The glycans of soybean peroxidase

James S.S.Gray, Byung Yun Yang, Steven R.Hull,
David P.Venzke and Rex Montgomery

Department of Biochemistry, College of Medicine, University of Iowa,
Iowa City, IA 52242 USA

To whom correspondence should be addressed

Soybean hull peroxidase (SBP, E.C. 1.11.1.7), an anionic glycoprotein, was found to contain 18.2% carbohydrate with the average composition: 2 mol GlcNAc, 3.3 mol Man, 0.9 mol Fuc, and 0.7 mol Xyl. The oligosaccharides of SBP, after release with glycopeptidase A, were investigated by a combination of high pH anion exchange chromatography with pulsed amperometric detection, methylation analysis and matrix assisted laser desorption/ionization-time-of-flight mass spectrometry. The structure of the major oligosaccharide, accounting for 60 to 65% of the total, is Man\(_2\) → 6(Man\(_1\)β1 → 3)(Xyl\(_1\)β1 → 2)Man\(_1\)β1 → 4GlcNAcβ1 → 4(Fuc\(_1\)α1 → 3)GlcNAc. A further 20 to 25% of the released oligosaccharides belong to the (Xyl)\(_m\)Man\(_f\)(Fuc)\(_m\)GlcNAc\(_{m}\) family. The rest of the oligosaccharides were of a 2-mannose type. Investigation of the six tryptic fractions containing carbohydrate revealed considerable heterogeneity in the N-linked oligosaccharides present in each fraction. The major glycan (4, Table III) was present in each fraction. Two of the fractions contained the major part of the 2-mannose type glycans, Man\(_m\)GlcNAc\(_m\) (m = 5-9), the major species being Man\(_6\)GlcNAc\(_6\). The other four fractions contained mainly members of the (Xyl)\(_m\)Man\(_f\)(Fuc)\(_m\)GlcNAc\(_{m}\) family. Methylation analysis of the holo- and apo-SBP provide support for the structures proposed for the oligosaccharides as well as for the heterogeneity of the glycopeptide fractions.

Key words: glycan/soybean peroxidase/MALDI-TOF/methylation analysis/N-linked oligosaccharides

Introduction

Peroxidases (E.C. 1.11.1.7) are heme-containing proteins, widely distributed in nature, where they play a role in a variety of physiological processes (van Huystee, 1987; Rodriguez Marañon and van Huystee, 1994). A number of soybean peroxidases have been described (Sessa and Anderson, 1981). Soybean hull peroxidase (SBP), an anionic glycoprotein with an isoelectric point of 4.1, is found in substantial amounts in soybean hulls, a byproduct of the soybean industry (Gillikin and Graham, 1991). It exists essentially as a single isozyme in the hull (Gillikin and Graham, 1991) and has unusually high thermal stability (McEldoon, 1995) even at low pH (McEldoon et al., 1995). These properties, together with the enzymatic activity of SBP in organic solvents, make it useful to the synthetic organic chemist and to industry (Klibanov et al., 1981; Blinkovsky et al., 1994).

This study reports an analysis of the N-linked glycans, released from apo-SBP and its tryptic glycopeptides by glycopeptidase A, by a combination of high performance anion exchange chromatography (HPAEC-PAD), matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF), and methylation analysis. This work was presented in part at the 23rd Annual Meeting of the Society for Glycobiology, University of Notre Dame, Notre Dame, Indiana, November 9-12, 1994.

Results

Molecular weight and monosaccharide composition

A molecular weight of 40,662 Da was determined for apo-SBP by MALDI-TOF and 33,250 Da after deglycosylation with glycopeptidase A. These values compare favorably with the molecular weights of 37,000 Da for the native enzyme and 30,000 for the deglycosylated enzyme determined by SDS–PAGE and reported by Gillikin and Graham (1991). The glycan therefore constitutes 18.2% of the mass of the apo-SBP molecule. Acid hydrolysis of the deglycosylated apo-SBP and HPAEC-PAD analysis of the released monosaccharides indicated that better than 90% of the carbohydrate was released by glycopeptidase A digestion.

The monosaccharide composition of FPLC-purified holo-SBP, as determined by hydrolysis and HPAEC-PAD analysis, is 2 mol GlcNAc, 3.3 mol Man, and 0.9 mol Fuc and 0.7 mol Xyl, corresponding to 17.6% carbohydrate. These non-stoichiometric values indicate heterogeneity of the glycans present on SBP. A millimolar extinction coefficient of 102 mM\(^{-1}\) cm\(^{-1}\) at 403 nm and pH 5.8 for the heme moiety (Aibara et al., 1982) was used to calculate the amount of protein used for the analyses.

Tryptic maps

Six peptides containing carbohydrate were identified in the tryptic digest of apo-SBP by hydrolysis and HPAEC-PAD analysis of the released monosaccharides (Figure 1). Glycopeptide fractions GP1, GP2, GP3, and GP5 contain substantial amounts of Fuc and Xyl in addition to Man and GlcNAc (Tables I, II). Glycopeptide fractions GP4 and GP6 contain reduced amounts of Fuc and Xyl and greatly increased amounts of Man relative to GlcNAc (Tables I, IV). Glycopeptide fractions GP3 and GP5 contribute more than 50% of the total carbohydrate recovered; the high-mannose oligosaccharide fractions (GP4 and GP6) comprise about 21% of the total carbohydrate.

Methylation analyses of SBP

Methylation analyses of holo-SBP and of a hydrazinolyzate of the apo-enzyme are presented in Table II. The assignments of the methyl alditol acetates were confirmed by
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Fig. 1. Tryptic map of apo-SBP. Apo-SBP was digested with TPCK-treated trypsin and the glycopeptides isolated by HPLC on a C8 column as described in the text.

Table I. Monosaccharide composition of tryptic glycopeptides isolated from SBP. The tryptic glycopeptides were isolated by HPLC on a Q column and the monosaccharide composition determined by HPAEC-PAD after hydrolysis with 2 M TFA for 3 h at 100°C.

<table>
<thead>
<tr>
<th>Glycopeptide</th>
<th>% of Total carbohydrate</th>
<th>Fuc</th>
<th>Xyl</th>
<th>GlcN</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.6</td>
<td>1.1</td>
<td>0.7</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>6.4</td>
<td>1.2</td>
<td>1.1</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>34.6</td>
<td>1.1</td>
<td>1.0</td>
<td>2.1</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>10.5</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.1</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>20.1</td>
<td>1.0</td>
<td>0.7</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>10.7</td>
<td>0.3</td>
<td>&lt;0.1</td>
<td>1.1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

GLC-MS and by the analysis of standards derived as described below. GLC-FID and GLC-MS analyses of the methyl alditol acetates prepared from undermethylated α-methyl mannoside, as described by Honda and Montgomery (1969), were used to obtain the retention times and relative response factors (GLC-MS total ion and selected ion) of the methyl mannositol acetates. The methyl alditol acetates prepared from asparaginyl carbohydrates A and B (Huang et al. 1970), were used to obtain standards for terminal- and 1,4-linked GlcNAc. The methyl alditol acetates from fetuin provided standards for 1,4-linked Gal; those from lacto-N-difucohexaose II provided standards for 1-linked Gal, 1-linked Fuc, 1,3-linked Gal, 1,3,4-linked Glc, and 1,3,4-linked GlcNAc. Methylation of lacto-N-difucohexaose II was also used to determine a response factor for 1,5-di-O-acetyl-2,3,4-tri-O-methyl fucitol (total ion and selected ion) on the GLC-MS.

In addition to the methyl alditol acetates characteristic of Manα 1 → 6 (Manα 1 → 3)(Xylβ 1 → 2) Manβ 1 → 4 GlcNAcβ 1 → 4 (Fucα 1 → 3) GlcNAc, substantial amounts of 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannotol and 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl mannotol are also present, indicating the presence of the higher homologues of (Xyl),Manα, 3,Fuc,GlcNAc, and Manα,GlcNAc, where f = 0 or 1 and x = 0 or 1 and m ≥ 5. A significant amount of 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl mannotol is also present indicative of 2,3-disubstituted mannosyl residues. Small amounts of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl and 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl mannotol show 1,3- and 1,6-linked mannosyl residues. Between 50 and 70% of the proximal GlcNAc of the chitobiose core is substituted, presumably with Fuc by analogy with the structures of the glycans described for other plant glycoproteins. Similarly, about 60% of the β-linked Man of the conserved core is 2-O substituted by xylose. No 2,6-disubstituted Man (1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl mannotol) or terminal GlcNAc (1,5-di-O-acetyl-(2-N-methylacetamido)-3,4,6-tri-O-methyl glucitol) residues were observed after methylation of either holo- or apo-SBP.

Oligosaccharide maps

No oligosaccharides were released by digestion of holo-SBP with either PNGase F or glycopeptidase A. Treatment of apo-SBP with PNGase F released only a small part of the oligosaccharides whereas glycopeptidase A digestion released greater than 90% of the oligosaccharides from the protein, as determined by monosaccharide analysis of the deglycosylated protein.

Analysis of the oligosaccharides released by glycopeptidase A from apo-SBP by HPAEC-PAD revealed a major, broad peak, of characteristic shape, eluting at the same position as the (Xyl)Manα,Fuc,GlcNAc, standard. A series of peaks eluting in the high-mannose region (Figure 2) were also observed. Some peaks eluted earlier than 15 min, but none of their retention times corresponded to the...
Together, the members of the (Xyl)Man\textsubscript{n}(Fuc)GlcNAc\textsubscript{m} family constitute about 66% of the total oligosaccharides of SBP, those of the (Xyl)Man\textsubscript{n}GlcNAc\textsubscript{2} family about 16% of the total and those of the Man\textsubscript{n}(Fuc)GlcNAc\textsubscript{2} family about 1% of the total. The high-mannose types of oligosaccharides make up about 17% of the total.

**Methylation analyses of the tryptic glycopeptides**

The methyl alditol acetates detected in the methylation analyses of holo- and apo-SBP are also present in the methylation analyses of the glycopeptides (Tables II, IV), indicating that no changes occurred in the glycans during the trypsin digestion and subsequent fractionation of the peptides produced.

The major differences are observed in glycopeptide fractions GP4 and GP6, which contain high proportions of 1,2- and 1,3,6-linked mannose residues and low proportions of terminal fucose and xylose residues (Table IV). Glycopeptide fraction GP5 contains a higher proportion of 1,3,6-linked mannose compared to GP1, GP2, and GP3 whereas no 1,3,6-linked mannose was detected in GP1 and GP2 and only a small amount in GP3.

Glycopeptide fractions GP2 and GP3 contain high proportions of terminal xylosyl residues, and 1,2,3- and 1,2,3,6-linked mannosyl residues.

**MALDI-TOF MS analysis of the glycan of soybean peroxidase released by glycopeptidase A from the tryptic glycopeptides**

Each of the tryptic fractions containing carbohydrate were digested with glycopeptidase A and the released glycans analysed by MALDI-TOF MS with 2,5-dihydroxybenzoate as matrix. The results are tabulated in Table V; the spectra obtained from GP4, GP5, and GP6, as examples, are presented in Figure 3B–D.

The major oligosaccharide in GP1, GP2, and GP3 is (Xyl)Man\textsubscript{n}(Fuc)GlcNAc\textsubscript{2} and oligosaccharides containing either Fuc or Xyl or both of these residues constituted over 95% of the glycan in these fractions. GP4 and GP6 contain substantial amounts of high-mannose type oligosaccharides (Table V, Figure 3B–D); these make up over 50% of the total carbohydrate in GP4 (Table V, Figure 3B) and over 36% of the carbohydrate in GP6 (Table V, Figure 3D).

**HPAEC-PAD analysis of the glycan released by glycopeptidase A from tryptic glycopeptides**

A HPAEC-PAD analysis of the glycans released from GP1, GP2, and GP3 revealed mainly a broad peak eluting at the same position as a (Xyl)Man\textsubscript{n}(Fuc)GlcNAc\textsubscript{2} standard (data not shown). No other significant oligosaccharide peaks were observed in these analyses. The oligosaccharides from GP4 and GP6, presented in Figure 4, are characterized by the lower amounts of (Xyl)Man\textsubscript{n}(Fuc)GlcNAc\textsubscript{2} and substantial amounts of high-mannose type glycans (Figure 4A,C). The major oligosaccharide eluting in the high-mannose region of the chromatogram and corresponding to one of the Man\textsubscript{n}GlcNAc\textsubscript{2} isomers (cf. Figure 2) does not elute in the same position as the major Man\textsubscript{n}GlcNAc\textsubscript{2} isomer present in a RNase B standard. The glycans...
Fig. 2. HPAEC-PAD analysis of a mixture of Man$_3$GlcNAc$_2$ (1) and a RNase B standard (2-5, 8, A), and the oligosaccharides released from apo-SBP by glycopeptidase A (B). Full scale was 300 nA (1000 mV).

Table III. MALDI-TOF analysis of the oligosaccharides released from apo-SBP by glycanase A. Apo-SBP was digested with glycanase A and the oligosaccharides were analyzed by MALDI-TOF MS with 2,5-dihydroxybenzoic acid as a matrix.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Oligosaccharide</th>
<th>Observed molecular weight</th>
<th>Calculated molecular weight</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GlcNAc$_3$Man$_2$FucXyl-Na</td>
<td>1048.7</td>
<td>1048.4</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>GlcNAc$_3$Man$_2$Xyl-Na</td>
<td>1065.6</td>
<td>1064.4</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>GlcNAc$_3$Man$_2$FucXyl-$K$</td>
<td>1080.4</td>
<td>1078.4</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>GlcNAc$_3$Man$_2$Xyl-$K$</td>
<td>1211.4</td>
<td>1210.4</td>
<td>60.8</td>
</tr>
<tr>
<td>5</td>
<td>GlcNAc$_3$Man$_2$FucXyl-$K$</td>
<td>1227.3</td>
<td>1226.4</td>
<td>12.0</td>
</tr>
<tr>
<td>6</td>
<td>GlcNAc$_3$Man$_2$Fuc-$K$</td>
<td>1257.2</td>
<td>1257.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>7</td>
<td>GlcNAc$_3$Man$_2$FucXyl-$K$</td>
<td>1373.0</td>
<td>1372.5</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>GlcNAc$_3$Man$_2$Xyl-$K$</td>
<td>1388.6</td>
<td>1388.4</td>
<td>1.3</td>
</tr>
<tr>
<td>9</td>
<td>GlcNAc$_3$Man$_2$Na</td>
<td>1419.2</td>
<td>1418.5</td>
<td>2.6</td>
</tr>
<tr>
<td>10</td>
<td>GlcNAc$_3$Man$_2$FucXyl-$K$</td>
<td>1534.2</td>
<td>1534.5</td>
<td>0.3</td>
</tr>
<tr>
<td>11</td>
<td>GlcNAc$_3$Man$_2$Fuc-$K$</td>
<td>1564.8</td>
<td>1564.5</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td>GlcNAc$_3$Man$_2$Na</td>
<td>1581.1</td>
<td>1580.5</td>
<td>9.5</td>
</tr>
<tr>
<td>13</td>
<td>GlcNAc$_3$Man$_3$-$K$</td>
<td>1597.8</td>
<td>1596.5</td>
<td>0.9</td>
</tr>
<tr>
<td>14</td>
<td>GlcNAc$_3$Man$_3$Na</td>
<td>1743.1</td>
<td>1742.6</td>
<td>2.8</td>
</tr>
<tr>
<td>15</td>
<td>GlcNAc$_3$Man$_3$-$K$</td>
<td>1904.4</td>
<td>1904.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>
from apo-SBP by glycopeptidase A, to be performed. The semiquantitative analysis of the oligosaccharides, released dihydroxybenzoic acid as matrix is proportional to the adducts of oligosaccharides containing Man and GlcNAc (1993) that the intensity of the ions produced using 2,5- and frequently Fuc and Xyl. The observation by Harvey sis of the glycans released by glycopeptidase A digestion of apo-SBP excludes O-linked glycosyl sites. obtained from the holo-SBP and the hydrazinolysis of the glycosylation sites on the protein. The identical results of SBP, consistent with the presence of five or six N-linked zyme, the carbohydrate contributes 17.7-18.2% to the mass well as the monosaccharide composition of the holo-en-

**Table IV.** Methylation analysis of glycopeptides derived from SBP. Tryptic glycopeptides GP1 to GP6 (5-10 nmol oligosaccharide) were subjected to methylation analysis as described in the text. The major methylated alditol acetates were quantitated by GLC with FID detection and by GLC-MS with integration of the total ion chromatogram. The minor peaks were quantitated from the extracted selected-ion chromatograms as described in the legend.

<table>
<thead>
<tr>
<th>Methylated alditol acetate</th>
<th>Relative mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP1</td>
<td>GP2</td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; Xyl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6</td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; Fuc&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6</td>
</tr>
<tr>
<td>2,3,4,6-Me&lt;sub&gt;4&lt;/sub&gt; Man&lt;sup&gt;h&lt;/sup&gt;</td>
<td>2.0</td>
</tr>
<tr>
<td>3,4,6-Me&lt;sub&gt;4&lt;/sub&gt; Man&lt;sup&gt;h,k&lt;/sup&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td>2,4,6-Me&lt;sub&gt;3&lt;/sub&gt; Man&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>3,4,6-Me&lt;sub&gt;3&lt;/sub&gt; Man&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4,6-Me&lt;sub&gt;2&lt;/sub&gt; Man&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,4-Me&lt;sub&gt;2&lt;/sub&gt; Man</td>
<td>0.0</td>
</tr>
<tr>
<td>4-Me Man</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-N-Me-3,6-Me&lt;sub&gt;2&lt;/sub&gt; GlcNAc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.0</td>
</tr>
<tr>
<td>2-N-Me-6-Me GlcNAc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*2,3,4-Me<sub>3</sub> Xyl = 1,5-di-O-acetyl-(1-deutero)-2,3,4-tri-O-methyl xylitol etc.

Quantitation from GLC-FID using equivalent carbon response factor derived by Sweet et al. (1975)
Quantitation from integrated total ion chromatogram (GLC-MS).
Quantitation from extracted and integrated m/z 118 chromatogram (GLC-MS).
Quantitation from extracted and integrated m/z 129 chromatogram (GLC-MS).
Quantitation from extracted and integrated m/z 130 chromatogram (GLC-MS).
Quantitation from extracted and integrated m/z 161 chromatogram (GLC-MS).
Quantitation from extracted and integrated m/z 159 chromatogram (GLC-MS).

from GP5 consist mainly of (Xyl)Man<sub>3</sub>(Fuc)GlcNAc<sub>2</sub> with a trace of Man<sub>2</sub>GlcNAc<sub>2</sub>.

**Discussion**

SBP is unusually stable to thermal denaturation (McEldoon, 1995) and is thermally stable at low pH (McEldoon et al., 1995). It is also active in organic solvents (McEldoon, 1995; McEldoon et al., 1995). Unlike horseradish peroxi-
dase, where many isoenzymes have been detected and purified (Shannon et al., 1966; Delincée and Radola, 1975; Aibara et al., 1982), SBP exists as a single anionic isozyme in soybean hulls (Gillikin and Graham, 1991), from which the enzyme is readily extracted and purified. Little is known about the N-linked glycans of SBP and their role in the stability of the enzyme. The aim of this study was to investigate the nature and distribution of the major and minor oligosaccharides of SBP.

From the difference in the molecular weight of apo-SBP (40,662 Da) and the deglycosylated enzyme (33,250) as well as the monosaccharide composition of the holo-enzyme, the carbohydrate contributes 17.7-18.2% to the mass of SBP, consistent with the presence of five or six N-linked glycosylation sites on the protein. The identical results obtained from the holo-SBP and the hydrazinolysis of the apo-SBP excludes O-linked glycosyl sites.

Fifteen peaks were detected by MALDI-TOF MS analysis of the glycans released by glycopeptidase A digestion of apo-SBP that could be assigned to the sodium or potassium adducts of oligosaccharides containing Man and GlcNAc and frequently Fuc and Xyl. The observation by Harvey (1993) that the intensity of the ions produced using 2,5-
dihydroxybenzoic acid as matrix is proportional to the amount of the oligosaccharide in the sample enabled a semiquantitative analysis of the oligosaccharides, released from apo-SBP by glycopeptidase A, to be performed. The major oligosaccharide present in apo-SBP, accounting for 61% of the total, is (Xyl)Man<sub>3</sub>(Fuc)GlcNAc<sub>2</sub>. Oligosaccharides containing either Fuc or Xyl or both sugars account for about 84% of the total. About 16% of the oligosaccharides are of the high mannose type, of which the major glycans is Man<sub>4</sub>GlcNAc<sub>2</sub> with smaller amounts of Man<sub>5</sub>GlcNAc<sub>2</sub>, Man<sub>6</sub>GlcNAc<sub>2</sub>, Man<sub>7</sub>GlcNAc<sub>2</sub> and a trace of Man<sub>5</sub>GlcNAc<sub>2</sub>. HPAEC-PAD analysis reveals that the major Man<sub>4</sub>GlcNAc<sub>2</sub> isomer of SBP is not the same as the major Man<sub>4</sub>GlcNAc<sub>2</sub> isomer present in the RNase B standard but elutes earlier in the analysis. A minor Man<sub>4</sub>GlcNAc<sub>2</sub> isomer of SBP elutes at the same position as the major Man<sub>4</sub>GlcNAc<sub>2</sub> isomer of RNase B. HPAEC-PAD analysis also reveals the presence of a third Man<sub>5</sub>GlcNAc<sub>2</sub> isomer in small amounts, together with traces of Man<sub>3</sub>GlcNAc<sub>2</sub> and Man<sub>4</sub>GlcNAc<sub>2</sub>. The other high-mannose species are not seen in the HPAEC-PAD analyses; Man<sub>4</sub>GlcNAc<sub>2</sub> is not detected in either MALDI-TOF or HPAEC-PAD analyses.

Methylation, MALDI-TOF and HPAEC-PAD analyses provide evidence for considerable heterogeneity in the distribution of the oligosaccharides along the peptide chain. Over 97% of the oligosaccharides present in GP1, GP2, and GP3 and over 93% present in GP5 are of the (Xyl), Man<sub>n</sub>(Fuc)GlcNAc<sub>m</sub> family (m = 2, f and x = 0, 1). In contrast, these oligosaccharides represent less than 33% of the glycans in GP4 and 65% of the oligosaccharides in GP6. GP4 and GP6 are clearly distinguished from the other glycopeptide fractions by the presence of a significant proportion of high-mannose type oligosaccharides. The high proportion of 1,2-linked- and 1,3,6-linked Man confirm the presence of these oligosaccharide species in GP4 and GP6. Glycopeptide fraction, GP5, contains a small amount of Man<sub>4</sub>GlcNAc<sub>2</sub> which may arise from contamination by either GP4 or GP6. Similarly, GP6 contains a low proportion of Fuc which may arise by contamination with GP5.

The absence or low concentration of 1,2,6-linked Man
residues in holo- and apo-SBP and in any of the glycopeptide fractions indicate that the β-linked mannose of the core GlcNAc2Man3 oligosaccharide is rarely substituted at the 2-O position by Xyl and at the 6-O position with Man, in contrast to stem bromelain where this substitution pattern is common (Ishihara et al., 1979). Usually, the β-linked Man residue is substituted at the 2-O position with Xyl and at the 3-O position with Man. No terminal GlcNAc residues were detected by any of the methylation analyses.

The high levels of 1,5-di-O-acetyl-2,3,4-tri-O-methyl xyllitol, 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl mannnitol and 1,2,3,5,6-penta-O-acetyl-4-O-methyl mannnitol in the methylated fragments of glycopeptide fractions GP2 and GP3 cannot be explained at this time.

No peak with a molecular weight corresponding to the sodium or potassium adduct of Man3GlcNAc2 (932 or 948 Da, respectively) was observed in the MALDI-TOF analysis of the SBP oligosaccharides. The smallest species observed was (Xyl)Man3(Fuc)GlcNAc2.

It has been observed previously (Yang et al., unpublished observations) that HPAEC-PAD analysis of the (Xyl)Mann(Fuc)GlcNAc2 (m = 2, f = 1, x = 0.1) family poses problems in the alkaline degradation of the fucosyl residues. The required structural information was obtained by the addition of methylation and MALDI-TOF analyses. HPAEC-PAD and MALDI-TOF complement each other well in the analysis of the high-mannose type oligosaccharides. Whereas the MALDI-TOF analysis showed the presence of a Man5GlcNAc2 species, HPAEC-PAD provided information about the distribution of the isomers present, clearly indicating that the major Man6GlcNAc2 isomer present on SBP is different to that from RNase B.

Summary

It is interesting to note from the present study that none of the glycanes have a nonreducing terminal GlcNAc residue, which is not unusual in the high-mannose glycan structures but is frequently observed in those glycans with Xyl linked to the β-D-mannose or Fuc to the chitobiosyl-core. Furthermore, the minor glycan 11 (Table III) with Fuc and six Man residues without a terminal GlcNAc is contrary to the processing pathway in the Golgi. This supports the proposal of Tezuka et al. (1992) of an extra-Golgi degradation system that produces glycans that are not in the Golgi-processing pathway.

Materials and methods

Materials

The materials for this study were purchased as follows: PNGase F (Peptide-β-N-acetyl-β-glucosaminidase) asparagine amidase), Genzyme (Boston, MA); Glycopeptidase A (almond), Seikagaku America, Inc. (Rockville, MD); L-1-tosylamido-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, Worthington Biochemicals (Malvern, PA); Soybean peroxidase, Enzymol, Inc. (2543 Westbelt Drive, Columbus, OH) and Sigma (St Louis, MI); lacto-N-fucophacchoxose II, MannGlcNAc2, (Xyl)Man(Fuc)GlcNAc2 and a RNase B oligosaccharide library, Oxford Glycosystems (Rosedale, NY).

Methods

Purification of SBP

Soybean hull peroxidases were purified from two preparations purchased from Sigma (St. Louis, Missouri). The initial RZ value for both preparations was 1.3. Commercial SBP (15 mg) in water was chromatographed on a column of DEAE-cellulose (1 × 8 cm) equilibrated in 50 mM NaOAc buffer, pH 5. All of the peroxidase bound to the column. The column was washed sequentially with 80 ml equilibration buffer and 80 ml equilibration buffer containing 50 mM NaCl, then 80 ml equilibration buffer containing 100 mM NaCl, which eluted most of the activity. The column was then washed with 80 ml each of starting buffer containing 250 and 500 mM NaCl in which no significant amount of peroxidase was eluted. Fractions (4 ml) were collected, and the absorbances at 280 and 403 nm were measured. Fractions in which the RZ was between 2 and 2.6 were pooled and dialyzed against water and purified by fast protein liquid chromatography (Pharmacia, Piscatway, NJ) on a Mono Q HR5/5 column. The protein (up to 3 mg) was loaded onto the column in 50 mM NaOAc buffer, pH 5. After 3 ml of equilibration buffer had passed over the column, the SBP was eluted with a NaCl gradient of 0–350 mM NaCl over 10 ml (i.e., 10 column volumes), 0.5 ml fractions being collected. The soybean hull peroxidase eluted at about 200 mM NaCl in which the absorbance at 403 nm. were pooled, dialyzed, and used as the source of peroxidase for further studies. The final RZ value was 2.42. SBP purchased from Enzymol was similarly purified. The final RZ value was 2.65.

Preparation of apo-SBP

The hem was extracted from SBP either by cold acidified acetone (2% HCl in acetone) as described by Chibbar and van Huystee (1983) or with 2-butanone at pH 2 as described by Theale (1959).

Monosaccharide composition

Hydrolysis conditions

The neutral monosaccharides and amino sugars were routinely released from the protein or glycopeptide (containing up to 5 mg carbohydrate) by hydrolysis with 2 M TFA for 3 h at 100°C either in acid washed 1.5 ml Sarstedt screw-cap vials (Hardy et al., 1988) or in 2 ml glass screw...
Fig. 4. HPAEC-PAD analysis of the oligosaccharides released from glycopeptide fractions GP4 (A), GP5 (B), and GP6 (C) by glycopeptidase A. Full scale was 100 nA (1000 mV).

Table V. MALDI-TOF analysis of the oligosaccharides released from the tryptic glycopeptides by glycanase A treatment. The tryptic glycopeptides, after separation by HPLC on a C4 column, were digested with glycanase A and the oligosaccharides were analyzed by MALDI-TOF MS with 2,5-dihydroxybenzoic acid as a matrix.

<table>
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<th>Peak number</th>
<th>Oligosaccharide</th>
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<th>GP1</th>
<th>GP2</th>
<th>GP3</th>
<th>GP4</th>
<th>GP5</th>
<th>GP6</th>
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<td>0.2</td>
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vials fitted with a Teflon-lined cap. The vials were cooled to room temperature and briefly centrifuged to collect all the condensate in the bottom of the vial, and the TFA was evaporated under a stream of nitrogen at 40°C. The final trace of TFA was removed by coevaporation with methanol or 2-propanol.

The neutral monosaccharide composition of holo-SBP was determined by hydrolyzing triplicate samples of the FPLC-purified enzyme with 2 M TFA for 1 h, 3 h, and 5 h at 100°C, selecting the maximum values for each sugar.

The amino sugars were released by hydrolysis with 4 M HCl at 100°C for 6 h. After cooling, an equal volume of i-butanol was added to the vial and the HCl removed under a gentle stream of N₂ at 40°C. The addition of the i-butanol and evaporation was repeated to ensure complete removal of the acid.

**HPAEC-PAD analysis**

**Monosaccharides**

The monosaccharides were dissolved in water (1–5 µg ml⁻¹) and analyzed by HPAEC-PAD on a Dionex BioLC (Sunnyvale, California) and a CarboPac PA1 analytical column (4.0 x 250 mm) and guard column (4 mm x 50 mm) in a Dionex DX-300 BioLC. Detection was by pulsed amperometry (PAD-2) after post-column mixing of the column effluent with 300 mM NaOH. The following PAD settings were used: E1, +0.05V; E2, +0.60V; E3, -0.60V; T1, 480 msec; T2, 120 msec; T3, 60 msec. All eluents were degassed by a Dionex eluent degas module and maintained under an atmosphere of helium. Data were collected and processed by the Dionex AI-450 automation system.

The monosaccharides were analyzed with the following eluent programs:

1. Isocratically with 18 mM NaOH. This program did not resolve Man and Xyl.
2. Isocratically with 22 mM NaOH. This program resolves Man and Xyl but gives poor resolution of Glc and Man.
3. Isocratically with 1 mM NaOH as described by Lee (1990) except that the 30 µM sodium acetate was omitted from the eluent. This program resolves all the monosaccharides present in the hydrolysis mixture including Man and Xyl (Yang et al., unpublished data). However, it cannot be used for monosaccharide mixtures containing GaLNA, Ara and Rha which all coelute. Nonetheless, it clearly resolves Puc, GlcN, Xyl, and Man, the monosaccharides present in SBP.

**Oligosaccharides.**

Neutral oligosaccharides released chemically or enzymatically from protein or glycopeptides, were analyzed using a CarboPac PA1 column on the Dionex BioLC equipment described above with the following gradient: 0–5 min, 100 mM NaOH; 5–60 min, 100 mM NaOH, 0–100 mM NaOAc; 60–80 min, 100 mM NaOH, 100–300 mM sodium acetate; 80–90 min, hold 100 mM NaHCO₃-300 mM NaOAc. No post-column addition of 300 mM NaOH was added to the column effluent.

**Trypsin digestion**

Apo-protein (10 mg ml⁻¹) was digested with TPCK-treated trypsin (substrate–trypsin ratio of 25:1) in 100 mM ammonium bicarbonate, pH 8 and 37°C for 16 h in the presence of toluene as a bacteriostatic agent. After removal of the toluene under a stream of nitrogen, the sample was lyophilized and the glycopeptides separated by reversed phase HPLC as described below.

**PNGase F and glycopeptidase A digests**

PNGase F digestions were carried out for 18 h in 12.5 mM sodium phosphate buffer, pH 7.5 and 37°C, as described by Hardy and Townsend (1994). Fetuin was digested with PNGase F as a positive control; buffer plus PNGase F was incubated as the negative control. Glycopeptidase A digestions were performed under the conditions specified by the manufacturer. Briefly, apo-SBP or glycopeptide (10–100 µg) was digested for 16–18 h with glycopeptidase A (at a final concentration of 0.20 mU/ml) in 100 mM citrate-phosphate buffer, pH 5.45 at 37°C. All digestions were performed under an atmosphere of toluene. The extent of digestion was determined by acid hydrolysis of the deglycosylated protein and HPAEC-PAD analysis of the released monosaccharides.

**Hydrazinolysis**

Oligosaccharides were released from apo-SBP and glycopeptides by hydrazinolysis for 5 h as described by Patel et al. (1993) and Patel and Parekh (1994). After hydrazinolysis, the oligosaccharides were separated from the peptides by chromatography on a short column of microcrystalline cellulose (Whatman, pre-wetted with butanol/ethanol/water 8:2:1). Under these conditions, the oligosaccharides are retained by the column whereas the peptides, which are not retained, appear in the initial wash. Care was taken to develop the column at a low flow rate for efficient absorption of the oligosaccharides to occur. The oligosaccharides were eluted with water and treatment of the hydrazinolysate with copper acetate and subsequent purification of the glycans were as described by Patel and Parekh (1994).

**Reversed phase HPLC and purification of glycopeptides**

Trypsin digests were fractionated by reversed phase chromatography on a Spherisorb (Phase Separations, Norwalk, CT) SS C8 column (5 µM packing, 4.6 x 250 mm) in an Altex HPLC consisting of dual model 110A piston pumps, a model 420 system controller, and a Rheodyne model 7125 injector. Detection at 210 nm was by Hitachi model 155 variable wavelength detector fitted with a 20 µl Altex spectrophotometer flow cell. The column was equilibrated at a flow rate of 1 ml min⁻¹ with 95% buffer A (0.1% TFA) and 5% buffer B (0.1% TFA in 90% acetonitrile). The following gradient was used: 0–5 mm, 5% B, 5–90 mm, 5–75% B. The flow was equilibrated for 30 min with starting buffer before the next injection.

Peaks were collected manually and after evaporation of the acetonitrile under a stream of nitrogen, the fractions were lyophilized. The peptides were dissolved in water (1–10 mg ml⁻¹) and the glycopeptides identified by the presence of monosaccharides after hydrolysis with 2 M TFA for 5 h and HPAEC-PAD analysis.

**Methylation analyses**

Methylation of the samples, hydrolysis and subsequent conversion to the methylated alditol acetates was performed in a single vial by a modification of the methods of Anumula et al. (1992) and York et al. (1985) as follows. Samples (5–10 µg (5–10 nmol) oligosaccharide) were lyophilized in 2 ml silanized glass screw-cap vials. Dry dimethyl sulfoxide (50 µl) was added, and the vial was sealed with a Teflon-faced septum. After sonication for 5 min, the vials were kept at room temperature for a further 30 min. Fifty microliters of NaOH/DMSO reagent, prepared as described by Anumula et al. (1992), was added, and the sample was mixed (vortex mixer) and then sonicated for 5 min. After low-speed centrifugation (bench-top centrifuge) to collect the sample in the bottom of the vial, methyl iodide (50 µl) was added and the sample was mixed (vortex mixer) and then sonicated for 7–10 min. The vial, after brief centrifugation to collect all the sample in the bottom of the vial, was opened and the excess methyl iodide and some of the DMSO was evaporated under a stream of nitrogen at room temperature. Water and water-saturated chloroform (750 µl each) was added to the vial. Sufficient solid NaCl was then added to saturate the aqueous phase and the methylated oligosaccharides were extracted into the chloroform by extensive and vigorous mixing (vortex mixer). After separation of the phases by centrifugation, the aqueous layer was carefully removed without disturbing the organic layer interface and discarded. The organic layer interface was carefully rinsed three times with water (1 ml) without disturbing the interface, and the water discarded. Water (1 ml) was added, and after mixing and separating the phases by centrifugation, the interface was again rinsed three times with water (1 ml) as described above. The whole process was repeated once more. After removal of the last water rinse, enough methanol was added to the chloroform to form a single phase and the methanolic chloroform was evaporated at room temperature under a stream of nitrogen to give the methylated oligosaccharide.

Hydrolysis, reduction of the O-methylated monosaccharides to the alditols with sodium borodeuteride and acetylation was performed as described by York et al. (1985). Briefly, the samples were hydrolyzed by 2 M TFA at 121°C for 1 h. After addition of 2-propanol (200 µl) as an aid to complete removal of the acid, the TFA was evaporated at 40°C under a stream of N₂. Thereafter, the partially methylated monosaccharides were dissolved in 220 µl ethanol and reduced by adding 200 µl of sodium borodeuteride (10 mg ml⁻¹ in 1 M ammonium hydroxide) and incubating at room temperature for 1–2 h. The excess sodium borodeuteride was decomposed by the addition of glacial acetic acid and the sample dried under a stream of nitrogen. Borate was removed, first by coevaporat-
ing with 10% acetic acid in methanol (150 μl) and then with methanol (150 μl). The evaporation was repeated three times from 10% acetic acid in methanol (150 ml) and twice from methanol (150 μl). Acetic anhydride (50 μl) was added, and the vial was closed with a Teflon-faced septum, and heated for 3 h at 121°C with occasional mixing. The vial was cooled, water (500 μl) was added, and the acid was neutralized by the addition of small portions of solid sodium carbonate. After effervescence ceased, an additional 500 μl of water and 500 μl of water-washed chloroform was added to the vial and the methylated alditol acetates were partitioned into the organic phase as described for the methylated oligosaccharides. The chloroform phase was washed twice with water as described above. The final aqueous wash was not removed but the organic phase was removed with a Pasteur pipette which had been drawn to a fine point, transferred to a clean vial, and carefully dried under a stream of N2. The methylated alditol acetates were dissolved in dichloromethane for analysis by gas chromatography (GLC) and gas chromatography-mass spectrometry (GLC-MS).

The methylated alditol acetates could be quantitated by GLC-FID only when the peaks were pure, as determined by examination of the mass spectra across the peak. When the peaks were contaminated with non-carbohydrate components, the carbohydrate was quantitated by using selected ions from GLC-MS that are characteristic of the methylated alditol acetate. Relative response factors determined from GLC-MS analyses of mixtures of methylated alditol acetates of known composition were used for quantitation. When the values from the integrated ion chromatograms agreed within 10%, they were averaged and reported. Thus, in Table II, the value for 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl mannitol is an average of the values determined from the integrated m/z 118 and 129 chromatograms. Similarly, the values for 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl mannitol in GP5 and GP6 (Table IV) are the averaged values from the integrated total ion chromatogram and the integrated m/z 129, 130, and 161 selected ion chromatograms.

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was performed on a Hewlett Packard G2520 instrument operating in the positive mode with an accelerating voltage of 28.0 kV. Single shots with a Ni laser operating at 337 nm (3 nsec pulses) were used for quantitation. The molecular weight of SBP was determined under similar conditions with both N- and O-linked oligosaccharides from glycoproteins.

References


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