed that the purified Gp-1 β -b6 was composed of Fuc, GalNAc,

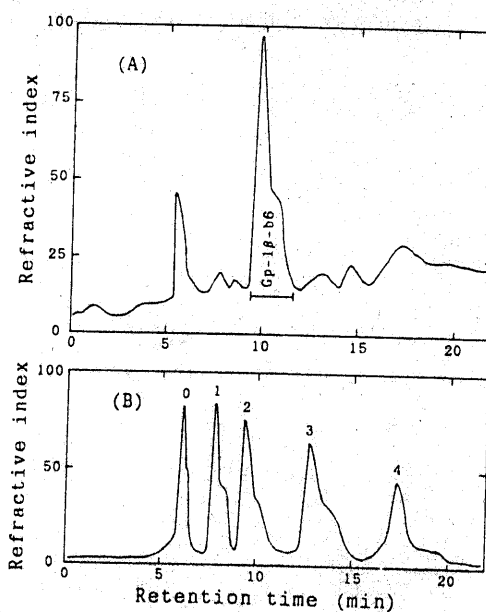


Fig. 2. Purification of Gp-1 β -b6. (A) Gp-1 β -b6 in Fig. 1 was rechromatographed on an HPLC column (0.6 $\times$ 15 cm) of YMC-Pack AL-312 ODS with deionized water at a flow rate of 1 ml/min under monitoring with a differential refractometer at 16 attenuation. (B) A mixture of 50 μ g each of standard chitooligosaccharitols, (GlcNAc β 1-4) $_n$ GlcNAc-ol ($n=0-4$), was also chromatographed under the same conditions as for (A).

TABLE III. Contributions of non-reducing terminal α - and β -GalNAc to the allergenicity of Gp-1 β -b6. Methods of evaluation and expression of allergenicity were the same as in Table II.

Sample	Skin reaction detected on patient					
	m	n	o	p	q	r
Intact Gp-1 β -b6	-	-	-	-	-	-
β -HexNAcase digest of Gp-1 β -b6	-	-	-	-	-	-
α -GalNAcase digest of Gp-1 β -b6	-	-	-	-	-	-
Gi-rep as reference	-	-	-	-	-	-

TABLE IV. Methylation/GC-MS analyses of Gp-1 β -b6 and β -N-acetylhexosaminidase digest of the ABEE derivative of equimolar periodate-oxidized Gp-1 β -b6. Molar contents of partially methylated sugar components were calculated by assuming that each sample contained 1 mol of 1,2,5-tri-O-acetyl-3,4-di-O-methylfucitol.

Component	Molar content in	
	Gp-1 β -b6	β -HexNAcase digest of ABEE-derivatized Gp-1 β -b6
1,5-Di-O-acetyl-3,4,6-tri-O-methyl-2-N-methylacetamido-2-deoxygalactitol	2.2	1.3
1,2,5-Tri-O-acetyl-3,4-di-O-methylfucitol	1.0	1.0
1,3,4,5-Tetra-O-acetyl-6-O-methyl-2-N-methylacetamido-2-deoxyglucitol	1.0	0.1
3-O-Acetyl-1,4,5,6-tetra-O-methyl-2-N-methylacetamido-2-deoxygalactitol	1.1	n.d.
1,3,5-Tri-O-acetyl-4,6-di-O-methyl-2-N-methylacetamido-2-deoxyglucitol	n.d.	0.8

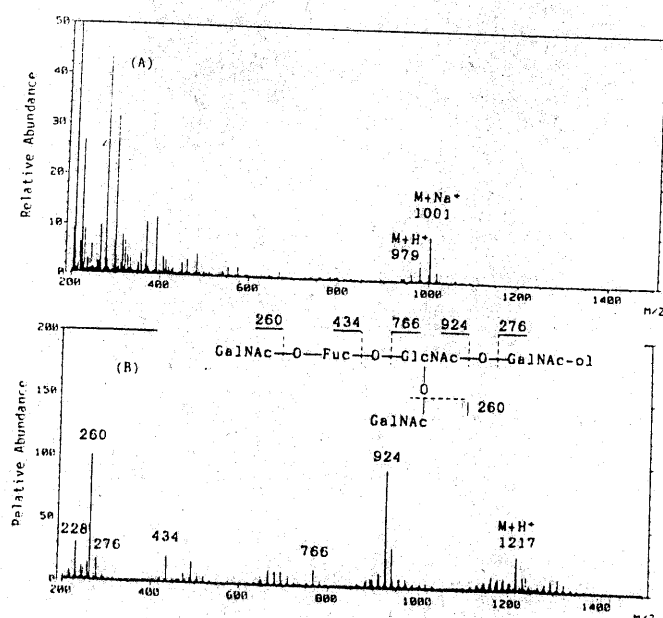


Fig. 3. Positive ion FAB-MS of intact Gp-1 β -b6 (A) and permethylated Gp-1 β -b6 (B).

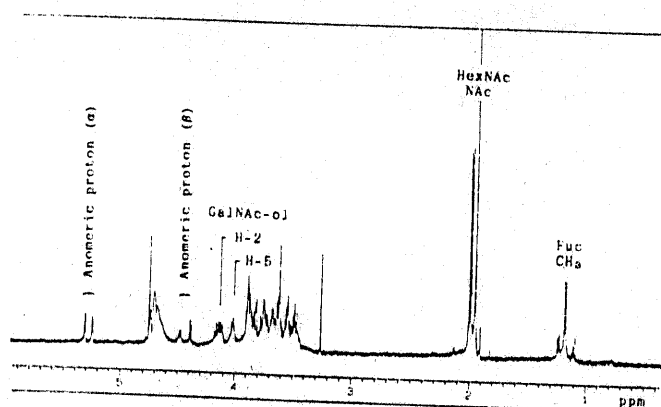


Fig. 4. ^1H -NMR spectrum of Gp-1 β -b6.

GlcNAc, and GalNAc-ol at a molar ratio of 1.1 : 2.3 : 1.1 : 1.0. The purified Gp-1 β -b6 was confirmed to be allergenic by means of the skin reaction test (Table III).

Methylation GC-MS and FAB-MS Analyses—Methylation GC-MS analysis showed that Gp-1 β -b6 was composed of 2 mol of non-reducing terminal GalNAc, 1 mol of 2-O-substituted Fuc, 1 mol of 3,4-di-O-substituted GlcNAc, and 1 mol of 3-O-substituted GalNAc-ol (Table IV). Thus, the sugar sequence of Gp-1 β -b6 was expected to be GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ (GalNAc1 $\rightarrow$)3,4GlcNAc1 $\rightarrow$ 3GalNAc-

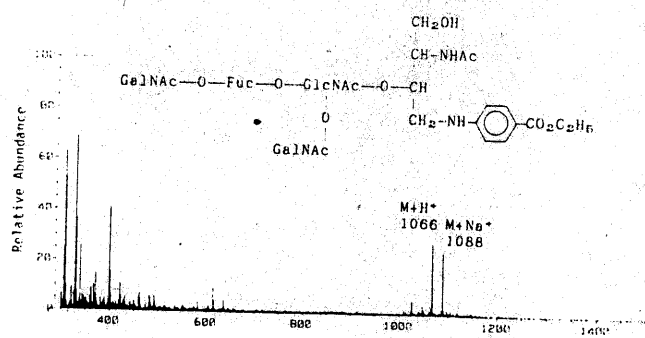


Fig. 5. Positive ion FAB-MS of the ABEE derivative of Gp-1 β -b6.

ol or GalNAc1 $\rightarrow$ (GalNAc1 $\rightarrow$)3,4GlcNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ 3GalNAc-ol.

In FAB-MS analysis, intact Gp-1 β -b6 gave two pseudomolecular ions at m/z 979 and 1,001 corresponding to $[M+H]^+$ and $[M+Na]^+$ of the pentasaccharitol, respectively (Fig. 3A). The pseudomolecular ion at m/z 1,217 from the permethylated Gp-1 β -b6 is consistent with the $[M+H]^+$ of m/z 979 from the intact sample (Fig. 3B). The fragment ions at m/z 260, 434, and 924 from the permethylated sample are ascribed to non-reducing terminal GalNAc-, GalNAc1 $\rightarrow$ 2Fuc-, and GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ (GalNAc1 $\rightarrow$)3,4GlcNAc-, respectively. The ions at m/z 276, and 766 are ascribed to the reducing terminal -GalNAc-ol, and -(GalNAc1 $\rightarrow$)3,4GlcNAc1 $\rightarrow$ 3GalNAc-ol, respectively. From these results, the sugar sequence of Gp-1 β -b6 is identified to be GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ (GalNAc1 $\rightarrow$)3,4GlcNAc1 $\rightarrow$ 3GalNAc-ol.

NMR Analysis—In NMR analysis of Gp-1 β -b6 (Fig. 4), the signals at 5.315 ppm ($J=3.66$ Hz) and 5.251 ppm ($J=4.03$ Hz) indicate that the saccharitol contained 2 mol of sugars in α -form, while the signals at 4.482 ppm ($J=7.33$ Hz) and 4.388 ppm ($J=8.42$ Hz) indicate that the saccharitol also contains 2 mol of sugars in β -form (15). Three singlet proton signals at 2.005, 1.991, and 1.954 are ascribed to the acetyl protons in 2 mol of terminal GalNAc, 1 mol of 3,4-di-O-substituted GlcNAc, and 1 mol of 3-O-substituted GalNAc-ol (15, 16). The signal at 1.181 ppm is ascribed to the methyl proton of 2-O-substituted Fuc (15, 17). The signals at 4.112 and 4.017 ppm are ascribed to H-2 and H-5 of 3-O-substituted GalNAc-ol, respectively (15, 17).

Labeling with *p*-Aminobenzoic Acid Ethyl Ester (ABEE)—For structural analysis of the allergenic pentasaccharitol in more detail by means of sequential exoglycosidase digestion, Gp-1 β -b6 was labeled with a UV-absorbing substance, *p*-aminobenzoic acid ethyl ester (ABEE), via

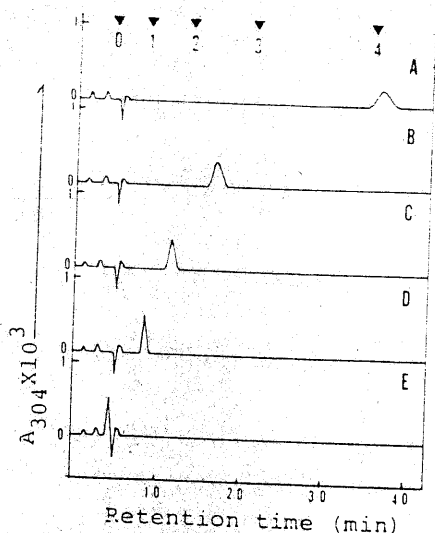


Fig. 6. Sequential exoglycosidase digestion of the ABEE derivative of Gp-1 β -b6. After each step of the sequential glycosidase digestion, the digest was heated at 100°C for 5 min and purified on a Sep-Pak C₁₈ cartridge. The purified digests were chromatographed on an HPLC column of Amide 80 (0.4 $\times$ 25 cm) with acetonitrile/water, 7 : 3 in volume, at a flow rate of 0.5 ml/min under monitoring by UV-absorption measurement at 304 nm. (A) ABEE derivative of Gp-1 β -b6; (B) β -HexNAcase digest of ABEE derivative of Gp-1 β -b6; (C) α -GalNAcase digest of the β -HexNAcase digest; (D) α -Fucose digest of the β -HexNAcase α -GalNAcase digest; (E) β -HexNAcase digest of the β -HexNAcase α -GalNAcase α -Fucose digest. Marks, $\blacktriangledown$, show the elution volumes of ABEE-labeled (GalNAc1 $\rightarrow$ 4) $_n$ GalNAc-ol ($n=0-4$) as calibration standards, which were prepared from the corresponding saccharitols by the same method as for the ABEE derivatization of Gp-1 β -b6.

equimolar NaIO₄ oxidation to convert its GalNAc-ol terminal to an aldehyde.

According to the method of Lenten and Ashwell (18) for preferential oxidation of sialic acid in some conjugate saccharides, Gp-1 β -b6 was first oxidized with equimolar NaIO₄. In a brown glass vial (1 $\times$ 10 cm), 100 μ g of Gp-1 β -b6 (0.1 μ mol) was reacted with 0.1 μ mol of NaIO₄ in 10 μ l of 0.1 M acetate buffer, pH 5.6, containing 0.15 M NaCl at 0°C for 10 min, and the reaction mixture was freeze-dried with 10 μ l of 10 mM ethyleneglycol. At this stage, it was expected that the 3-*O*-substituted GalNAc-ol in Gp-1 β -b6 would be converted to 3-*O*-substituted 4-acetamido-4-deoxy-L-lyxose (L-LyxNAc) or 2-*O*-substituted 3-acetamido-3-deoxy-L-threose (L-ThrNAc) by preferential oxidation at the C₅-C₆ or C₄-C₅ glycol, respectively.

According to the method of Wang *et al.* (19) and Matsuura and Imaoka (20), the partial oxidation product was reacted with 120 μ l of the ABEE-labeling reagent [a mixture of 35 mg (210 μ mol) of ABEE, 3.5 mg (55 μ mol) of NaBH₃CN, 40 μ l of glacial acetic acid, and 350 μ l of absolute methanol] at 80°C for 1 h in a vial tightly sealed with a screw cap. After addition of 1 ml of deionized water, the reaction mixture was extracted five times with 1 ml each of diethyl ether, and the aqueous layer was freeze-dried. The reaction product, dissolved in 2 ml of deionized water, was applied onto a Sep-Pak C₁₈ cartridge. After washing with deionized water, and cartridge was eluted repeatedly with water/acetonitrile, 19 : 1, 9 : 1, and 4 : 1 by volume, under monitoring of the UV absorption at 304 nm. A UV-absorbing substance recovered in the eluate with

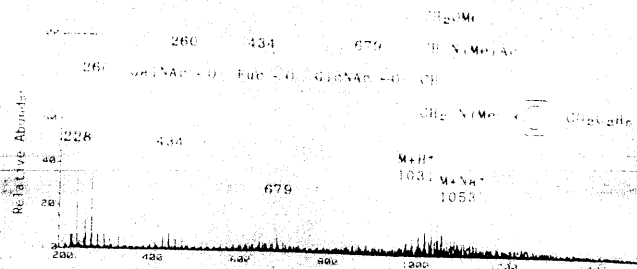


Fig. 7. Positive ion FAB-MS of the permethylated sample of β -HexNAcase digest of the ABEE-labeled Gp-1 β -b6.

water/acetonitrile, 9 : 1, were evaporated as the ABEE derivative of the partially oxidized Gp-1 β -b6. The recovery of the ABEE derivative was 80% as estimated from the UV-absorption at 304 nm based on the molecular absorption coefficient of the ABEE derivative from the equimolar-periodate-oxidized chitobitol.

In FAB-MS analysis of the ABEE derivative, the pseudomolecular ions at m/z 1,066 [$M+H$]⁺ and 1,088 [$M+Na$]⁺ were both consistent with the structure of GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ (GalNAc1 $\rightarrow$)3,4-GlcNAc1 $\rightarrow$ 2-L-ThrNAc-ABEE (Fig. 5), indicating that the GalNAc-ol terminal was converted to L-ThrNAc by the equimolar NaIO₄ oxidation.

Exoglycosidase Digestion of the ABEE Derivative—The ABEE derivative of Gp-1 β -b6 (0.25 μ mol) was sequentially treated with β -HexNAcase (0.5 unit, pH 5.0), α -Fucose (0.1 unit, pH 4.0), α -GalNAcase (0.5 unit, pH 4.5), α -Fucose (0.1 unit, pH 4.0), and β -HexNAcase (0.5 unit, pH 5.0) in 0.1 M citrate buffer at 37°C for 72 h. After every step of the glycosidase digestion, the digest was heated at 100°C for 5 min and purified on a Sep-Pak C₁₈ in the same way as in the purification of the ABEE derivative of Gp-1 β -b6. Each of the purified digests was analyzed on an HPLC column of TSK gel Amide 80 under monitoring with UV-absorption measurement at 304 nm. The ABEE derivative of Gp-1 β -b6 gave a single peak at a retention time of 36 min (Fig. 6A), suggesting that the allergenic saccharitol in Fig. 2A was substantially pure.

After the treatment of the ABEE derivative with β -HexNAcase, the β -HexNAcase digest gave an elution peak at 17 min in the HPLC analysis, showing that at least 1 mol of non-reducing terminal β -GalNAc was eliminated from the substrate (Fig. 6B). Methylation/GC-MS analysis also showed that the β -HexNAcase digest contained 1 mol each of terminal GalNAc, 2-*O*-substituted Fuc, and 3-*O*-substituted GlcNAc, indicating that the 4-*O*-hydroxyl on 3,4-di-*O*-substituted GlcNAc in the substrate has been substituted with 1 mol of terminal β -GalNAc (Table IV). In FAB-MS analysis of the permethylated β -HexNAcase digest, the pseudomolecular ions detected at m/z 1,031 and 1,053 are assigned to be [$M+H$]⁺ and [$M+Na$]⁺ from the permethylated derivative of GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ 3GlcNAc1 $\rightarrow$ 2-L-ThrNAc-ABEE, respectively. Furthermore, the series of ions detected at m/z 260, 434, and 679 indicates the presence of a linear sequence, GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ 3GlcNAc- (Fig. 7).

Subsequently, the β -HexNAcase digest was treated with α -Fucose, but no shift was detected in the retention time of the peak (data not shown), suggesting that 2-*O*-substituted Fuc would not be substituted with terminal β -GalNAc. This

suggestion is consistent with the methylation GC-MS analysis finding that 2-*O*-substituted Fuc was still retained in the β -HexNAcase digest (Table IV).

On the other hand, the treatment with α -GalNAcase apparently shifted the retention time of the β -HexNAcase digest from 17 to 12 min, suggesting that the other 1 mol of terminal α -GalNAc had been linked to 2-*O*-substituted Fuc (Fig. 6C). Then, the β -HexNAcase/ α -GalNAcase digest was again treated with α -Fucase, and it was observed that the peak shifted from 12 to 7 min (Fig. 6D). This result indicates that 2-*O*-substituted Fuc is substituted with the non-reducing terminal α -GalNAc.

Finally, the peak of the β -HexNAcase/ α -GalNAcase/ α -Fucase digest was shifted from 7 to 4 min by treatment with β -HexNAcase (Fig. 6E). The peak at 4 min could corresponded to ThrNAc-ABEE as the final residue from the ABEE derivative of Gp-1 β -b6 after the sequential glycosidase digestion. From these results, the allergenic pentasaccharitol, Gp-1 β -b6, is identified as GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ 3(GalNAc β 1 $\rightarrow$ 4)GlcNAc β 1 $\rightarrow$ 3GalNAc-ol.

Allergenicity and Terminal Structures—As the allergic reaction in a patient's skin is considered to depend on cross-linking of IgE antibodies by two or more epitopes on an allergenic molecule, the allergenic Gp-1 β -b6 is expected to contain at least two epitopes capable of reacting with the IgEs specific to sea-squirt allergy. In the above-mentioned structure of Gp-1 β -b6, two of the non-reducing terminal disaccharide units, GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ and GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$, might correspond to the possible epitopes.

Thus, intact Gp-1 β -b6 was treated with β -HexNAcase or α -GalNAcase, and each of the digests was examined for allergenicity. It was found that the allergenicity was mostly eliminated from Gp-1 β -b6 by treatments to remove either the terminal β -GalNAc or the terminal α -GalNAc (Table III). This finding supports the suggestion that the allergenic pentasaccharitol contains two epitopes, one GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ and the other GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$.

The 2-*O*-substituted Fuc-containing structure, GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$, is considered to be quite unusual as a naturally occurring carbohydrate. However, the disaccharide unit was also characterized as a possible epitope in the *N*-linked carbohydrate chains of Gp-2 from Gi-rep (5). Furthermore, the GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ unit detected in the *O*-linked carbohydrate chains of H-antigen (6) might also correspond to the GalNAc α 1 $\rightarrow$ 2Fuc α 1 $\rightarrow$ unit.

On the other hand, the GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ unit might correspond to the GalNAc1 $\rightarrow$ (3-4)GlcNAc1 $\rightarrow$ unit previously detected in Gp-2 (5) and H-antigen (6). In H-antigen, GalNAc1 $\rightarrow$ (3-4)GalNAc1 $\rightarrow$ was also characterized as an additional possible epitope (6). These disaccharide structures in Gp-2 and H-antigen should be further analyzed in more detail by the present exoglycosidase digestion/HPLC method and/or NMR analysis.

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