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The substrate specificity of the enzyme Endo-*α*-*N*-acetyl-D-galactosaminidase from *Diplococcus pneumonia*

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The substrate specificity of the enzyme endo-*α*-*N*-acetyl-D-galactosaminidase from *Diplococcus pneumonia* was re-examined using bovine submaxillary mucin and remodelled antifreeze glycoprotein as substrates. Incubation with desialylated bovine submaxillary mucin, which contains six O-linked core types, indicated that the disaccharide Gal*β*1-3GalNAc, which is present in very small amount, was the only glycan released, while the disaccharide GlcNAc*β*1-3GalNAc, which is the major structure present, and other disaccharides, were not released. To test whether the core disaccharide Gal*β*1-3GalNAc with sialic acid linked *α*2-3 to the Gal or linked *α*2-6 to the GalNAc was released, the enzyme was incubated with remodelled antifreeze glycoprotein containing (1) [^{3}H]NeuAc*α*2-3Gal*β*1-3GalNAc and (2) Gal*β*1-3[[^{14}C]NeuAc*α*2-6]GalNAc as substrates. No NeuAc-containing trisaccharide was released. These results serve to clarify the doubts of many researchers regarding the activity of this enzyme on some newly-described core types and on sialylated substrates.

***Keywords*: mucin, enzyme, Endo-*α*-*N*-acetyl-D-galactosaminidase, release, O-linked**

Introduction

Protocols for the structural analysis of the carbohydrate chains of glycoproteins usually employ, as a first step, the release of the oligosaccharide chains from the protein backbone. Both chemical and enzymatic methods have been described; the former have an advantage over enzymatic methods in that they are less selective [1]. However, chemical methods such as hydrazinolysis [2] for *N*-linked chains and *β*-elimination [3] for O-linked chains, unlike the enzymatic methods, suffer from the disadvantage of possible chemical degradation of the oligosaccharide and the protein.

While several enzymes that cleave *N*-linked oligosaccharides have been reported [4–6], there is no known enzyme capable of completely deglycosylating *O*-glycoproteins. The removal of O-linked glycan chains from the protein backbone by enzymatic methods is restricted by the few enzymes available and their limited substrate specificities.

Endo-α-*N*-acetylgalactosaminidase (*O*-glycanase, EC 3.2.1.97) which catalyses the hydrolysis of the linkage between GalNAc and Ser/Thr in glycoproteins [7] has been isolated from a number of sources. The *O*-glycanase from *Alcaligens* sp. (Endo-GalNAcase-A), which is commercially available, specifically hydrolyses the link between Gal*β*1-3GalNAc and Ser/Thr [8] with a higher affinity for Ser rather than Thr as aglycon [9]. Endo-GalNAcase-C from *Clostridium perfringens* [10] releases the disaccharide Gal*β*1-3GalNAc from Ser/Thr. Endo-GalNAcase-S from *Streptomyces* sp. [11] has been reported to liberate both the disaccharide Gal*β*1-3GalNAc and the tetrasaccharide Gal*β*1-3Gal*β*1-4[GlcNAc*β*1-6]GalNAc and may preferentially liberate an oligosaccharide linked to serine rather than to threonine [12].

Endo-GalNAcase-D from *Diplococcus pneumonia*, which is commercially available, has been reported to have substrate specificity limited to the release of the disaccharide Gal*β*1-3GalNAc from Ser/Thr [9, 13]. However, the specificity of this enzyme with regard to some newly-reported core-types has not been reported. In addition, some researchers have suggested that this enzyme had in fact a much broader substrate specificity than previously thought. In order to clarify the exact substrate specificity of this enzyme we have used bovine submaxillary mucin [BSM], which contains six different core types [14–18], along with remodelled and radiolabelled antifreeze glycoprotein [19], which contains both α2-3- and α2-6-linked sialic acid, as substrates for the enzyme.

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Materials and methods

Materials

Cetyltrimethylammonium bromide (Cetavlon) was obtained from BDH (Poole, UK), Dowex 50-X8, *N*-acetylgalactosamine and *N*-acetylneuraminic acid from Sigma Chemical Company (Poole, England); AG1-X2, BioGel P-2 and BioGel P-4 from BioRad Laboratories (CA); liquid scintillation cocktail from Beckman (Ireland); Spherisorp S5 NH_2 column from Phase Sep (Queensferry, Clwyd, UK) and HPLC solvents from Rathburn Chemicals Limited (UK). Silica gel 60 thin layer chromatography plates were obtained from Merck (Darmstadt, Germany).

Enzymes

Endo-α-*N*-acetyl-D-galactosaminidase (*O*-glycosidase) from *D. pneumonia* (EC 3.2.1.97) was generously provided by Boehringer Mannheim Biochemica (Germany).

Substrates

Bovine submaxillary mucin was also generously provided by Boehringer Mannheim Biochemica. Radiolabelled and remodelled antifreeze glycoprotein containing (1) [^{3}H]NeuAcα2-3Galβ1-3GalNAc and (2) Galβ1-3[[^{14}C]NeuAcα2-6]GalNAc were generous gifts from Professor D.H. van den Eijnden, Vrije Universiteit, Amsterdam.

Bovine submaxillary mucin

Desialylation of BSM

Three hundred mg of the mucin was dissolved in 11 ml of 0.1 M H_2SO_4 (2% solution) and incubated at 80 °C for 1 h. Free *N*-acetylneuraminic acid was detected using the thiobarbituric acid assay. After 1 h the desialylated glycoprotein was neutralized and then purified by gel permeation chromatography on a column of BioGel P-4 (1.4 × 200 cm, 200–400 mesh) which was equilibrated with 50 mM ammonium acetate (pH 5.2). The desialylated glycoprotein-containing fractions were combined and desalted by dialysis for 24 h against ~20 volumes of water and then lyophilized.

Enzyme digestion of BSM

Two hundred mg desialylated mucin and 100 mU endo-α-*N*-acetyl-D-galactosaminidase from *D. pneumonia* were incubated in 6 ml sodium phosphate buffer, 200 mM (pH 7.0) at 37 °C, for 24 h. The enzyme digestion was stopped by heating at 90 °C for 3 min. The released oligosaccharide(s) were isolated by gel permeation chromatography on a column of BioGel P-4 (1.4 × 200 cm, 200–400 mesh) which was equilibrated and eluted with 50 mM ammonium acetate (pH 5.2). Fractions were collected and assayed using the phenol sulphuric acid assay. The oligosaccharide-containing fraction was pooled and lyophilized.

Reduction of released oligosaccharide(s)

The enzyme-released product was reduced by incubation with 1 M $NaBH_4$ in 0.1 M NaOH at 25 °C for 14 h in a final volume of 5 ml. The solution was neutralized by dropwise addition of acetic acid (4 M) at 0 °C until pH 6 was reached. To remove the sodium ions, the resulting solution was applied to a column of Dowex 50-X8 (H^+, 20–50 mesh, 2.0 × 12 cm) at 4 °C which was eluted with four column volumes of 0.01 M formic acid. The eluant was collected, lyophilized and the boric acid volatilized as methyl borate by evaporation under reduced pressure with the addition of methanol containing 5% acetic acid, followed by three additions of methanol. The resulting residue was dissolved in water and lyophilized.

Desalting of reduced oligosaccharide(s)

The oligosaccharide mixture was desalted by applying to BioGel P-2 (1.4 × 30 cm, 200–400 mesh) which was equilibrated and eluted with water. The oligosaccharide fractions were combined and lyophilized.

HPLC separation of reduced oligosaccharide(s)

HPLC was carried out on a Milton Roy CM4000 system equipped with a Rheodyne 7125 injection valve and a SM4000 programmable wavelength detector operating at 195 nm. Chromatograms were recorded with a LDC/Milton Roy CI-10 integrator. Chromatography was performed on a column of Spherisorb S5 NH_2 (25 cm × 4.6 mm i.d.). A pre-column filled with silica was used. All solvents were filtered through 0.45 μm millipore filters and degassed using helium gas in the solvent reservoirs. Elution was performed with a mobile phase consisting of a mixture of acetonitrile and deionized distilled water containing 15 mM potassium phosphate (pH 5.2) [20]. The isocratic solvent system; 80% acetonitrile and 20% 15 mM potassium phosphate (pH 5.2), was used. A flow rate of 2 ml min^{-1} was maintained. The oligosaccharide alditol(s) were dissolved in distilled water before injection.

270-MHz^1 H-NMR spectroscopy

For ^{1}H-NMR spectroscopic analysis the oligosaccharide product was treated at least three times with 0.4 ml ^2H_2O at room temperature, with intermediate lyophilization. Finally, the sample was dissolved in 400 μl of ^2H_2O [21].

^{1}H-NMR analysis was performed on a Jeol GX-270 spectrometer at a probe temperature 293K, operating in the Fourier transform mode. For one-dimensional experiments 700–1200 transients were collected and a pulse (PW1) of 9 μs was used. Resolution enhancement of the spectra was achieved by applying a Gaussian weighting function to the time domain data prior to Fourier-transformation.

Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl silapentane-1-sulphonate (DSS) but were actually measured by reference to internal acetone (2.225 ppm).

FAB-MS

Fast atom bombardment mass spectrometry was carried out in the laboratory of Professor A. Dell, Imperial College, London. FAB-MS spectra were acquired using a ZAB-2SE FPD mass spectrometer fitted with a caesium ion gun operated at 20–25 kV. Data acquisition and processing were performed using a VG Analytical Opus software. The spectra were recorded, after deuteroacetylation, according to published procedures [22]. The derivatized *O*-glycans were aliquoted in methanol and loaded in a 1-monothioglycerol matrix.

Antifreeze glycoprotein

Enzyme digestion of antifreeze glycoprotein

(a) Antifreeze glycoprotein containing [^{3}H]NeuAcα2-3Galβ1-3GalNAc 6.5 mM (5.4 Ci mol^{-1}) and 25 milliunits of endo-α-*N*-acetyl-D-galactosaminidase from *D. pneumonia* were incubated in 250 µl sodium phosphate buffer (20 mM, pH 6.5) at 37 °C for 13 h. The enzyme digestion was stopped by heating at 90 °C for 3 min. (b) Antifreeze glycoprotein containing Galβ1-3[[^{14}C]NeuAcα2–6]GalNAc 7 mM (5.0 Ci mol^{-1}) and 25 milliunits of endo-α-*N*-acetyl-D-galactosaminidase from *D. pneumonia* were incubated in 250 µl sodium phosphate buffer (20 mM, pH 6.5) at 37 °C for 13 h. The enzyme digestion was stopped by heating at 90 °C for 3 min.

Separation of products by BioGel P-4

The released products were isolated by gel permeation chromatography on a column of BioGel P-4 (1.4 × 200 cm, 200–400 mesh) which was equilibrated and eluted with 50 mM ammonium acetate (pH 5.2). One ml fractions were collected and 500 µl fractions were diluted 1:6 with scintillation cocktail fluid (total volume 3.5 ml) and counted. The fractions from the oligosaccharide region of the column were then combined and lyophilized.

Desalting of radioactive fractions

The radioactive fractions were desalted by applying to BioGel P-2 (1.4 × 30 cm, 200–400 mesh) which was equilibrated and eluted with water. The radioactive fractions were combined and lyophilized.

HPLC of released radioactive products

HPLC was carried out on a Milton Roy CM4000 system as described above. Elution was performed with a mobile phase consisting of a mixture of acetonitrile and deionized distilled water containing 15 mM potassium phosphate, (pH 5.2) [20]. The gradient used started with 80% acetonitrile for 30 min; this was then decreased at a rate of 0.5%, min^{-1} to 50% acetonitrile with a flow rate of 2 ml min^{-1}. The radiolabelled products were dissolved in distilled water before injection. The elution position of the radioactive material was established by collecting 1 ml effluent fractions in counting vials followed by liquid scintillation counting.

Table 1. Core structures of O-linked oligosaccharides in BSM.

Core type	*Structure*	*Selected reference*
1	Galβ1-3GalNAc	14
	GlcNAcβ1	
	6	15
2	GalNAc	
	3	
	Galβ1	
3	GlcNAcβ1-3GalNAc	16
	GlcNAcβ1	
	6	15
4	GalNAc	
	3	
	GlcNAcβ1	
5	GalNAcα1-3GalNAc	17
7	GalNAcα1-6GalNAc	18

Table 2. Structures of sialylated trisaccharides found in remodelled antifreeze glycoprotein.

Core type	*Structure*	*Selected reference*
1	NeuAcα2-3Galβ1-3GalNAc	19
1	Galβ1-3[NeuAcα2-6]GalNAc	19

Results and discussion

Bovine submaxillary mucin has been shown to contain oligosaccharides with six different core types as shown in Table 1. The major oligosaccharide present is the disaccharide GlcNAcβ1-3GalNAcol (Table 1).

Remodelled antifreeze glycoprotein has been shown to contain the oligosaccharide structures shown in Table 2.

Bovine submaxillary mucin

HPLC

Since the HPLC retention times and ^{1}H-NMR data were available for the oligosaccharides released from BSM by β-elimination [14–17, 23], the oligosaccharide(s) released by the enzyme were reduced to give the corresponding oligosaccharide alditol(s).

The component(s) of the enzyme-released mixture were compared to those released by β-elimination by their HPLC retention times. Table 3 shows the retention times of some oligosaccharide alditol standards and the relative amounts of these oligosaccharides found in BSM. The structures of these chemically-released oligosaccharides were confirmed previously by ^{1}H-NMR and methylation analysis [14–17, 24].

Table 3. HPLC retention times of the smaller oligosaccharides alditols previously released from BSM by β-elimination.

Standard	*Structure*	*Retention time (min)*	*Relative amounts*[a]
1	GalNAcol	5.48	50
2	GalNAcα1-3GalNAcol	10.90	3
3	GlcNAcβ1-3GalNAcol	12.04	40
4	Galβ1-3GalNAcol	12.98	4
5	Fucα1-2Galβ1-3GalNAcol	14.62	3

[a] Approximate relative amounts present in desialylated BSM [14–17, 23].

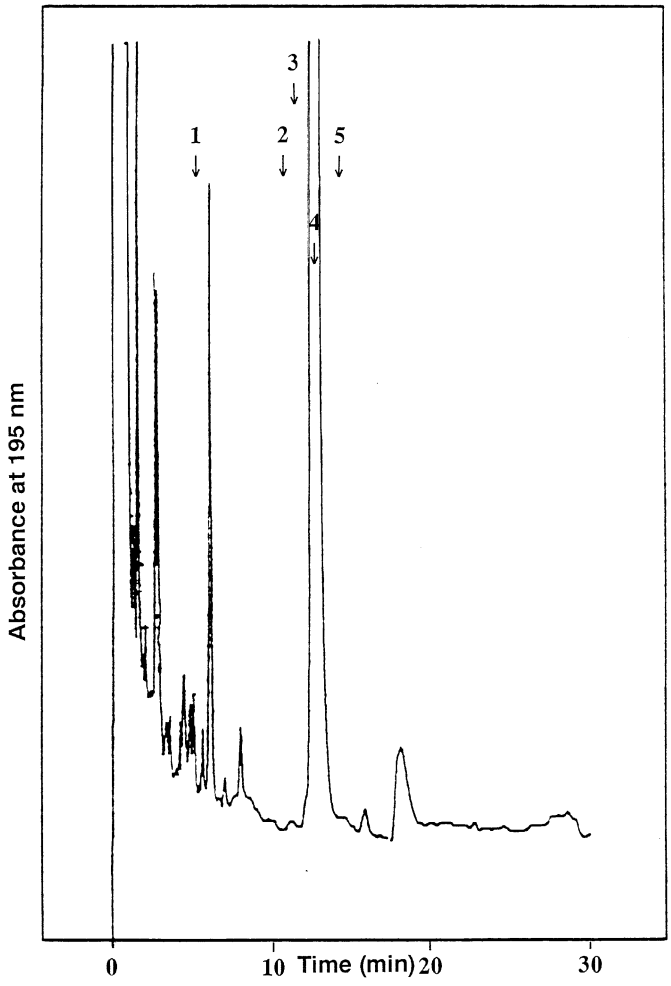

Figure 1. Chromatogram showing the HPLC separation of the enzyme-released oligosaccharide-alditol(s). The standards are numbered according to Table 1.

The oligosaccharide(s) released by the enzyme, when reduced and applied to HPLC, showed one major peak and several minor peaks (Figure 1). The elution time for the major peak corresponded to that of Galβ1-3GalNAcol. It should be noted that although the disaccharide GlcNAcβ1-3GalNAcol is the predominant disaccharide present in desialylated BSM, no evidence for its release is seen by HPLC. In addition, no peaks with retention times corresponding to GalNAcol, GalNAcα1-3GalNAcol or Fucα1-2Galβ1-3GalNAcol were seen. The retention times of the minor peaks, some of which eluted earlier than Galβ1-3GalNAcol and two which eluted later, do not correspond to the elution times of any oligosaccharides known to be present in BSM. These minor peaks are considered to be either impurities or artefacts of sample preparation, for example, *N*-deacetylated Galβ1-3GalNAcol (which would be expected to elute later).

^{1}H-NMR analysis of enzyme-released oligosaccharide(s)

The 270 MHz ^{1}H-NMR spectrum showed the presence of a number of structural reporter groups. The main signals, assigned with the aid of DQF-COSY spectrum to the disaccharide Galβ1-3GalNAcol, were as follows: GalNAcol: H-2, 4.397 ppm; H-3, 4.066 ppm; H-4, $\approx$ 3.5 ppm; H-5, 4.198 ppm; H-6, 3.60–3.65 ppm; H-6′, 3.60–3.65 ppm; NAc, 2.051 ppm; Gal[3]: H-1, 4.478; H-2, 3.5–3.6 ppm and H-4, 3.902. These assignments are in agreement with earlier data [25, 26]. The presence of signals due to additional structural reporter groups of other oligosaccharides known to be present in BSM were not observed.

FAB-MS

Fast atom bombardment mass spectra were recorded after deuteroacetylation of the reduced mixture. The analysis was carried out using both positive and negative FAB-MS.

The major molecular ion at 768 in the positive FAB spectrum (Figure 2) is the [M + Na] signal for HexHexNAcol (reduced GalGalNAc, core 1) and is accompanied by an [M + H] signal at 746. Signals appearing at intervals of 45 mass units below 768 and 746 are due to underacetylated species. The presence of signals 3 mass units higher than 768 and 746 is thought to be due to *N*-deacetylation during sample preparation that is now deuteroacetylated.

There are no clear signals present at 742 and 764 indicating that neither GalNAc-GalNAcol (core 5 or 7) or GlcNAc-GalNAcol (core 3) are present in the sample. There is a very minor signal at 1004 indicating possible FucHexHexNAcol [M + Na]; however, there is no signal 3 mass units higher as with GalGalNAc and it is felt that one component of the mixture is unlikely not to be affected if other components show signals for *N*-deacetylation. The signal at 840 is thought to correspond to glycerol glycoside formed during the enzyme incubation between glycerol (in which the enzyme is supplied) and the newly released disaccharide.

The negative FAB spectrum is shown in Figure 3. Major [M + Cl] molecular ions are observed at 780 for HexHexNAcol (reduced GalGalNAc) and 852, again thought to be

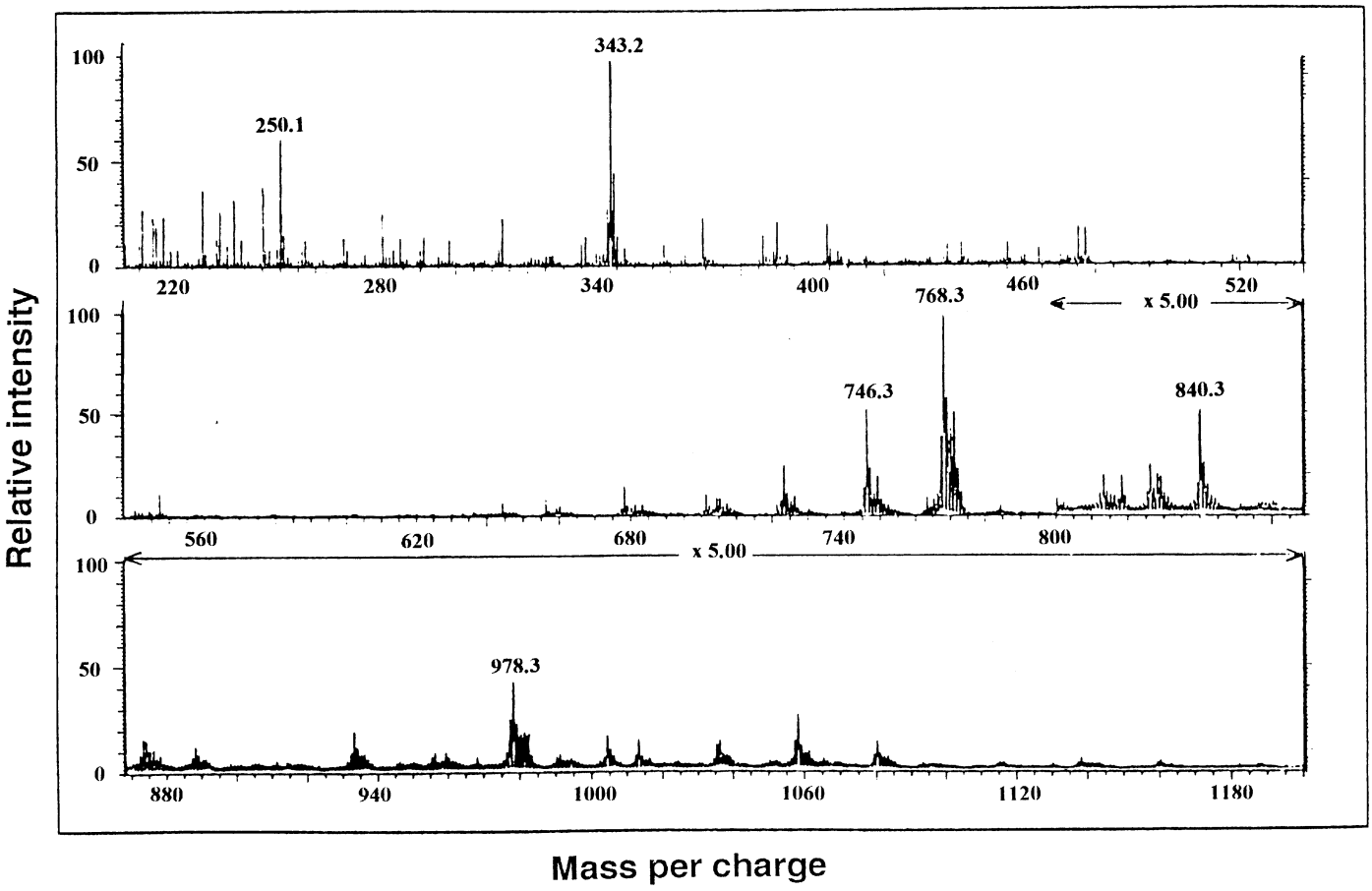

Figure 2. Positive FAB-MS spectrum of the enzyme-released oligosaccharide-alditol(s).

due to glycerol glycoside. There is no signal in the negative ion spectrum corresponding to FucHexHexNAcol.

It is clear therefore, from both NMR and FAB-MS spectra that the only glycan released by the enzyme is the disaccharide Galβ1-3GalNAc.

Antifreeze glycoprotein

Incubation of antifreeze glycoprotein with endo-α-*N*-acetyl-D-galactosaminidase from *D. pneumonia* resulted in release of radiolabelled product(s) which were separated from glycoprotein and desalted by BioGel chromatography. Chromatography of a pre-incubation sample of both glycoproteins indicated that each was intact since there was no radioactivity present in the oligosaccharide elution region.

On the other hand BioGel chromatography after incubation indicated that some radioactivity was released in both incubations. The oligosaccharide fractions from the BioGel P-4 columns were combined, lyophilized and desalted prior to analysis by HPLC.

Each sample was applied to HPLC, with counting of collected fractions so as to locate the radiolabelled product. Figures 4 and 5 show HPLC profiles (DPM vs min) of the incubations. Although standards for the expected products [^{3}H]NeuAcα2-3Galβ1-3GalNAc and Galβ1-3[[^{14}C]NeuAcα2-6]GalNAc were not available, the retention times were predicted using available standards (GalNAcol, *N*-acetylneuraminic acid and Galβ1-3[NeuAcα2-6]GalNAc) and published data for reduced and reducing sugars [20, 27, 28].

Figure 4 shows an HPLC profile of the enzyme-released product after incubation of the ^{3}H labelled antifreeze glycoprotein with *O*-glycosidase (DPM vs elution time). The trisaccharide NeuAcα2-3Galβ1-3GalNAc would be expected to elute after *N*-acetylneuraminic acid. Only one peak occurs in this profile which appears at an elution time earlier than NeuAc. Since there are no other peaks occurring in the profile it is clear that the trisaccharide NeuAcα2-3Galβ1-3GalNAc is not present.

The trisaccharide, NeuAcα2-3Galβ1-3GalNAc would be expected to elute at a time similar (*ie* within 2–3 min) time to the reduced trisaccharide which occurs at $\sim$45 min (Figure 5). Since no peak occurs in this region, it is clear that the trisaccharide Galβ1-3[NeuAcα2-6]GalNAc is not

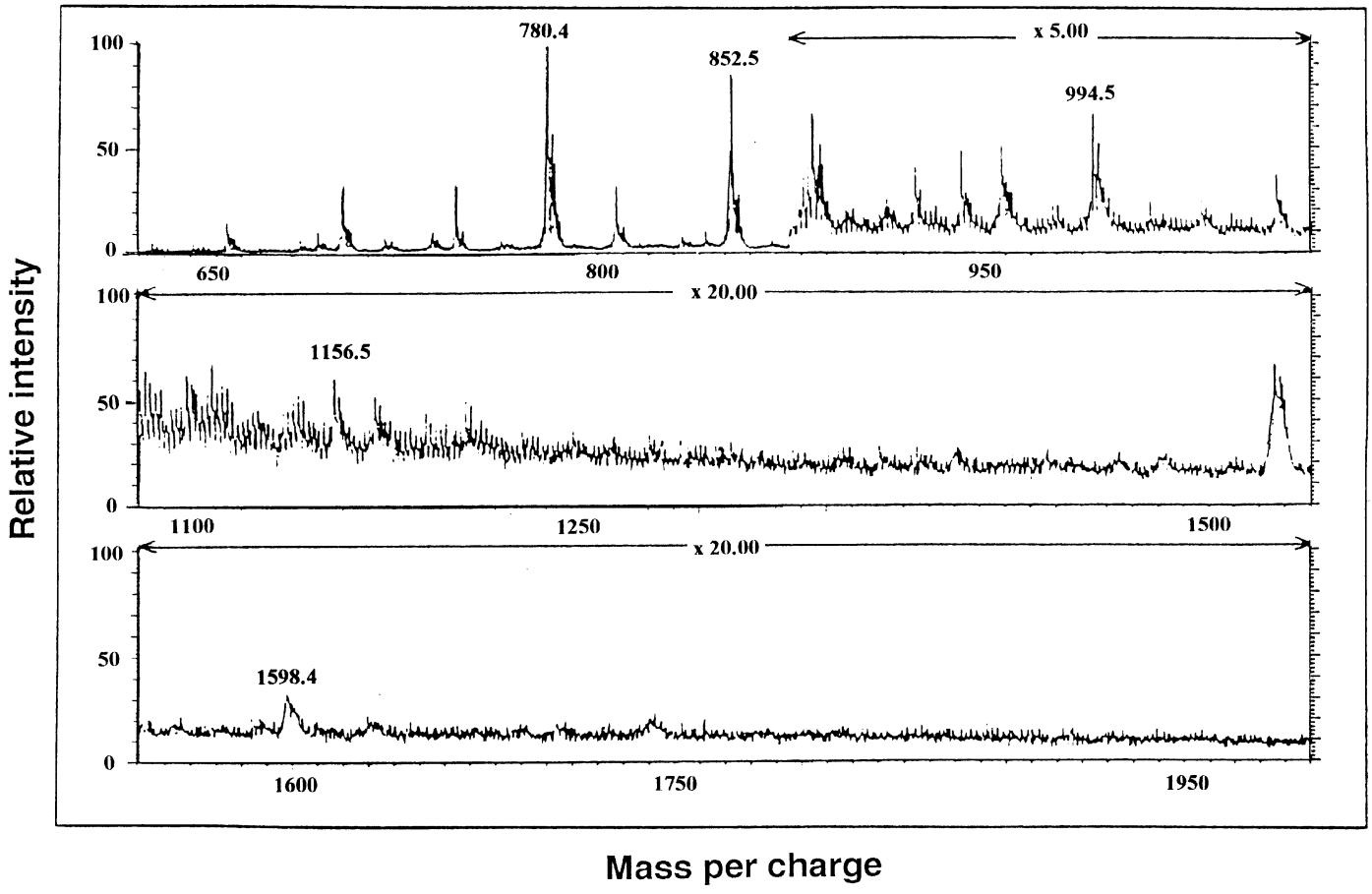

Figure 3. Negative FAB-MS spectrum of the enzyme-released oligosaccharide-alditol(s).

present. A minor peak occurs at ~19 min which corresponds to *N*-acetylneuraminic acid. This may have been released on heating the sample at the end of the incubation. In addition, a peak which occurs in both profiles at approximately 12 min could not be assigned to any of the expected products. Nonetheless, the results here indicate that neither the trisaccharide NeuAcα2-3Galβ1-3GalNAc nor the trisaccharide Galβ1-3[NeuAcα2-6]GalNAc were released by the enzyme *O*-glycosidase.

From the results obtained, using HPLC retention times, ^{1}H-NMR spectroscopy and FAB-MS, it is clear that only the disaccharide containing core type 1, Galβ1-3GalNAc, was released by the enzyme *O*-glycosidase. This disaccharide, which is one of the minor components present in desialylated BSM (Table 1) was released while the disaccharide GlcNAcβ1-3GalNAcol, which accounts for about 40% of the smaller structures found is desialylated BSM, was not released. In addition, none of the other four core types which are present in BSM in amounts similar to GlcNAcβ1-3GalNAcol were released by the enzyme. It is also clear that when the Gal of this disaccharide is substituted by Fucα1-2 or NeuAcα2-3 or when the GalNAc is substituted by NeuAcα2-6, the oligosaccharide was not released by the enzyme.

The enzyme *O*-glycosidase from *D. pneumonia* has been shown to exhibit transferase activity in the presence of acceptors such as glycerol [29]. Therefore, before incubation with the enzyme, it is advisable to remove glycerol (which is present in the enzyme preparation as a protectant against denaturation) by dialysis. In this study, the presence of a glycerol glycoside is apparent from the mass spectra.

To understand the functional role of carbohydrate moieties associated with glycoproteins, their contribution to the structure and function of the protein, and the precise alignment of sugars within oligosaccharide chains, a non-destructive means of releasing intact oligosaccharides from their associated proteins is essential. An enzyme-based approach provides an ideal solution to this problem. However, enzyme release of *O*-glycans is severely limited by the enzymes available and their restricted substrate specificity.

As *O*-glycosidase from *D. pneumonia* is an enzyme which is widely used to cleave the *O*-glycosidic linkage between *N*-acetylgalactosamine and the serine or threonine residue, it is important that its exact substrate specificity is known.

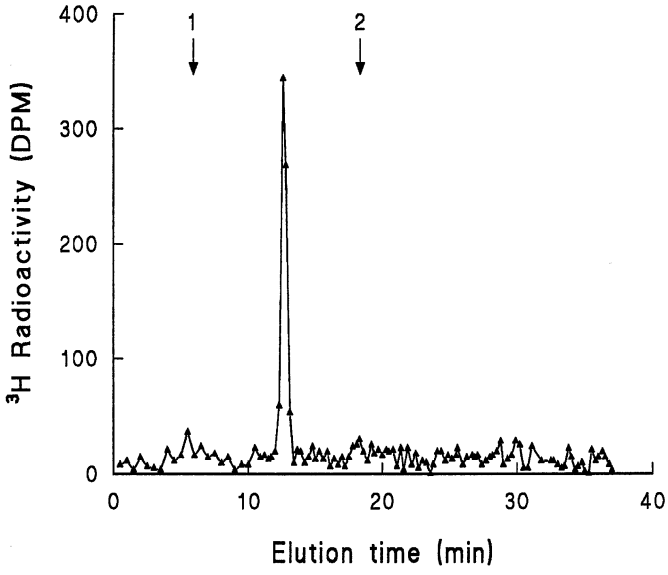

Figure 4. HPLC of the enzyme-released radiolabelled product after incubation of the ^{3}H labelled antifreeze glycoprotein with *O*-glycosidase. The positions of the standards are indicated by arrows: (1) GalNAcol; and (2) *N*-acetylneuraminic acid.

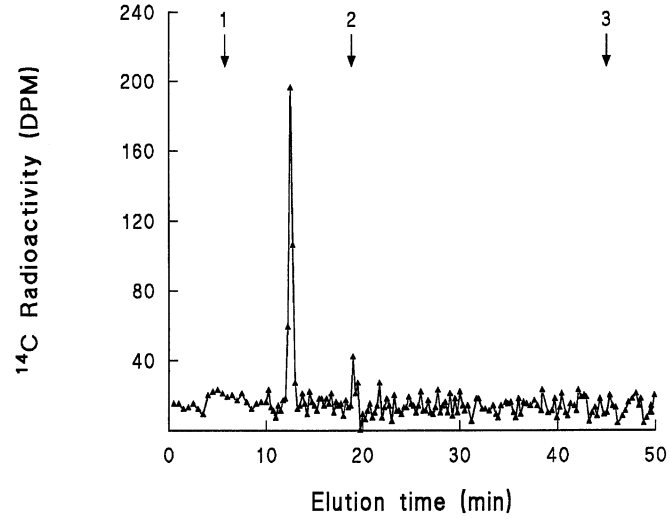

Figure 5. HPLC of the enzyme-released radiolabelled products after incubation with the ^{14}C labelled antifreeze glycoprotein. The positions of the standards are indicated by arrows: (1) GalNAcol; (2) *N*-acetylneuraminic acid; and (3) Galβ1-3[NeuAcα2-6]GalNAc.

The results of this study, using three approaches to structure determination, are clear and serve to clarify the doubts of many researchers as to the exact substrate specificity of *O*-glycosidase from *D. pneumonia*. The search, therefore, continues to find an enzyme that acts on O-linked glycoproteins, which has a specificity that enables the release of a broad range of oligosaccharides from the protein backbone.

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