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Removal of the *N*-linked glycan structure from the peanut peroxidase prxPNC2: Influence on protein stability and activity

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Abstract

Lines of transgenic tobacco have been generated that are transformed with either the wild-type peanut peroxidase *prxPNC2* cDNA, driven by the CaMV35S promoter (designated *35S::prxPNC2-WT*) or a mutated *PNC2* cDNA in which the asparagine residue (Asn_{189}) associated with the point of glycan attachment (Asn_{189}) has been replaced with alanine (designated *35S::prxPNC2-M*). PCR, using genomic DNA as template, has confirmed the integration of the *35S::prxPNC2-WT* and *35S:prxPNC2-M* constructs into the tobacco genome, and western analysis using anti-PNC2 antibodies has revealed that the *prxPNC2-WT* protein product (PNC2-WT) accumulates with a molecular mass of 34,670 Da, while the *prxPNC2-M* protein product (PNC2-M) accumulates with a molecular mass of 32,600 Da. Activity assays have shown that both PNC2-WT and PNC2-M proteins accumulate preferentially in the ionically-bound cell wall fraction, with a significantly higher relative accumulation of the PNC2-WT isoenzyme in the ionically-bound fraction when compared with the PNC2-M isoform. Kinetic analysis of the partially purified PNC2-WT isozyme revealed an affinity constant (apparent K_m) of 11.2 mM for the reductor substrate guaiacol and 1.29 mM for H_2O_2, while values of 11.9 mM and 1.12 mM were determined for the PNC2-M isozyme. A higher Arrenhius activation energy (E_a) was determined for the PNC2-M isozyme (22.9 kJ mol^{-1}), when compared with the PNC2-WT isozyme (17.6 kJ mol^{-1}), and enzyme assays have determined that the absence of the glycan influences the thermostability of the PNC2-M isozyme. These results are discussed with respect to the proposed roles of *N*-linked glycans attached to plant peroxidases.

Keywords: *Arachis hypogaea* (L.); Leguminosae; Peanut; *N*-linked glycosylation; Peroxidase

1. Introduction

Evidence is accumulating in plants that *N*-linked glycan structures influence protein targeting, stability and function (Lerouge et al., 1998; van Huystee and McManus, 1998). For the peroxidases, where these have been examined, all enzymes have been shown to be glycosylated, with the first characterised, horseradish peroxidase (HRP) known to be a glycoprotein for many years (Shannon et al., 1966). It is only comparatively recently, however, that some structures of the carbohydrate chains attached to these important plant enzymes have been solved: information that acts a prelude to ascertaining the function of these *N*-linked sugars.

In terms of *N*-glycan structure, the major peroxidase iso-enzyme of horseradish, iso-enzyme C (HRP-C) has

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received much research attention. Examination of the amino acid sequence revealed 8 carbohydrate attachment sites as determined by the occurrence of the Asn–X–Ser/Thr motif (Welinder, 1976), and each of these putative *N*-linked sites has been shown to have a sugar structure attached (Welinder, 1979). The major glycan, comprising 80% of the carbohydrate, has been characterised as Manα1 → 6(Manα1 → 3)(Xylβ1 → 2) Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 3) GlcNAc after release from HRP-C by hydrazinolysis, Bio-Gel P4 gel filtration, composition analysis and high-resolution ^{1}H NMR spectroscopy (McManus et al., 1988). This structure has now been confirmed by Kurosaka et al. (1991) and Yang et al. (1996). A detailed structural analysis of anionic peroxidases isolated from roots of horseradish and from those secreted from root cell cultures has also been undertaken (Harthill and Ashford, 1992), with a major structure again identified as Manα1 → 6(Manα1 → 3)(Xylβ1 → 2)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 3) GlcNAc. Six other xylose/fucose complex structures with slight variations were also identified.

Structural analysis has also been undertaken on an anionic soybean hull peroxidase (Gray et al., 1996; Welinder and Larsen, 2004), a major peroxidase from barley seed, designated as BP-1 (Johansson et al., 1992), and two anionic and a cationic peroxidase from Korean radish (Kim and Kim, 1996).

The spent medium of peanut cell suspension cultures has provided an excellent experimental source with which to study plant peroxidases, and a combination of Con A affinity chromatography and ion-exchange chromatography has identified at least three distinct isoenzymes that are secreted by the peanut cells into the medium with the major iso-enzyme, identified as a cationic form, designated as CPRx (O'Donnell et al., 1992). In terms of elucidation of the glycan structures attached to these isoforms, most effort has centred on the CPRx isoform, and putative structures of the three glycans have been proposed at Asn_{60}, Asn_{144} and Asn_{185} (van Huystee et al., 1992; Shaw et al., 2000).

Thus far, there have been comparatively few studies that directly address the role of the *N*-linked glycan structures attached to peroxidase. For these studies, three major experimental approaches have been used: (i) de-glycosylation of peroxidases using chemical or enzymatic digestion, (ii) expression of the enzyme in a prokaryote background, and (iii) ablation, using site-directed mutagenesis, of strategic Asn residues and expression of the mutated peroxidase in a higher plant background. For the first approach, partial de-glycosylation of an anionic peroxidase purified from peach seeds (Tigier et al., 1991), an avocado peroxidase (Sanchez-Romero et al., 1994), and a neutral peroxidase from turnip (Duarte-Vazquez et al., 2003) has been shown to influence the activity and stability of each enzyme, although chemical glycosylation of HRP-C did not alter the specific activity of the enzyme (Tams and Welinder, 1995). As the second approach to determine the role of *N*-linked glycans on plant peroxidases, genes encoding peroxidases from horseradish have been expressed in *Escherichia coli* (where no glycosylation will occur) and refolding experiments in the presence of heme have suggested that the glycans may not be critical for refolding/activity (Smith et al., 1990; Gazaryan et al., 1994). However, a potential drawback of both the first and second approaches is that all *N*-linked glycosylation chains are wholly or partially removed. Plant peroxidases can contain only one, or up to eight glycan structures, and so it may be that each structure imparts specific properties to the enzyme.

For the third approach, therefore, Lige et al. (2001) used site-directed mutagenesis to ablate each of three *N*-linked sites individually (*N*-60, *N*-144, *N*-185) on the major cationic peroxidase of peanut (CPRx) coded for by *prxPNC*1 (Buffard et al., 1990). Purification of the mutated proteins from a transgenic tobacco leaf background revealed that ablation at each site did influence protein function with ablation of *N*-60 or *N*-144 decreasing the specific activity of the protein, ablation of *N*-185 decreasing the thermal stability of the enzyme, and ablation of any of the three sites influenced the rate of protein folding (Lige et al., 2001).

Examination of the sites of *N*-linked glycosylation of peroxidases reveals some conservation around the site of glycan attachment (Buffard et al., 1990). While the number of glycan chains attached to peroxidases varies from one to eight, if only one chain is attached, then it is at Asn_{185} (using HRP-C as reference) or the corresponding residue attached to other peroxidases. Thus it is reasonable to postulate that the *N*-linked glycan corresponding to Asn_{185} may confer significant properties to the enzyme.

In this study, therefore, we report on the influence of de-glycosylation of the peanut peroxidase isoform coded for by the gene corresponding to *prxPNC2*. This isoenzyme contains a single glycosylation at Asn_{189}, and the effect of removal of this structure has been examined in terms of enzyme targetting, stability and activity.

2. Results

2.1. Expression of wild-type (WT) and mutated (M2) peanut peroxidase in tobacco

Lines of transgenic tobacco were generated that were transformed with either the wild-type peanut peroxidase *prxPNC2* cDNA, driven by the CaMV35S promoter (designated *35S::prxPNC2-WT*) or a mutated *PNC2* cDNA in which the asparagine residue (Asn_{189}) at the point of glycan attachment was replaced with alanine (Fig. 1). The mutated gene was also driven by the

AAC AGA TTG TTC AAC TTC AAT GGA ACT GCT GCT GCT GAC CCT

Asn_{183}Arg Leu Phe Asn Phe Asn Gly Thr Ala Ala Ala Asp Pro_{196} *prxPNC2-WT*

AAC TTC **GC**T GGA ACT GCT

Asn Phe Ala Gly Thr Ala *prxPNC2-M*

Fig. 1. Diagrammatic representation of the *35S::prxPNC2-WT* and *35S::prxPNC2-M* constructs, as indicated, with the site-directed modified sequence highlighted for the *35S::prxPNC2-M* construct.

CaMV35S promoter and designated as *35S::prxPNC2-M*.

PCR, using genomic DNA as template, was used to confirm that integration of the *35S:prxPNC2-WT* or *35S::prxPNC2-M* constructs into the tobacco genome, and a representative selection is shown as Fig. 2. If positive, the primer design used should amplify a 990 bp sequence, and in the transgenic plants shown, a ca. 1000 bp product was observed (Fig. 2). Genomic DNA, isolated from non-transformed tobacco plants, was also used as template in the PCR reactions, but no products of any size were amplified (Fig. 2).

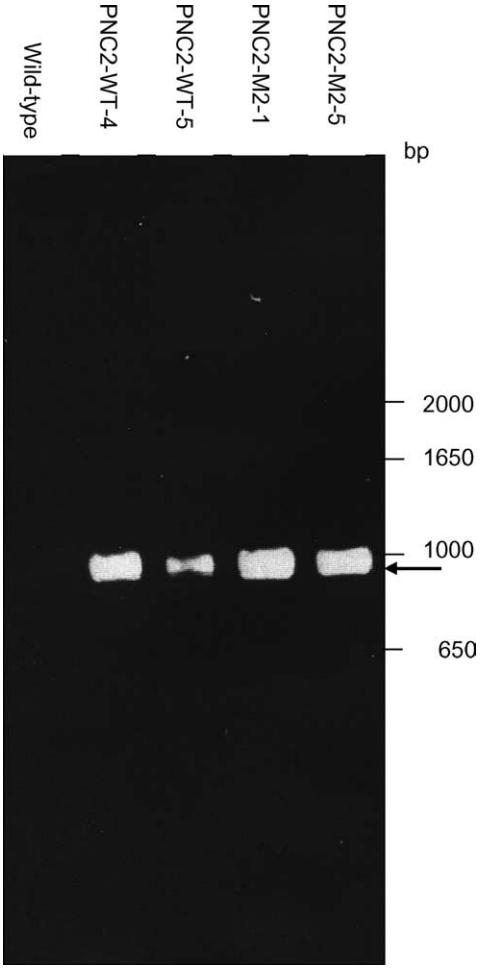


Fig. 2. PCR amplification of the wild-type or mutated *prxPNC2* peroxidase cDNA. One μg of genomic DNA from the plant lines, as indicated, was used as template. The amplification product at the expected size in the transformants (*ca.* 1000 bp) is indicated by the arrow.

Accumulation of peroxidase protein in the transgenic tobacco background was determined by western analysis using antibodies raised against PNC2 protein expressed in *E. coli.* In two of the *35S::prxPNC2-WT* lines examined (WT-4, WT-5), a protein with a molecular mass of 34,670 Da accumulated as determined by gradient SDS–PAGE, while a protein with a molecular mass of 32,600 Da accumulated in the two *35S::prxPNC2-M* lines tested (M2-1, M2-5) (Fig. 3). No proteins of any molecular mass were recognised by the anti-PNC2 antibody in leaf extracts of non-transgenic leaves.

To localise the accumulation of the PNC2-WT isozyme and PNC2-M isozyme, leaf extracts were divided into a water-soluble fraction, and an ionically-bound

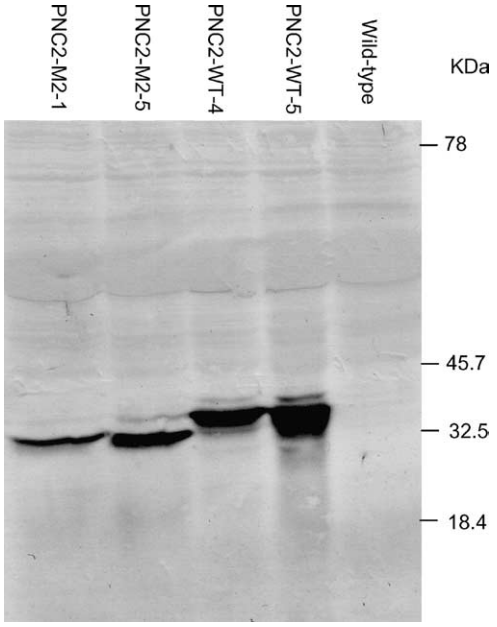


Fig. 3. Western analysis, using a gradient (10–20%) SDS–PAGE separation, of extracts of leaves excised from non-transformed wild-type, *35S::prx-PNC2-WT* or *35S::prx-PNC2-M* transformants, as indicated. The PVDF membranes were incubated with anti-PNC2 antibodies, and antibody recognition visualized using alkaline phosphatase conjugated secondary antibodies. The masses of standard proteins are as indicated.

cell wall fraction. In terms of specific activity (as determined by guaiacol oxidase activity), the rate in the soluble fraction of the PNC2-WT4 extract (3.12 ± 0.09 nmol min^{-1} mg^{-1}) was broadly similar when compared with the PNC2-M2-5 extract (4.2 ± 0.17 nmol min^{-1} mg^{-1}) (Fig. 4(a)). In the ionically-bound cell-wall fraction, specific activity had increased significantly in both the PNC2-WT4 extract (24.8 ± 0.7 nmol min^{-1} mg^{-1}) and the PNC2-M2-5 extract (13.08 ± 0.4 nmol min^{-1} mg^{-1}), with the value in the PNC2-WT extract significantly higher than the PNC2-M2-5 rate (Fig. 4(a)). To confirm that the difference in activity was due to a difference in accumulation of the PNC2-WT protein, western analysis was performed using the anti-PNC2 antibody. Here the intensity of recognition was highest in the PNC2-WT-5 ionically-bound cell wall extract where a protein with a molecular mass of ca. 34,500 Da was recognised, when compared with a protein with a molecular mass of ca. 32,500 Da in the corresponding PNC2-M2-5 extract (Fig. 4(b)).

2.2. Partial purification of WT and M2 peanut peroxidase

As a prelude to a more detailed characterisation of the PNC2-WT and PNC2-M isozymes, each was partially purified using hydrophobic interaction and then ion-exchange chromatography (Fig. 5). After each method of separation, antibody detection was used to determine the elution of the PNC2-WT or PNC2-M proteins.

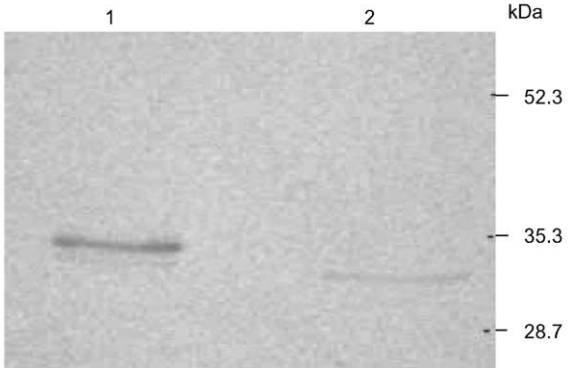
After hydrophobic interaction column chromatography, the PNC2-WT5 isozyme eluted in fractions 33–34 (at 93–95% buffer B; 100–140 mM ammonium sulfate), while the PNC2-M2-5 isozyme eluted in fractions 30–31 (at 92–96% buffer B; 80–160 mM ammonium sulfate) (Fig. 5(b)). Western analysis confirmed that proteins of the expected size were present in each fraction (data not shown). After ion-exchange, the PNC2-WT5 isozyme eluted in fractions 15–16 (at 440–470 mM NaCl) (Fig. 6), while the PNC2-M2-5 isozyme eluted predominantly in fraction 15 (460 mM NaCl) (Fig. 7) with proteins of the expected molecular masses in each fraction determined by western analysis with the anti-PNC2 antibody (Figs. 6(b) and 7(b)). Silver staining of SDS–PAGE separations of fraction 15 from the PNC2-WT5 and PNC2-M2-5 purifications revealed that the peroxidase isoforms represented the major protein present in each fraction, with only very minor contaminants of other molecular masses evident (data not shown).

2.3. Enzyme stability and function of PNC2-WT and PNC2-M2 peroxidases

The partially purified PNC2 peroxidases obtained after ion-exchange chromatography were then subjected to enzyme assays to compare some kinetic properties of the PNC2-WT and PNC2-M isozymes.

WT-5 (Sol)	M2-5 (Sol)	WT-5 (Wall)	M2-5 (Wall)
3.12 ± 0.09	4.2 ± 0.17	24.8 ± 0.7	13.08 ± 0.4

a



b

Fig. 4. (a) Peroxidase activity (as nmol min^{-1} mg^{-1} protein) in the soluble cellular (Sol) and ionically-bound cell wall (wall) extracts of leaves excised from the *prxPNC2-WT-5* and *prxPNC2-M2-5* transformants,as indicated. Values are ±SE; $n = 3$. (b) Western analysis, using 12.5% SDS–PAGE, of the ionically-bound cell wall extracts of the *35S::prx-PNC2-WT* (lane 1) or *35S::prx-PNC2-M* (lane 2) transformants, as indicated. The PVDF membranes were incubated with anti-PNC2 antibodies, and antibody recognition visualized using alkaline phosphatase conjugated secondary antibodies. The masses of standard proteins are as indicated.

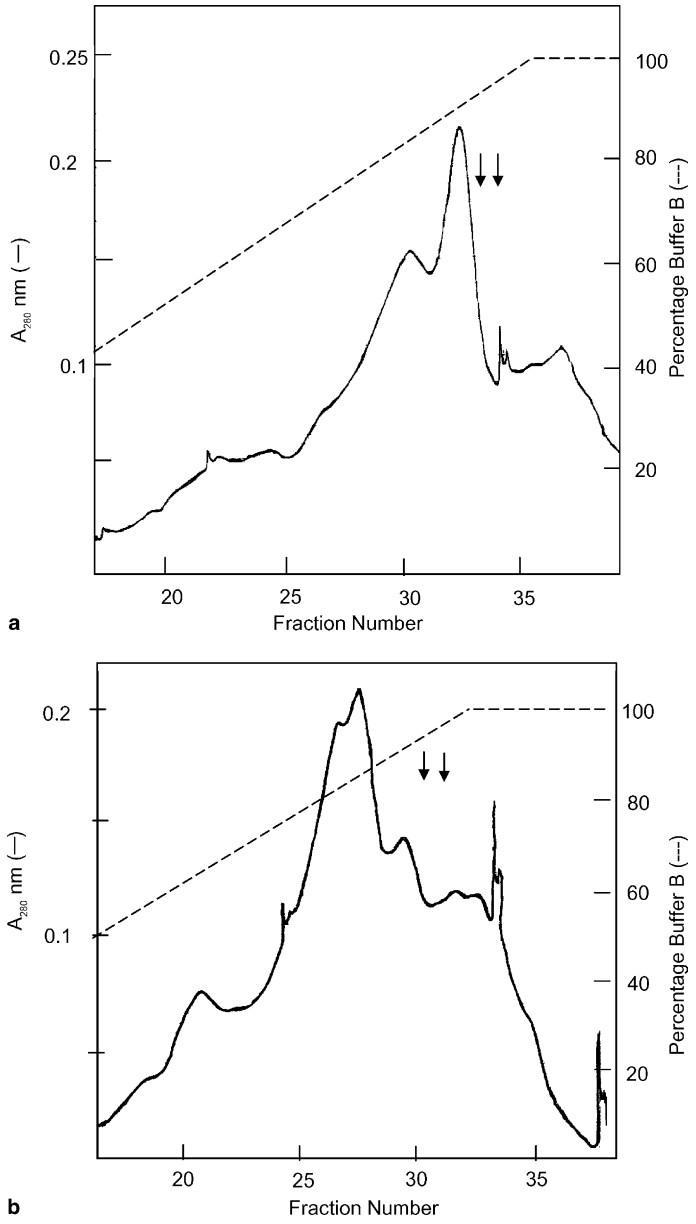


Fig. 5. Separation of protein extracts of leaves excised from the *35S::prx-PNC2-WT* transformant line PNC2-WT-5 (a) or the *35S::prx-PNC2-M* transformant line PNC2-M2-5 (b) using hydrophobic interaction chromatography. After extraction with 50 mM sodium phosphate, pH 6.0, containing 100 mM NaCl, precipitation with 85% (saturated) ammonium sulfate, Sephadex G-25 column chromatography and then adjustment of the eluate to 2.0 M ammonium sulfate, protein samples were applied to a Phenyl-Superose HR 5/5 column. Bound proteins eluted with a linear gradient of 100% buffer A (2.0 M ammonium sulphate, pH 5.6):0% buffer B (20 mM potassium phosphate, pH 7.0) to 0% buffer A:100% buffer B. The arrows denote fractions with maximum peroxidase activity that were purified further.

In terms of affinity for the substrate guaiacol, an apparent K_m value of 11.2 mM was determined for the PNC2-WT5 isoform, and 11.9 mM was determined for the PNC2-M2-5 isozyme. For H_2O_2 an apparent K_m value of 1.29 mM was determined for the PNC2-WT5 isoform, and 1.12 mM was determined for the PNC2-M2-5 isozyme (Table 1).

Arrenhius activation energy values (E_a) were also calculated for the PNC2-WT and PNC2-M isozymes, with values (kJ mol^{-1}) of 17.6 for the PNC2-WT5 isoform, and 22.9 for the PNC2-M2-5 determined (Table 1).

For the thermal stability of each PNC2 isoform, extracts were heated for 15 min at temperatures ranging from 25 to 60 °C, after which four volumes of 15 mM EDTA were added and peroxidase activity assayed at 25 °C. For the PNC2-WT5 isozyme, peroxidase activity increased from 25 to 50 °C, after which activity decreased sharply at 55 and 60 °C (Fig. 8). For the PNC2-M2-5 isozyme, the peroxidase rate increased sharply between 25 and 40 °C, after which the activity declined to be lower than the rate at 25 °C over the range of 50–60 °C (Fig. 8).

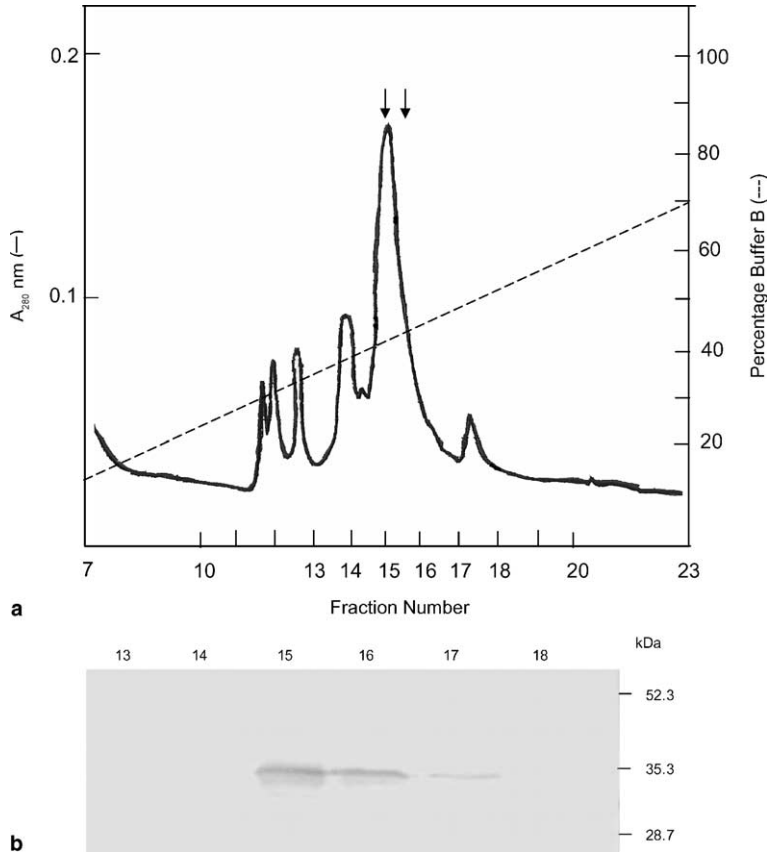


Fig. 6. (a) Separation, using Mono-S ion-exchange column chromatography, of peroxidase fractions identified after hydrophobic interaction chromatography of the *35S: prx-PNC2-WT* transformant line PNC2-WT-5. After hydrophobic interaction chromatography, peroxidase-containing fractions were dialysed against 50 mM sodium acetate, pH 4.5, applied to a Mono-S HR 5/5 ion-exchange column and bound protein were eluted within a gradient of 100% buffer A (50 mM sodium acetate, pH 4.5):0% buffer B (50 mM sodium acetate, pH 4.5, containing 1.0 M NaCl) to 0% buffer A:100% buffer B. The arrows denote fractions with maximum peroxidase activity that were used for further experiments. (b) Western analysis of the fractions, as indicated, of the Mono-S separation. The PVDF membrane was incubated with anti-PNC2 antibodies, and antibody recognition visualized using alkaline phosphatase conjugated secondary antibodies. The masses of standard proteins are as indicated.

3. Discussion

This study has sought to investigate the consequences of removing a potentially strategic *N*-linked sugar structure from an isozyme of peanut peroxidase. The sequence used in this study, *prxPNC2* (Buffard et al., 1990) contains only one site of glycosylation attachment, at the potentially strategic Asn_{189} residue (Buffard et al., 1990). To ablate the point of *N*-linked glycan attachment, the motif Asn_{189}–Gly–Thr_{191} was altered to Ala_{189}–Gly–Thr_{191} using site-directed mutagenesis. While no direct assays were performed to confirm the loss of the sugar (apart from sequence confirmation of the *prxPNC2-M* construct), the use of gradient SDS–PAGE has revealed a molecular mass of ca. 32,600 Da for the PNC2-M isozymes examined (PNC2-M2-4, PNC2-M2-5). This is in good agreement with the theoretical molecular mass of the mature PNC2 protein of 32,954 Da (Buffard et al., 1990), and suggests, additionally, that the predicted 22 amino acid hydrophobic signal sequence of PNC2 has been cleaved in the tobacco cell background. In contrast, a molecular mass of ca. 34,670 Da was calculated for the PNC2-WT isozymes that were studied (PNC2-WT-4, PNC2-WT-5) suggesting that the glycan structure accounts for ca. 2000 Da of the molecular mass. This difference in mass is in good agreement with the theoretical masses calculated for the glycan structures known to be attached to plant glycoproteins. For example, a molecular mass range of 1296 Da can be determined for the more commonly occuring Manα1 → 6(Manα1 → 3)(Xylβ1 → 2)Manβ1 → 4GlcNAcβ1 → 4(Fucα1 → 3) GlcNAc structure (McManus et al., 1988; Harthill and Ashford, 1992; van Huystee et al., 1992; Gray et al., 1996; Welinder and Larsen, 2004), designated as the paucimannosidic type (Lerouge et al., 1998), while a mass of 2622 Da can be calculated for a larger complex-type glycan structure (Lerouge et al., 1998) proposed to be attached to Asn_{185} on the PNC1 peanut peroxidase (van Huystee et al., 1992). Given that the larger complex-type sugar chains are proposed to be attached to the secreted glycoproteins (rather than the vacoule-bound paucimannosidic

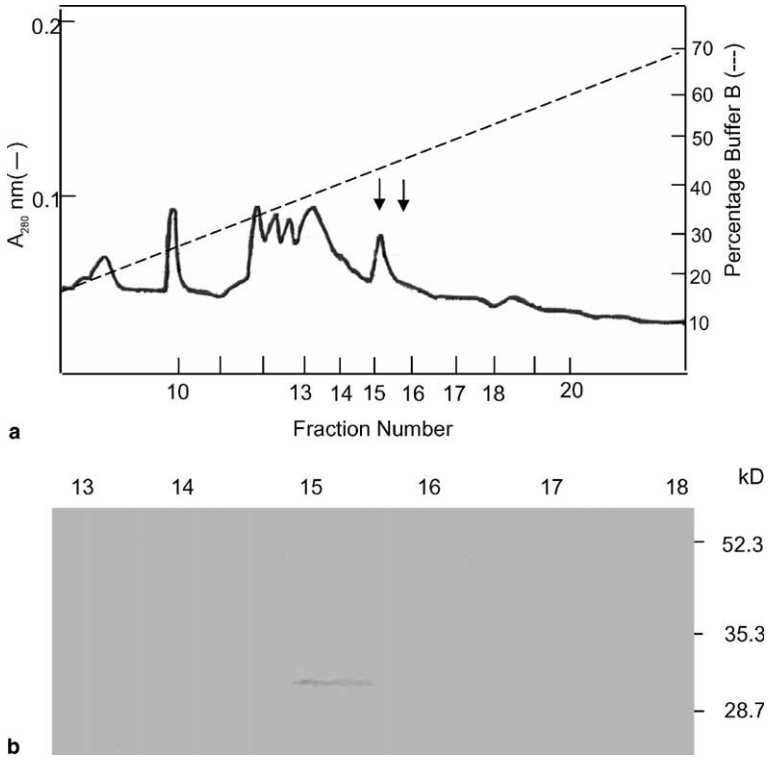


Fig. 7. (a) Separation, using Mono-S ion-exchange column chromatography, of peroxidase fractions identified after hydrophobic interaction chromatography of the *35S::prx-PNC2-M* transformant line PNC2-M2-5. After hydrophobic interaction chromatography, peroxidase-containing fractions were dialysed against 50 mM sodium acetate, pH 4.5, applied to a Mono-S HR 5/5 ion-exchange column and bound protein were eluted within a gradient of 100% buffer A (50 mM sodium acetate, pH 4.5):0% buffer B (50 mM sodium acetate, pH 4.5, containing 1.0 M NaCl) to 0% buffer A:100% buffer B. The arrow denotes the fraction with maximum peroxidase activity that was used for further experiments. (b) Western analysis of the fractions, as indicated, of the Mono-S separation. The PVDF membrane was incubated with anti-PNC2 antibodies, and antibody recognition visualized using alkaline phosphatase conjugated secondary antibodies. The masses of standard proteins are as indicated.

Table 1
Summary of properties of the PNC2-WT and PNC2-M peroxidase isozymes

	PNC2-WT	PNC2-M
Chromatographic properties		
Hydrophobic interaction elution [mM ammonium sulfate]	100–140 mM	80–160 mM
Ion-exchange elution [mM NaCl]	440–470 mM	460 mM
Kinetic parameters		
Apparent K_m		
Guaiacol (mM)	11.2	11.9
H_2O_2 (mM)	1.29	1.12
Activation energy (kJ mol^{-1})	17.6	22.9

structures; Lerouge et al., 1998), this suggests that a sugar moeity of ca. 2000 Da may be attached to the PNC2 isoenzyme.

To characterise the PNC2-WT and PNC2-M isozymes further, we have partially purified each isozyme. To begin with, the use of the CaMV35S promoter will induce a high level of gene transcription, and should result in significant accumulation of the PNC2-WT and PNC2-M isozymes in the tobacco extracts. Comparison of the specific activities of soluble extracts from the *35S::prxPNC2-WT* and *35S::prxPNC2-M* transformants with similar extracts from wild-type tobacco revealed that the activity was ca. 50-fold higher in the transformants (data not shown). Further, the extracted peroxidases were partially purified using a strategy that separated proteins based on two independent properties, surface hydrophobicity and surface charge (ion-exchange), and anti-PNC2 antibodies were used to detect the peroxidases during separation. Extracts of wild-type tobacco have been separated using hydrophobic interaction chromatography, and little or no peroxidase activity has eluted at the same point within the gradient as observed for the PNC2-WT isozyme. We conclude, therefore, that the purification process has produced fractions that are very highly enriched for the PNC2-WT and PNC2-M isozymes.

The role of *N*-linked glycans on plant peroxidases has been brought into focus with the characterisation of the *cgl* mutant of *Arabidopsis* that contains (as determined by antiserum binding) no complex-type *N*-linked glycans (von Schaewen et al., 1993). Complementation studies revealed that the mutant lacks a functional GNT1 enzyme, and so the first step in the conversion

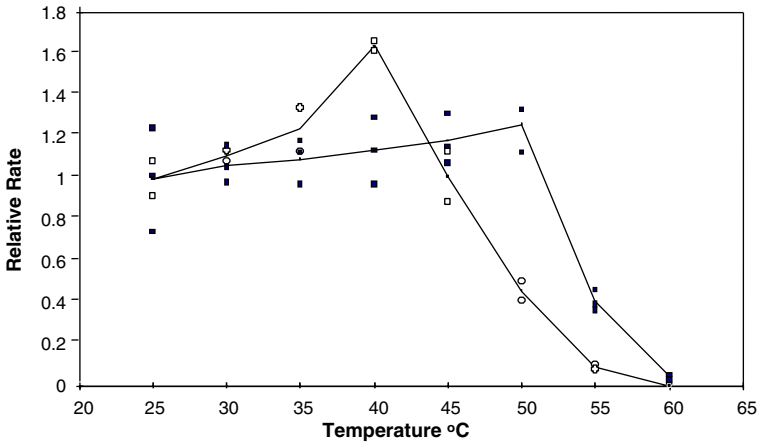


Fig. 8. Thermostability of the partially purified peroxidases after ion-exchange column chromatography. Fractions were incubated for 15 min at the temperature indicated, after which 4 volumes of 15 mM EDTA were added and activity assayed under standard conditions at 25 °C. Values are scatter plots of the data from one assay. The assay was repeated three times. PNC2-WT-5 (-■-); PNC2-M2-5 (-□-).

of high-mannose chains to complex types is blocked (Gomez and Chrispeels, 1994). However, the mutant can complete its life cycle normally, suggesting that glycosylation per se is important, but the addition of high-mannose instead of complex chains appears to have little effect on protein function, as determined by plant phenotype.

In terms of plant peroxidases, the published evidence suggests a role for the *N*-linked glycan structures. Using enriched preparations, we have shown that complete removal of the *N*-linked glycan resulted in an increased activation energy and a decrease in the thermostability of the PNC2-M isozyme. The observation of the increased activation energy is consistent with the observation using the anionic peroxidase of peach seed (Tigier et al., 1991), while the decrease in thermostability observed for PNC2-M is consistent with similar findings for the partially deglycosylated peach seed anionic peroxidase, the avocado leaf peroxidase, and the neutral peroxidase of turnip (Tigier et al., 1991; Sanchez-Romero et al., 1994; Duarte-Vazquez et al., 2003). In a study of the major cationic peroxidase of peanut, PNC1, attachment of the sugar at the equivalent *N*-linked site (Asn_{185}) has also resulted in a decrease in the thermostability of the protein (Lige et al., 2001).

Partial or complete deglycosylation of peroxidases has also been shown to influence the affinity of the enzyme for various substrates (as determined by changes in the measured apparent K_m), although there is no commonly emerging trend. For H_2O_2, partial deglycosylation of the peroxidase of avocado leaf, and complete deglycosylation of the peroxidase of turnip resulted in a decrease in the K_m (Sanchez-Romero et al., 1994; Duarte-Vazquez et al., 2003), while partial glycosylation of an anionic peroxidase from peach seeds resulted in an increase in K_m. For the reductor substrates, a decrease in K_m has been observed for a partially deglycosylated peroxidase of avocado (Sanchez-Romero et al., 1994), and a fully deglycosylated peroxidase of turnip (Duarte-Vazquez et al., 2003). For PNC2, no difference in the apparent K_m for H_2O_2 or the reductor substrate assayed, guaiacol, was observed when PNC2-WT is compared with PNC2-M, suggesting that the glycan attached to the Asn_{191} residue is important primarily in enzyme stability, as determined by the observed differences in thermostability.

In terms of glycoproteins generally in plants, another well established role for the *N*-linked glycan structure is in mediating the efficient secretion of the glycoprotein into the cell wall (Ravi et al., 1986; Driouich et al., 1989; Faye and Chrispeels, 1989; Lerouge et al., 1996; Pagny et al., 2003). In these experiments, the role of these sugar moeites for secreted proteins was determined principally using specific inhibitors of glycan processing, including tunicamycin, that abolishes *N*-linked glycosylation within the secretory system. Further, experiments with the glycan processing inhibitor, castanospermine suggest that glycosylation per se is important for extracellular secretion of glycoproteins, but this process may be independent of oligosaccharide structure (Lerouge et al., 1996). The use of site-directed mutagenesis, therefore, affords the possibility of investigating the role of *N*-linked glycosylation on glycoprotein secretion without the use of externally applied inhibitors.

For the major secreted cationic peroxidase of peanut, Lige et al. (2001) were able to purify recombinant peroxidases (each with any one of three *N*-linked sites ablated) from cell suspensions made from transformed tobacco tissue. The occurence of these mutated forms of peroxidase in the growth media suggest that ablation of a single *N*-linked site (of the three) is not sufficient to prevent secretion, although these researchers did not determine whether any reduction in accumulation had occurred (Lige et al., 2001). In the current study, where we have

ablated the single *N*-linked site attached to PNC2, both the wild-type PNC2-WT and mutated (de-glycosylated) PNC2-M isoforms have been determined to accumulate preferentially in the ionically-bound cell wall fraction when compared with the soluble fraction. However, the relative accumulation of PNC2-WT in the ionically-bound fraction when compared with the soluble fraction (ca. 8-fold) is higher than the relative accumulation of PNC2-M in the ionically-bound and soluble fractions (ca. 3-fold). This suggests that the PNC2-M isoform is being degraded at a faster rate in the ionically-bound fraction than the PNC2-WT protein. It would be relevant, therefore, to extend this investigation into comparing the turnover rate of PNC2-WT with PNC2-M, perhaps by using protein labelling in vivo, or to establish cell suspension cultures from both transformants to determine if the PNC2-M protein is secreted into the medium.

Taken together, our study has shown that the loss of the single, potentially strategic *N*-linked glycan (equivalent to Asn_{185} on HRP-C) has primarily influenced the thermostability of the PNC2 isoenzyme, as well as the thermal activation energy. Deglycosylation may also have influenced the relative accumulation of PNC2-M in the cell wall when compared with the PNC2-WT isoform. The loss of the glycan has not resulted in complete loss of activity suggesting that for this enzyme, at least, *N*-linked glycosylation is part of a probable array of post-translational 'fine-tuning' of the enzyme that together optimise enzyme targeting, stability and function.

4. Experimental

4.1. Site-directed mutagenesis and plant transformation

Site-directed mutagenesis of the *prxPNC*2 cDNA was carried out using the method as described by Dong and Nickoloff (1992) to modify the codon coding Asn_{189} (AAT) to Ala_{189} (GCT) (Fig. 1). Sequencing was used to confirm the modified sequence. After this modification, the 990 bp coding sequences (including the 69 bp signal sequence) of *prxPNC2-WT* and *prxPNC2-M* were then amplified by PCR using MMM-7 as forward primer (5′-CGGAATTCACAATG GAGGGTGTTT-TCAAC-3′) to provide a 5′ *Eco*RI site, and MMM-2 as the reverse primer (5′-CGGTCTAGATTAGT-TAAAAGCAGAACAAATCTTGC-3′) to provide a 3′ *Xba*I site. After amplification, the coding sequence were then cloned into the pART7 plant transformation vector (Gleave, 1992) to provide the plant transformation vectors *CaMV35S::prxPNC2-WT* and *CaMV35S::prx-PNC2-M* (Fig. 7). Details of transformation into *Agrobacterium tumefaciens* LBA 4404, and transformation of tobacco was carried out as described in McManus et al. (1999).

4.2. Genome DNA isolation and PCR amplification

Genomic DNA was isolated from young tobacco leaf tissue using the PLANT-DNA™ kit (Sigma Chemicals, St Louis, Mo, USA). One microgram of DNA was used as template for PCR, with MMM-7 and MMM-2 used as primers. PCR conditions were: 92 °C for 10 min, then 30 cycles of 94 °C for 40 s, 60 °C for 30 s, 72 °C for 1 min, then 72 °C for 10 min, after which the amplified products were separated through a 1.0% (w/v) agarose gel and revealed using ethidium bromide staining.

4.3. Peroxidase extraction and partial purification

To extract peroxidases as 'soluble' and 'ionically-bound cell wall' fractions, leaf tissue from transformants or wild-type plants was homogenised with 25 mM 2-mercaptoethanol, and the slurry centrifuged at 14,000*g* for 5 min at 4 °C. The supernatant was then decanted and designated the 'soluble' fraction, and pellet resuspended with fresh 25 mM 2-mercaptoethanol, and then centrifuged as before. The supernatant was again reserved, and the pellet resuspended and centrifuged as described for a further 6 to 8 times. After the final wash, the pellet was resuspended with 1.0 M NaCl, incubated at 37 °C for 60 min, centrifuged as before and the supernatant reserved and designated as the 'ionically-bound cell wall' fraction. For each supernatant, including the 2-mercaptoethanol washes, the protein content was measured using the method of Bradford (1976).

For extraction of peroxidases for gradient SDS–PAGE and for further purification, leaf tissue was extracted in 50 mM sodium phosphate, pH 6.0, containing 100 mM NaCl, at a ratio of 1.0 g FW: 3.0 mL buffer, and the slurry centrifuged at 20,000*g* for 15 min. Extracts in the supernatant were either subjected to gradient SDS–PAGE, or purified further. For further purification, the supernatant was adjusted to 80% saturated ammonium sulfate, incubated on ice for 30 min and then the precipitated protein collected by centrifugation at 20,000*g* for 15 min. The protein pellet was then exchanged into 20 mM potassium phosphate, pH 7.0 using Sephadex G-25 (Amersham Pharmacia Biotech, Uppsala, Sweden), and protein in the eluate adjusted to 2.0 M ammonium sulphate, before application to a Phenyl-Superose HR 5/5 column (Amersham Pharmacia Biotech). Bound proteins eluted with a linear gradient of 100% buffer A (2.0 M ammonium sulphate, pH 5.6):0% buffer B (20 mM potassium phosphate, pH 7.0) to 0% buffer A:100% buffer B. Fractions containing maximum peroxidase activity were dialysed against 50 mM sodium acetate, pH 4.5, applied to a Mono-S HR 5/5 column (Amersham Pharmacia Biotech) and bound protein were eluted within a gradient of 100% buffer A (50 mM sodium acetate, pH 4.5):0% buffer B (50 mM sodium acetate, pH 4.5, containing 1.0 M

NaCl) to 0% buffer A:100% buffer B. Fractions containing peroxidase activity were identified and used for further experiments.

4.4. SDS–PAGE and western analysis

SDS–PAGE was carried out essentially as described by Laemmli (1970). For gradient electrophoresis, the methodology and molecular weight determination was carried out as described in Hames and Rickwood (1981). Western analysis and the production of antibodies to PNC2 was carried out as described in Watson et al. (1998).

4.5. Peroxidase enzyme assays

To identify peroxidase activity in column eluates, assays were conducted in 96-well plates using 0.05% (w/v) *o*-phenylenediamine and 0.03% (v/v) H_2O_2 essentially as described in Watson et al. (1998). For studies with the partially purified enzyme, and unless specified otherwise, the standard assay comprised 20 mM guaiacol, 10 mM H_2O_2 in 50 mM sodium phosphate buffer, pH 6.0, at 25 °C. Accumulation of tetraguaiacol was measured at 470 nm, where $\epsilon_{470} = 26.6\ mM^{-1}\ cm^{-1}$. To determine the Arrhenius constant, standard assays were performed over a temperature range of 10–35 °C, and the log of each reaction rate plotted against 1/Absolute temperature (K). The Arrhenius constant was then calculated using the formulae outlined in Morris (1968).

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