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CARBOHYDRATE MOIETIES OF THREE RADISH PEROXIDASES

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Key Word Index—*Raphanus sativus*; cruciferae; radish; peroxidases; carbohydrate moieties.

Abstract—The carbohydrate moieties of two anionic peroxidases, termed A_1 and A_2, and one cationic peroxidase, named C_3, from Korean radish (*Raphanus sativus*) were studied. For profiling of *N*-glycans, each peroxidase was treated with peptidyl *N*-glycosidase F and hydrazine. These peroxidases were more susceptible to hydrazine than to peptidyl *N*-glycosidase F. When these three peroxidases were subjected to trifluoroacetic acid treatment, mannose, fucose and *N*-acetylglucosamine were released. Two major *N*-glycans of peroxidase C_3 were isolated and treated with several glycohydrolases. Analysis of digested products of the two major *N*-glycans on polyacrylamide gel suggested that core-fucosylated trimannosylchitobiose may contain a different linkage from the typical α-1,6 of native N-linked oligosaccharide.

INTRODUCTION

Plant peroxidases (EC 1.11.1.7) are glycoproteins that contain N-linked oligosaccharide chains and exist as multiple forms in a large number of different species [1–3]. In recent years, the carbohydrate moieties of plant peroxidases have received considerable attention. In contrast to the polypeptide chain of a glycoprotein, which exhibits precise homology in amino acid sequence and length, the carbohydrate moiety shows heterogeneity in content, in the number and degree of branching, and in distributions along the polypeptide chains [4, 5]. Furthermore, the carbohydrate units in plant peroxidases play a critical role for the acquisition of their catalytic activities, stabilization and secretion [6–8].

Six isoperoxidases C_1, C_3, A_1, A_2, A_{3n} and A_3 from Korean radish (*Raphanus sativus*) have been purified and identified as glycoproteins composed of a single polypeptide chain [9–11]. When peroxidases A_2 and C_3 were deglycosylated using trifluoromethanesulfonic acid the deglycosylated peroxidases did not cross-react with antibodies against the native enzymes [9]. The carbohydrate moieties of cationic and anionic peanut peroxidase isozymes have also been demonstrated to contribute to the antigenicity of the native enzymes [12, 13].

The purpose of the present study was to elucidate the carbohydrate structure of two anionic peroxidases, A_1 and A_2, and one cationic peroxidase, C_3, by *N*-glycan profiling, monosaccharide composition analysis and sequencing of major *N*-glycans. These studies provide information in clarifying the roles of carbohydrate moieties for radish peroxidases.

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RESULTS AND DISCUSSION

Profiling of N*-glycans*

To profile the *N*-glycans of the three peroxidases, each peroxidase was treated with PNGase F (peptidyl *N*-glycosidase F) and hydrazine as described in Experimental. PNGase F is a glycosidase which recognizes the *N*-glycosidic bonds of high mannose, complex and hybrid types with high specificity [14]. Hydrazine cleaves the *N*-glycosidic bond indiscriminately [15]. When each peroxidase was treated with PNGase F and hydrazine the released *N*-glycans showed differences in number. PNGase F released only one glycan from peroxidase A_1 and two from peroxidases A_2 and C_3 (Table 1), whereas hydrazine released several glycans from all peroxidases (Table 2). The DP (degree of polymerization) values of major glycans of the three peroxidases after PNGase F treatment are in the range 4–5 (Table 1), which is small compared with 8.8 for horseradish peroxidase, β-glucosidase and pectin methyltransferase, and 7.3 for α-glucosidase and β-amylase [16]. Table 2 shows the DP values for the released glycans after hydrazine treatment. The number of released glycans was larger in the three peroxidases

Table 1. DP values of released *N*-glycans of peroxidases A_1, A_2 and C_3 after PNGase F treatment

Standards*	A_1	A_2	C_3
G_6	—	—	—
G_5	—	5.1	5.3
G_4	4.4	4.5	4.5
G_3	—	—	—

*Oligomers of glucose; subscript gives degree of polymerization.

Table 2. DP values of released *N*-glycans of peroxidases A_1, A_2 and C_3 after hydrazine treatment

Standards*	A_1	A_2	C_3
G_7	—	—	—
G_6	6.4	—	6.5
	6.2	6.1	6.3
G_5	5.3	5.3	5.3
G_4	4.3	4.3	4.5
	3.9	3.9	4.0
G_3	3.4	3.5	3.4

*See Table 1 footnote.

than in PNGase F, indicating that the carbohydrate moieties of the three peroxidases are more readily cleaved by hydrazine. Comparison with data for N-linked oligosaccharide pools released from hen egg ovalbumin, porcine thyroglobulin and human α_1-acid glycoprotein by hydrazinolysis and PNGase F show similar gel-filtration chromatograms [15]. However, we only obtained partial cleavage with PNGase F even though we increased the amount of enzyme used several-fold. It is known that oligosaccharides carrying fucose-linked α-1-3 to GlcNAc(*N*-acetylglucosamine) are resistant to PNGase F [17]. The monosaccharide analysis of the three peroxidases indicated the presence of a large percentage of fucose (Table 3). Furthermore, endoglycosidase H digests showed no released oligosaccharides in peroxidase A, and only a very light band around DP3 value in the peroxidase C_3 (data not shown). These results suggest that the *N*-glycans of the three peroxidases are not high mannose or hybrid-type N-linked oligosaccharides.

Monosaccharide composition

To investigate the monosaccharide compositions of peroxidases A_1, A_2, and C_3, each peroxidase was treated with 4 M trifluoroacetic acid (TFA) for neutral and amine sugar release. The released monosaccharides of the three peroxidases were composed of mannose, fucose and *N*-acetylglucosamine (Table 3). When each peroxidase was treated with 0.2 M TFA no band co-migrated with the *N*-acetylneuraminic acid standard, indicating that the three peroxidases do not have sialic acid (data not shown). The quantity of each monosaccharide released was measured using an imager, and the percentage glycosylation was calculated using the quantities of total monosaccharides and the amount of peroxidase initially used. Percentage glycosylation was 18.1 in peroxidase C_3, while it was 6.6 and 8.9 in peroxidases A_1 and A_2, respectively (Table 3). Thus, the monosaccharides contained in the three peroxidases were the same, even though their percentage glycosylation showed differences.

Table 3. Molar ratios of monosaccharides and percentage glycosylation of peroxidases A_1, A_2 and C_3

	Molar ratios (%)			Glycosylation (%)
	Mannose	Fucose	GlcNAc	
A_1	43.4	36.8	19.8	6.6
A_2	53.2	37.2	9.6	8.9
C_3	43.7	27.1	29.2	18.1

Sequencing of two major N*-glycans of peroxidase* C_3

In order to determine the carbohydrate sequences of the two major glycans of peroxidase C_3 which were released during PNGase F treatment, the two glycans with DP values of 5.3 and 4.5 were eluted from high percentage polyacrylamide gel. The isolated glycans were digested sequentially with various glycohydrolases. The digestion patterns of the two glycans using neuraminidase, β-galactosidase and β-*N*-acetylhexosaminidase suggest that neither contain any terminal sialic acid, galactose or *N*-acetylglucosamine. Also, both glycans did not migrate differently when treated with alkaline phosphatase, indicating that there was no phosphate on the oligosaccharides released (data not shown). However, both were digested by α-mannosidase, indicating the presence of terminal mannose (Table 4). Analysis of the digested bands using image analysis indicates that the DP 4.5 glycan contains one or two mannoses α-linked at the C-1 position (Table 4). Likewise, the DP 5.3 glycan may also contain at least two or three mannoses α-linked at the C-1 position. We only obtained partial cleavage with α-mannosidase even though we increased the amount of enzyme used. Furthermore, the bands obtained after digestion with α-mannosidase did not co-migrate with the two core standards (Man(β1,4)GlcNAc$_2$ and Man(β1,4)GlcNAc[Fuc(α1,6)]GlcNAc). These results suggest that the DP 5.3 glycan of peroxidase C_3 has two or three terminal mannoses linked to GlcNAc and, since this glycan contains fucose, the core seems to be fucosylated rather than α-1,6 linked. Recently, the structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum was determined [18]. Table 5 shows that the proposed sequence of the DP5.3 glycan of radish peroxidase C_3 is rather similar to that of

Table 4. Sequence analysis of isolated DP 4.5 and DP 5.3 glycans of isoperoxidase C_3 after digestion with α-mannosidase

Standards*	DP 4.5	DP 5.3	Core standards†
G_5	—		—
		4.77	
G_4			—
	3.86	3.89	
	3.11		3.24
G_3	—	—	
			2.57

†Man(β1,4)GlcNAc$_2$ and Man(β1,4)GlcNAc[Fuc(α1,6)]-GlcNAc.

*See Table 1 footnote.

Table 5. Comparison of sequences of major *N*-glycans of some peroxidases

Peroxidases	Sequences
Radish C_3*	$Man_3GlcNAc(Fuc)GlcNAc$
Horseradish†	$Man_2(Xyl)ManGlcNAc(Fuc)GlcNAc$
Coprinus cinereus‡	GlcNAc
Coprinus macrorhizus‡	$Man_5GlcNAc_2$
Arthromyces ramosus‡	$Man_7GlcNAc_2$

*DP 5.3 glycan.
†Data taken from ref. [18].
‡Data taken from ref. [19].

horseradish, but not to the sequences of fungal peroxidases [19].

EXPERIMENTAL

Purification of isoperoxidases. One cationic peroxidase, C_3, and two anionic peroxidases, A_1 and A_2, were purified from Korean radish (*R. sativus* L.) by CM-Cellulose and DEAE-Sephacel ion-exchange chromatography [10, 11]. The purity of each peroxidase was verified by SDS-PAGE and starch gel electrophoresis.

Profiling of N*-glycans.* The *N*-glycan profile of each peroxidase was determined using PNGase F (Glyko, Inc.) and N_2H_4 (Oxford Glycosystems). First, 100 μg of each peroxidase was treated with 1 μl 5% SDS and 1.5 μl 1.44 M β-mercaptoethanol for complete denaturation; 5 μl 7.5% Nonidet P-40 was added prior to 2 μl (5 mU) of PNGase F treatment. The reaction mixt. was then incubated at 37° for 2 hr. The protein fr. was pptd by subsequent addition of 100% cold EtOH and the *N*-glycan supernatant collected by pipette. Each peroxidase was also treated with anhyd. N_2H_4 (5 mg/ml^{-1}) at 95° for 5 hr following the method of ref. [15]; released glycans were collected using an Oxford *N*-glycan recovery kit (Oxford Glycosystems). Profiling of *N*-glycans was performed using 40% PAGE according to the manufacturer's procedures (Glyko, Novato, CA).

Monosaccharide composition. Each peroxidase (20 μg 50 μl^{-1} H_2O) was treated with the same vol. of 0.2 M TFA at 80° for 30 min for sialic acid release and with 4 M TFA at 100° for 5 hr for neutral and amine sugar release. Released monosaccharides were compared with the sugar standards, GalNAc (*N*-acetylgalactosamine), mannose, fucose, glucose, galactose and GlcNAc—on PAGE (Glyko). The quantity of each monosaccharide released was measured using a Glyko SE 100 imager, and percentage glycosylation was calcd using quantities of total monosaccharides and amount of peroxidase initially used.

Sequencing of N*-glycans of peroxidase* C_3. The two major *N*-glycans identified by *N*-glycan profiling were excised from the polyacrylamide gel and soaked in a minimum vol. of H_2O. They were then stored at 4° overnight. The supernatant containing 200 pmol of each *N*-glycan was collected and lyophilized, then subjected to glycohydrolase treatment (2 μl) with alkaline phosphatase (20 mU), neuraminidase (10 mU), β-galactosidase (10 mU), β-*N*-acetylhexosaminidase (84 mU) and α-mannosidase (20 mU). The digestion pattern was compared with two core standards (Man(β1,4)-$GlcNAc_2$ and Man(β1,4)GlcNAc[Fuc(α1,6)]GlcNAc) using PAGE (Glyko).

Visualization of carbohydrate moieties on SDS-PAGE. N-glycans released after treatment with PNGase F and N_2H_4, monosaccharides released after treatment with TFA, and the *N*-glycans subjected to glycohydrolases, were labelled with aminonaphthalenetrisulphonic acid (100 pmol in 10 μl) for 3 hr at 45° and dried under a seed vacuum evaporator. Samples were resuspended in 20 μl de-ionized H_2O and 20 μl of 2 × loading buffer; 4 μl of sample was loaded on to each gel well and run on 40% PAGE gel (Glyko) for 90 min at 15 mA below 10°. Gels were visualized by exposure to long wavelength UV (360 nm). DP values and quantities of each band were determined using a Glyko SE 100 imager.

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REFERENCES

1. Welinder, K. G. (1985) *Eur. J. Biochem.* **151**, 497.
2. van Huystee, R. B. and Cairns, W. L. (1982) *Phytochemistry* **21**, 1843.
3. Gaspar, T., Penel, C., Thorpe, T. and Greppin, H. (1982) *Peroxidases 1970–1980. A Survey of Their Biochemical and Physiological Roles in Higher Plants*. University of Geneva, Center of Botany, Geneva.
4. Nathan, S. (1984) *Trends Biochem. Sci.* **9**, 198.
5. Wan, L. and van Huystee, R. B. (1993) *Biochem. Biophys. Res. Commun.* **194**, 1398.
6. Hu, C. and van Huystee, R. B. (1989) *Biochem. J* **263**, 129.
7. Ravi, K., Hu, C., Reddi, P. S. and van Huystee, R. B. (1986) *J. Exp. Botany* **37**, 1708.
8. Woo, K., Lee, M. Y. and Kim, S. S. (1987) *Korean Biochem. J.* **20**, 58.
9. Kim, S. S., Kim, B. J., Yoo, S. B. and Lee, M. Y. (1993) in *Plant Peroxidases: Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K.,

Penel, C. and Greppin, H., eds), pp. 75–80. University of Geneva, Geneva.
10. Lee, M. Y. and Kim, S. S. (1994) *Phytochemistry* **35**, 287.
11. Lee, M. Y., Choi, Y. and Kim, S. S. (1994) *Plant Physiol. Biochem.* **32**, 259.
12. Hu, C. and van Huystee, R. B. (1988) *Biochem. Biophys. Res. Commun.* **156**, 500.
13. Hu, C. and van Huystee, R. B. (1989) *Biochem. Biophys. Res. Commun.* **163**, 689.
14. Tarentino, A. L., Gomez, C. M. and Plummer, T. H., Jr., (1985) *Biochemistry* **24**, 4665.
15. Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A. and Parekh, R. (1993) *Biochemistry* **32**, 679.
16. Macmanus, M. T., Mckeating, J., Secher, D. S. and Rademacher, T. W. (1988) *Planta* **175**, 506.
17. Dwek, R. A., Edge, C. J., Harvey, D. J. and Wormald, M. R. (1993) *Annu. Rev. Biochem.* **62**, 65.
18. Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nokagawa, J. and Kawasaki, T. (1991) *J. Biol. Chem.* **7**, 4168.
19. Kjalke, M., Anderson, M. B., Schneider, P., Christensen, B., Schule, M. and Welinder K. (1992) *Biochim. Biophys. Acta* **1120**, 248.