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The glycans of horseradish peroxidase [1]

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Abstract

Horseradish peroxidase (E.C. 1.11.1.7) isozyme c (HRPc) is a glycoprotein found to contain 21.8% carbohydrate with the average composition: 2 mol GlcNAc, 2.6 mol Man, and 0.8 mol each of Fuc and Xyl. The oligosaccharides of HRPc 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 released by digestion with glycopeptidase A, accounting for between 75 and 80% of the total, was confirmed to be α-Man-(1 → 6)[α-Man-(1 → 3)][β-Xyl-(1 → 2)]-β-Man-(1 → 4)-β-GlcNAc-(1 → 4)[α-Fuc-(1 → 3)]-GlcNAc. Most of the remaining oligosaccharides were found to belong to the $(Xyl)_x Man_m (Fuc)_f GlcNAc_2$ ($m = 2, 4, 5, 6$; $f = 0$ or 1; $x = 0$ or 1) family. Less than 5% of the oligosaccharides were of the $Man_m GlcNAc_2$ ($m = 4$ to 7) type. Methylation analysis of holo- and apo-HRPc and its tryptic glycopeptides support the structures proposed for the oligosaccharides. Furthermore, methylation analysis of the tryptic glycopeptides provides evidence for the heterogeneity of the oligosaccharides occurring at each of the *N*-linked sites. © 1996 Elsevier Science Ltd.

Keywords: Glycan; Horseradish peroxidase; MALDITOF; Methylation analysis; *N*-linked oligosaccharides

Abbreviations: FPLC, Fast Protein Liquid Chromatography; GLC, Gas Liquid Chromatography with FID detector; GLC–MS, Gas Liquid Chromatography with Mass Selective Detector; GP, Glycopeptide-containing fractions; HPAEC–PAD, High pH Anion-Exchange Chromatography with Pulsed Amperometric Detection; HRPc, Horseradish peroxidase isozyme c; MALDITOFMS, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; TPCK, L-(tosylamido 2-phenyl)ethyl chloromethyl ketone; RZ, Ratio of the absorbance at 403 and 280 nm.

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1. Introduction

Horseradish peroxidase is one of the most extensively studied plant peroxidases. Shannon et al. [1] originally purified seven isozymes from horseradish, three anionic species and four cationic species. Subsequently, more isozymes have been detected and purified [2,3]. Horseradish peroxidase c (HRPc) has been sequenced [4], cloned, and expressed in *Escherichia coli* [5]. As isolated from horseradish, it is a glycosylated protein with eight *N*-linked glycans attached [4]. The major glycan, comprising 80% of the carbohydrate, was reported by Kurosaka et al. [6] to be: α-Man-(1 → 6)[α-Man-(1 → 3)][β-Xyl-(1 → 2)]-β-Man-(1 → 4)-β-GlcNAc-(1 → 4)[α-Fuc-(1 → 3)]GlcNAc. Antisera raised against this glycan recognizes a neural-specific carbohydrate antigen in *Drosophila* and other insects [6].

This communication supports the structure of the carbohydrate of HRPc proposed by Kurosaka et al. [6]. It also presents an analysis of the oligosaccharides released by glycopeptidase A and hydrazinolysis from apo-HRPc and its tryptic glycopeptides by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDITOFMS).

2. Experimental

Materials.—The enzymes were obtained as follows: PNGase F (Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase) was obtained from Genzyme (Boston, MA); Glycopeptidase A (almond), Seikagaku America, Inc. (Rockville, Maryland); horseradish peroxidase, Type VIA, Sigma (St. Louis, MO); L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (bovine pancreas), Worthington Biochemical Corporation (Freehold, NJ).

Methods.—Purification of HRP. Unless stated otherwise, HRP type VIA with a RZ value of 3.3 was used in most of the work described here without further purification. For some studies, HRP was further purified by Fast Protein Liquid Chromatography (FPLC) using a Pharmacia (Pharmacia Biotech, Inc., Piscataway, NJ) FPLC system and a Mono-S HR5/5 column. The HRP (3 mg in 1 mL of 10 mM NaOAc buffer, pH 5) was loaded onto the column, which had been pre-equilibrated in the same buffer. The column was washed with 3 mL of starting buffer (3 column volumes) and the buffer concentration was changed stepwise to 16 mM NaOAc, pH 5, and the column eluted with a further 2 mL of buffer under these conditions. An 8-mL linear gradient of 16 to 20 mM NaOAc was then run. The eluent was monitored continuously at 280 nm; fractions (0.5 mL) were collected, pooled appropriately, and dialyzed against water for use in further experiments.

The monosaccharides were analyzed by HPAEC–PAD on a Dionex PA-1 column (4 × 250 mm) using 1 mM NaOH isocratically, which resolves all the sugars in HRPc.

The monosaccharide composition of the FPLC-purified HRPc, as determined by hydrolysis and HPAEC–PAD analysis, is 2 mol GlcNAc, 2.6 mol Man, and 0.8 mol each of Fuc and Xyl.

The percentage of carbohydrate in HRPc from acid hydrolysis and HPAEC–PAD

analysis was 21.8%. A millimolar extinction coefficient of 102 mM^{-1} cm^{-1} at 403 nm at pH 5.8 was used to calculate the amount of protein for the analyses [3].

Analytical methods.—The methods for determination of monosaccharide composition, HPAEC–PAD analysis, preparation of apo-HRPc, trypsin digestion and neutral oligosaccharides release from tryptic peptides or HRP, reverse-phase HPLC and purification of glycopeptides, hydrazinolysis, defucosylation, and methylation procedures are described previously [7].

Matrix-Assisted Laser Desorption / Ionization Time-of-Flight Mass Spectrometry (MALDITOFMS).—MALDITOFMS was performed on a Voyager-RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) operating in the positive mode with an accelerating voltage of 30.0 kV. Between 100 and 256 shots aimed at different parts of the target with a N_2 laser, operating at 337 nm (3 ns pulses), were accumulated until an acceptable signal/noise ratio was attained. Data was obtained at a vacuum of 1×10^{-6} Torr or better.

1 μL of oligosaccharide (0.1–10 pmol) was mixed with an equal volume of 2,5-dihydroxybenzoic acid (10 mg mL^{-1} in 10% EtOH) directly on the target and allowed to air-dry (10–15 min). Where the analysis was performed in the presence of KCl, 0.3 μL of a 0.25 M solution was added to the sample before addition of the matrix. The sample/matrix was recrystallized by adding 0.5 μL of absolute EtOH to the sample on the target and allowing it to air-dry. A set of oligosaccharides prepared from RNAse B and pure $Man_3GlcNAc_2$, purchased from Oxford Glycosystems (Rosedale, NY), were used as external standards.

The molecular weight of HRPc was determined under similar conditions except that sinapinic acid (10 mg mL^{-1} in 1:2 CH_3CN/0.1% CF_3COOH) was used as a matrix and the sample/matrix was not recrystallized from EtOH. Bovine serum albumin (66431 Da) and cytochrome C (12360 Da) were used as internal standards.

3. Results

Tryptic maps.—Eight fractions containing carbohydrate were identified in the tryptic map of HRPc (Fig. 1). Each tryptic glycopeptide fraction contains similar amounts of Fuc, Xyl, GlcN, and Man. These data are the result of a single hydrolysis time without

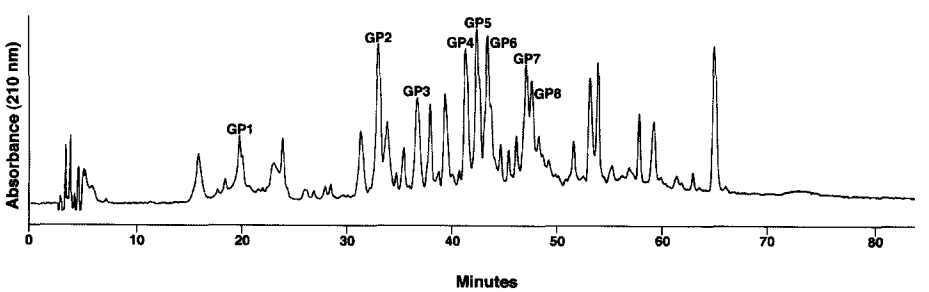

Fig. 1. Tryptic map of apo-HRPc. Apo-HRPc was digested with TPCK-treated trypsin and the glycopeptides purified by reverse-phase chromatography on a C_8 column as previously described [7].

optimization and correction for the losses of any monosaccharides. The total carbohydrate recovered in each fraction is variable with fractions 1, 4, and 5 containing over 60% and fractions 2 and 6 each containing $< 5\%$ of the total carbohydrate. The amount of oligosaccharide recovered in fraction 2 was low, although the monosaccharide composition of this fraction is similar to the others.

Oligosaccharide maps.—Attempts to release oligosaccharides from either holo- or apo-HRPc with PNGase F digestion were unsuccessful. Glycanase A digestion of apo-HRPc, but not of holo-HRPc, released $> 90\%$ of the carbohydrate as determined by hydrolysis and monosaccharide analysis of the residual protein. Analysis of the released oligosaccharides by HPAEC–PAD revealed a single broad peak, which co-eluted with a (Xyl)Man_3(Fuc)$GlcNAc_2$ standard. There was also evidence for some material eluting close to the leading base of the major peak as well as a shoulder on the trailing edge of the peak. This latter shoulder was variable and was present in some preparations and not in others; it was also observed in the peak obtained in the analysis of a (Xyl)Man_3(Fuc)$GlcNAc_2$ standard. A minor peak, eluting in the high-mannose region of the chromatogram, as determined by chromatography of a set of oligosaccharides derived from RNAse B, was also observed. No peaks eluting in the $Man_3GlcNAc_2$ region or in the Man_3(Fuc)$GlcNAc_2$ were observed. Although the latter oligosaccharide has the inner GlcNAc of the chitobiose core substituted at the 6-O position and not at the 3-O position as found in HRPc, it does illustrate that substitution of the proximal GlcNAc of the chitobiose core with Fuc shifts the elution to an earlier time.

The broad, major peak was collected manually from the CarboPac PA-1 column with and without desalting of the effluent by passage through a micromembrane anion suppresser. After the chromatography, the oligosaccharide collected directly without desalting was neutralized with 1 N HCl. The solvent was evaporated under a stream of N_2, the oligosaccharides were dissolved in water, and a portion of each was rechromatographed on the CarboPac PA-1 column under the same conditions. A further portion of each was hydrolyzed with 2 M TFA and the monosaccharide composition determined by HPAEC–PAD.

There was a substantial loss of the major HRPc oligosaccharide after chromatography on the CarboPac PA-1 column eluted with 100 mM NaOH, particularly if the effluent was not immediately neutralized. About 30% of the oligosaccharide was lost if the oligosaccharide was collected after on-line desalting, whereas 75% was lost if the collected fraction was allowed to stand for a short time before neutralization. These changes were reflected in the monosaccharide analysis of the samples in which it was found that Fuc was greatly reduced in the sample collected without on-line neutralization, whereas the loss was lower in the case of on-line neutralization.

Defucosylation with concentrated TFA at room temperature of the oligosaccharides released by glycanase A led to the conversion of the major, broad peak to a sharp peak eluting 1–2 min earlier. A minor peak eluting where $Man_3GlcNAc_2$ elutes was also observed. Analysis of the defucosylated oligosaccharide mixture by HPAEC–PAD using a monosaccharide program detected only free Fuc; free Xyl, Man, and GlcNAc were not present. Methylation analysis of the defucosylated oligosaccharide showed that there was a decrease in the amount of 1,3,4,5-tetra-*O*-acetyl-(2-*N*-methylacetamido)-6-*O*-methyl glucitol and a corresponding increase in the amount of 1,4,5-tri-*O*-acetyl-(2-*N*-

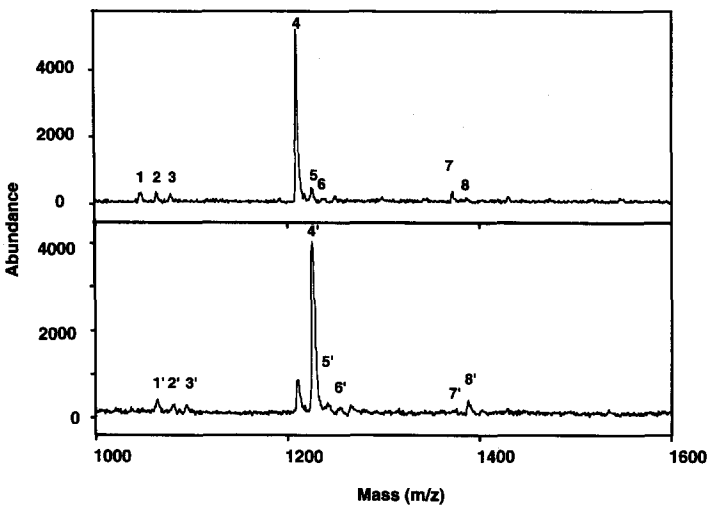

Fig. 2. MALDITOFMS analysis of the oligosaccharides released from apo-HRPc by glycopeptidase A in the absence of (top panel) and presence of KCl (bottom panel). The numbered species are identified in Table 1. The primed numbers refer to the K-adducts of the oligosaccharides.

methylacetamido)-3,6-di-*O*-methyl glucitol, as expected if the fucose was removed from position 3 of the proximal GlcNAc of the chitobiose core.

MALDITOFMS analysis.—MALDITOFMS analysis of the glycopeptidase A-released oligosaccharides revealed 8 peaks (Fig. 2) for which either the Na- or K-adducts of oligosaccharides containing a combination of Fuc, Xyl, Man, and GlcNAc were assigned (Table 1). The assumption that the structures of the oligosaccharides released by glycopeptidase A from apo-HRPc were similar in motif to those described for other

Table 1
MALDITOF analysis of the oligosaccharides released from apo-HRP by glycanase A. Apo-HRPc was digested with glycanase A and the oligosaccharides were analyzed by MALDITOFMS with 2,5-dihydroxybenzoic acid as a matrix

Peak number	Oligosaccharide	Na-adduct			K-adduct		
		Observed M_r	Calculated M_r	Intensity [a] (% of total)	Observed M_r	Calculated M_r	Intensity [b] (% of total)
1	(Xyl)Man_2(Fuc)$GlcNAc_2$	1047.9	1049.4	4.6 ± 0.5	1064.28	1065.33	5.8 ± 0.6
2	(Xyl)$Man_3GlcNAc_2$	1064.7	1065.4	4.8 ± 0.5	1080.1	1081.3	4.5 ± 0.5
3	Man_3(Fuc)$GlcNAc_2$	1079.0	1079.4	4.0 ± 0.4	1094.8	1095.3	3.9 ± 0.6
4	(Xyl)Man_3(Fuc) $GlcNAc_2$	1210.8	1211.4	70.0 ± 1.1	1226.9	1227.4	70.4 ± 2.4 [c]
5	(Xyl)$Man_4GlcNAc_2$	1226.5	1227.4	6.8 ± 0.6	1242.2	1243.4	4.5 ± 0.5
6	Man_4(Fuc)$GlcNAc_2$	1241.3	1241.4	2.6 ± 0.5	1255.4	1257.4	3.4 ± 0.5
7	(Xyl)Man_4(Fuc)$GlcNAc_2$	1371.6	1373.5	4.7 ± 0.7	1388.2	1389.5	4.9 ± 0.7
8	(Xyl)$Man_5GlcNAc_2$	1387.3	1389.4	2.6 ± 0.4	1402.9	1405.4	2.5 ± 0.7

[a] Mean ± SE (Mean), $n = 11$.
[b] Mean ± SE (Mean), $n = 12$.
[c] Sum of the intensity at m/z 1211 and 1227.

plant glycoproteins [8–10], and that the major oligosaccharide was the same as that proposed for HRP by Kurosaka et al. [6], allowed a tentative identification to be made from the MALDITOFMS data (Table 1).

The major oligosaccharide, contributing between 70 and 75% of the signal, had a molecular weight of 1211 Da, which corresponds well with the calculated molecular weight of the Na-adduct of (Xyl)Man_3(Fuc)$GlcNAc_2$ (1211 Da) (Fig. 2, top panel). Peaks corresponding to further members of this family were also detected, viz. (Xyl) Man_2(Fuc)$GlcNAc_2$-Na (1048 Da) and (Xyl)Man_4(Fuc) $GlcNAc_2$-Na (1372 Da) (Fig. 2, top panel, and Table 1). Related peaks in which either the Fuc or the Xyl was absent were also detected (Fig. 2, top panel, and Table 1). Between 80 and 85% of the total oligosaccharides released from HRPc belong to the (Xyl)Man(Fuc)$GlcNAc_2$ family; the rest consists of oligosaccharides in which either the Fuc or the Xyl has been removed. No oligosaccharides belonging to the high-mannose family were observed in our analyses of the oligosaccharides released from apo-HRP.

The addition of KCl to the sample shifted the masses of the oligosaccharides to 16 Da higher (Fig. 2, bottom panel, and Table 1). Peaks obscured by the K-adducts, such as (Xyl)$Man_4GlcNAc_2$-Na (1227 Da), are now clearly observed (Fig. 2, bottom panel) and can be quantitated. The peak at 1227 Da in Fig. 2, top panel, in the analysis without added KCl therefore contains both (Xyl)$Man_4GlcNAc_2$-Na and (Xyl)Man_3(Fuc)$GlcNAc_2$-K.

The quantitation of both the Na- and the K-adducts of the observed oligosaccharides is similar (Table 1), except the values of the Na-adducts whose masses coincide with the

Table 2
MALDITOF analysis of the oligosaccharides released from the tryptic glycopeptides of HRPc by glycanase A. Apo-HRPc was digested with trypsin and the glycopeptides purified by reverse-phase chromatography on a RP C_8 column. The purified glycopeptides were digested with glycanase A and the oligosaccharides analyzed by MALDITOFMS with 2,5-dihydroxybenzoic acid as a matrix.

Peak number	Oligosaccharide	Calculated M_r	Intensity (%) [a]							
			GP1	GP2	GP3	GP4	GP5	GP6	GP7	GP8
1	(Xyl)Man_2(Fuc)$GlcNAc_2$-Na	1049.4	0	+	+	0	3	2	2	3
2	(Xyl)$Man_3GlcNAc_2$-Na	1065.4	51	+	+	+	3	3	3	6
3	Man_3(Fuc)$GlcNA_2$-Na	1079.4	14	+	+	0	3	4	3	6
4	(Xyl)Man_3(Fuc)$GlcNAc_2$-Na	1211.4	27	51	75	78	78	71	60	58
5	(Xyl)$Man_4GlcNAc_2$-Na	1227.4	8	28	25	22	10	13	14	15
6	Man_4(Fuc)$GlcNAc_2$-Na	1241.4	0	22	+	0	0	2	0	4
7	(Xyl)Man_4(Fuc)$GlcNAc_2$-Na	1373.5	0	0	+	0	2	2	10	5
8	(Xyl)$Man_5GlcNAc_2$-Na	1389.5	+	+	+	0	1	0	4	4
9	Man_5(Fuc)$GlcNAc_2$-Na	1403.5	+	+	0	0	0	0	0	0
10	$Man_6GlcNAc_2$	1419.5 [b]	0	0	0	0	0	0	<1	0
11	(Xyl)$Man_6GlcNAc_2$-Na	1551.5	0	0	0	0	+	3	2	+
12	$Man_7GlcNAc_2$-Na	1581.5	0	0	0	0	0	+	1	0

[a] A plus indicates that the signal for the oligosaccharide species is present, but at a concentration too low to measure the intensity with confidence.

[b] The major peak was at m/z 1413 Da; the value reported here was from a signal at 1418 Da. All oligosaccharide species in the table were within 1 Da of the theoretical mass.

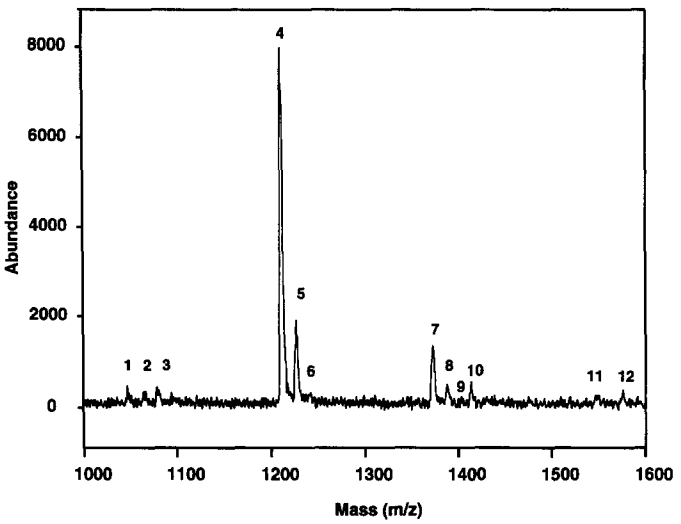

Fig. 3. MALDITOFMS analysis of the oligosaccharides released from glycopeptide seven (GP7) by digestion with glycopeptidase A. The numbered species are identified in Table 2.

K-adduct of another oligosaccharide species are slightly higher [e.g. (Xyl)$Man_4GlcNAc_2$-Na (1227 Da), 6.8% vs. (Xyl)$Man_4GlcNAc_2$-K (1242 Da), 4.5%].

MALDITOFMS analyses of the glycans released from the glycopeptides by digestion with glycopeptidase A revealed a similar series of oligosaccharides (Table 2). The distribution of the oligosaccharides among the various glycopeptides is different. The major oligosaccharide present in all the glycopeptides, except for GP1, is (Xyl)Man_3(Fuc)$GlcNAc_2$. The predominant oligosaccharide of GP1 is (Xyl)$Man_3GlcNAc_2$ with lesser amounts of Man_3(Fuc)$GlcNAc_2$ and (Xyl)Man_3(Fuc)$GlcNAc_2$. GP2 contains proportionately higher amounts of (Xyl)$Man_4GlcNAc_2$ and Man_4(Fuc)$GlcNAc_2$ than the other glycopeptides. Although GP3 has a wide distribution of oligosaccharides, only (Xyl)$Man_3GlcNAc_2$ and (Xyl) $Man_4GlcNAc_2$ were present in sufficient amounts to quantitate.

Glycopeptides 5–8 are characterized by the presence of the higher homologues of the (Xyl)$_x$$Man_m(Fuc)_f$$GlcNAc_2$ family ($x = 0$ or 1; $m = 4$, 5, 6; $f = 0$ or 1) (Table 2). Moreover, low amounts of $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ are present in GP6 and GP7 (Fig. 3), while trace amounts are present in GP5 and GP8. Glycopeptides 5, 6, and 7 contain a significant peak with an m/z value corresponding to (Xyl)$Man_6GlcNAc_2$ (1552); GP8 contains a trace of this species.

4. Discussion

Although the structure of the major oligosaccharide of HRPc has been reported by Kurosaka et al. [6], little is known about the minor species present or the distribution of these oligosaccharides along the peptide chain. The principal aim of this study was to

investigate the nature and distribution of the minor oligosaccharide species found on HRPc. In the course of the study the structure of the major oligosaccharide $(Xyl)Man_3(Fuc)GlcNAc_2$ was confirmed.

Sigma HRP Type VIA consists primarily of HRPc. This was confirmed in our laboratory by FPLC analysis, where the two isozymes, HRPc1 and HRPc2 described by Aibara et al. [3], were detected. A minor peroxidase species with a lower pI was also detected in the Sigma preparation. In addition, an arabinose-containing glycan was found to elute close to the latter peak, which was well separated from the two HRPc isozymes. Neither HRPc1 nor HRPc2 was found to contain arabinose, but the presence of an arabinan accounted for methylated arabinitol acetates in our preliminary studies, calling for added caution in accepting samples as pure without careful analysis.

The molecular weight of HRPc was determined by MALDITOF to be 43540 Da. The molecular weight of HRPc, predicted from its amino acid sequence, is 33890 Da [4]. The difference in molecular weight of 9650 Da represents the contribution of the carbohydrate to the molecule and is calculated to be 22.2% of the total mass of HRPc. The value of 21.8% determined experimentally by HPAEC–PAD of the monosaccharides after hydrolysis and summation is in good agreement with that determined by a combination of MALDITOF and sequence data.

Eight fractions containing carbohydrate were detected in the tryptic digest of HRPc. Three fractions, viz. GP1, GP4, and GP5, contain over 60% of the carbohydrate. The monosaccharides of the eight glycopeptides are similar, containing Man, GlcNAc, Xyl, and Fuc in the approximate molar ratio of 3:2:1:1 as expected for a glycoprotein containing predominantly $(Xyl)Man_3(Fuc)GlcNAc_2$. Divergence from this ratio indicates the degree of heterogeneity of the oligosaccharides attached to each of these glycopeptides.

The reason for the variability in the recovery of carbohydrate in each glycopeptide is not known.

MALDITOFMS analysis of the oligosaccharides released by glycopeptidase A from apo-HRPc detected eight peaks. Previous work by Harvey [11] has shown that with 2,5-dihydroxybenzoic acid as a matrix for MALDITOFMS, the intensity of the ions produced is proportional to the concentration of the oligosaccharides. This observation allows one to semi-quantitate the amount of the different oligosaccharides released by glycopeptidase A from apo-HRPc. The major oligosaccharide, $(Xyl)Man_3(Fuc)GlcNAc_2$, was determined to constitute between 70 and 75% of the total, a value in good agreement with that determined by Kurosaka et al. [6].

Na and K as well as Fuc and Man differ in mass by 16 Da. Most carbohydrates are detected in MALDITOFMS analyses as their Na-adducts together with a small proportion of their K-adduct. This creates a problem when analyzing a major species containing Fuc and a minor species in which the Fuc is substituted by Man since the K-adduct of the major species may overwhelm the signal of the Na-adduct of the minor species. This situation occurs in the analysis of the glycans from apo-HRPc where the masses of $(Xyl)Man_3(Fuc)GlcNAc_2$-K and $(Xyl)Man_4GlcNAc_2$-Na (1227 Da) are practically identical. This dilemma can be overcome by analyzing the sample in an excess of KCl. This strategy successfully revealed the presence of $(Xyl)Man_4GlcNAc_2$-K which, in the absence of KCl, co-migrated with $(Xyl)Man_3(Fuc)GlcNAc_2$-K. Moreover, the

shift in mass by 16 Da of peaks clearly identified them as carbohydrate and not as some non-carbohydrate contaminant.

Three pairs of peaks in the MALDITOFMS analysis of the oligosaccharides from apo-HRPc were differentiated in this manner: those at 1049 and 1065, 1211 and 1227, and 1374 and 1390 Da. These peaks correspond to the Na- and K-adducts of $(Xyl)Man_2(Fuc)GlcNAc_2$ and $(Xyl)Man_3GlcNAc_2$, $(Xyl)Man_3(Fuc)GlcNAc_2$ and $(Xyl)Man_4GlcNAc_2$, $(Xyl)Man_4(Fuc)GlcNAc_2$ and $(Xyl)Man_5GlcNAc_2$, respectively. These species were successfully differentiated and quantified in apo-HRPc in the presence of KCl.

The amount of information obtained from the HPAEC–PAD analyses of the glycans is not nearly as detailed as that obtained from the MALDITOFMS analyses. The major peak chromatographs on the CarboPac PA-1 column as a broad peak, which co-elutes with a $(Xyl)Man_3(Fuc)GlcNAc_2$ standard, together with a number of other minor peaks. The broad peak observed in the HPAEC–PAD analysis of the HRPc oligosaccharides contains incompletely resolved oligosaccharides. Moreover, the breadth of the peak may reflect the continual alkaline degradation of the oligosaccharides, which are substituted at 3-O of the proximal GlcNAc of the chitobiose core, a configuration that is favorable for β-elimination to occur. Such a β-elimination would explain the difficulty experienced in this study of purifying $(Xyl)Man_3(Fuc)GlcNAc_2$ by HPAEC–PAD.

Each glycopeptide contained six to nine different glycans, only two or three in each case representing more than 10% of the mixture. Together with their incomplete chromatographic separation and the sensitivity of the fucosyl residues to alkali, their unique structures could not be established by our methodologies. However, preliminary methylation studies of the glycopeptide fractions allowed for some structural conclusions:

(1) The predominance of $(Xyl)Man_3(Fuc)GlcNAc_2$ in GP5 and GP6 gave rise to a methylation analysis that supported the structure proposed.

(2) The complete absence of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol in any of the methylation analyses of HRPc indicates that in $(Xyl)Man_2(Fuc)GlcNAc_2$, the 3-O of the β-linked branch Man is always substituted and the 6-O of this species is never substituted as has been reported for stem bromelain [12].

(3) Significant amounts of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, particularly in GP6, was observed. The latter methylated alditol acetate, characteristic of 1,3-linked mannose residues, indicates the presence of $(Xyl)_xMan_4(Fuc)_fGlcNAc_2$ (f, $x \leq 1$) in which the mannose residue on the 6-branch of the β-linked core mannose is substituted mainly at the 3-O position and rarely at the 6-O position.

MALDITOFMS analyses of the oligosaccharides released by glycopeptidase A from the tryptic glycopeptides reveal considerable heterogeneity. The major glycan species present in all the glycopeptides, except for GP1, is $(Xyl)Man_3(Fuc)GlcNAc_2$. The major oligosaccharide species in GP1 is the defucosylated derivative of this species. There are only small amounts of the high-mannose type glycan and these are concentrated mainly in GP7. Members of the $(Xyl)_xMan_m(Fuc)_fGlcNAc_2$ family (f, $x \leq 1$; $m = 2$–6) make up more than 95% of the total glycans present in HRPc, in contrast to the situation in soybean hull peroxidase, where there is a substantial portion of the high-mannose type oligosaccharides [7].

The (Xyl)$Man_6GlcNAc_2$-Na (1552 Da) detected in GP6 and GP7 by MALDITOFMS analysis cannot be explained in terms of the current knowledge of the processing pathway of glycoproteins in the Golgi apparatus. It has been shown by Tezuka et al. [9] that $Man_5GlcNAc_2$ is a substrate for GlcNAc transferase I and that the product of this reaction, (GlcNAc)$Man_5GlcNAc_2$, is a substrate for xylosyl transferase. However, these workers found that neither $Man_6GlcNAc_2$ nor $Man_7GlcNAc_2$ were substrates.

In conclusion, MALDITOFMS analysis of the glycans from the separated glycopeptides of HRPc demonstrated significant heterogeneity at each glycosylated site. Preliminary methylation analysis permitted limited conclusions of the structure of any but the principal glycan, which was 78% of two of the glycopeptides. Some characteristics are evident, however, such as the total absence of terminal *N*-acetyl-D-glucosamine residues.

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