Characterization of acetylated and acetolyzed glycoprotein high-mannose core oligosaccharides by fast-atom-bombardment mass spectrometry (Saccharomyces cerevisiae mannanproteins/endoglugosaminidase/trifluoroacetic anhydride/phosphorylated oligosaccharides)

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ABSTRACT Fast-atom-bombardment mass spectrometry has been applied to acetylated neutral and phosphorylated oligosaccharides from yeast glycoproteins and to their acetylation products. Although acetylation increases the sample molecular weight and the complexity of the spectra, it also enhances the sensitivity of detection, is applicable to samples that contain salt, and is especially useful for analysis of phosphorylated derivatives. Acetylation by trifluoroacetic anhydride/glacial acetic acid is particularly convenient and can be done rapidly on a small amount of material. Acetylation by acetic anhydride/glacial acetic acid/H2SO4 is done on the acetylated oligosaccharides, and the acetylated fragments are recovered by solvent extraction and immediately subjected to mass spectrometry. The methodology allows molecular weight determinations and sequence analysis by acetylation to be carried out on a few micrograms of isolated oligosaccharide in a few hours.

The technique of fast-atom-bombardment (FAB) mass spectrometry (1–3) has provided a renewed impetus to structural studies on complex carbohydrates, particularly for molecules in the molecular weight range of 1500–5000 (10–30 hexose units) (4–9). The obvious advantage of being able to obtain precise molecular weights is complemented, in FAB mass spectrometry, by sequence information that is available in the fragmentation pattern that results from disruption both of glycosidic bonds and sugar rings (6, 9).

In the present study, we have analyzed acetylated and acetolyzed high-mannose oligosaccharides obtained from yeast glycoproteins by endoglugosaminidase H digestion (10, 11). Our results show that such products give excellent mass spectra in positive and negative mode and in neutral or phosphorylated forms. Moreover, sequence information can be obtained by selective acetylation of oligosaccharide bonds (12–14). The usual procedure for characterizing the acetylation fragments has involved their isolation, deacetylation, and chromatography on paper (15) or by gel filtration (16). Here we show that FAB mass spectrometry of the acetylated fragments is a convenient method of analysis and is especially suitable for phosphorylated oligosaccharides. When the procedure is coupled with acetylation by the trifluoroacetic anhydride/glacial acetic acid method (16, 17), the analysis can be carried out in 2–3 h on a few micrograms of sample. Because it is fast and requires little material, the method should facilitate studies on the functional role of such carbohydrates in glycoproteins.

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MATERIALS AND METHODS

Acetylation. Oligosaccharides (10–100 µg), from previous studies (18, 19), were acetylated in a 2:1 (vol/vol) mixture of trifluoroacetic anhydride/glacial acetic acid (200 µl) at room temperature (20°C) for about 30 min (16). The sample usually dissolved within 5 min and, at the end of the reaction time, the solution was evaporated below 40°C to dryness, first on a rotary evaporator and then on an oil pump. The acetylated product was dissolved in methanol or acetonitrile for mass spectrometry. Although most glycosidic bonds are relatively stable to this procedure, glycosylphosphate linkages may be split. This was avoided by acetylation instead with pyridine/acetic anhydride (2:1, vol/vol), which was allowed to proceed until the oligosaccharide dissolved (8–48 hr), after which the solvent was removed by rotary evaporation. The acetylated product was dissolved in CHCl3, the solution was washed with dilute HCl to remove pyridine and convert acidic oligosaccharides to the protonated form, and the CHCl3 layer was evaporated to dryness for further analysis.

Acetylation. The acetylated product was dissolved in 500 µl of a 10:10:1 (vol/vol) mixture of acetic acid/acetic anhydride/concentrated sulfuric acid and kept at 40°C for 30–90 min (13). Two milliliters of CHCl3 and 2 ml of water were added to the reaction tube, which was mixed on a vortex mixer and centrifuged to separate the layers, and the upper water layer was removed with a Pasteur pipet. The lower CHCl3 layer was washed rapidly several times with water in this manner, after which it was evaporated to dryness first on a rotary evaporator and then on an oil pump. The acetylated fragments were dissolved in methanol and analyzed by mass spectrometry.

Mass Spectrometry. Mass spectra were obtained in the Biochemistry Department, Imperial College, London, on a VG Analytical ZAB HF mass spectrometer equipped with an FAB source and an M-scan FAB gun following published procedures (6, 9).

RESULTS AND DISCUSSION

The high-mannose core oligosaccharides of yeast glycoproteins typically contain 8–14 mannose units (11, 19), and the products obtained by endoglugosaminidase H digestion have a single N-acetylglucosamine unit. Whereas the sizes of such molecules can be determined approximately by chromatography (15) or by integration of proton nuclear magnetic resonances (20), the FAB mass spectra (Table 1) of underiva-
Acetylation of the oligosaccharides has been found to overcome some of the problems associated with the complex-type oligosaccharides. The use of carboxypeptidase A for the desalting of the samples also reveals the presence of minor components. Thus, for Man$_5$GlcNAc(OAc)$_9$, ions were seen at (M – 59), (M – 101), and (M – 119). See Figs. 1 and 2 for the interpretations.

Table 1. FAB mass spectral data for neutral and phosphorylated oligosaccharides

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Observed ion*</th>
<th>Proposed composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral oligosaccharides, mmn1 mmn2 core†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1678 (+)</td>
<td>Man$_5$GlcNAc – H$^+$</td>
</tr>
<tr>
<td></td>
<td>1840 (+)</td>
<td>Man$_5$GlcNAc – H$^+$</td>
</tr>
<tr>
<td>Acetolyzed 30 min‡</td>
<td>2922 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{19}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>2634 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{12}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>2346 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{23}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>2058 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{13}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>1770 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{19}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>1482 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{13}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>1194 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{19}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>906 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{19}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>618 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{19}$ – OAc$^–$</td>
</tr>
<tr>
<td></td>
<td>1482 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{13}$ – OAc$^–$</td>
</tr>
<tr>
<td>Acetolyzed 90 min§</td>
<td>2226 (+)</td>
<td>Man$_5$GlcNAcP + H$^+$</td>
</tr>
<tr>
<td></td>
<td>2408 (+)</td>
<td>Man$_5$GlcNAcP + H$^+$</td>
</tr>
<tr>
<td></td>
<td>717 (+)</td>
<td>Man$<em>3$P(OAc)$</em>{11}$ + H$^+$</td>
</tr>
<tr>
<td></td>
<td>1005 (+)</td>
<td>Man$<em>3$P(OAc)$</em>{10}$ + H$^+$</td>
</tr>
<tr>
<td></td>
<td>1047 (+)</td>
<td>Man$<em>3$P(OAc)$</em>{11}$ + H$^+$</td>
</tr>
<tr>
<td></td>
<td>1482 (+)</td>
<td>Man$<em>5$GlcNAc(OAc)$</em>{11}$ – OAc$^–$</td>
</tr>
</tbody>
</table>

*The sign indicates whether the spectrum was obtained in positive or negative mode.
†The two homologs were separated by gel filtration on Bio-Gel P-4 before analysis.
‡For most acetolysis products, a group of three ions was observed. Thus, for Man$_5$GlcNAc(OAc)$_{19}$, ions were seen at (M – 59), (M – 101), and (M – 119). See Figs. 1 and 2 for the interpretations.
§Only the major ion is listed. Other ions at lower mass are also seen, representing degradation products, although none remains at higher mass.

Plausible structures for the ions observed from the peracetylated parent molecules are presented in Figs. 1 and 2. Acyclic molecules such as (M + 103) are reasonable, because such compounds are isolable from acetylation reactions (21). Normally, the majority of the ion current is carried by two or three of the ions shown in Figs. 1 and 2; and the relatively complicated spectral pattern does not pose interpretation problems but is actually advantageous when spectra are weak, because the presence of several related signals allows a more confident assignment.

For Man$_5$GlcNAc acetylated with pyridine/acetic anhydride (M, 2981), the ion most commonly observed in the positive mode is (M – 59) (Fig. 3B), probably because the Cl-acetate anion is a good leaving group. If the oligosaccha-

![Fig. 1. Postulated structures for ions observed in the positive FAB spectra of acetylated or acetolyzed oligosaccharides. In any one spectrum, only two or three major ions are observed for a particular molecular species.](image-url)
ride is acetylated with trifluoroacetic anhydride/acetic acid, the result is often different with the ion \((M - 41)\) (Fig. 3A) being preponderant. This probably is a consequence of loss of the C1-acetyl because of the acidic conditions of the reaction. This difference is most significant when ions are analyzed in the negative mode because the completely acetylated molecule can

\[
\begin{align*}
\text{Man}(1 \to 6)\text{Man}(1 \to 6)\text{Man}(1 \to 6)\text{Man}(1 \to 4)\text{GlcNAc} & \quad \text{and} \quad \text{Man}(1 \to 6)\text{Man}(1 \to 6)\text{Man}(1 \to 6)\text{Man}(1 \to 4)\text{GlcNAc}. \\
2 & \quad 2 \quad 2 \quad 3 \quad 3 \\
\uparrow & \quad \uparrow & \quad \uparrow & \quad \uparrow & \quad \uparrow \\
1 & \quad 1 & \quad 1 & \quad 1 & \\
\text{Man} & \quad \text{Man} & \quad \text{Man} & \quad \text{Man} & \\
2 & \quad \uparrow & \quad \uparrow & \quad \uparrow & \quad \uparrow \\
1 & \quad 1 & \quad 1 & \quad 1 & \\
\text{Man} & \quad \text{Man} & \quad \text{Man} & \quad \text{Man} & \\
2 & \quad \uparrow & \quad \uparrow & \quad \uparrow & \quad \uparrow \\
1 & \quad 1 & \quad 1 & \quad 1 & \\
\text{Man} & \quad \text{Man} & \quad \text{Man} & \quad \text{Man} & \\
& \quad 2 & \quad 2 & \quad 2 & \quad \uparrow \\
& \quad 1 & \quad 1 & \quad 1 & \\
& \quad \text{Man} & \quad \text{Man} & \quad \text{Man} & \\
\end{align*}
\]

An unusual feature of the peracetylated Man\(_3\)P positive ion spectrum was the presence in the freshly prepared acetylsate of an ion at \((M + 43)\) that disappeared from the spectrum after the sample had been stored several days in methanol. We interpret the ion to be the acetylyphosphate mixed anhydride that was solvolysed during storage to give the monooester.

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{O} \\
\text{CH}_3\text{C}-\text{O} & \quad \text{P}-\text{OR} & \quad \text{HO}-\text{P}-\text{OR} \\
\text{OH/H}^+ & \quad \text{OH/H}^+ \\
\end{align*}
\]

Studies on a diphosphorylated oligosaccharide from Saccharomyces cerevisiae mnn1 mnn9 strain with the following structure (next page) reveals the real power of this technique. In agreement with this structure, the molecular ion region of the negative FAB spectrum of the underivatized oligosaccharide (Fig. 4) shows two ion clusters of equal intensity.
assignable to \((M - H^+)\) and \((M + Na^+ - 2H^+)\) at \(m/z\) 2324 and 2346. Following mild acid hydrolysis to cleave the mannosylphosphate linkages, the major ion was seen at \(m/z\) 2000, which corresponds to \((M - H^+)\) for a Man\(_9\)GlcNAcP\(_2\) \((M, 2001)\). Thus, two of the mannosides are linked in an acid-labile manner.

The relative locations of the two phosphate groups were investigated by partial acetylation. The characteristic ions of interest are those that demonstrate the presence of Man\(_5\)P, Man\(_4\)GlcNAcP, and Man\(_6\)GlcNAcP\(_2\). The partial acetylating of the intact oligosaccharide diphosphate (Man\(_{12}\)GlcNAcP\(_2\)) gave the expected ions for Man\(_5\)P(OAc)\(_2\) - H\(^+\) at \(m/z\) 715 and for Man\(_4\)GlcNAcP(OAc)\(_3\) - H\(^+\) at \(m/z\) 1578, but an ion for Man\(_6\)GlcNAcP\(_2\)(OAc)\(_3\) - H\(^+\) at 2192 was not observed in this spectrum (Fig. 5). This result appears to have been due to the ready loss of a mannosylphosphate group during the bombardment process because a strong ion is seen for Man\(_9\)GlcNAcP(OAc)\(_2\) - H\(^+\) at \(m/z\) 2154.

This conclusion was confirmed when the mild acid-hydrolyzed oligosaccharide (Man\(_9\)GlcNAcP\(_2\)) was acetylated, for ions at \(m/z\) 2192 (Man\(_9\)GlcNAcP\(_2\)(OAc)\(_3\) - H\(^+\)) and at \(m/z\) 2234 (Man\(_9\)GlcNAcP\(_2\)(OAc)\(_2\) - H\(^+\)) are observed (Fig. 6). These two ions correspond to \((M - 1)\) and \((M + 42)\), the latter probably reflecting formation of some acetylated mixed anhydride. Such pairs of ions are also seen at \(m/z\) 2154/2196, 1578/1620, 1579/1621, 1291/1333, 1003/1045, 901/943, and 715/757; and in all instances they are correlated with the presence of one or two phosphate groups in the

![Fig. 3. FAB spectra for the molecular ion regions of the acetylated Man\(_x\)GlcNAc oligosaccharide from S. cerevisiae mnn1 mnn2 man92 manprotein. (A) Acetylation with pyridine and acetic anhydride; (B) acetylation with trifluoroacetic anhydride/acetic acid. The Inset shows the structure of the oligosaccharide and the postulated structures of the major ions.](image)

![Fig. 4. Negative FAB spectrum of the molecular ion region of S. cerevisiae mnn1 mnn9 oligosaccharide diphosphate. The postulated structure is Man\(_9\)GlcNAcP\(_2\) \((M, 2325)\), and the ions at 2324 \((M - H)\) and 2346 \((M + Na - 2H)\) are consistent with this assignment.](image)

![Fig. 5. Negative FAB spectrum of acetylated intact oligosaccharide diphosphate. Characteristic ions are at \(m/z\) 715/757 (Man\(_5\)P), at 1578/1620 (Man\(_4\)GlcNAcP) and at 2154/2196 (Man\(_9\)GlcNAcP). Other major assignable ions are at \(m/z\) 1579/1621 (Man\(_5\)P) and 2155/2197 (Man\(_7\)P).](image)
m/z 2154/2196 (Man6GlcNAcP), and ions fragment produced 1578/1620 (Man4GlcNAcP), 1579/1621 (Man5P), 1867/1909 (Man6P), groups 2192/2234 m/z acetolyzed oligosaccharide diphosphate. Characteristic FIG. 6. Negative FAB spectrum of mild acid hydrolyzed and acetolyzed oligosaccharide diphosphate. Characteristic ions are at m/z 2192/2234 (Man6GlcNAcP2) and confirm that both phosphate groups are positioned so that they can be retained on a Man6GlcNAc fragment produced by partial cleavage of α1 → 6 linkages. Other assignable ions are at m/z 1003/1045 (Man4P), 1291/1333 (Man5P), 1578/1620 (Man6GlcNAcP), 1579/1621 (Man5P), 1867/1909 (Man6P), and 2154/2196 (Man6GlcNAcP). Above m/z 2300, ions are seen at m/z 2442/2484 (Man6GlcNAcP), 2480/2522 (Man6GlcNAcP2), 2688/2730/2772 (Man4GlcNAcP), and 2768/2810 (Man6GlcNAcP2).

molecule. The detection of an ion for Man6GlcNAcP in the spectrum of acetolyzed Man6GlcNAcP produced by mild acid hydrolysis may also reflect the fact that cleavage of a single bond was necessary to form this ion, whereas three bonds would have to be broken to form the same ion from the parent Man6GlcNAcP2.

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