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Glycans of higher plant peroxidases: recent observations and future speculations

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Plant peroxidases are composed of a peptide and associated heme, calcium and glycans. The 3D structure of the major cationic peanut peroxidase has revealed the sites of the heme and calcium. But the diffraction of the glycans was not sufficient to show their structure. This review presents research that has been executed to obtain putative glycans and their binding sites, and to gain an indirect insight into these glycans. It also offers approaches that will be used to determine the function of the glycans on the peanut peroxidase. Some comparisons are made with other plant glycoproteins including peroxidases from plants other than peanut.

***Keywords*: plant peroxidases, glycans**

***Abbreviations:* CPrx, major cationic peanut peroxidase; APrx anionic peanut peroxidase; CPz minor cationic peanut peroxidase; Con-A, concanavalin-A; NMR, nuclear magnetic resonance; GlcNAc, *N*-acetyl-glucosamine; Asn, asparagine.**

Introduction

While plant peroxidases have been known to be glycoproteins for many years [1], information on their glycan structure is comparatively scarce. The advent of molecular biological techniques has allowed the cloning of several peroxidase genes, and examination of the sequences reveal putative N-linked oligosaccharide attachment sites.

In this review, we summarize the literature pertaining to the glycosylation of peroxidases from higher plants. Particular emphasis is placed on the nature of the glycans attached to the major cationic peroxidase purified from peanut cell suspension cultures. Some speculation on the function of the glycan moieties on this enzyme is also included. There is evidence that glycosylation is important in determining animal glycoprotein structure and function [2] but a definitive answer to whether glycans are important in plant glycoprotein structure and function, particular peroxidase, is still elusive.

Plant peroxidases are oxidoreductases. The heme and surrounding amino acids participate in a two step enzymatic reaction with H_2O_2 and a large array of hydrogen donors [3]. The enzyme function has been reported to be associated with, for example, plant growth and disease resistance.

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The N-linked glycans of peanut peroxidase

Even though much work has been done on a few selected plant glycoproteins, both *O*- [4] and *N*-glycosylated [5], more research on their structure and function is needed.

Peroxidase activity has been detected in the spent medium of peanut cell suspension cultures, and a combination of ion-exchange and Concanavalin-A (Con-A) affinity chromatography has isolated three distinct isoenzymes. Early identification of genetic variation among these three was made by peptide mapping [6] suggesting that three distinct genes are expressed. The major iso-enzyme, identified as a cationic form (designated CPrx) can accumulate up to 5 $mg l^{-1}$ in the spent medium [7], and the structures of its glycans have been tentatively determined [8] (Figure 1).

The second isoenzyme, the anionic form (designated APrx), has a single glycan of large (8 kDa) mass [9]. Due to the large mass of the glycan structure and the low occurrence (1/10 of the cationic isoenzyme) of the protein in the spent medium [7], very little of the glycan composition is known at present.

The third isoenzyme [6], another cationic form (designated CPz), is the least represented isoform and no characterization of the glycans has been undertaken.

Determination of the glycan structure of CPRX

The cDNA clone prxPNC-1 [10] was identified as the homolog for the gene copy for CPrx by protein cleavage using formic acid and amino acid sequencing of the

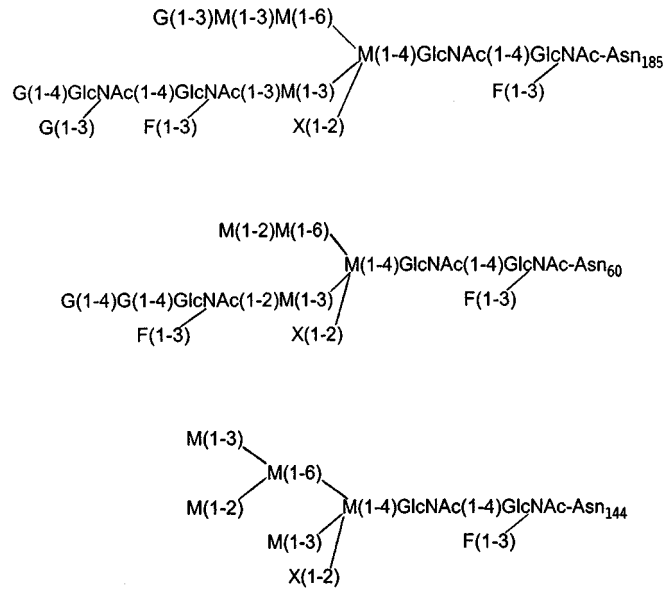

Figure 1. The three putative glycans from the major cationic peanut peroxidase. Determinations were done by sugar composition and methylation assays [8]. Abbreviations: G, galactose; M, mannose; F, fucose; GlcNAc, *N*-acetylglucosamine; X, xylose; Asn, asparagine.

resulting peptides [11]. Further confirmation was obtained by amino acid sequence after trypsin cleavage [12] and by comparison of the cDNA with the amino acid sequence derived from the X-ray diffraction of the CPrx protein crystal [13]. A faint indication of the glycans was seen.

Examination of the nucleotide sequence of *prxPNC-1* revealed four putative glycan attachment sites. The hypothesis of four glycans attached to CPrx [8] was incorrect in that only three out of those four binding sites are used [12]. The open site has a proline in the third position following the Asn_{209} binding site which will prevent *N*-glycosylation [14]. Structures of the three glycans from CPrx have been proposed [8], and the structure of the largest glycan has been verified by Nuclear Magnetic Resonance (NMR) spectroscopy (G. Shaw personal communication). The linkage site for this glycan is Asn_{185}, with the others at Asn_{60} and Asn_{144} [12].

The use of immunological techniques is a useful approach in the study of glycosylated forms of peroxidase and has provided some indirect evidence as to the nature of the glycans attached to peanut peroxidase. In early experiments, a combination of immunoprecipitation of the protein from peanut cell extracts and fluorography revealed a 'laddering' of three products with slightly varying molecular mass [15]. This can now be interpreted as three distinct glycosylated forms of peroxidase. Cell treatment with tunicamycin confirmed that all binding sites were *N*-glycosylated [15]. When monoclonal antibodies were raised to CPrx, four different hybridoma lines were isolated, but only antibodies from one of these inhibited the enzymatic activity [16]. However, after prior treatment of the CPrx antigen with periodate, this monoclonal antibody was shown, along with two others tested, to reduce recognition of the CPrx by half as determined by ELISA binding [17]. The Asn_{144} linked glycan is reportedly located near the active site [13].

Antibodies have been raised against three trypsin-generated glycopeptides from CPrx linked as haptens to albumin and with each peptide containing one of the three glycans [18]. Competition ELISA revealed that these antibodies cross-reacted, suggesting shared components [18]. Competition ELISA with fetuin, a glycoprotein with three *N*-linked glycans, that differ from the complex glycans of the CPrx, because they lack xylose and fucose on the core structure [19], demonstrated no cross-reactivity suggesting no shared epitopes. However, competition with bromelain resulted in severe inhibition of binding for all three glycopeptides, as did competition with 1 M concentrations of fucose, xylose and *N*-acetylglucosamine (GlcNAc) [18]. Competition with mannose and galactose demonstrated variable results for the three glycopeptides. The glycan structure of bromelain is: Manα1 $\rightarrow$ 6 (Xylβ1 $\rightarrow$ 2) Manβ1 $\rightarrow$ 4GlNacβ1 $\rightarrow$ 4 (Fucα1 $\rightarrow$ 3) GlcNac [20, 21]. This, together with the free sugar inhibition data suggests that all three glycan chains have a xylose/fucose core structure with variations in the peripheral structures (Figure 1). The high immunogenicity of complex glycans exhibited in these experiments has been observed before [19, 20], and such immunological evidence supports the putative structures proposed for the three glycans attached to CPrx (Figure 1).

Further studies on CPRX glycans using specific glycosidases

When peroxidase is treated with PNGase F, less than 1 kDa of the glycan structure is lost [24]. Each of the putative glycans is well in excess of that mass [8]. What could this indicate? It may mean that the locations of some of the glycan binding sites occur in the hydrophobic areas of the protein and are therefore inaccessible to the glycosidase [12], or is it due to $\alpha(1 \rightarrow 3)$ fucose linked to proximal GlcNAc? PNGase F is capable of cleaving chains with $\alpha(1 \rightarrow 6)$ fucose but is incapable of cleaving those with proximal $\alpha(1 \rightarrow 3)$ linked fucose [25, 26] which occur commonly in plant glycoproteins with complex glycan chain [5]. This lack of an expected PNGase F action may indirectly confirm the site and the nature of fucose in the complex antennae on the putative glycan (Figure 1). It may also explain the reason why mannosidase treatment of the cationic peroxidase did not reduce the overall CPrx mass (van Huystee, unpublished observations).

What causes the partial loss of CPrx mass after the PNGase F treatment [24, 27]? No protease activity was detected in the PNGase F [27]. Could it be that the enzyme

cleaves other GlcNAc residues postulated to occur further along the glycan of CPrx (Figure 1)? Furthermore, a severe reduction in heme absorbance was noted after treatment of peroxidase with PNGase F, and this may account for loss of enzyme activity that is observed. Circular dichroism spectra revealed an absorption change at 280 nm [27] indicating an alteration in the protein moiety. A loss of sugars could cause an irreversible alteration of the protein folding [22, 28, 29] which in turn could cause a loss of heme. Further studies on the PNGase F effect on these glycans are needed.

Glycan microheterogeneity

Cationic peanut peroxidase accumulated in either the plant cell wall or the spent medium of cultured peanut cells, depending on the type of assay used, *ie* whole plants or cultures [7, 30]. While no protease activity was detected in the medium [31], there were two forms (40 and 37 kDa) of CPrx. These had identical protein mass, but differed in the lenths of glycan chains [11]. However, this loss of carbohydrates in one of the forms, did not cause a loss in specific activity in the same way as that observed after PNGase F treatment. This raises the possibility that specific sugars may be involved in the maintenance of activity and conformation of the enzyme [27, 28].

Microheterogeneity in the carbohydrate structures was proposed to be the cause of these two forms of the media CPrx, because although they both have three glycans and the same single formic acid cleavage site [12], they exhibited different binding capacities to Con-A [11]. The largest putative glycan from the 40 kDa form had several terminal galactose residues on the antennae (Figure 1), but there was a possibility that these residues either may not have been added in the biosynthetic process [32] or may have been removed by a galactosidase. A wide variety of glycosidases have been reported in the extracellular compartment of plant cell walls [33] and one could propose that such cell wall or medium glycosidases alter the glycan chain length. To test this idea, assays for mannosidase and *N*-acetyl-glucosaminidase in the medium were undertaken throughout the normal 14 d growth period, but no activity was found. However, β-galactosidase activity was identified [34]. This enzyme is quite common in plants [35, 36]. It was isolated from the spent medium and incubated with the 40 kDa, Con-A nonbinding, form of CPrx at pH 3.5. Most of that form was transformed to the smaller, Con-A binding form [34]. Therefore, β-galactosidase cosecreted in the medium with the peroxidase could alter some of the glycans. The reason that in the cell suspension medium only 10% of the larger glycans [11] were transformed by galactosidase, was probably due to the pH of the medium being close to 5.0 [37]. The pH optimum for β-galactosidase is 3.5 [34].

Glycan variability and stability

There is evidence from studies with animal systems that glycosylation of the same protein varies in different tissues [38, 39]. In plants, an anionic form of horse-radish peroxidase isolated from cell cultures has been shown to contain a total of 11 glycosylation sites based on tritium incorporation into the released glycans and the mass of the protein, while amino acid sequencing of the similar anionic form isolated from roots revealed only seven [40]. For such reasons a steady state plant cell culture may be ideal for studies of glycan peroxidases. The use of peanut cell suspension cultures provides cells at a relatively constant physiological state. Moreover, there is a much higher expression, as determined by Northern analysis, of cationic peroxidase genes in cultured cells when compared with intact tissue [41]. In plants, the expression of the peroxidase genes is not always constitutively expressed, but may be tissue specific, such as observed in the seedcoat of soybean [42]. These are reasons for plant glycoprotein studies to be done on CPrx from cultured peanut cells. These studies could be followed by examinations of glycan chains in specific tissues.

Model studies of plant glycoproteins are carried out on relatively obscure proteins. In studies on protein folding, barnase has been used extensively [43]. The reasons for such an approach are because of its well-known structure, a single chain RNase from *B. amyloliquefaciens*, and its ease of handling [44]. It may be possible to study the behaviour of plant glycoproteins using peanut peroxidase as a model system. The three-dimensional structure of peanut peroxidase is available from X-ray diffraction studies [13]. Peroxidase production by cultures of different plant species varies greatly [45, 46]. Peanut cells in suspension culture appear to produce significantly larger amounts of peroxidase [46] with the major cationic form, CPrx isolated at 5 $mg\,l^{-1}$ spent medium every 14 days [7]. What causes this high accumulation? Could this high yield for the enzyme be due to its longer half-life [47]? Could it be the glycans and the glycosidases are controlling this process?

II Peroxidase from other plant species

In terms of protein structure horseradish peroxidase is one of the more extensively studied plant peroxidases. Originally seven major iso-enzymes were identified [48], but subsequently more have been identified [49]. These have been traditionally arranged in five major groups [50] although sequence comparison with a recently isolated cDNA suggests that a sixth group may exist [51].

Of the major horseradish iso-enzymes, HRP-C is the best studied. The determined protein sequence showed that there were eight carbohydrate attachment sites (1), each *N*-glycosylated [52]. The major glycan, comprising 80% of the carbohydrate, has been identified as: Manα1→6(Manα1→3)(Xylβ1 → 2)Manβ1 → 4GlcNAcβl → 4(Fucα1 → 3)GlcNAc, [53, 54]. Most of the remaining oligosaccharides have been

identified as mixed xylose/fucose-type variants, with a smaller fraction of high-mannose types.

A detailed structural analysis of the anionic peroxidases secreted from root cell cultures has also been undertaken [40]. In this study, the major structure has also been identified as: Manα1 → 6(Manα1 → 3)(Xylβ1 → 2)Manβl → 4GlcNAcβ1 → 4 (Fucα1 → 3)GlcNAc with 6 other xylose/fucose complex structures that vary slightly.

Other peroxidases

Carbohydrate analysis of the soybean seedcoat peroxidase showed that te major structure, accounting for 60 to 65% of the total carbohydrate, was the same as that determined for the major glycan of the horseradish peroxidase, Manα1 → 6 (Manβ1 → 3)Xylβ1 → 2)Manβl → 4GlcNAcβ1 → 4 (Fucα1 → 3) GlcNAc [55]. There were also a further 20 to 25 variants of the complex-type, with a small number being of the high mannose type. The structure of the major glycan attached to the predominant peroxidase from barley seed, BP-1 has also been identified as: Manα1 → 6(Xylβ1 → 2)Manβl → 4GlcNAcβ1 → 4(Fucα1 → 3)GlcNAc [56].

Some analysis of the *N*-linked glycans from 2 anionic and a cationic peroxidase from Korean radish has been reported. No detailed structural analysis has been undertaken but the sequence attached to the cationic form has been tentatively identified as Man_3GlcNAc(Fuc)GlcNAc [57]. Glycan structures with cores such as: [Xyl]$(Man)_3$[Fuc]$(GlcNac)_2$Asn (square brackets indicate branching) are common in plant glycoproteins [58].

III The function of glycans on peroxidase

The evidence from studies with animal glycoproteins overwhelmingly supports the concept that glycan moieties play important roles in macromolecular function. In animal development, complex *N*-glycosylation plays a major part in development of the normal embryo. GlcNAc-transferase 1 appears not to be essential for the survival of cell lines but is essential for the development of the nervous system in mice [59]. Previously, Olden *et al.* [60] summarized five key areas in which glycosylation was probably important: (1) maintenance of protein conformation and solubility, (2) proteolytic processing and stabilization of the polypeptide against uncontrolled proteolysis, (3) mediation of biological activity, (4) intracellular sorting and externalization of glycoproteins, and (5) embryonic development and differentiation. Today, the evidence suggests that there is no single unifying function for oligosaccharides (2) but there is more support for each of the roles outlined by Olden and colleagues.

There is some evidence for glycans having some of the above roles in plants [22], including a role in plant cell development [61]. In considering plant peroxidases, it is relevant to review the literature of the influence of glycan structures on the function of other plant proteins.

Site-directed mutagenesis

The advent of site-directed mutagenesis to selectively ablate N-linkage sites and subsequent plant transformation has increased our understanding of the potential function of glycan moieties on plant glycoproteins. Voelker *et al.* [62] introduced an asparagine-linkage mutated phytohaemagglutinin (*PHA*) gene into tobacco and showed that alterations in the glycans may influence accumulation of the lectin in the seed. They showed that while the protein was still being targetted to the seeds of the transgenic tobacco, the loss of the glycan decreased its accumulation. In other studies with seed storage proteins, there is evidence that the glycans attached to lupin conglutin aid folding rather than protein stability [63].

The deletion of the N-linked glycosylation site on rice α-amylase, iso-enzyme Amyl A, influenced the thermostability and kinetics of the enzyme when it was expressed in yeast [64]. However, in these experiments, the structure of the glycans attached to the non-mutated (control) enzyme were not determined. Even though heterologous expression of plant genes is frequently executed in yeast [65], it is well-known that these cells produce molecules with excessively mannose-rich chains [66]. In studies in which plant glycoproteins were expressed in heterologous systems, the nature of the glycans attached in the new genetic background were not determined [67, 68].

In other experiments, where bean PHA lectin cDNA was transfected into tobacco cells, the complex glycan structure attached to the lectin appeared to be unmodified. However, some modifications of the high mannose chains were observed [69]. Such experiments underline the need to determine the structure of the glycosylated (control) protein against the new genetic background of the transgenic plant. It may be that the information contained within the glycan structure is not reproduced faithfully. Complex glycan analysis by lectins of the expression of a complex glycan protein from animals in Baculovirus/insect cells indicated that neither galactose nor sialic acid were added [70].

A mutant plant of *Arabidopsis thaliana* has been isolated that contains (as determined by antiserum binding) no complex-type N-linked glycans [71]. Furthermore, complementation studies revealed that the mutant lacked a functional *N*-acetyl-glucosaminyl transferase I and so the first step in the conversion of high-mannose chains to complex types was blocked [72]. Since the mutant could complete its normal life cycle, the possibility was raised that the information contained in the complex glycan was needed only on a small subset of glycoproteins, perhaps those induced by particular stress conditions [71]. In animals, the complex glycans are needed for normal development [59].

Studies with peroxidases

So far, there have been few studies that directly address the role of glycans attached to peroxidase. Tams and Welinder

Table 1. Chronological events in glycan studies of peanut peroxidase.

Event	*Reference*
Detection of 20% sugars in peanut peroxidases (CPrx)	[79]
CPrx glycan sites are N-linked (tunicamycin)	[15]
Three CPrx immuno-precipitation products	[15]
Three sugar binding noncompetitive monoclonal antibodies	[16]
A cDNA clones for cationic peanut peroxidase	[10]
Two glycan forms of the cationic peroxidase (CPrx)	[11]
Putative identification of the three glycans	[8]
Identification of the three glycan binding sites on CPrx	[12]
Isolation of polyclonal antibodies for three glycans	[18]
Galactosidase as agent for two CPrx glycan forms	[34]
The 3-D structure of the cationic peroxidase (CPrx)	[13]

[73] deglycosylated HRP-C with trifluoromethane sulfonic acid under mild conditions, and recovered enzyme activity with no alterations in its kinetics parameters. They concluded, therefore, that the glycans were not required for activity. Partial de-glycosylation of an avocado peroxidase using PNGase F caused a decrease in the K_m for its substrate and a decrease in thermostability [74]. In another approach, genes encoding peroxidase have been expressed in *E. coli* and folding experiments performed to reconstitute activity [75, 76]. These *in vitro* reconstitution experiments suggested that the glycans may not be important for folding/activity. However, direct comparisons with native enzymes and under *in vivo* conditions were not undertaken.

Site directed mutagenesis is commonly used in studies with animal glycoproteins. For example, for the four *N*-glycosylation sites of the human lecithin cholesterol acyltransferase, Qu *et al.* [77], used selective ablation, and determined that each structure influenced a different aspect of enzyme function. As yet, there has been no similar systematic study on plant glycoproteins. The cationic iso-enzyme of peanut, CPrx, with three *N*-linkage sites and its corresponding cDNA clone prxPNC-1, may be a particularly amenable protein for such a study in plants, using selective N-linked ablation and expression in transgenic plant cells. This is particularly true for a protein of which the 3-D structure is known.

Table 1 summarizes glycan related studies done with peanut peroxidase over the last 10 years. Further studies are underway to determine the structures of the two glycan chains not yet analysed by NMR. (Removal of the glycans by PNGase A shows promise for clean glycan cleavage!) Once confirmation of the structure of these glycans is made they could be modelled on the protein as done for animal glycoproteins [29]. Peanut peroxidase may be harvested readily within a 14 day culturing period and purified in only a few steps [78]. Thus, the supply of the enzyme is relatively abundant for any type of assay. In addition, experiments underway to search for glycosidases in spent peanut medium, that may attack the glycan chain and cause a loss of enzyme activity. Also, the loss of specific sugars from the glycan chain may indicate that these sugars are responsible for the half-life of this rugged enzyme [47].

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