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A detailed structural characterization of ribonuclease B oligosaccharides by ^{1}H NMR spectroscopy and mass spectrometry

Daotian Fu *, Ling Chen, Roger A. O'Neill

Perkin Elmer Applied Biosystems Division, 850 Lincoln Centre Dr., Foster City, CA 94404, USA

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Abstract

The structures of ribonuclease B oligosaccharides have previously been shown to be high mannose type by methylation analyses and sequential exoglycosidase digestion. Due to the unique nature of these oligosaccharides, in that all mannosyl residues are attached by α-(1 → 2)-linkages beyond the branch points, methylation analysis fails to solve the exact structures beyond Man5. Therefore, we have undertaken this study using ^{1}H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. In this study, bovine pancreatic ribonuclease B was first reduced and carboxymethylated, and was then deglycosylated by peptide/*N*-glycosidase F (PNGase F). The released oligosaccharides were fractionated by Bio-Gel P-4 chromatography to give five pools, Man5 through Man9. The structures of the oligosaccharide pools were then studied by laser desorption time of flight mass spectrometry and ^{1}H NMR spectroscopy at 300 MHz. For Man5, Man-A and Man-B are attached in α-(1 → 3)- and α-(1 → 6)-linkages to the α-(1 → 6)-linked Man-4' of the pentasaccharide core structure. For Man6, Man-C is linked α-(1 → 2) to the α-(1 → 3)-linked Man-4. Man7 exists as three structural isomers, and has the additional mannosyl residue (Man-D) linked α-(1 → 2) to Man-A, Man-B, and Man-C is linked α-(1 → 2) to the α-(1 → 3)-linked Man-4. Man-7 exists as three structural isomers, with the additional two mannosyl residues linked α-(1 → 2) to Man-A, Man-B, and Man-C. For each position, Man-A, Man-B, and Man-C, the extent of occupancy by one of the additional α-(→)-linked mannosyl residues was 15, 94, and 91%, respectively. Man9 is a single component, with the three additional mannosyl residues linked α-(1 → 2) to Man-A, Man-B, and Man-C, respectively. The relative molar proportions of Man5 to Man9 are 57, 31, 4, 7, and 1%, respectively. This report presents for the first time the complete structural characterization of the oligosaccharides from ribonuclease B.

* Corresponding author.

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Key words: Ribonuclease B; Oligosaccharides; Mass spectrometry; NMR spectroscopy

1. Introduction

Bovine pancreatic ribonuclease B is a glycoprotein with a molecular weight of 15500 daltons. It contains 124 amino acid residues and one glycosylation site (Asn_{34}) [1]. Its carbohydrate structures have previously been studied [2–4] and its crystal structure at 2.5 Å resolution has been solved [5]. In the previous study of ribonuclease B oligosaccharide structures [2], the carbohydrate chains of RNase B were released from the polypeptide moiety by hydrazinolysis and by digestion with *endo*-β-*N*-acetylglucosaminidase H. The oligosaccharide mixtures thus released were fractionated by Bio-Gel P-4 column chromatography, and their structures were studied by sequential exoglycosidase digestion, methylation analysis, and by periodate oxidation. The results indicated that the oligosaccharides present were of the high mannose type. Due to the unique nature of these high mannose type oligosaccharides, in that all mannosyl residues beyond the branch points were linked by α-(1 → 2)-linkages, methylation analyses and exoglycosidase digestion failed to solve the exact structures of the oligosaccharides having more than five mannosyl residues. Therefore, the exact structures of the oligosaccharides beyond Man5 remained undetermined.

As the biological functions of the carbohydrate chains of glycoproteins are being increasingly recognized and understood, detailed characterization of their structures has presented a new level of analytical challenge. With the possible exception of high field NMR, no single analytical technique is capable of the complete elucidation of an oligosaccharide structure. As a consequence, complete carbohydrate characterization is usually performed using several complementary techniques, which may include serial lectin affinity chromatography [6,7], exoglycosidase digestion [8], mass spectrometry [9–11], NMR spectroscopy [12,13], and composition and linkage analyses [14].

Of all available methods, mass spectrometry and NMR spectroscopy are the two most commonly used tools in the elucidation of detailed carbohydrate structures. Both techniques are complementary to each other in many ways, each with its own set of advantages and disadvantages. The main advantage of mass spectrometry is its sensitivity and its ability to give an unequivocal molecular weight of an oligosaccharide [9,10]. With tandem mass spectrometry, sequence information may be derived [11]. NMR, on the other hand, is the single most powerful technique for carbohydrate structure characterization [12,13]. It, by itself, can be used to solve the complete structure of an oligosaccharide. The most widely used approach to the NMR of carbohydrates from glycoproteins is the structural reporter-group concept introduced by Vliegenthart and co-workers [12]. In this approach, the interpretation of a ^1H NMR spectrum of an oligosaccharide is based on the chemical shifts and coupling constants of protons of the structural reporter groups. These groups include all of the anomeric protons, H-2 and H-3 of mannose, H-3

and H-4 of galactose, H-5 and the methyl protons of fucose, H-3 (axial and equatorial) of sialic acid, and the *N*-acetyl protons [12].

In this study, bovine pancreatic ribonuclease B was reduced and carboxymethylated, and then deglycosylated by peptide/*N*-glycosidase F (PNGase F). The released oligosaccharides were fractionated by Bio-Gel P-4 chromatography to give five pools. Their identities were characterized to be $Man_5GlcNAc_2$ through $Man_9GlcNAc_2$ by composition analysis and laser desorption time of flight mass spectrometry. The detailed structures were further elucidated using the structural reporter-group approach of ^1H NMR spectroscopy. By combining mass spectrometry and ^1H NMR spectroscopy, we have for the first time solved the exact structures of nine distinct oligosaccharides from ribonuclease B.

2. Experimental

Materials.—Bovine pancreatic ribonuclease B, dithiothreitol, and iodoacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). PNGase F was purchased from Genzyme Co. (Boston, MA). Bio-Gel P-4 (extra fine) and P-6 (medium) were purchased from Bio-Rad Lab., Inc. (Hercules, CA). Amicon G16 × 1000 columns and water jackets were purchased from Amicon, Inc. (Berverly, MA). Two G16 × 1000 columns were used to make a 2-m column (16 × 2000 mm) by joining them together. All other reagents were of analytical grade and were available commercially.

Reduction and carboxymethylation.—Bovine pancreatic ribonuclease B (650 mg) was reduced with dithiothreitol and *S*-carboxymethylated with iodoacetic acid as previously described by Carr and Roberts [15].

PNGase F release and isolation of oligosaccharides.—The reduced and carboxymethylated ribonuclease B (650 mg) was dissolved in 50 mL of 100 mM ammonium acetate (pH 7.5) with 10 mM EDTA. Approximately 14 units of PNGase F in 50% glycerol were added (7 units initially and 7 units after 12 h of incubation). The mixture was incubated at room temperature on a shaker for 24 h. This procedure was shown to release all oligosaccharides from ribonuclease B. The released oligosaccharides were separated from the deglycosylated protein using a Bio-Gel P-6 column (30 × 1000 mm) with 500 mM ammonium acetate as the eluant. The carbohydrate containing fractions, as assayed by the anthrone method [14], were pooled and lyophilized.

Gel permeation chromatography.—Bio-Gel P-4 column chromatography was performed using a 16 × 2000 mm column equipped with water jackets. The oligosaccharide sample was loaded onto the column in 0.2 mL of water. The column was eluted with 500 mM ammonium acetate, and 3-mL fractions were collected and lyophilized. During operation, the column was kept at 40°C by circulating warm water through the jackets.

Composition analysis.—The glycosyl residue composition of the isolated oligosaccharides was determined as 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives by reverse phase HPLC, as previously described by Honda et al. [16].

Anthrone assay of total carbohydrate.—The carbohydrate contents of the oligosaccharide pools from Bio-Gel P-4 chromatography were determined by the anthrone method according to York et al. [14]. Neutral sugars were determined using mannose as a standard. Since *N*-acetyl-D-glucosamine did not give any color by the anthrone method, its quantity could not be directly measured. However, the mass spectra (Fig. 2) and the compositional analysis data (Table 1) indicated that the oligosaccharide pools, Man5 through Man9, contained two *N*-acetyl glucosaminyl and five to nine mannosyl residues, respectively. The total carbohydrate of each pool was, therefore, calculated from the anthrone assay results, taking into account the assumed presence of two *N*-acetyl glucosaminyl residues per oligosaccharide molecule.

Mass spectrometry.—Mass spectra were recorded on an Applied Biosystems prototype matrix-assisted laser desorption mass spectrometer, equipped with a linear time-of-flight analyzer and a pulsed nitrogen laser (337 nm). The pulse duration was 3 ns and the pulse energy was 120 μJ. The acceleration voltage of the mass spectrometer was maintained at 15 kV. 2,5-Dihydroxybenzoic acid (15 mg/mL) dissolved in 9:1 H_2O–EtOH was used as the matrix. The samples were prepared by mixing the oligosaccharide sample (50 ng/μL) with the matrix in a 1:1 ratio. The sample (1 mL) was loaded onto the target, which was then air-dried at room temperature. The spectra were recorded by accumulation of data from 4 to 10 laser pulses.

^{1}H NMR spectroscopy.—For ^{1}H NMR spectroscopy, the oligosaccharides isolated from the gel permeation column were exchanged three times with 99.96% D_2O at room temperature with intermediate lyophilization. The ^{1}H NMR spectra were recorded at 300 MHz on a Varian Unity 300 NMR spectrometer at room temperature. Chemical shifts were expressed in ppm by reference to internal acetone (δ 2.225 ppm) and are accurate to $\pm$0.001 ppm.

3. Results

Bovine pancreatic ribonuclease B (650 mg) was reduced with dithiothreitol and *S*-carboxymethylated with iodoacetic acid, and after digestion of the glycoprotein with PNGase F, the released oligosaccharides were separated from the deglycosylated protein on a Bio-Gel P-6 column (data not shown). The oligosaccharide-containing fractions were pooled and lyophilized, and the lyophilized oligosaccharide mixture (20 mg) was dissolved in 0.2 mL of water and chromatographed on a Bio-Gel P-4 column. Fractions (3 mL) were collected and assayed for carbohydrate by the anthrone method [14]. The absorbance at 650 nm vs. fraction number is plotted in Figure 1. The oligosaccharide mixture from ribonuclease B was resolved into five peaks, designated Man5 through Man9. Each peak was pooled, as indicated by the bars in Fig. 1, and lyophilized to give five pools, Man5 through Man9. The total carbohydrate for each pool was assayed by the anthrone method to be 9.2 mg for Man5, 5.7 mg for Man6, 0.8 mg for Man7, 1.6 mg for Man8, and 0.3 mg for Man9. The relative molar proportions of the five pools, as calculated

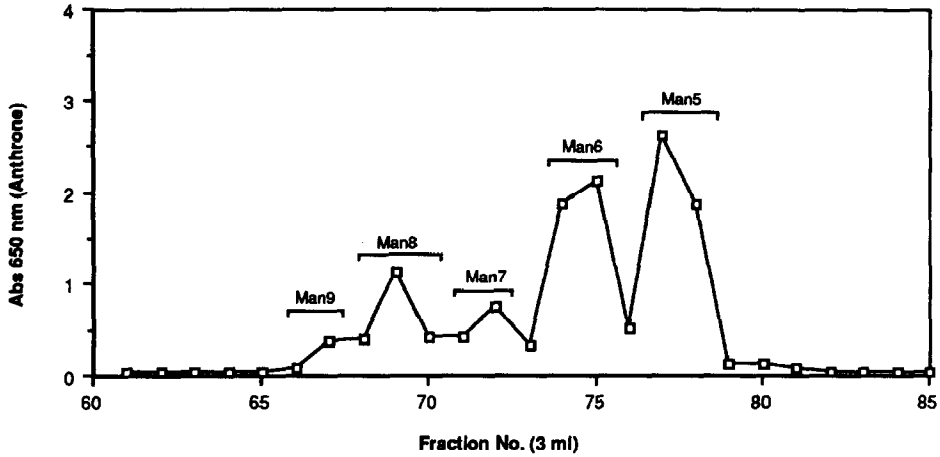
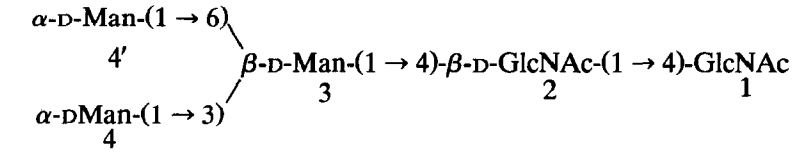


Fig. 1. Bio-Gel P-4 column chromatogram of the ribonuclease B oligosaccharide mixture released by PNGase F. The column was eluted with 500 mM ammonium acetate, and 3-ml fractions were collected and assayed for carbohydrate using the anthrone assay. Carbohydrate-containing fractions were pooled as indicated by the bars.

from their total carbohydrates and their molecular weights as determined below, were 57, 31, 4, 7, and 1%, respectively.

In order to confirm their identities and assess their purities, the five oligosaccharide pools were subjected to compositional analysis and laser desorption time of flight mass spectrometry. The mass spectra (Fig. 2) show that the five pools contain ions that correspond respectively to $(M + Na)^+$ of $Hex_5HexNAc_2$, $Hex_6HexNAc_2$, $Hex_7HexNAc_2$, $Hex_8HexNAc_2$, and $Hex_9HexNAc_2$. The compositional analysis data (Table 1) clearly indicate that they are of the high mannose type with five to nine mannosyl residues, respectively. As judged by their mass spectra, all pools are pure except Man8, which is contaminated by a small amount of Man9.

Once the identities of the five pools had been characterized and their purities assessed, they were subjected to ^1H NMR spectroscopy at 300 MHz for detailed structure characterization. Fig. 3 shows the H-1 and H-2 regions of the ^1H NMR spectra, and Table 2 lists the chemical shifts of the structural reporter groups. As reported previously [2–4], the ^1H NMR spectra indicate that all five pools contain an identical pentasaccharide ($Man_3GlcNAc_2$) core structure, as follows:



The characteristic structural features of this pentasaccharide core have been extensively studied and documented [17,18]. Briefly, the two H-1 signals of GlcNAc-1 at δ 5.19 for GlcNAc-1(α) and at δ 4.70 for GlcNAc-1(β), the H-1 signal of

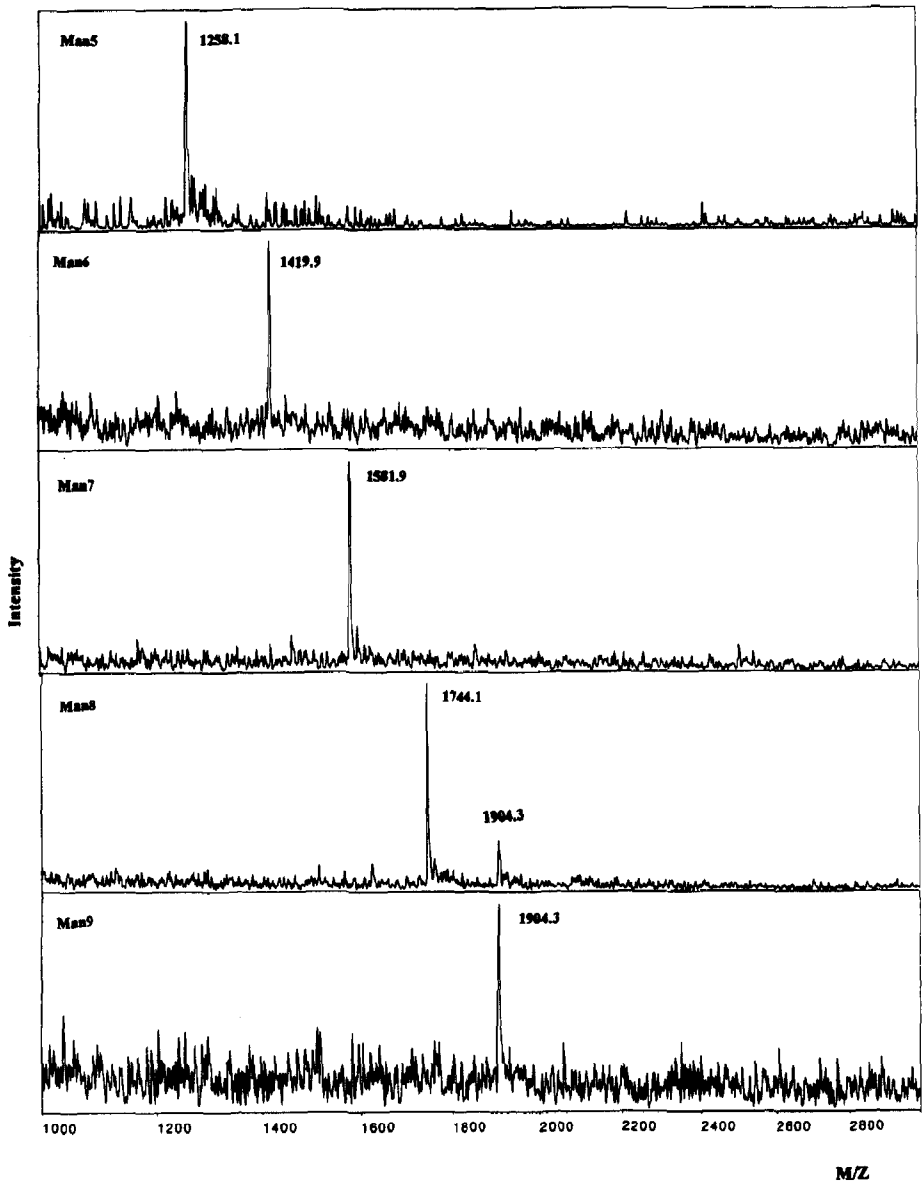


Fig. 2. Laser desorption time of flight mass spectra of the pooled oligosaccharide pools, Man5 through Man9, from ribonuclease B. The peaks at m/z 1258.1 (1258.2), 1419.9 (1420.4), 1581.9 (1582.5), 1744.1 (1744.7), and 1904.3 (1906.8) correspond to the $(M+Na)^+$ of $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$, respectively. The numbers in parentheses represent their calculated molecular masses.

Glc-NAc-2 at δ 4.61, and the *N*-acetyl methyl singlets at δ 2.05 (GlcNAc-1) and at δ 2.08 (GlcNAc-2) are typical of the reducing *N,N'*-diacetylchitobiose unit. The occurrence of the three H-1 signals at δ 4.78 (Man-3), δ 5.10 (Man-4), and δ 4.92

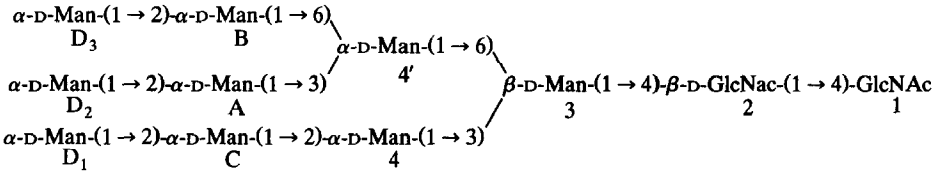
Table 1
Monosaccharide composition of fractions Man5 through Man9

Oligosaccharide	Normalized molar ratio [a]	
	GlcNAc	Man
Man5	2.0	5.1
Man6	2.0	6.1
Man7	2.0	6.9
Man8	2.0	7.9
Man9	2.0	8.8

[a] Numbers normalized to 2.0 GlcNAc.

(Man-4′), and the three Man H-2 signals (in the δ 4.0 to 4.3 region) is further evidence for the presence of the pentasaccharide core structure.

For the spectral interpretation of pool Man5, it can be conceived of as an extension of the pentasaccharide core with two mannosyl residues. They can be both linked to either Man-4 or Man-4′, or one mannosyl residue can be linked to each. A comparison to the spectrum of the pentasaccharide core shows that the chemical shifts of H-1 and H-2 of Man-4 and Man-3 are essentially unaltered, indicating that the two mannosyl residues are not likely to be linked to Man-4. However, the H-1 signal of Man-4′ is shifted upfield to δ 4.875 relative to that in the pentasaccharide core (δ 4.92). This chemical shift decrease of 0.045 ppm is due to the substitution of Man-4′ at O-6, and possibly at O-3 as well, since the substitution at O-3 of Man-4′ hardly changes its H-1 signal noticeably [12,13]. Evidence for the presence of a disubstituted Man-4′ derives from the H-1 and H-2 signals of the additional two mannosyl residues. The signals at δ 4.911 and 3.987 are attributed to H-1 and H-2 of the terminal α-(1 → 6)-linked mannosyl residue (Man-B) [12,13], indicating substitution of Man-4′ at O-6. The H-1 signal at δ 5.093 and the H-2 resonance at δ 4.074 clearly indicates the presence of a terminal α-(1 → 3)-linked mannosyl residue (Man-A) [12], further confirming that Man-4′ is indeed disubstituted at both O-6 and O-3 with Man-B and Man-A, respectively. The numbering system used for denoting glycosyl residues in the high mannose type oligosaccharides is as follows:



The structure of Man5, derived from the above evidence, is shown in Table 2. Pool Man5 accounts for 57 mol% of the total ribonuclease B oligosaccharides according to the anthrone assay.

Pool Man6 has one more mannosyl residue than Man5, and can be considered an extension of Man5. A brief spectral comparison of Man6 with Man5 reveals a new set of chemical shifts at δ 5.050 and 4.066, which the H-1 and H-2 signals of a

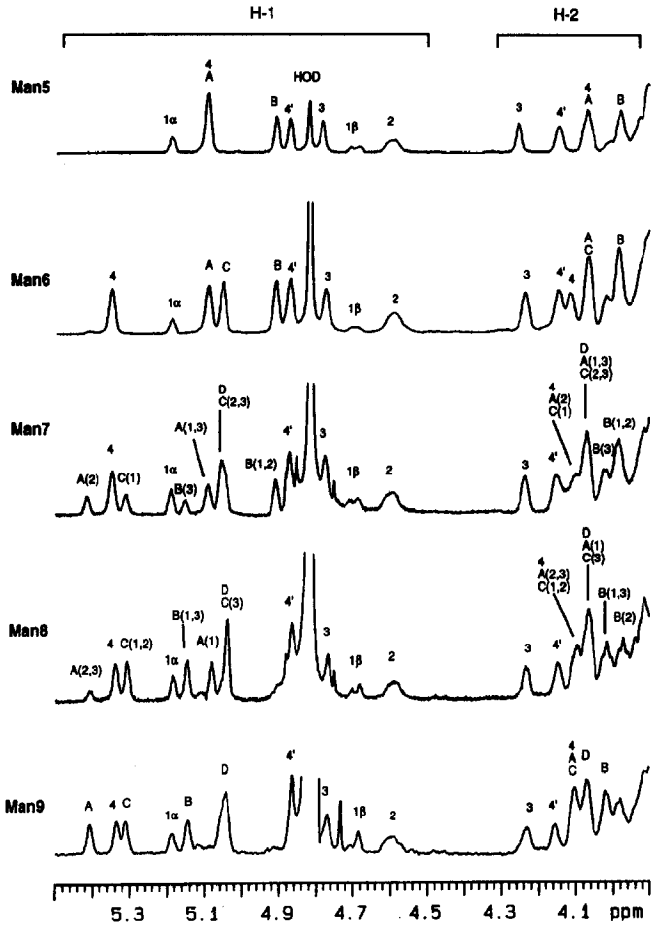


Fig. 3. ^{1}H NMR spectra (300 MHz, D_2O, 25°C) of the oligosaccharide pools, Man5 through Man9, from ribonuclease B. The peaks were assigned using symbolism described in the text. For Man7 and Man8, the number(s) in parentheses indicate the contributing isomers in the mixture. The H-1 and H-2 signals, as assigned in the figure, indicate that they are of high mannose type with the structures listed in Table 2.

terminal α-(1 → 2)-linked mannose [12,13]. The terminal α-(1 → 2)-linked mannosyl residue (Man-C) is unambiguously linked to Man-4 (Table 2), as the H-1 and H-2 signals of Man-4 are shifted downfield by 0.25 and 0.033 ppm, respectively. The H-1 and H-2 signals of Man-3 are both shifted slightly upfield ($\Delta\delta_{H\text{-}1} = -0.015$ and $\Delta\delta_{H\text{-}2} = -0.023$ ppm). The chemical shifts of the structural reporter groups of other residues remain essentially identical to those of Man5. The structure of Man6 is shown in Table 2. Pool Man6 accounts for 31 mol% of the total ribonuclease B oligosaccharides according to the anthrone assay.

For the spectral interpretation of pool Man7, the H-1 and H-2 signals at δ 5.057 and 4.071 prove that the additional mannosyl residue is terminally linked in an

Table 2
Oligosaccharide structures of Man5 through Man9

Name	Structure	Mol%
Man5	α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 3)	57
Man6	α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	31
Man7(1)	α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	1.5
Man7(2)	α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 2)-α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	1.5
Man7(3)	α-D-Man-(1 → 2)-α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	1.0

Table 2 (continued)

Name	Structure	Mol%
Man8(1)	α-D-Man-(1 → 2)-α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	5.9
Man8(2)	α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 2)-α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	0.4
Man8(3)	α-D-Man-(1 → 2)-α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 2)-α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	0.7
Man9	α-D-Man-(1 → 2)-α-D-Man-(1 → 6) α-D-Man-(1 → 6) α-D-Man-(1 → 2)-α-D-Man-(1 → 3) β-D-Man-(1 → 4)-β-D-GlcNAc-(1 → 4)-GlcNAc α-D-Man-(1 → 2)-α-D-Man-(1 → 2)-α-D-Man-(1 → 3)	1.0

Table 3
^{1}H Chemical shifts of the structural reporter groups of Man5 through Man9

Group	Residue [a]	Chemical shifts (ppm)								
		Man5	Man6	Man7(1)	Man7(2)	Man7(3)	Man8(1)	Man8(2)	Man8(3)	Man9
H-1	GlcNAc-1(α)	5.191	5.187	5.190	5.190	5.190	5.187	5.187	5.187	5.191
	GlcNAc-1(β)	4.700	4.704	4.701	4.701	4.701	4.693	4.693	4.693	4.690
	GlcNAc-2	4.606	4.589	4.601	4.601	4.601	4.586	4.586	4.586	4.596
	Man-3	4.789	4.774	4.774	4.774	4.774	4.771	4.771	4.771	4.775
	Man-4	5.099	5.349	5.348	5.348	5.348	5.341	5.341	5.341	5.343
	Man-4′	4.875	4.870	4.874	4.874	4.874	4.870	4.870	4.870	4.869
	Man-A	5.093	5.090	5.091	5.415	5.091	5.085	5.415	5.415	5.414
	Man-B	4.911	4.908	4.909	4.909	5.135	5.151	4.886	5.151	5.153
	Man-C		5.050	5.310	5.050	5.050	5.309	5.309	5.050	5.317
	Man-D_1			5.057			5.042	5.042		5.047
	Man-D_2				5.057			5.042	5.042	5.047
	Man-D_3					5.057	5.042		5.042	5.047
H-2	Man-3	4.260	4.237	4.237	4.237	4.237	4.234	4.234	4.234	4.238
	Man-4	4.084	4.117	4.096	4.096	4.096	4.099	4.099	4.099	4.108
	Man-4′	4.152	4.149	4.147	4.147	4.147	4.149	4.149	4.149	4.149
	Man-A	4.074	4.066	4.071	4.100	4.071	4.069	4.099	4.099	4.108
	Man-B	3.987	3.987	3.985	4.025	4.025	4.021	3.974	4.021	4.025
	Man-C		4.066	4.100	4.071	4.071	4.099	4.099	4.069	4.108
	Man-D_1			4.071			4.069	4.069		4.074
	Man-D_2				4.071			4.069	4.069	4.074
	Man-D_3					4.071	4.069		4.069	4.074
NAc	GlcNAc-1	2.044	2.041	2.043	2.043	2.043	2.037	2.037	2.037	2.042
	GlcNAc-2	2.070	2.065	2.068	2.068	2.068	2.063	2.063	2.063	2.072

[a] Refer to the numbering system given in the text.

α-(1 → 2)-linkage. In addition to the signals of all the structural reporter groups of Man6, which remain essentially unchanged, a new set of signals appears at δ 5.415, 5.310, and 5.135. These correspond to H-1 resonances of Man-A, Man-C, and Man-B when each is substituted with a terminal α-(1 → 2)-linked Man. Since there is only one more mannosyl residue in Man7 than in Man6, and the integration values of all three signals together constitute one proton, we concluded that pool Man7 was a mixture of three structural isomers, namely Man7(1), Man7(2), and Man7(3) with the additional mannose (as Man-D_1, Man-D_2, and Man-D_3) linked to Man-C, Man-A, and Man-B, respectively (Table 2). Based on the integration of the H-1 signals, Man7(1), Man7(2), and Man7(3) exist in a molar ratio of 38:37:25, and together constitute 4 mol% of the total oligosaccharides according to the anthrone assay.

The spectral interpretation of pool Man8 is similar to that of Man7. In comparison to that of Man7, the chemical shifts of all the structural reporter groups of pool Man8 remain essentially the same with the exception of the integration values of the signals. The presence of the extra terminal α-(1 → 2)-linked Man-D is evident from the integrations of the H-1 and H-2 signals at δ

5.042 and 4.069. It is clear that pool Man8 is again a mixture of three structural isomers (Table 2), namely Man8(1) with Man-D_1 and Man-D_3 linked to Man-C and Man-B, Man8(2) with Man-D_1 and Man-D_2 linked to Man-C and Man-A, and Man8(3) with Man-D_2 and Man-D_3 linked to Man-A and Man-B. As estimated by their NMR integrations, the three isomers, Man8(1), Man8(2), and Man8(3), exist in a molar ratio of 84:6:10, and together account for 7 mol% of the total ribonuclease B oligosaccharides according to the anthrone assay (Table 2).

As is evident from the composition analysis and mass spectrometry data, pool Man9 contains two GlcNAc and nine Man residues. In comparison to other pools, the NMR spectral interpretation of Man9 is straightforward. The occurrence of the additional mannosyl residue is once again confirmed by the integrations of the H-1 and H-2 signals at δ 5.047 and 4.074. However, the signals for terminal Man-A, Man-B, and Man-C have all disappeared from the spectrum, as expected for this structure. Man-C, Man-B, and Man-A are, therefore, all terminated with α-(1 → 2)-linked mannosyl residues, namely Man-D_1, Man-D_2, and Man-D_3 (Table 2). Pool Man9 is, therefore, a single component accounting for 1 mol% of the total ribonuclease B oligosacchairdes according to the anthrone assay.

4. Discussion

Widespread use of bovine pancreatic ribonuclease B as a source of high mannose type oligosaccharides has generated considerable interest in the structures of its carbohydrate moieties. Despite the previous work on its structural characterization [2–4,18,19], detailed structural assignments have, until now, remained incomplete.

Bovine pancreatic ribonuclease occurs as at least four distinct molecules; A, B, C, and D. All appear to have the same polypeptide chains, and differ only in their carbohydrate moieties [19,20]. Ribonuclease A was reported to be free of any carbohydrate, and ribonuclease B, C, and D all had a single sugar chain attached to Asn_{34} [18,19]. Ribonuclease B was shown to contain, on average, approximately six residues of mannose and two residues of glucosamine [21]. Ribonuclease C and D, however, were reported to contain mannose, fucose, and galactose in a molar ratio of 3.84:0.88:2.0 and 2.8:0.93:2.0, respectively [20]. The oligosaccharides of ribonucleases C and D are apparently more highly processed than ribonuclease B toward complex types of structures. It is not completely clear why the carbohydrate moiety of ribonuclease B is not further processed to complex type oligosaccharides. Studies in two cell free systems provided evidence that some aspects of the native ribonuclease B conformation prevented one or more of the processing enzymes of bovine pancreas from acting on the high mannose oligosaccharide chains [22].

It is well known that ribonuclease B contains high mannose type oligosaccharides with five to nine mannosyl residues [2,21]. However, the occurrence of three structural isomers of both Man7 and Man8 has not been shown before. Our data indicated that, as reported previously [2], Bio-Gel P-4 column chromatography could not resolve the three structural isomers of Man7 and Man8. Recently, Rudd

et al. studied the glycoform populations of ribonuclease B, both by direct analysis of the intact glycoprotein using capillary electrophoresis (CE), and by indirect analysis of the hydrazine released oligosaccharides using high pH anion exchange (HPAE) and Bio-Gel P-4 chromatographies [23]. Unfortunately, neither CE or HPAE could resolve the three structural isomers of Man7, or the three of Man8. It should be noted that the relative population of each glycoform reported by Rudd et al. [23] was significantly different from that of our study here. This discripancy may be due to differences of the ribonuclease B preparations used in these two studies, as glycoproteins are known to vary significantly from one preparation to another in their carbohydrate populations. Our results also indicated that, as reported by Rudd et al. [23], the ribonuclease B preparation from Sigma Chemical Co. contained as much as 50% of ribonuclease A (data not shown). We were able to show that the molar ratios of the nine oligosaccharide structures from the two different lots of ribonuclease B (Sigma Chemical Co.) we have used were essentially the same.

In conclusion, this study has refined our insight into the structures of ribonuclease B oligosaccharides. We have elucidated the complete structures and determined the relative proportions of nine distinct high mannose type oligosaccharides (Table 2) from ribonuclease B by ^{1}H NMR spectroscopy. This reflects a greater heterogeneity than had previously been appreciated for this glycoprotein [2,21], and represents the first complete description of all oligosaccharides present in ribonuclease B. Further, our finding that terminal α-(1 → 2)-linked mannosyl residues are preferentially linked to some branches over others in high mannose type oligosaccharides contributes to the understanding of the biosysthesis and processing of glycoprotein oligosaccharides.

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