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A NOVEL MASS SPECTROMETRIC PROCEDURE FOR THE RAPID DETERMINATION OF THE TYPES OF CARBOHYDRATE CHAINS PRESENT IN GLYCOPROTEINS: APPLICATION TO α-GALACTOSIDASE I FROM VICIA FABA SEEDS

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A fast atom bombardment mass spectrometric protocol has been developed to determine the type of oligosaccharide chain present in glycoproteins. The procedure is based on acetolysis of the intact glycoconjugate, extraction of the peracetylated carbohydrate fragments and analysis by fast atom bombardment mass spectrometry. The molecular ions present in the FAB spectra uniquely define the composition of the oligosaccharides with respect to hexose, aminohexose and sialic acid content. High mannose oligosaccharides yield a series of peracetylated hexose oligomers whereas complex-type oligosaccharides afford a series of N-acetyl-lactosamine containing species. Fucosylation is usually not detected but sialylated oligosaccharides are readily identified and the type of sialic acid is also defined. The method has been tested on three glycoproteins of known structure - fetuin, ribonuclease B and erythrocyte Band 3 - and on a glycoprotein of unknown structure - α-galactosidase I, an enzyme lectin from Vicia faba. The latter is shown to contain high mannose carbohydrate chains.

It is not always necessary, or indeed possible when sample size is limited, to undertake a full structural study on many carbohydrates and glycoconjugates; a simple but definitive test for oligosaccharide class may often suffice to establish strategies for further biochemical analyses.

In the past few years fast atom bombardment mass spectrometry (FAB-MS) has been successfully applied to a variety of carbohydrate problems including plant cell wall oligosaccharides, bacterial antigens and mammalian glycoproteins ([1-4]). We report here the development of a

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microchemical/FAB-MS assay for the rapid and sensitive determination of the type of carbohydrate chains present in glycoproteins. This novel protocol is applicable to any glycoprotein and requires only 10-50 µg of sample. The technique involves acetolysis of the intact glycoprotein followed by FAB-MS analysis of the peracetylated carbohydrate fragments generated. Experimental protocols have been devised using fetuin (which contains complex-type carbohydrate chains), ribonuclease B (a high mannose glycoprotein) and erythrocyte Band 3 (a lactosaminoglycan containing glycoprotein). The methods were then applied to α-galactosidase I, a glycoprotein isolated from Vicia faba seeds (5). This enzyme (EC 3.2.1.22) is a tetrameric protein (M_r 160,000) which displays D-glucose/D-mannose-specific lectin activity (6).

MATERIALS AND METHODS

Materials: All chemicals were of Analar grade. Xenon for FAB-MS was obtained from BOC plc. Fetuin and Ribonuclease a were purchased from Sigma and Band 3 glycoproteins were a gift from Dr. M. Fukuda, La Jolla Cancer Research Foundation.

Preparation of α-galactosidase I and α-galactosidase I glycopeptide: The enzyme was purified according to an earlier procedure (5). S-Carboxymethylation of α-galactosidase I was achieved by the method of Konigsberg (8). The product was then digested with Streptomyces griseus protease according to the method of Allen et al (9) and the hydrolysate applied to a Biogel P-4 column (5.0 cm x 80 cm) in water. The column was eluted with water and the fractions assayed for carbohydrate by the anthrone method (10). The fractions (elution vol 100-150 ml), representing the major glycopeptide peak, which were eluted from the column were pooled and lyophilised. A sample of the material was hydrolysed (6 M HCl, 100° for 16 h) and analysed for amino acids. Other samples were subjected to acetolysis.

β-N-Acetylglucosaminidase H digestion: The conditions reported by Tarentino et al (11) were used for digestion of α-galactosidase I for a period of 15 h. The digest was then applied to a Sephacryl S-200 column (2.5 cm x 90 cm) which was eluted with McIlvaine buffer (pH 5.5). The resulting fractions were assayed for a α-galactosidase activity (12) and carbohydrate (10).
Acetolysis: Samples (10-50 µg) were dissolved in glacial acetic acid/acetic anhydride/sulphuric acid (10:1:1, v/v/v, 1 ml) and incubated at 60°C. Aliquots (0.2 ml) were removed at 0.5 h, 2 h, 5 h, 10 h and 18 h and quenched with 1 ml of water. The solution was extracted into chloroform which was washed three times with water and dried under a stream of nitrogen and finally under vacuum.

FAB-MS: FAB was carried out on a VG analytical ZAB-HF mass spectrometer fitted with an M-Scan FAB gun. Xenon was used to generate the primary ionising beam and the gun was operated at 10 µA, 10 kV. Samples were dissolved in methanol (5-10 µl) and 1-2 µl aliquots were loaded into the glycerol/monothioglycerol (1:1, v/v) matrix on the FAB target.

RESULTS AND DISCUSSION

(1) Protocol

Acetolysis is a widely used procedure for generating peracetylated carbohydrate fragments from oligosaccharides and glycoproteins (7). We have previously shown that FAB-MS is an excellent procedure for identifying the number of residues present in each of the products of acetolysis of high mannose carbohydrates isolated from yeast glycoproteins (13). During these studies we recognised that the high sensitivity achieved in the FAB-MS analysis of peracetylated oligosaccharides coupled with its ability to unambiguously define the exact number of hexoses, aminohexoses, sialic acids etc present in each component of the acetolysis mixture, could form the basis of a rapid and sensitive procedure for probing the carbohydrate structures in glycoproteins. Our objective was to devise a general protocol which would be applicable to any glycoprotein, irrespective of its size or carbohydrate structure and which could be applied directly to the intact glycoprotein. A variety of experiments were carried out on fetuin, ribonuclease B and erythrocyte Band 3, glycoproteins which encompass complex, high mannose and lactosaminoglycan structures, from which we established a general protocol which, we believe, should be applicable to a wide variety of unknown samples. Details are given in Materials and Methods.

The speed at which peracetylated oligosaccharides are released from a glycoprotein is governed by the types of linkages present and by the sequence. The time course we recommend permits the detection of fragments derived from cleavages of both labile and resistant bonds. Interestingly, sialyl linkages are relatively resistant to acetolysis and the method allows the detection of sialic acid and also the identification of their structures since different sialic acids have different residue masses. Fucosyl residues are, however, rapidly removed and may not be detected if impurity signals obscure the low mass region of the spectrum where the acetylated fucose molecular ion appears. The reaction is terminated by

quenching in water and the peracetylated oligosaccharides are extracted into chloroform. Any peptide fragments generated by the acetolysis, in addition to any salt, remain in the water layer and do not interfere with the subsequent mass spectrometric analysis. The original glycoprotein sample need not, therefore, be salt-free, and the results are not affected by the presence of contaminating proteins provided they are not glycosylated.

(2) Fetuin, ribonuclease B and Band 3 Glycoprotein

Interpretation of the FAB mass spectra of the acetolysis products is straightforward. Three ion species account for the major signals present. These are (i) molecular ions of the fully peracetylated species, (ii) molecular ions of molecules with a free OH at the reducing end but otherwide fully acetylated, and (iii) A-type oxonium ions resulting from glycosidic cleavages (14). If nitrogen is absent from the oligosaccharide the the sample ionises as the catioised $(M+NH_4)^+$ species (15); nitrogen containing samples ionise predominantly as protonated $(M+H)^+$.

The data obtained from fetuin is illustrative of complex-type structures. FAB-MS of the acetolysed sample yielded a spectrum containing molecular ion $(M+H)^+$ and $(M+NH_4)^+$ species as shown in Table 1. Additionally a-type fragment ions were observed at m/z 1035 (NeuNAc-Hex-HexNAc$^+$), 745 (NeuNAc-Hex$^+$), 618 (Hex-HexNAc$^+$), 460 (NeuNAc$^+$) and 331 (Hex$^+$). The 18 h acetolysed sample produced a similar spectrum, the only difference being that molecular ions containing sialic acid were less intense than at 10 hours.

Ribonuclease B is a high mannose glycoprotein and was thus used to test the method for such structures. The spectrum obtained from a 30 min acetolysis contained three low intensity molecular ion $(M+NH_4)^+$ signals at m/z 1272 (Hex_4), 1560 $(Hex)_5$ and 1848 (Hex_6). The presence of $(M+NH_4)^+$ species plus the readily recognisable pattern of 288 mass differences makes the identification of hexose polymers especially simple. Further aliquots of the reaction mixture were analysed after 2 h, 4 h and 18 h. All spectra contained the same signals with the intensity of the high mass signals

TABLE 1

Major molecular ions observed in the positive FAB mass spectrum of Fetuin after 10 hour acetolysis

m/z	Composition
1958	NeuNAc-Hex_3-$HexNAc_2$-OAc/H^+
1828	Hex_3-$HexNAC_3$-OAc/H^+
1670	NeuNAc-Hex_2-$HexNAc_2$-OAc/H^+
1541	Hex_3-$HexNAc_2$-OAc/H^+
1499	Hex_3-$HexNAc_2$-OH/H^+
1383	NeuNAc-Hex_2-HexNAc-OAc/H^+
1341	NeuNAc-Hex_2-HexNAc-OH/H^+
1253	Hex_2-$HexNAc_2$-OAc/H^+
1095	NeuNAc-Hex-HexNAC-OAc/H^+
966	Hex_2-HexNAC-OAc/H^+
924	Hex_2-HexNAc-OH/H^+
825	NeuNAc-Hex-OAc/H^+
808	NeuNAc-Hex-OAc/H^+
678	Hex-HexNAc-OAc/H^+
636	Hex-HexNAc-OH/H^+
408	Hex-OAc/NH_4^+

declining at 18 h. Molecular ions were present at m/z 2424 (Hex_8), 2136 (Hex_7), 1848 (Hex_6), 1560 (Hex_5), 1272 (Hex_4), 948 $(Hex)_3$ and 696 $(Hex)_2$. These signals were accompanied by A-type fragment ions at m/z 2347, 2057, 1771, 1483, 1195, 907 and 619. The presence of a series of hexose polymers after acetolysis is absolutely diagnostic of high mannose structures provided that there is no contamination of the sample with hexose containing polysaccharides from, for example, column gels.

Band 3 glycoprotein from human erythrocytes has recently been structurally characterised (4). It contains lactosaminoglycan chains which are branched in the adult but not in the fetus(16). Evidence was obtained after only 25 min acetolysis, of the presence of lactosamine containing chains; the spectrum showed a single peak at m/z 1211 corresponding to the molecular ion of peracetylated oligosaccharide of composition $Hex_2HexNAc_2$-OH. The polylactosaminoglycan nature of the sugar chains

was readily apparent after further acetolysis. After 4 h acetolysis as series of ions was observed at m/z 2116 ($Hex_4HexNAc_3^+$), 1828 ($Hex_2HexNAc_3^+$), 1541 ($Hex_3HexNAc_2^+$), 1253 ($Hex_2HexNAc_2^+$) 1095 ($NeuNAcHexHexNAc^+$), 966 ($Hex_2HexNAc^+$) and 678 ($HexHexNAc^+$). Similar results were obtained at 18 h, except that the higher mass signals were less intense. Both fetal and adult samples gave the same results indicating that the method is not able to give information on detailed aspects of structure such as branching.

(3) α-Galactosidase I

FAB-MS of the 2 h acetolysed sample of V.faba α-galactosidase I afforded a single peak at m/z 907 which corresponds to an A-type ion of composition Hex_3^+. The 5 h and 10 h acetolysis mixtures yielded signals 288 m.u. apart (acetylated hexosyl) at m/z 1771, 1483, 1195, 907 and 619 which are A-type ions of composition Hex_6, Hex_5, Hex_4, and Hex_3 respectively. Ammonium cationised molecular ions formed the related series at m/z 1848, 1560, 1272 and 984. The generation of acetylated hexose oligomers (and the presence of ammonium cationised species) is very good evidence that the glycoprotein contains high mannose chains.

In view of the importance of this finding further evidence for the high mannose structure was sought. Firstly, the glycoprotein was treated with endo β-N-acetylglucosaminidase H and the digest examined by Sephacryl S-200 gel filtration. A new active enzyme species corresponding to a dimeric form (M_r 80,000) was detected. In addition, carbohydrate-rich fragments which were presumably the released glycan chains, were observed. Secondly, a glycopeptide isolated from the glycoprotein after pronase digestion was shown to contain only four amino acids - Gly, Asx, Ser, and Glx - in approximately equal ratios; this result is consistent with the requirement for glycosylation at Asn, i.e. the sequence X-Asn-Y-Ser. Acetolysis of the glycopeptide again afforded hexose polymers. These observations clearly show that α-galactosidase I is a member of the high mannose family of glycoproteins in addition to possessing two types of

carbohydrate-binding sites (6). A further dimension to the intriguing physiological role of this enzyme in seeds has, therefore, been introduced.

CONCLUSION

FAB mass spectrometry coupled with acetolysis can offer a rapid, simple and sensitive method for determining the class of carbohydrate chains present in glycoproteins. The technique can yield valuable information at the early stages of a larger structural study.

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