Carbohydrate structures of hen ovomucoid

A mass spectrometric analysis

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The apparently homogenous *N*-glycosidically-linked glycans **1**, **7**, **11** and **14** released by hydrazinolysis from hen ovomucoid were analysed by fast atom bombardment and electron-impact mass spectrometry after reduction and permethylation. The spectra support the primary structures established independently [FEBS Letters (1983) 152, 145–152] using methylation analysis, partial acid hydrolysis and 500 MHz ^{1}H NMR spectroscopy. In addition to the major constituents present in fractions **1**, **7**, **11** and **14**, four minor components not detected by other methods could be characterized with the aid of the mass spectrometry data as: $Man_2GlcNAcGlcNAc$-ol, $GlcNAc_4Man_3GlcNAc$-ol, $GlcNAc_6Man_3GlcNAc$-ol and $GalGlcNAc_6$-$Man_3GlcNAc$-ol. Our results show that the physical techniques used provide valuable data on the structure of complex glycans. In addition they can be employed to ascertain the homogeneity of the compounds examined as well as to detect trace amounts of homologs that might not be noticed by other methods.

Ovomucoid *Carbohydrate structure* *Fast atom bombardment* *Heterogeneity* *Mass spectrometry*

1. INTRODUCTION

The multibranched structures of the glycans of hen ovomucoid were first recognized by Stacey and Wooley [1,2]. The heterogeneity of the oligosaccharide moieties linked *N*-glycosidically to hen ovomucoid is well documented [3]. After hydrazinolysis 17 major fractions could be separated by HPLC. The structures of several of the major components were determined by combined methylation analysis, partial acid hydrolysis and 500 MHz ^{1}NMR spectroscopy [4,5]. Fast atom bombardment and electron-impact mass spectrometry [6–8] of the 4 permethylated fractions **1**, **7**, **11** and **14** [3] homogenous by chromatographic criteria furnished additional data supporting the proposed structures [4,5] but showing also the presence of minor amounts of homologs that were not yet detected by other methods.

Dedicated to Professor Eckhard Buddecke on the occasion of his 60th birthday

Abbreviations: amu, atomic mass units; FAB, fast atom bombardment; EI, electron impact; MS, mass spectrometry; NMR, nuclear magnetic resonance; MIKES, mass analysed ion kinetic energy spectrometry

2. MATERIALS AND METHODS

2.1. *Preparation, purification and permethylation*

The preparation, purification and permethylation of the fractions **1**, **7**, **11** and **14** from hen ovomucoid followed the procedures in [3,9–13].

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2.2. *Mass spectrometry*

Mass spectrometry was performed on a VG analytical ZAB-HF reversed geometry mass spectrometer. For fast atom bombardment [7,8], samples were dissolved in methanol to ~5 μg/μl. The stainless steel target was first loaded with 1 μl of a 0.1% solution of sodium acetate in methanol. After drying, 3–4 μl 1-mercapto-2,3-propanediol (EGA Chemie, Steinheim) and 0.5–1.0 μl sample solution was applied to the target. The target was bombarded with xenon atoms having a kinetic energy equivalent to 9.0–9.5 kV. Spectra were recorded routinely at 7 kV acceleration voltage giving a mass range of ~3800 amu in a mass-controlled scan. The resolution was set to 250 ppm. Normally, 5 μg sample furnished molecular or pseudo-molecular ions with a 1000-fold intensity as compared to that of the background signals produced by the thioglycerol matrix. Spectra were recorded in the positive ion mode by a mass controlled scan of 100–500 s duration depending on the M_r of the sample. The spectra were evaluated by counting thus giving whole mass numbers that are presented in section 3. The mass numbers indicated are therefore lower by about one mass unit at M_r 2000 than calculated ones based on: C = 12.000; H = 1.008; O = 15.995; and N = 14.003.

Electron impact spectra were obtained on the same instrument. The samples were heated indirectly by raising the temperature of the ion source (150–350°C) until relevant spectra were obtained. Spectra were recorded at an ionization energy of 20 eV. Fomblin oil was used for the calibration of the mass marker. For linked-scan measurements mass analysed ion kinetic energy spectrometry [14], B/E and B^2/E measurements were used.

3. RESULTS AND DISCUSSION

The FAB spectra of the fractions **1, 7, 11** and **14** showed intense pseudo-molecular ions M + Na^+ besides ions produced by the reduced and non-reducing terminal carbohydrate constituents like mannose, *N*-acetyl-D-galactosamine, D-galactose and *N*-acetyl-D-glucosaminol.

Structurally important ions were also produced by the cleavage of the glycosidic bond of the chitobiose unit with the positive charge remaining on the large non-reducing oligosaccharide fragment, thus furnishing another series of ions of diagnostic value.

The major fragment ions obtained by FAB MS from the 4 permethylated fractions are gathered in table 1.

Thanks to the high intensity of the pseudo-molecular ions, FAB MS is especially sensitive and well-suited for the detection of accompanying homologs that are not easily revealed by other spectroscopic or degradative methods. This is exemplified by the pseudo-molecular ions at *m/e* 983, *m/e* 1922, *m/e* 2412 and *m/e* 2616 in fractions **1, 7, 11** and **14**, respectively, which indicate the presence of lower homologs in all 4 fractions. In **1** the pseudo-molecular ion *m/e* 983, 204 amu below the major component, is accompanied by *m/e* 668, which is produced by fission of the chitobiose moiety, thus indicating that here one of the terminal mannose residues is missing. In **7, 11** and **14** the pseudo-molecular ions of the lower homologs all differ by 245 amu from the major components, thus indicating a difference of one *N*-acetyl-D-glucosamine residue. Whereas in **7, 11** and **14** all 3 major pseudo-molecular ions are accompanied by ions 315 amu lower than are produced by fission of the chitobiose linkage, no such ions were found for the lower homologs. Hence, it is concluded that in these components one of the *N*-acetyl-D-glucosamine residues of the chitobiose moiety has been lost.

The EI spectrum of **1** furnishes valuable additional structural information. The base peak *m/e* 182 indicates a 4-substituted GlcNAc residue. Like in the FAB spectrum the ion *m/e* 521 represents the chitobiose residue. The branched structure of the mannotriose can be deduced from the absence of dihexosyl fragments (*m/e* 423, 391). A typical feature of EI spectra of oligomannosides is the high intensity of the M-45 ion *m/e* 1119 [15]. Like in the higher oligomannosides the α-1–6 bond of one of the terminal mannose residues is documented by the rearrangement ion *m/e* 989 as in fig.1a. The fragment *m/e* 1034 is produced by cleavage of the C_2–C_3 bond of the reduced GlcNAc residue.

For the lower homolog $Man_2GlcNAc_2$ detected in the FAB spectrum only weak signals are present in the EI spectrum obtained at 250°C. From the relative intensity of the fragment *m/e* 785 over 725

Table 1

Carbohydrate composition and relative intensity of major ions of diagnostic importance observed in the FAB MS spectra of reduced and permethylated oligosaccharide fractions **1, 7, 11** and **14** obtained after hydrazinolysis from hen ovomucoid glycopeptides

Carbohydrate composition	Atomic mass number (amu)	Relative intensity (%) of base peak			
		1	7	11	14
Gal^+	219	33			
Man^+	219				
$GlcNAc^+$	260		100	100	100
$GlcNAc\text{-}ol^+$	276	32	6	7	6
$GlcNAcMan^+$	464				
$GalGlcNAc^+$	464		1		22
$GlcNAcGlcNAc\text{-}ol^+$	521	4	0.5	1	0.5
$Man_2GlcNAc^+$	668	8			
$Man_3GlcNAc^+$	872	24			
$Man_2GlcNAcGlcNAc\text{-}ol + Na^+$	983	26			
$Man_3GlcNAcGlcNAc\text{-}ol + Na^+$	1187	90			
$GlcNAc_4Man_3GlcNAc^+$	1852		1		
$GlcNAc_4Man_3GlcNAc\text{-}ol + Na^+$	1922		1		
$GlcNAc_4Man_3GlcNAcGlcNAc\text{-}ol + Na^+$	2167		3		
$GlcNAc_6Man_3GlcNAc^+$	2342			3	
$GlcNAc_6Man_3GlcNAc\text{-}ol + Na^+$	2412			7	
$GalGlcNAc_6Man_3GlcNAc^+$	2546				0.5
$GalGlcNAc_6Man_3GlcNAc\text{-}ol + Na^+$	2616				1
$GlcNAc_6Man_3GlcNAcGlcNAc\text{-}ol + Na^+$	2657			10	
$GalGlcNAc_6Man_3GlcNAcGlcNAc\text{-}ol + Na^+$	2861				2

found however in EI spectra obtained at 230°C probe temperature a preponderance of the structure shown in fig.1b can be deduced.

The EI spectra of fractions **7, 11** and **14** are, as a consequence of the highly branched structure, extremely complex. In contrast to EI spectra of permethylated oligosaccharides with linear chains which show series of intense sequence ions produced by fission of the glycosidic linkages, here secondary fragmentation and recombination mechanisms are prevailing. As an example, the EI spectrum of fraction **11** is shown in fig.2. The terminal constituents are easily recognized by *m/e* 260, 228 and *m/e* 276. The reduced chitobiose unit gives rise to *m/e* 521. In the middle and high mass range only a few and not always the most intense fragments are produced by fission of one of the glycosidic bonds like *m/e*: 709; 1419; 1664; 2113; 2342; and 2358. The primary fragments in the high mass range furnish series of secondary ions that are produced predominantly by loss of GlcNAc residues with concomitant elimination or recombination of hydrogen, oxygen or methyl groups leading to ions 2, 14 or 16 amu apart (schemes 1a,b). Thus instead of producing a series of intense ions as in linear chains, clusters of ions are present whose centers are 260 amu apart. The lower homolog $GlcNAc_6$-$Man_3GlcNAc$-ol, shown to be present by the FAB MS can hardly be recognized in the EI spectrum by specific fragments because in some cases they are coinciding with or are buried in the ion clusters produced by the major compound.

In oligosaccharides containing 1–6 bonds, rather intense rearrangement ions are normally observed 60 amu higher than the ions produced by the cleavage of the glycosidic bond [15]. In fig.2, two such pairs can be recognized at *m/e* 2113; 2173 and *m/e* 2358; 2418, respectively, which can be attributed to the two components present in **11.** Some of the major degradative pathways are depicted in the schemes 1a and 1b.

FAB MS combined with EI MS provides valuable data on the structure of complex glycans. Certainly the complete structural elucidation has

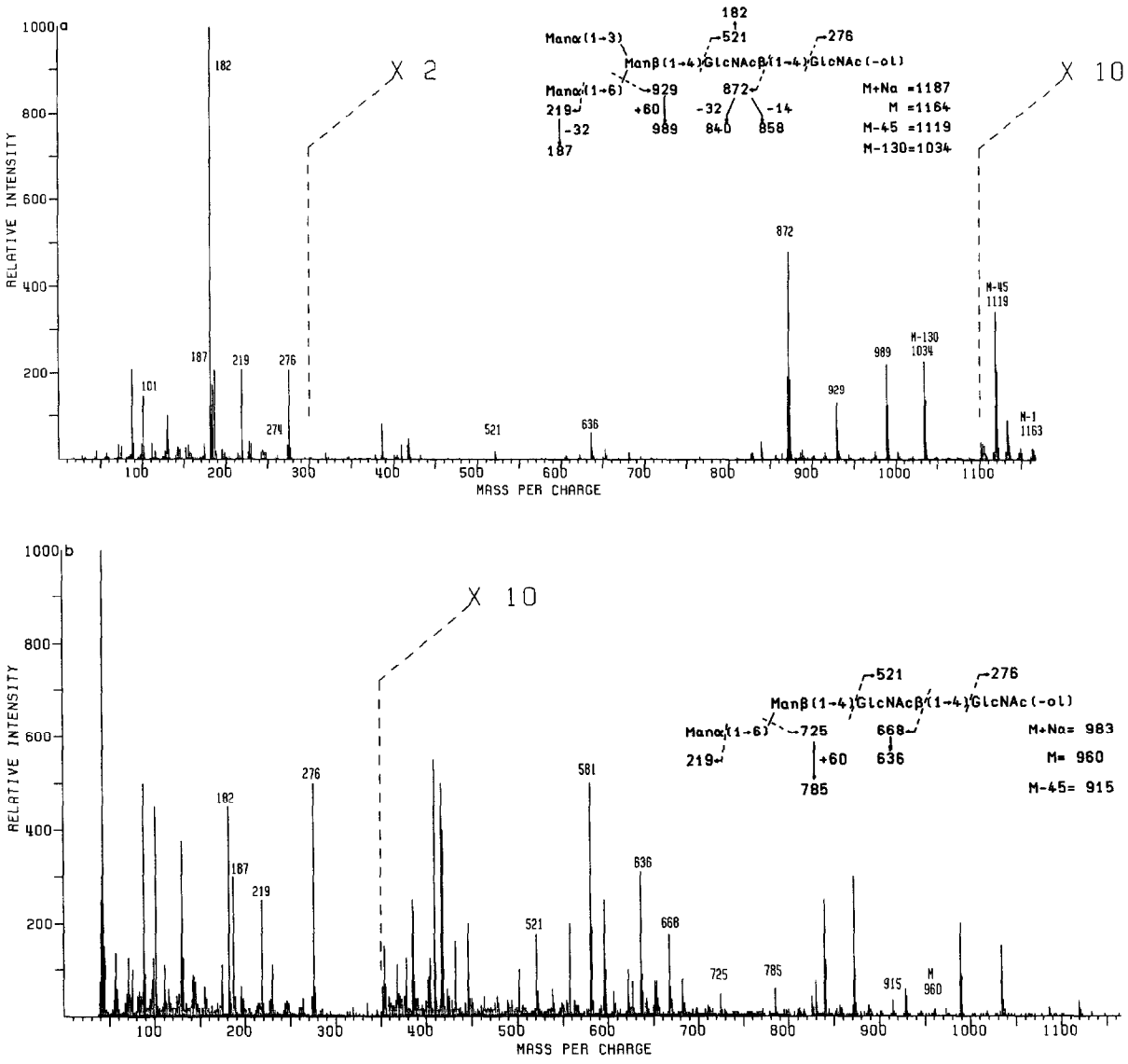


Fig.1. Electron impact mass spectrum obtained at 20 eV ionization energy and (a) 250°, (b) 230° probe temperature from compound **1**. The major fragmentation pathways for both constituents are shown in the inserts.

to take resort to additional techniques, preferably high resolution NMR. However, due to the high intensity of pseudo-molecular ions and the highly selective fragmentation, FAB MS may be used with great advantage to ascertain the homogeneity of the compounds under investigation or, on the other hand, to detect trace amounts of homologs or isomers that would possibly escape determination by other methods. With the aid of pre-established spectra–structure relationship, also complete structural analyses can be conducted on μg amounts of oligosaccharide mixtures.

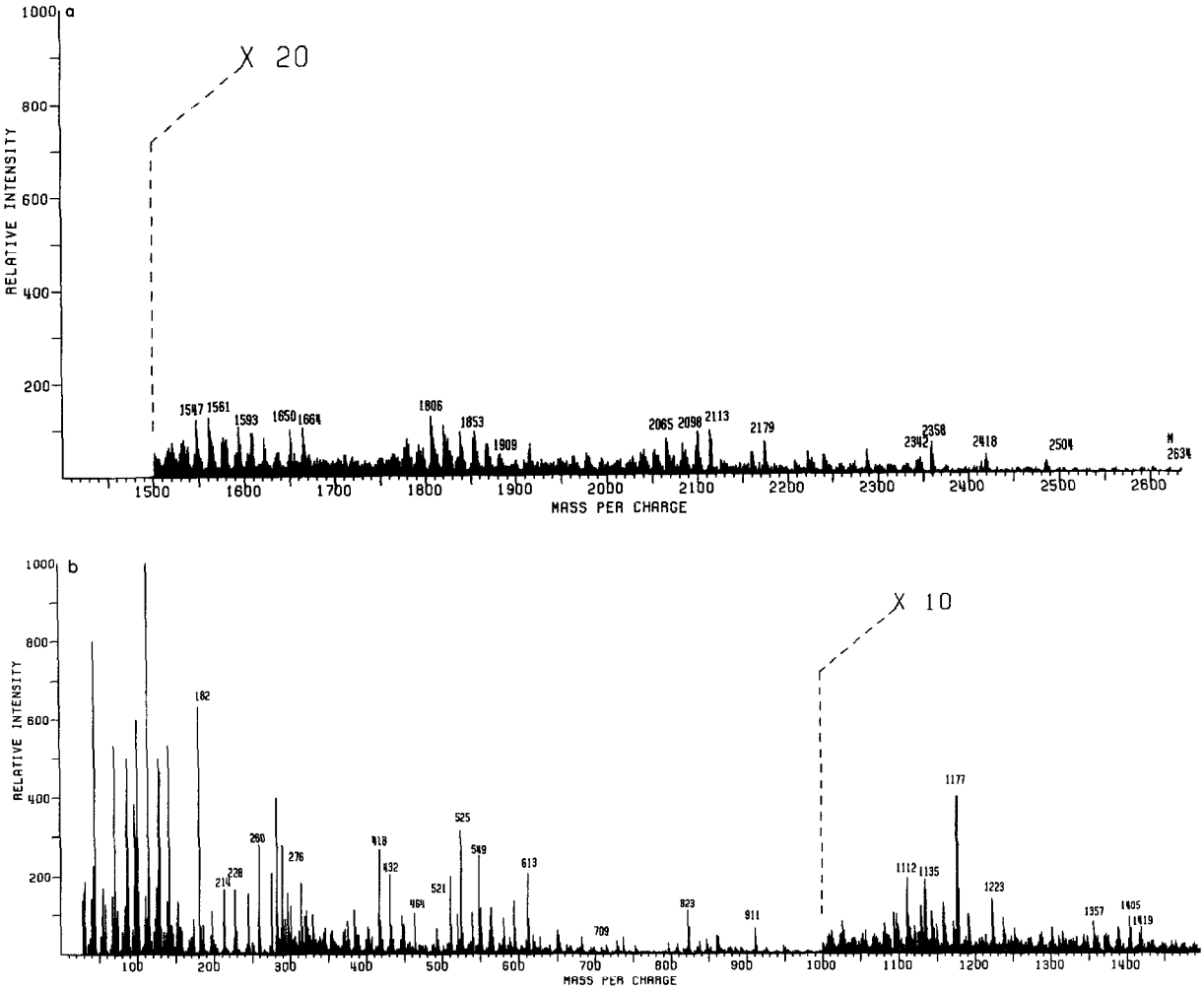
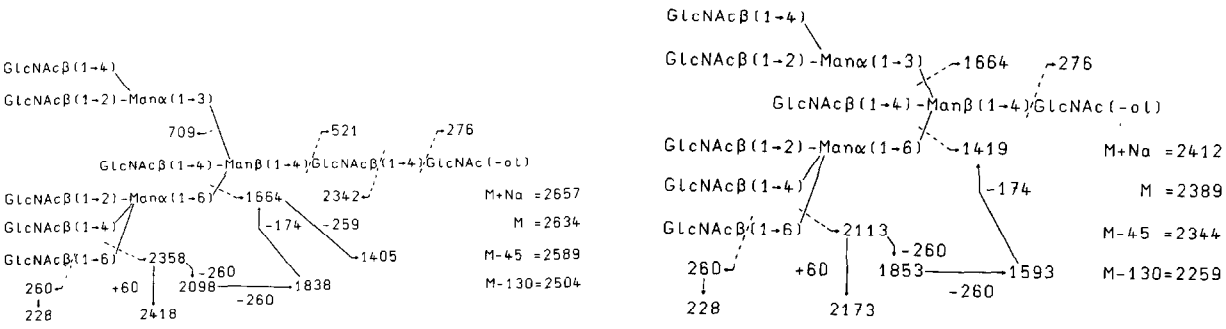


Fig.2. EI mass spectrum of compound **11** obtained at 20 eV ionization energy and 300°C probe temperature.



Schemes 1a, b

Major fragmentation pathways of the two components present in fraction **11**, as observed under electron impact

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