Structural Analyses of Asparagine-Linked Oligosaccharides of Porcine Pancreatic Kallikrein†

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ABSTRACT: The structures of asparagine-linked oligosaccharides of porcine pancreatic β-kallikrein are reported. Asparagine-linked neutral oligosaccharides were released by *N*-oligosaccharide glycopeptidase digestion, and the reducing ends of the oligosaccharides were derivatized with a fluorescent reagent, 2-aminopyridine. The mixture of pyridylamino oligosaccharides was separated by reverse-phase and amide-adsorption high-performance liquid chromatography. The pyridylamino oligosaccharides were separated into more than 50 kinds of oligosaccharides. The structures of 5 kinds of triantennary and 12 kinds of tetraantennary oligosaccharides were determined by the use of high-resolution proton nuclear magnetic resonance spectroscopy and methylation analysis. Furthermore, the structures of five kinds of oligomannose-type oligosaccharides were elucidated by a combination of exoglycosidase digestion and high-performance liquid chromatography. ^{1}H NMR data for 14 out of the 17 kinds of *N*-acetyllactosamine-type oligosaccharides reported here have not previously been described in the literature. (1) It has been shown that fucose containing tri- and tetraantennnary oligosaccharides is predominant in porcine pancreatic β-kallikrein B. (2) It has also been shown that the heterogeneity of the structure in these types of oligosaccharides is derived from the variety of the positions of galactose residues linked to outer *N*-acetylglucosamine residues. (3) The distribution of oligosaccharides into two glycosylation sites, asparagine-95 and asparagine-239, of β-kallikrein B was determined. It has been found that oligomannose-type oligosaccharides are exclusively present at asparagine-239, although *N*-acetyllactosamine-type oligosaccharides occur at both glycosylation sites. (4) The structures of oligosaccharides of porcine pancreatic β-kallikrein A, which has one glycosylation site at asparagine-95, were compared with the results obtained for β-kallikrein B. It is of interest that oligomannose-type oligosaccharides significantly exist at asparagine-95 of β-kallikrein A; in marked contrast, this type of oligosaccharides is scarcely present at asparagine-95 of β-kallikrein B.

Tissue kallikreins are serine proteinases that release a vasodepressor peptide called Lys-bradykinin or kallidin from high or low molecular weight kininogens (Werle & Berek, 1948; Habermann, 1966; Yano et al., 1971). Tissue kallikreins occur mainly in pancreas, pancreatic juice, salivary glands, saliva, kidney, and urine. A tissue kallikrein best characterized so far in terms of structure is the porcine pancreatic β-kallikrein. It consists of two polypeptide chains, which are bonded by a disulfide bridge. Porcine pancreatic β-kallikrein can be separated into several molecular species, designated A, B, C, and X (Habermann, 1962; Fiedler & Werle, 1967; Kutzbach & Schmidt-Kastner, 1972), which differ in their carbohydrate content (Fiedler & Hirschauer, 1981; Ikekita et al., 1980).

The amino acid sequences of porcine pancreatic β-kallikreins A (Bode et al., 1983) and B (Tschesche et al., 1979) are identical. β-Kallikrein A possesses only one carbohydrate chain at Asn-95 (Ikekita et al., 1980; Bode et al., 1983), whereas β-kallikrein B has carbohydrate chains at Asn-95 and at Asn-239.

Moriya et al. obtained the neutral oligosaccharides from β-kallikreins A and B and separated the oligosaccharides into several groups according to their molecular sizes. On the basis of the results on the carbohydrate compositions of oligosaccharides they suggested that oligomannose-type and *N*-acetyllactosamine-type oligosaccharides are present in the β-kallikreins (Moriya et al., 1981). The detailed carbohydrate structure, however, has not yet been elucidated. This is due to the extreme heterogeneity of carbohydrate chains and the difficulty of isolation of each oligosaccharide.

In the present paper, we report the detailed structures of more than 80% of N-linked oligosaccharides of β-kallikrein B. To precisely determine the structure, we separated completely neutral oligosaccharides by two kinds of HPLC[1] col-

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[1] Abbreviations: Fuc, L-fucose; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Sia, Sialic acid; HPLC, high-performance liquid chromatography; PA, pyridylamino; NMR, nuclear magnetic resonance.

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umns, reverse phase and amide adsorption, and each oligosaccharide of β-kallikrein B was then subjected to ^{1}H NMR analyses.

Experimental Procedures

Materials. The following enzymes and chemicals were purchased from the sources indicated: *N*-oligosaccharide glycopeptidase (EC 3.5.1.52) from almond (obtainable as glycopeptidase A), β-galactosidase from *Charonia lampas*, β-*N*-acetylhexosaminidase and α-mannosidase from jack bean were from Seikagaku Kogyo Co.; pepsin was from Sigma Chemical Co.; Sephadex G-15, Sephadex G-75, DEAE-Sepharose CL-6B, and phenyl-Sepharose CL-4B were from Pharmacia Fine Chemicals; Bio-Gel P-4 (200–400 mesh) was from Bio-Rad Laboratories; sodium cyanoborohydride was from Aldrich; 2-aminopyridine was from Wako Pure Chemical Industries.

Preparation of β-Kallikreins A and B. Crude powder of pancreatic kallikrein prepared from fresh porcine pancreas was obtained from Seikagaku Kogyo Co. Isolation of β-kallikreins A and B from the crude powder was carried out by ionic exchange chromatography using a DEAE-Sepharose column (Kutzbach & Schmidt-Kastner, 1972).

β-Kallikrein A thus obtained was further purified by hydrophobic chromatography using a phenyl-Sepharose CL-4B column. β-Kallikreins A and B preparations obtained as above were confirmed to be homogeneous by SDS–polyacrylamide gel electrophoresis. It was confirmed that the purity of preparations of β-kallikreins A and B used in the present structural analyses is satisfactory by determining the sequence of N-terminal residues Ile-Ile-Gly for both of these proteins.

Isolation of Light Chain and Heavy Chain of Porcine Pancreatic β-Kallikrein B. Three milligrams of porcine pancreatic β-kallikrein B was reduced with 2-mercaptoethanol and then carboxymethylated with iodoacetic acid. The light chain and the heavy chain were separated by gel filtration using a Sephadex G-75 column.

Preparation of Oligosaccharides from β-Kallikrein. Porcine pancreatic β-kallikrein A (25 mg), β-kallikrein B (65 mg), and the light chain (1.3 mg) and the heavy chain (1.9 mg) of β-kallikrein B were used for the structural analysis of olgisaccharides. Each sample was desialylated by mild acid hydrolysis at pH 2, 90 °C, for 1 h and then was digested with pepsin. Resultant glycopeptides thus obtained were treated with *N*-oligosaccharide glycopeptidase as described previously (Tomiya et al., 1987). The oligosaccharide fraction was purified by gel filtration on a Bio-Gel P-4 column.

Derivatization of Oligosaccharides. Oligosaccharides thus obtained were reductively aminated with a fluorescent reagent, 2-aminopyridine, by the method of Hase et al. (1984). Before HPLC analyses, the pyridylamino (PA) derivatives of oligosaccharides were purified by gel filtration on a Sephadex G-15 column.

Preparation of PA Derivatives of Standard Oligosaccharides. Standard oligosaccharides described below were prepared by glycopeptidase digestion as described previously (Tomiya et al., 1987). Oligomannose-type oligosaccharides were prepared from chicken egg albumin ($Man_5GlcNAc_2$ and $Man_6GlcNAc_2$; Tai et al., 1975), Japanese quail egg albumin ($Man_7GlcNAc_2$; Mutsaers et al., 1985), and porcine thyroglobulin ($Man_8GlcNAc_2$ and $Man_9GlcNAc_2$; Tsuji et al., 1981). Biantennary *N*-acetyllactosamine-type oligosaccharides with or without fucose residues were prepared from human immunoglobulin G (Takahashi et al., 1987). Tri- and tetraantennary oligosaccharides without fucose residues were prepared from human α_1-acid glycoprotein (Yoshima et al., 1981). The isomaltooligosaccharides of 4–11 glucose units were prepared from the acid hydrolysate of dextran (Nishigaki et al., 1978).

All standard oligosaccharides thus obtained were derivatized with 2-aminopyridine as described above. Degalactosylated triantennary and tetraantennary PA oligosaccharides without fucose residues were prepared by digestion with β-galactosidase. Trimannosyl core oligosaccharides, Manα1→6-(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc-PA and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→-4GlcNAc-PA, were prepared from biantennary oligosaccharides with or without fucose, respectively, by sequential digestion with β-galactosidase and β-*N*-acetylhexosaminidase. The structure of each oligosaccharide isolated by HPLC was elucidated by chemical analysis and ^{1}H NMR measurements.

Exoglycosidase Digestion of PA Oligosaccharides. About 100 pmol of PA oligosaccharide was incubated with β-galactosidase (5 milliunits) in 15 μL of 0.1 M citrate–phosphate buffer (pH 4.1), β-*N*-acetylhexosaminidase (20 milliunits) in 15 μL of 0.1 M citrate–phosphate buffer (pH 5.0), and α-mannosidase (50 milliunits) in 15 μL of 0.1 M ammonium acetate buffer (pH 5.0) containing 10 mM $ZnCl_2$, at 37 °C for 15 h.

High-Performance Liquid Chromatography (HPLC). The separation of pyridylamino oligosaccharides was carried out by HPLC using a Shimadzu LC-6A chromatograph with two kinds of columns, described previously (Tomiya et al., 1988) (1) Reverse-phase HPLC was performed with a Shim-pack CLC-ODS column (0.6 × 15 cm, Shimadzu). Elution was performed at a flow rate of 1.0 mL/min at 55 °C with two solvents, A and B. Solvent A was 10 mM sodium phosphate buffer (pH 3.8), and solvent B was 10 mM sodium phosphate buffer (pH 3.8) containing 0.5% 1-butanol. The column was equilibrated with solvent A. After injection of sample (0.5–100 μL, more than 100 pmol), the ratio of solvents B to A was increased to 45:55 in 120 min with a linear gradient. Then the ratio of solvents B to A was held constant for 20 min. Each oligosaccharide fraction (about 1–5 mL) separated as a peak was collected and evaporated to dryness in vacuo. The residue was dissolved in a small amount (about 10–100 μL) of solvent C, and 0.1–100 nmol of sample was injected on the second column. (2) Size-fractionation HPLC was performed with a TSK-GEL Amide-80 column (0.46 × 25 cm, Tosoh). Elution was performed at a flow rate of 1.0 mL/min at 40 °C using two solvents, C and D. Solvent C was composed of 3% acetic acid in water with triethylamine (pH 7.3) and acetonitrile, 35:65 by volume. Solvent D was composed of 3% acetic acid in water with triethylamine (pH 7.3) and acetonitrile, 50:50 by volume. The column was equilibrated with solvent C. After injection of a sample, the ratio of solvent D to solvent C was increased to 100% of solvent D in 50 min with a linear gradient. In both HPLC systems, PA oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively.

Carbohydrate Composition. Contents of neutral sugars in porcine pancreatic kallikreins were estimated as follows. One milligram of β-kallikreins A or B was hydrolyzed by 2.5 M trifluoroacetic acid at 100 °C for 6 h in an evacuated, sealed tube (Arakawa et al., 1976). The monosaccharides obtained were analyzed by HPLC on an ISA-07/S2504 column (0.4 × 25 cm) according to the method of Mikami and Ishida (1983). Contents of amino sugars were determined by the amino acid analyzer after hydrolysis of the sample with 4 N HCl for 4 h at 100 °C in an evacuated, sealed tube. Sialic acid was determined as the amount of *N*-acetylneuraminic acid

Table I: Carbohydrate Composition of Porcine Pancreatic β-Kallikreins A and B[a]

	β-kallikrein A	β-kallikrein B		β-kallikrein A	β-kallikrein B
Man	2.3	5.6	GlcNAc	3.3	6.5
Gal	0.6	1.9	GalNAc	nd[b]	nd[b]
Fuc	0.3	1.1	Sia	0.2	0.4

[a] Values are expressed as the number of each sugar residue per one molecule. [b] nd = not detected. Details are described under Experimental Procedures.

by the periodate–thiobarbituric acid method (Warren, 1963).

^1H NMR Measurements. ^1H NMR measurements were carried out as described previously (Takahashi et al., 1987). Prior to NMR measurements, each PA oligosaccharide (about 10–100 nmol) isolated by HPLC was desalted by gel filtration on a Sephadex G-15 column. Samples were dissolved in 99.8% D_2O, lyophilized, and dissolved again in 99.8% D_2O. NMR measurements were made at 23 and 60 °C on a Bruker AM-400 spectrometer operating at 400 MHz in the Fourier-transform mode.

Methylation Analysis. PA oligosaccharide (about 50–100 nmol each) isolated by HPLC was methylated by the method of Hakomori (1964). The permethylated products were analyzed as described by Takahashi et al. (1985) on a JEOL-DX 300 gas chromatograph–mass spectrometer.

Results and Discussion

Carbohydrate Composition. Carbohydrate compositions of β-kallikreins A and B are summarized in Table I. The sugar content per 1 mol of β-kallikrein B was about twice as much as that of β-kallikrein A. This result is quite consistent with the fact that β-kallikrein A has one carbohydrate chain and β-kallikrein B has two. The amount of *N*-acetylglucosamine was much more than that of mannose. This result suggests that the major parts of the carbohydrate chains of porcine pancreatic β-kallikreins have an *N*-acetyllactosamine-type structure. *N*-Acetylgalactosamine was not detected in both β-kallikreins A and B. The present results on the sugar composition of porcine pancreatic β-kallikreins are in good agreement with the previous report by Moriya et al. (1983).

Preparation of Oligosaccharides by N-Oligosaccharide Glycopeptidase Digestion. Oligosaccharides were released by sequential digestion of β-kallikrein B (2.25 μmol) with pepsin and *N*-oligosaccharide glycopeptidase (almond). It was confirmed on the basis of the results of the sugar content determination that less than 10% of the total carbohydrates remained as undigested materials (data not shown).

Characterization of PA Oligosaccharides by a Combination of Reverse-Phase HPLC and Exoglycosidase Digestion. PA oligosaccharides prepared from porcine pancreatic β-kallikrein B were separated into about 50 peaks by reverse-phase HPLC (Figure 1a). The structures of the standard oligosaccharides used as references are described in the legend for Figure 1. The elution positions of peaks A, B, C, D, and E were the same as those of standard oligomannose-type oligosaccharides 1, 2, 3, 4, and 5, respectively.

After a mixture of PA oligosaccharides (Figure 1a) was digested with β-galactosidase, the reaction mixture was analyzed again by the same column. Two major peaks (X and Y) appeared (Figure 1b). When the β-galactosidase digest was then treated with β-*N*-acetylhexosaminidase, a new single peak was observed, whose elution position coincided with that of the standard oligosaccharide $Man_3FucGlcNAc_2$ (no. 15) (Figure 1c). These results indicate that the major parts of

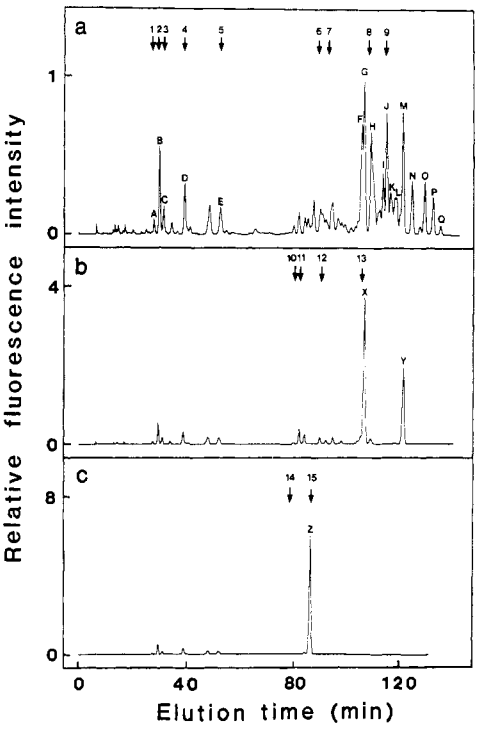
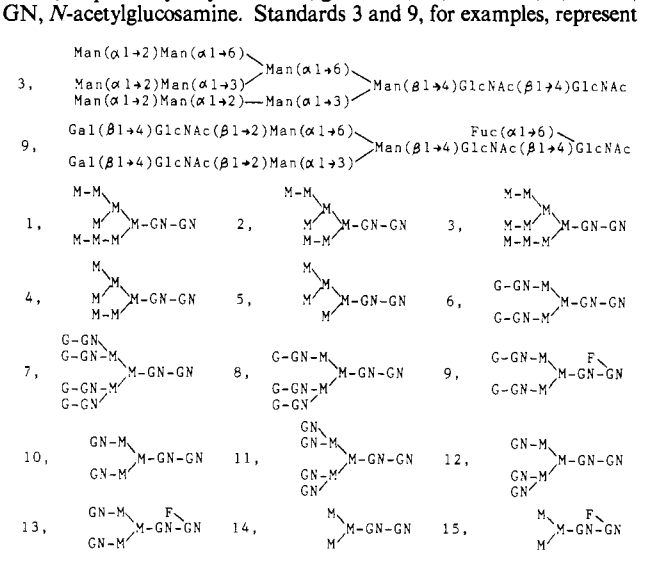


FIGURE 1: HPLC profiles on an ODS–silica column for pyridylamino derivatives of oligosaccharides obtained from porcine pancreatic β-kallikrein B. A mixture of PA oligosaccharides (about 100 nmol) was injected on an ODS–silica column. A gradient started at time 0. Details are described under Experimental Procedures. (a) Intact pyridylamino oligosaccharides obtained from porcine pancreatic β-kallikrein B; (b) β-galactosidase digest of (a); (c) β-*N*-acetylhexosaminidase digest of (b). Arrows indicate the elution positions of the standard pyridylamino oligosaccharides. The structures of the standards are shown below. The detailed structures of standards 7 and 8 are described in Tables IV and V, as reference compounds 3 and 4, respectively. Symbols: G, galactose; M, mannose; F, fucose; GN, *N*-acetylglucosamine. Standards 3 and 9, for examples, represent



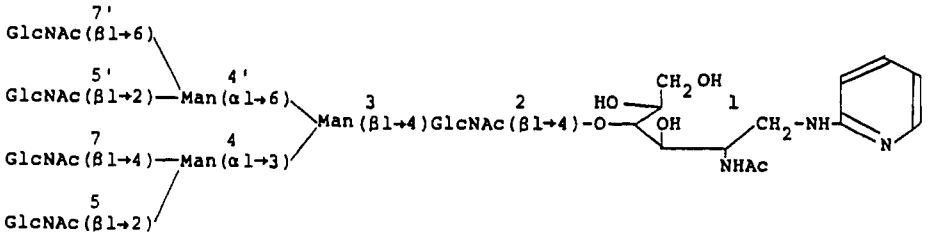
the oligosaccharides obtained from porcine pancreatic β-kallikrein B have a common core structure, Manα1→6-(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc.

Fractions F–Q were separately collected, and each fraction was digested with β-galactosidase. By this procedure fractions F–L were converted to oligosaccharide X, and fractions M–Q were converted to oligosaccharide Y (data not shown).

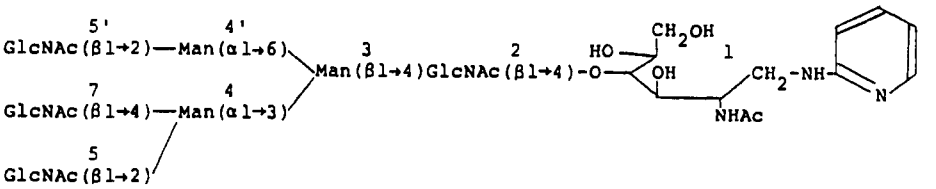
^1H NMR Analyses of Common Core Oligosaccharides X and Y. Chemical shift values for the anomeric protons and the methyl protons of oligosaccharides X and Y along with reference oligosaccharides without fucose, i.e., tetraantennary

Table II: Chemical Shifts of Anomeric Protons and Methyl Protons for Oligosaccharides X and Y

Reference Compound 1:



Reference Compound 2:

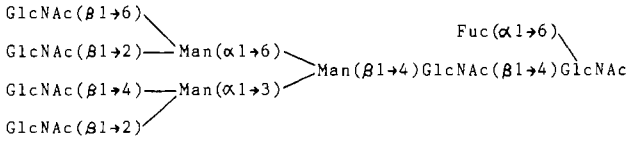


compd	chemical shifts[a] of anomeric protons								
	GlcNAc-2	Man-3	Man-4	Man-4′	GlcNAc-5	GlcNAc-5′	GlcNAc-7	GlcNAc-7′	Fuc
ref compd 1	4.631	(4.738)[b]	(5.118)	(4.848)	4.532	4.551	4.512	4.521	
ref compd 2	4.630	(4.736)	(5.108)	(4.898)	4.529	4.541	4.511		
X	4.677	(4.739)	(5.124)	(4.849)	4.537	4.552	4.515	4.522	(4.849)
Y	4.655	(4.741)	(5.118)	(4.900)	4.533	4.544	4.519		(4.855)

compd	chemical shifts[a] of methyl protons					
	GlcNAc-2	GlcNAc-5	GlcNAc-5′	GlcNAc-7	GlcNAc-7′	Fuc
ref compd 1	2.059	2.047	2.039	2.067	2.030	
ref compd 2	2.065	2.042	2.042	2.065		
X	2.070	2.051	2.040	2.070	2.031	1.176
Y	2.072	2.048	2.041	2.072		1.184

[a] Chemical shift values are expressed in ppm from internal DSS but were actually measured with internal acetone (δ = 2.2164 ppm in D_2O at 23 °C). [b] Values in parentheses were measured at 60 °C by using internal acetone (δ = 2.213 ppm in D_2O).

(compound 1) and triantennary (compound 2), are compiled in Table II. ^1H NMR spectral data for the H-1 and methyl groups clearly indicate that the chemical shifts of oligosaccharide X and the reference tetraantennary oligosaccharide (compound 1) were all in good agreement except for a fucose residue that does not exist in compound 1. Chemical shifts are observed for the H-1 and CH_3 protons of a fucose residue of oligosaccharide X. The chemical shifts of H-1 and CH_3 protons for GlcNAc-2 of oligosaccharide X are shifted from 4.631 (compound 1) to 4.677 ppm and from 2.059 (compound 1) to 2.070 ppm, respectively. These results indicate the existence of a fucose residue linked to GlcNAc-1 (Takahashi et al., 1987). These NMR data indicate that oligosaccharide X is a pyridylamino derivative of a fucose containing a tetraantennary oligosaccharide whose structure is as follows:



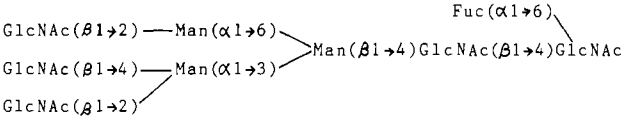
The ^1H NMR data for oligosaccharide Y reveal the characteristic features of fucose-containing triantennary oligosaccharide without galactose residues (Table II). The chemical shift values of oligosaccharide Y are in good agreement with those of reference compound 2, except for a fucose residue that does not exist in the latter. The presence of a fucose residue bonded to GlcNAc-1 through an α-1,6 linkage is shown on analogy to those described for oligosaccharide X. These NMR

Table III: Molar Ratios of Methylated Alditol Acetates in the Hydrolysates of the Permethylated Oligosaccharides X and Y

alditol acetates	molar ratio[a]	
	oligosaccharide X	oligosaccharide Y
2,4-Me_2Man	1.0	1.0
3,4-Me_2Man	1.1	0
3,6-Me_2Man	1.0	1.2
3,4,6-Me_3Man	0	1.1
3,6-Me_2GlcNAc(Me)	0.7	0.7
3,4,6-Me_3GlcNAc(Me)	3.9	2.7
2,3,4-Me_3Fuc	0.4	0.4

[a] Values are calculated on the basis of one residue of 2,4-Me_2Man.

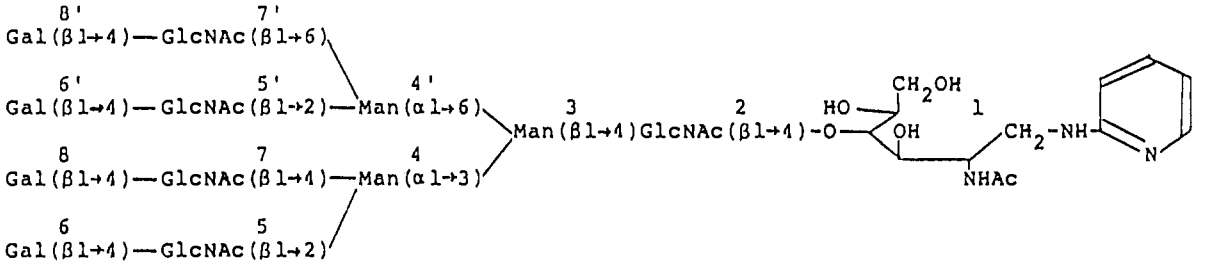
data indicate that oligosaccharide Y is a pyridylamino derivative of a fucose-containing triantennary oligosaccharide whose structure is as follows:



Methylation Analyses of Common Core Oligosaccharides X and Y. Table III summarizes the results of methylation analyses of oligosaccharides X and Y. Four *N*-acetylglucosamine residues are present in the nonreducing terminal of oligosaccharide X. The presence of a 3,4-di-*O*-methylmannose residue and a 3,6-di-*O*-methylmannose residue in oligosaccharide X indicates that two of the four *N*-acetylglucosamine residues link to one mannose residue in the C-2 and C-4

Table IV: Chemical Shifts of Anomeric Protons and Methyl Protons for Tetraantennary Oligosaccharides (Groups I–V)

Reference Compound 3:



	chemical shifts[a] of anomeric protons									chemical shifts[a] of methyl protons					
compd	GlcNAc-2	GlcNAc-5	GlcNAc-5′	GlcNAc-7	GlcNAc-7′	Gal-6	Gal-6′	Gal-8	Gal-8′	GlcNAc-2	GlcNAc-5	GlcNAc-5′	GlcNAc-7	GlcNAc-7′	Fuc
ref compd 1[b]	4.631	4.532	4.551	4.512	4.521					2.059	2.047	2.039	2.067	2.030	
ref compd 3	4.620	4.563	4.583	4.541	4.541	4.460	4.460	4.460	4.473	2.065	2.047	2.032	2.065	2.032	
G	4.672	4.535	4.550	4.518	4.518					2.071	2.049	2.039	2.071	2.030	1.178
F	4.681	4.541	4.585	4.519	4.519		4.461			2.071	2.051	2.036	2.071	2.036	1.178
H-1	4.694	4.532	4.550	4.532	4.521			4.459		2.069	2.049	2.039	2.069	2.030	1.166
H-1′	4.694	4.532	4.550	4.513	4.542				4.471	2.069	2.049	2.039	2.069	2.030	1.166
J-1	4.666	4.560	4.549	4.520	4.520	4.455				2.074	2.049	2.039	2.074	2.030	1.184
H-2	4.677	4.536	4.582	4.517	4.536		4.460		4.472	2.074	2.051	2.033	2.074	2.033	1.183
I	4.669	4.561	4.581	4.518	4.518	4.459	4.459			2.074	2.049	2.033	2.074	2.033	1.182
K-1	4.675	4.559	4.541	4.519	4.541	4.453			4.472	2.072	2.048	2.038	2.072	2.027	1.179
L-1	4.703	4.562	4.552	4.533	4.523	4.459		4.459		2.067	2.046	2.040	2.067	2.032	1.162
J-2	nd[c]	4.558	4.578	4.515	4.537	4.455	4.455		4.471	2.071	2.047	2.032	2.071	2.032	1.168
K-2	nd[c]	4.560	4.581	4.539	4.517	4.459	4.459	4.459		2.073	2.048	2.033	2.069	2.033	1.180
L-2	4.698	4.560	4.580	4.539	4.539	4.459	4.459	4.459	4.474	2.071	2.046	2.033	2.067	2.033	1.165

[a] Chemical shifts are expressed in ppm from internal DSS but were actually measured with internal acetone (δ = 2.2164 ppm in D_2O at 23 °C). [b] Structure of reference compound 1 is described in Table II. [c] The signal was not detectable due to the limited amount of sample.

positions and the other two *N*-acetylglucosamine residues link to another mannose residue in the C-2 and C-6 positions.

Three *N*-acetylglucosamine residues exist in the nonreducing terminal of oligosaccharide Y. The presence of a 3,6-di-*O*-methylmannose residue and a 3,4,6-tri-*O*-methylmannose residue in oligosaccharide Y indicates the presence of the mannose residues being substituted in the C-2 position and in the C-2 and C-4 positions.

All of these results support the structures for oligosaccharides X and Y that are proposed on the basis of the ^1H NMR data.

Size Fractionation of Oligosaccharides F–L. Oligosaccharides F–Q separated on a reverse-phase column were further purified by use of an amide-adsorption Amide-80 column (Figure 2). The number of galactose residues bonded to each oligosaccharide can also be estimated. It has been confirmed that the elution positions of oligosaccharides on the amide-adsorption column reflect primarily the molecular sizes, one galactose residue behaving like 1.0 glucose unit regardless of its linking position (Tomiya et al., 1988).

It was revealed that oligosaccharides F, G, I, and M–Q were homogeneous in terms of molecular size. By contrast, oligosaccharides H and J–L were separated into two fractions H-1 and H-2, J-1 and J-2, K-1 and K-2, and L-1 and L-2, respectively.

Eleven kinds of tetraantennary oligosaccharides thus purified with the two kinds of HPLC columns were classified into five groups on the basis of the differences of the number of galactose residues: group I (which does not have galactose residue), oligosaccharide G; group II (one galactose residue), oligosaccharides F, H-1, and J-1; group III (two galactose residues), oligosaccharides H-2, I, K-1 and L-1; group IV (three galactose residues), oligosaccharides J-2 and K-2; group V (four galactose residues), oligosaccharide L-2.

Five kinds of triantennary oligosaccharides were classified into four groups: group VI (which does not have any galactose residues), oligosaccharide M; group VII (one galactose residue), oligosaccharides N and O; group VIII (two galactose residues), oligosaccharide P; group IX (three galactose residues), oligosaccharide Q.

Determination by ^1H NMR Measurements of the Linking Positions of Galactose Residues in Oligosaccharide Groups I–IX. Table IV gives the relevant chemical shift data for tetraantennnary oligosaccharides (groups I–V), together with those of reference compounds 1 and 3, which have tetraantennary structure without galactose residues and tetraantennary structure with four galactose residues, respectively. Comparisons of the chemical shift data for reference compounds 1 and 3 clearly indicate that the substitutions of outer *N*-acetylglucosamine residues by galactose residues through $\beta1\rightarrow4$ linkages cause the anomeric proton signals to shift downfield, from 4.532 (compound 1) to 4.563 ppm (compound 3) for GlcNAc-5, from 4.551 to 4.583 ppm for GlcNAc-5′, from 4.512 to 4.541 ppm for GlcNAc-7, and from 4.521 to 4.541 ppm for GlcNAc-7′. The H-1 resonance observed at 4.473 ppm is characteristic for Gal-8′. H-1 chemical shifts for the other galactose residues were the same within the experimental error (4.460 ppm).

Group I (Oligosaccharide G). In oligosaccharide G, the signals of both anomeric protons for GlcNAc-5, GlcNAc-5′, GlcNAc-7, and GlcNAc-7′ showed essentially the same chemical shift values as those observed reference compound 1. These results indicate that oligosaccharide G does not contain any galactose residues (Figure 4).

Group II (Oligosaccharides F, H-1, and J-1). The chemical shifts of anomeric protons of GlcNAc-5′ (oligosaccharide F) and GlcNAc-5 (oligosaccharide J-1) shifted to 4.585 and 4.560 ppm, respectively. These NMR data indicate that oligo-

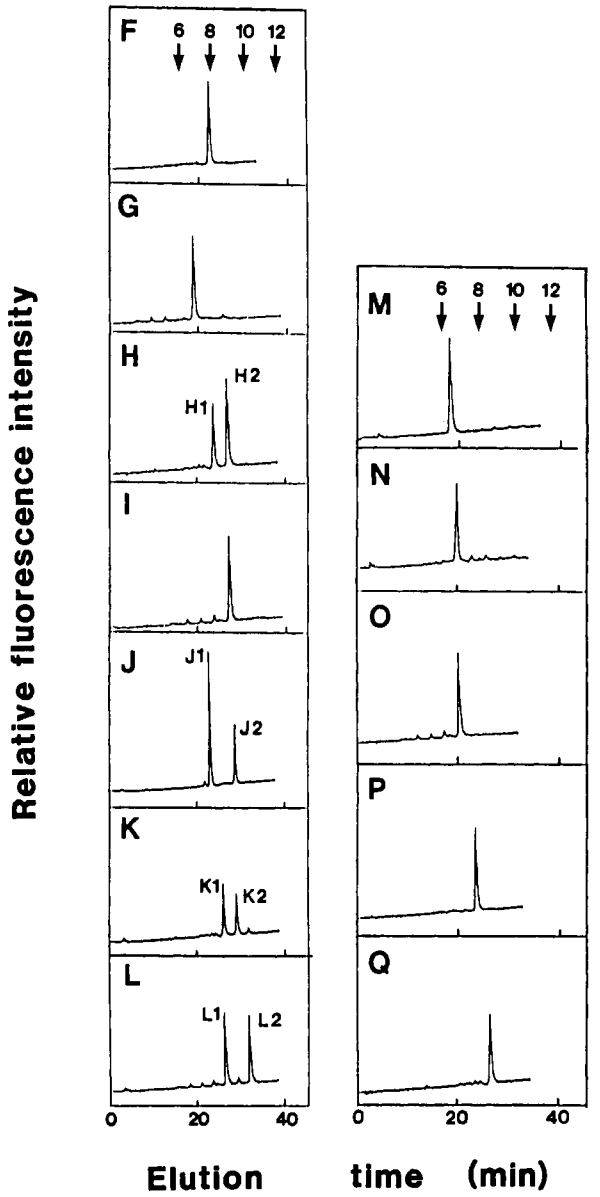


FIGURE 2: HPLC profiles on an amide–silica column for pyridylaminated oligosaccharides F–Q obtained from an ODS–silica column (Figure 1a). Each of the PA oligosaccharides (about 1 nmol) was injected on an amide–silica column. A gradient was started at time 0. Arrows indicate the elution positions of PA-glucose oligomers. Numbers represent the glucose unit.

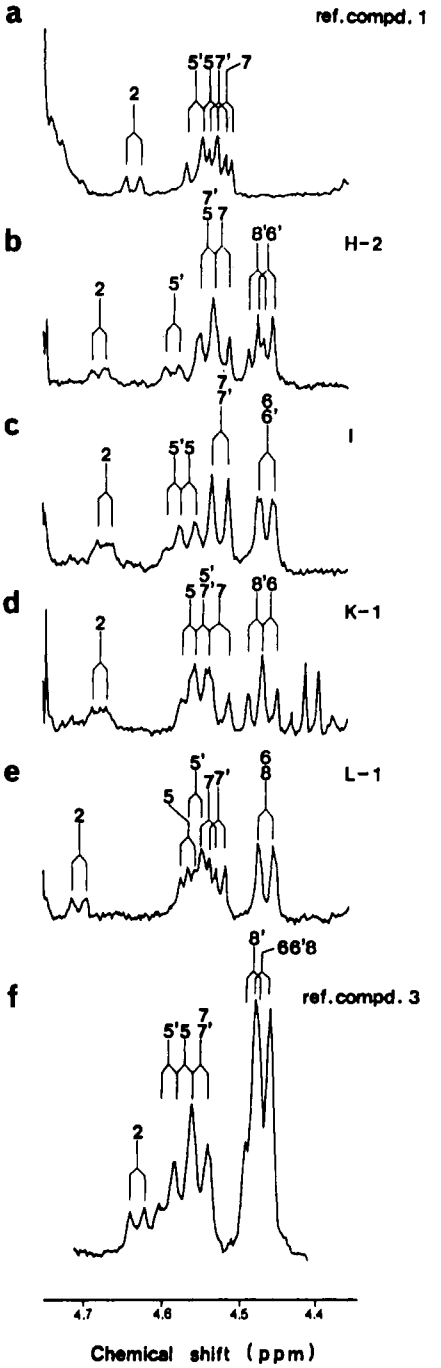


FIGURE 3: 400-MHz ^{1}H NMR spectra of anomeric protons of oligosaccharides H-2 (b), I (c), K-1 (d), and L-1 (e), from porcine pancreatic β-kallikrein B. Spectra of anomeric protons of reference compounds 1 (a) and 3 (f) are shown. Assignments: 2, GlcNAc-2; 5, GlcNAc-5; 5′, GlcNAc-5′; 7, GlcNAc-7; 7′, GlcNAc-7′; 6, Gal-6; 6′, Gal-6′; 8, Gal-8; 8′, Gal-8′.

saccharides F and J-1 have Gal-6′ and Gal-6, respectively. The NMR data showed that oligosaccharide H-1 is still a mixture of two components, designated H-1 and H-1′. The signals at 4.521 (H-1) and 4.513 ppm (H-1′) can be assigned to those of nonsubstituted GlcNAc-7′ (H-1) and GlcNAc-7 (H-1′), respectively. The assignment of the signals at 4.532 and 4.542 ppm can be done on the basis of the intensities of these signals. The characteristic signal of anomeric proton for Gal-8′ (4.471 ppm) was observed for oligosaccharide H-1′. This fact is in accord with the presence of substituted GlcNAc-7′ in oligosaccharide H-1′.

Group III (Oligosaccharides H-2, I, K-1, and L-1). The ^{1}H NMR spectra of the anomeric protons of group III oligosaccharides are reproduced in Figure 3b–e. The chemical shifts of anomeric protons due to two galactose residues were observed for all the oligosaccharides in this group, H-2, I, K-1, and L-1. In oligosaccharide H-2, the characteristic chemical shift of anomeric proton for Gal-8′ (4.472 ppm) was observed and the chemical shift of anomeric proton for GlcNAc-5′ shifted downfield (4.582 ppm). These NMR data indicate the presence of galactose residues bonded to GlcNAc-5′ and GlcNAc-7′. In oligosaccharide I, the signals of anomeric protons for GlcNAc-5 and GlcNAc-5′ shifted to 4.561 and 4.581 ppm, respectively. These results indicate that GlcNAc-5 and GlcNAc-5′ are substituted by Gal-6 and Gal-6′, respectively. In oligosaccharide K-1, the characteristic chemical shift of anomeric proton for Gal-8′ was observed and the chemical shift of the anomeric proton for GlcNAc-5 shifted downfield (4.559 ppm). It is concluded, therefore, that in oligosaccharide K-1 galactose residues link to GlcNAc-5 and GlcNAc-7′. In oligosaccharide L-1, the chemical shifts of anomeric protons for GlcNAc-5 and GlcNAc-7 shifted downfield (4.562 and

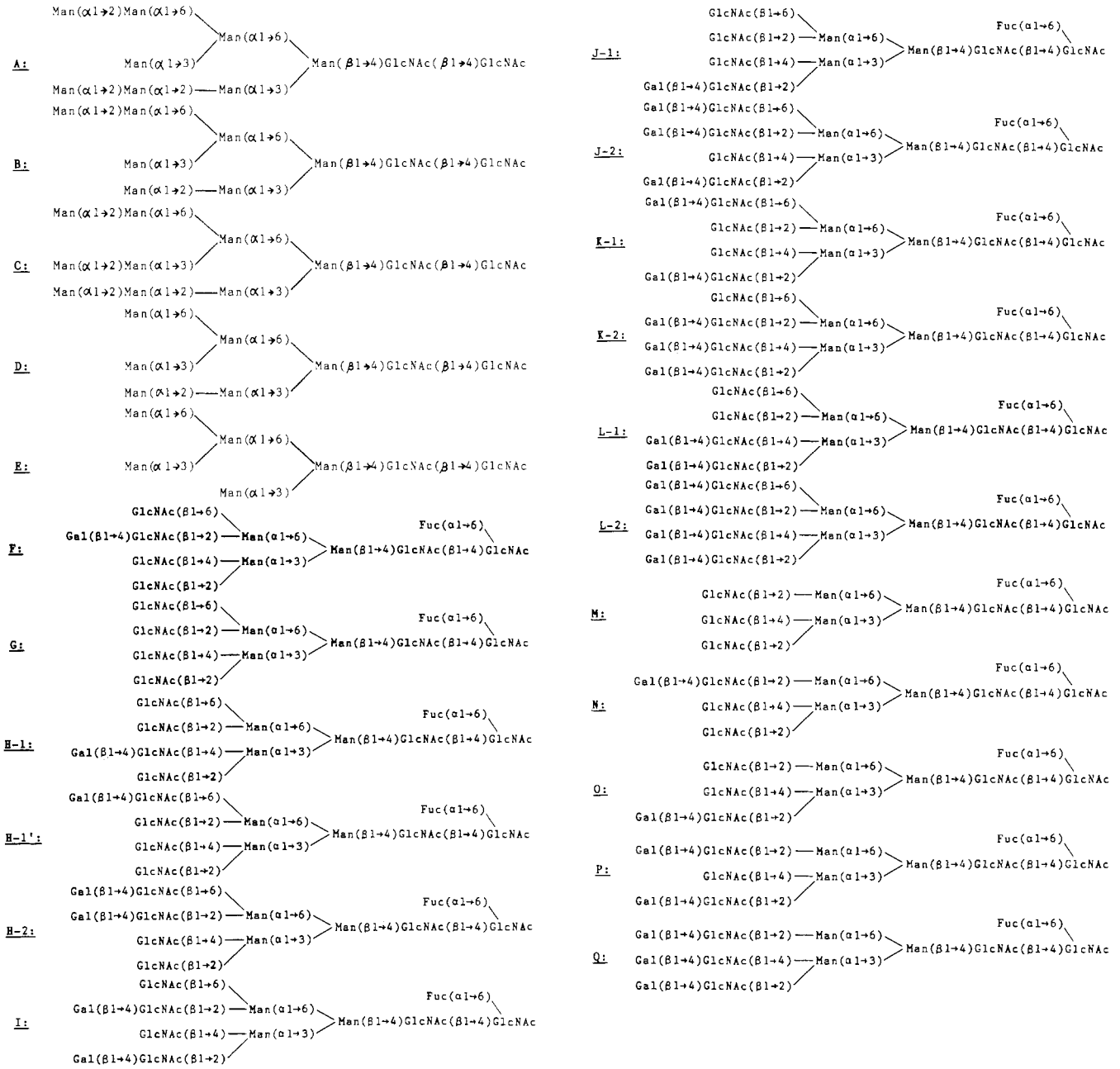


FIGURE 4: Proposed structures of the oligosaccharides of porcine pancreatic β-kallikrein B.

4.533 ppm, respectively). These results show that oligosaccharide L-1 has Gal-6 and Gal-8.

Group IV (*Oligosaccharides J-2 and K-2*). In oligosaccharide J-2, both GlcNAc-5 and GlcNAc-5′ are substituted by galactose residues. Furthermore, the presence of the signal at 4.471 ppm indicates the existence of Gal-8′. In oligosaccharide K-2, the chemical shifts of anomeric protons for GlcNAc-5 and GlcNAc-5′ showed downfield shifts to 4.560 and 4.581 ppm, respectively. These results indicate that GlcNAc-5 and GlcNAc-5′ are substituted by Gal-6 and Gal-6′, respectively. Since oligosaccharide K-2 does not show the characteristic H-1 chemical shift for Gal-8′, the NMR signal observed at 4.539 ppm can be assigned to the signal for GlcNAc-7 substituted by Gal-8.

Group V (*Oligosaccharide L-2*). The NMR data for oligosaccharide L-2 showed essentially the same features as obtained for reference compound 3 except for the values of chemical shifts for GlcNAc-2 and Fuc. These results indicate that oligosaccharide L-2 is a fucose-containing tetraantennary oligosaccharide that possesses four galactose residues.

The relevant chemical shift data for triantennary oligosaccharides (groups VI–IX), together with those of reference compounds 2 and 4, are listed in Table V. The H-1 chemical shifts for GlcNAc-5, GlcNAc-5′, and GlcNAc-7 shifted downfield by the introduction of galactose residues, Gal-6, Gal-6′, and Gal-8, respectively. The chemical shifts for the three galactose residues were identical within the experimental error (4.459 ppm).

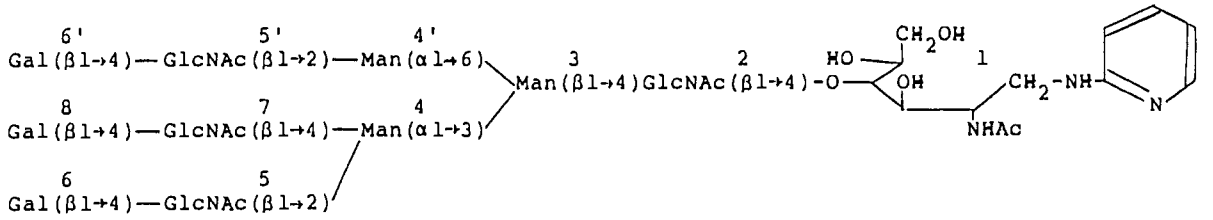
Group VI (*Oligosaccharide M*). The NMR data clearly indicate that oligosaccharide M does not have any galactose residues.

Group VII (*Oligosaccharides N and O*). The signals of H-1 protons for GlcNAc-5′ (oligosaccharide N) and GlcNAc-5 (oligosaccharide O) shifted downfield to 4.570 and 4.557 ppm, respectively. These show that the linking positions of galactose residues in oligosaccharides N and O are GlcNAc-5′ and GlcNAc-5, respectively.

Group VIII (*Oligosaccharide P*). In oligosaccharide P, the chemical shifts of H-1 protons for GlcNAc-5 and GlcNAc-5′ showed the same chemical shift values as those observed in

Table V: Chemical Shifts of Anomeric Protons and Methyl Protons for Triantennary Oligosaccharides (Groups VI–IX)

Reference Compound 4:



compd	chemical shifts[a] of anomeric protons							chemical shifts[a] of methyl protons				
	GlcNAc-2	GlcNAc-5	GlcNAc-5′	GlcNAc-7	Gal-6	Gal-6′	Gal-8	GlcNAc-2	GlcNAc-5	GlcNAc-5′	GlcNAc-7	Fuc
ref compd 2[b]	4.630	4.529	4.541	4.511				2.065	2.042	2.042	2.065	
ref compd 4	4.613	4.556	4.569	4.535	4.459	4.459	4.459	2.065	2.044	2.036	2.065	
M	4.675	4.528	4.542	4.515				2.073	2.046	2.040	2.071	1.179
N	4.631	4.532	4.570	4.515		4.462		2.071	2.047	2.037	2.071	1.177
O	4.669	4.557	4.540	4.517	4.453			2.072	2.042	2.042	2.072	1.181
P	4.674	4.557	4.571	4.516	4.457	4.457		2.072	2.045	2.037	2.072	1.181
Q	nd[c]	4.556	4.570	4.536	4.459	4.459	4.459	2.076	2.044	2.036	2.067	1.180

[a] Chemical shifts are expressed in ppm from internal DSS but were actually measured with internal acetone (δ = 2.2164 ppm in D_2O at 23 °C). [b] Structure of reference compound 2 is described in Table II. [c] The signal was not detectable due to the limited amount of sample.

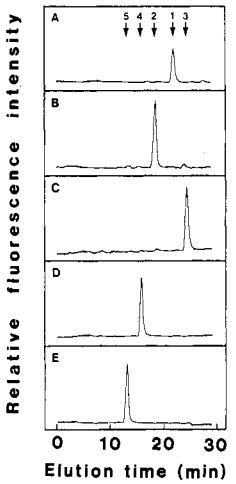


FIGURE 5: HPLC profiles on an amide–silica column for oligosaccharides A–E obtained from an ODS–silica column (Figure 1a). Each of the PA oligosaccharides (about 1 nmol) was injected on an amide–silica column. A gradient was started at time 0. (A) Oligosaccharide A; (B) oligosaccharide B; (C) oligosaccharide C; (D) oligosaccharide D; (E) oligosaccharide E. Arrows indicate the elution positions of standard oligosaccharides 1–5. The structures of the standards are described in the legend for Figure 1.

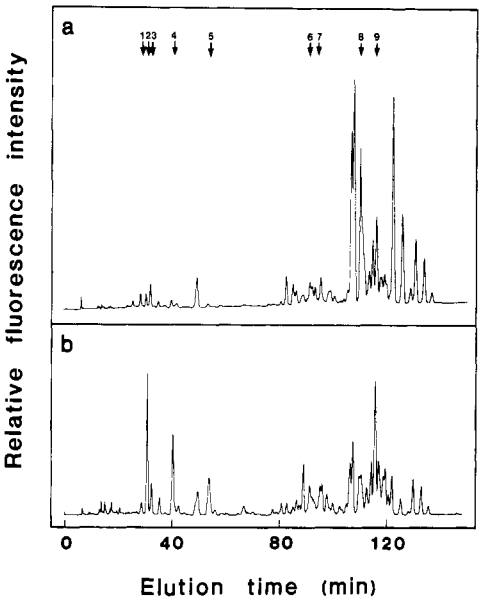


FIGURE 6: Comparison of the oligosaccharides linked to the two glycosylation sites of β-kallikrein B. (a) Asn-95 (light chain); (b) Asn-239 (heavy chain). A mixture of PA oligosaccharides (about 1 nmol) obtained from each of polypeptides was injected on an ODS–silica column. A gradient was started at time 0. Arrows indicate the elution positions of the standard oligosaccharides. The structures of the standards are described in the legend of Figure 1.

the reference compound 4. These results suggest that GlcNAc-5 and GlcNAc-5′ of oligosaccharide P are substituted by galactose residues.

Group IX (Oligosaccharide Q). The NMR data for oligosaccharide Q indicate that this oligosaccharide has three galactose residues at the nonreducing terminal.

The proposed structures for group I–IX oligosaccharides are summarized in Figure 4.

Characterization of Oligosaccharides A–E. As shown in Figure 1a, the elution positions of oligosaccharides A–E on an ODS–silica column coincided with those of standard oligomannose-type oligosaccharides 1–5, respectively. Oligosaccharides A–E were separately subjected to size fractionation HPLC (Figure 5). Oligosaccharides A–E were eluted again at the same elution positions as those of standard oligosaccharides 1–5, respectively. Although all of these oligosaccharides A–E were not susceptible to both β-galactosidase and β-*N*-acetylhexosaminidase, they were converted to Man(β1→4)GlcNAc(β1→4)GlcNAc-PA by jack bean α-mannosidase digestion (data not shown). The structures proposed on the basis of these results for oligosaccharides A–E are summarized in Figure 4.

Distribution of Carbohydrate Chains into Two Glycosylation Sites Asn-95 and Asn-239. The two glycosylation sites Asn-95 and Asn-239 of β-kallikrein B are present in the light chain and the heavy chain, respectively. The two polypeptide chains of β-kallikrein B were separated, and the oligosaccharides of each of these polypeptide chains were separately released by glycopeptidase digestion. The reverse-phase HPLC profiles of the PA oligosaccharides obtained from each of these polypeptide chains are given in Figure 6. It should be noted that oligomannose-type oligosaccharides exist exclusively at Asn-239, although tri- and tetraantennary *N*-acetyllactosamine-type oligosaccharides are present in both of the glycosylation sites.

Oligosaccharide Profile of Porcine Pancreatic β-Kallikrein

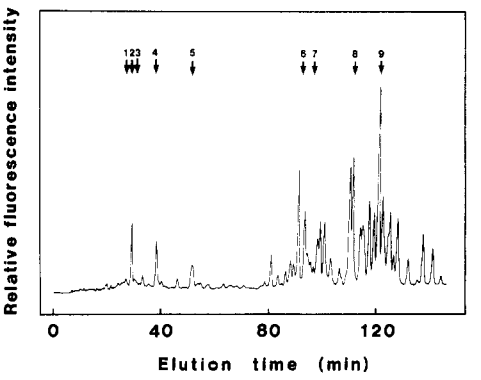


FIGURE 7: HPLC profiles on an ODS–silica column for pyridylaminated oligosaccharides obtained from porcine pancreatic β-kallikrein A. A mixture of PA oligosaccharides (about 1 nmol) was injected on an ODS–silica column. A gradient was started at time 0. Arrows indicate the elution positions of the standard oligosaccharides. The structures of the standards are described in the legend of Figure 1.

A. β-Kallikrein A has only one glycosylation site at Asn-95. Figure 7 gives the elution profile on a reverse-phase column for PA oligosaccharides prepared from porcine pancreatic β-kallikrein A. It is concluded that the pattern of oligosaccharides obtained from kallikrein A (Figure 7) is very similar to that for kallikrein B (Figure 1a).

It has been suggested that oligosaccharide moieties of pancreatic kallikreins play important roles such as participation in secretion of kallikrein from pancreas, protection against proteolytic degradation, or clearance from blood. At present, however, very little evidence is available about the role of asparagine-linked oligosaccharides present in pancreatic kallikreins. This is because little has been known concerning the exact structures of these oligosaccharides. It was difficult to isolate individual carbohydrate chains of porcine pancreatic kallikrein and to determine the structures in detail, because the carbohydrate chains of this protein were extremely heterogeneous (Moriya et al., 1981). Detailed structural analyses on the basis of the HPLC and ^{1}H NMR data reported in this paper will be of great help in discussing the role of oligosaccharides of porcine pancreatic kallikrein.

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REFERENCES

Arakawa, Y., Imanari, T., & Tamura, Z. (1976) *Chem. Pharm. Bull. 24*, 2032–2037.

Bode, W., Chen, Z., Bartels, K., Kutzbach, C., & Schmidt-Kastner, G. (1983) *J. Mol. Biol. 164*, 237–282.

Fiedler, F., & Werle, E. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 1087–1089.

Fiedler, F., & Hirschauer, C. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1209–1218.

Habermann, E. (1962) *Hoppe-Seyler's Z. Physiol. Chem. 328*, 15–23.

Habermann, E. (1966) *Naunyn Schmiedelbergs Arch. Pharmakol. 253*, 474–483.

Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.

Hase, S., Ibuki, T., & Ikenaka, T. (1984) *J. Biochem. (Tokyo) 95*, 197–203.

Ikekita, M., Moriya, H., Kizuki, K., & Ozawa, S. (1980) *Chem. Pharm. Bull. 28*, 1948–1950.

Kutzbach, C., & Schmidt-Kastner, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 1099–1106.

Mikami, H., & Ishida, Y. (1983) *Bunseki Kagaku 32*, e207–e210.

Moriya, H., Ikekita, M., & Kizuki, K. (1981) *Chem. Pharm. Bull. 29*, 1785–1788.

Moriya, H., Ikekita, M., & Kizuki, K. (1983) *Adv. Exp. Med. Biol. 156A*, 309–316.

Mutsaers, J. H. G. M., van Halbeek, H., Vliegenthart, J. F. G., Iwase, H., Kato, Y., & Hotta, K. (1985) *Biochim. Biophys. Acta 843*, 123–127.

Nishigaki, M., Yamashita, K., Matsuda, I., Arashima, S., & Kobata, A. (1978) *J. Biochem. (Tokyo) 84*, 823–834.

Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., & Kobata, A. (1975) *J. Biol. Chem. 250*, 8569–8575.

Takahashi, N., Ishihara, H., Tejima, S., Oike, Y., Kimata, K., Shinomura, T., & Suzuki, S. (1985) *Biochem. J. 229*, 561–571.

Takahashi, N., Ishii, I., Ishihara, H., Mori, M., Tejima, S., Jefferis, R., Endo, S., & Arata, Y. (1987) *Biochemistry 26*, 1137–1144.

Tomiya, N., Kurono, M., Ishihara, H., Tejima, S., Endo, S., Arata, Y., & Takahashi, N. (1987) *Anal. Biochem. 163*, 489–499.

Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., & Takahashi, N. (1988) *Anal. Biochem. 171*, 73–90.

Tschesche, H., Mair, G., Godec, G., Fiedler, F., Ehret, W., Hirschauer, C., Lemon, M., Fritz, H., Schmidt-Kastner, G., & Kutzbach, C. (1979) *Adv. Exp. Med. Biol. 120A*, 245–260.

Tsuji, T., Yamamoto, K., Irimura, T., & Osawa, T. (1981) *Biochem. J. 195*, 691–699.

Warren, L. (1963) *Methods Enzymol. 6*, 463.

Werle, E., & Berek, V. (1948) *Angew. Chem. 60A*, 53.

Yano, M., Magasawa, S., & Suzuki, T. (1971) *J. Biochem. (Tokyo) 69*, 471–481.

Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., & Kobata, A. (1981) *J. Biol. Chem. 256*, 8476–8484.