THE GLYCOSYLATION STATUS AND THE ROLE OF CARBOHYDRATE MOIETIES IN THE HETEROGENEITY OF CUCUMBER ANIONIC VIRUS-INDUCIBLE PEROXIDASE

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Summary. – Three forms of anionic peroxidase (PRX) from hypersensitively reacting cucumber cotyledons were purified to homogeneity and different methods were used to analyze the nature of their carbohydrate chains. Immunoblot analysis with βF1 antiserum showed that all three forms are highly glycosylated and contain asparagine N-linked glycans commonly found in other plant glycoproteins. Mobility shift analysis showed that chemical deglycosylation converted PRXs 1, 2 and 3 to the same-sized (35 K) products. Enzymatic deglycosylation with α-mannosidase converted PRX1 and PRX2 to immunoreactive products migrating in mobility shift polyacrylamide gels at the positions of PRX2 and PRX3, respectively. PRX3 treated with α-mannosidase yielded a product with Mr similar to that obtained with the chemical deglycosylation. Cleavage of the PRXs 1, 2 and 3 by formic acid at the Asp-Pro site resulted in peptide maps and the putative glycopeptide(s) were recognized using βF1 antiserum. Only one glycopeptide was observed for each of the forms. Lectin-affinity blot analysis using biotin-conjugated lectins suggested that virus-inducible PRX contains complex-type N-glycosyl carbohydrate chain(s). These results indicate that heterogeneity of cucumber virus-inducible PRX is not caused mainly by differences in the terminal α-linked mannose residues.

Key words: cucumber; Cucumis; deglycosylation; glycopeptides; immunoblot analysis; lectin-affinity blot analysis; polyacrylamide gel electrophoresis; tobacco necrosis virus

Introduction

PRXs (EC 1.11.1.7) are ubiquitous proteins and their numerous isoforms have been purified from higher plants and characterized (Aibara *et al.*, 1982; Hendriks and Van Loon 1990; O´Donnell *et al.*, 1991, Gazaryan and Lagrimini, 1996). In cucumber (*Cucumis sativus* L.), the fast-moving, virus-induced, anionic PRX is a soluble glycoprotein, most prominently present in the apoplast of hypersensitively-reacting cotyledons (Repka and Slováková, 1994). Upon polyacrylamide gel electrophoresis (PAGE) of intercellular fluid (ICF) from virus-inoculated cotyledons under native conditions this PRX is visualized as a group of three enzymatically active bands. More recently, it has been demonstrated that in addition to their accumulation after pathogen attack or other type of stress, these isoforms show distinct patterns of development-, organ- and cell-type-specific expression (Repka and Jung, 1995; Repka and Fischerová, 1996, 1998; Repka *et al.*, 1996, 1997, 2000).

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Abbreviations: Con A = concanavalin A; GSA = *Griffonia simplicifolia* agglutinin I; HR = hypersensitive response; HRP = horseradish peroxidase; ICF = intercellular fluid; NC = nitrocellulose; PAGE = polyacrylamide gel electrophoresis; PRX = peroxidase; RCA-I = *Ricinus communis* agglutinin I; SDS = sodium dodecyl sulfate; TBS = 10 mmol/l Tris-HCl pH 7.4 plus 150 mmol/l NaCl; TFMS = trifluoromethane sulfonic acid; TNV = tobacco necrosis virus; UEA-I = *Ulex europeans* agglutinin I; WGA = wheat serum agglutinin

The virus-inducible PRX of cucumber was purified and its three molecular forms (PRXs 1, 2 and 3) were shown to have the same isoelectric point (4.3), but they differed slightly in Mr (PRX1, 40.7 K; PRX2, 38 K; PRX3, 37.1 K) (Repka and Slováková, 1994). Since there was an asynchronous expression pattern observed either in pathological- or development-regulated situations, it was suggested that the molecular heterogeneity of a cucumber virus-inducible PRX must result from posttranscriptional modification of a primary gene product (s). So far nothing is known about the nature of this heterogeneity but there are data indicating that it may be related to the carbohydrate moieties of the isoforms, and that their developmentally- and/or stress-controlled modification might be, at least partially caused by the removal of sugar residues or after its secretion into the apoplast.

Here we tested this hypothesis by investigating the glycosylation status of purified molecular forms of virus-inducible PRX and tried to find out whether the molecular heterogeneity of this PRX could be abolished by removal of its carbohydrate chain (s).

Material and Methods

Plant material. Cucumber plants (*Cucumis sativus* L., *cv.* Laura) were grown from seeds in a greenhouse under controlled conditions for 7 days (Repka, 1996) unless otherwise stated.

Virus and inoculation procedure. Tobacco necrosis virus (TNV) strain D lacking satelite virus was used. At an age of 7 days, plants with well developed cotyledonary leaves were transferred to a growth chamber (22°C) and dusted with carborundum. Inoculation with a partially purified suspension of TNV was done essentially as described previously (Repka and Slováková, 1994). Control plants were treated either with virus isolation buffer alone or were not treated.

Protein extraction. Intercellular fluid (ICF) extracts were obtained either from healthy or virus-inoculated cotyledons by vacuum infiltration procedure as described elsewhere (Repka *et al.*, 1993). Then the ICF extracts were centrifugally concentrated using the Centriprep-3 concentrator (Amicon), passed through Sephadex G-25 PD-10 minicolumns (Pharmacia) and stored frozen at -20°C.

Protein concentration was determined according to Bradford (1976) with bovine serum albumine as a standard.

Purification of virus-inducible PRX isoforms. Immediately after isolation, ICF (120 ml) was centrifuged for 30 mins at 20,000 x *g* and the supernatant was adjusted to 80% saturation with solid ammonium sufate. Proteins were precipitated overnight at 4°C, collected by centrifugation at 20,000 x g for 10 mins and finally dissolved in 12 ml of sterile distilled water. The suspension was throughly dialyzed against sterile deionized water. After centrifugation at 20,000 x g for 10 mins, the supernatant was finally dialyzed against 200 ml of A buffer (500 mmol/l Tris-HCl pH 6.8). Individual isoforms of virus-inducible anionic PRX were further purified to electrophoretical homogeneity using the MiniPrep Cell device (Bio-Rad). A preparative tube containing 10% non-denaturing polyacrylamide gel was loaded with 0.35 ml of concentrated proteins and the separation was performed at 4°C for 8 hrs at 50 mA. Fractions of highly purified isoforms (RZ 3) of virus-inducible PRX thus obtained were stored frozen at -20°C until used.

PAGE of purified isoforms was performed under denaturing conditions on an 1.5 mm thick slab gel using a 4% stacking gel and a 12.5% separating gel according to Laemmli (1970). Each lane of the gel was loaded with equal amount of protein and bands were visualized by silver staining according to Blum *et al.* (1987).

Western blot analysis. Electrophoreticaly separated proteins were alternatively electroblotted onto a nitrocellulose membrane (0.45 μm, Protran BA-85, Schleicher and Schuell) using 0.04 mmol/l sodium phosphate pH 6.5 as a transfer buffer. The blots were blocked for 2 hrs in 1 x TEN buffer (50 mmol/l Tris-HCl pH 7.4, 5 mmol/l EDTA, and 150 mmol/l NaCl) containing 5% Blotto (non-fat dry milk) and incubated for 7 hrs with a cucumber PRX antiserum diluted 1:100 in the blocking buffer. The blots were then washed for 3 x 10 mins in 1 x TEN with 1% Tween 20, incubated for 1 hr with diluted (1:1000) swine anti-rabbit IgG HRP-conjugated secondary antibodies (SwAR-HRP, Sevac), and washed again with 1 x TEN buffer plus 1% Tween 20 for 4 x 10 mins. For antibody detection we used 0.03% DAB (Sigma), 0.03% H_2O_2 and 0.03% $CoCl_2$ as an intensifier. Low-molecular mass standards (Bio-Rad) on blots were stained with 0.1% Amido Black.

Detection of glycan residues attached to PRX. To detect glycan residues attached to PRX, the immunoblots prepared as described above were incubated with a 1:1000 dilution of Glyc antiserum for 7 hrs. A peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Labs, Gaithersburg, USA) was applied as a second antibody at a dilution of 1:1000 in 1 x TEN buffer for 1 hr. The immunoblots were developed with a mixture containing 0.02% 4-chloro-1-naphtol, 0.03% DAB, 0.03% $CoCl_2$, 5% methanol and 0.01% H_2O_2 in PBS. The reaction was terminated after 5–10 mins by several washes with distilled water.

Chemical deglycosylation. For chemical deglycosylation, portions of 30 μg of PRX isoforms were precipitated with five volumes of 80% acetone for 24 hrs at -20°C. Then the glycosidic side chains were removed using TFMS as described by Edge *et al.* (1981) with a minor modification. The precipitated PRXs were centrifuged at 10,000 x g for 10 mins and vacuum dried. Sixty μl of TFMS-anisole (2:1) cooled to 0°C was added to the dried pellets and nitrogen was bubbled through the mixture for 30 secs. The samples were placed on ice for 5 hrs. Sugars and reagents were removed by extracting the mixture 3 times with 2 volumes of ether and an 1 volume of 50% aqueous pyridine (vortexing was used to dissolve the pyridine salts each time). The upper ether phase was removed each time and the water phase, after three extractions, was dialyzed against distilled water. The deglycosylated proteins were then precipitated with 80% acetone for 2 hrs at -20°C. After centrifugation at 10,000 x g for 10 mins, the pellet was dissolved in the Laemmli's sample buffer (Laemmli, 1970), heated at 100°C for 5 mins and analyzed by SDS-PAGE and immunoblot analysis.

Enzymatic deglycosylation. Portions (10 μg) of acetone-precipitated PRXs (see above) were incubated with jack bean α1-2,3,6 mannosidase (2U per sample, Oxford Glycosciences,

Abingdon, UK) in 100 µl of 100 mmol/l sodium acetate buffer pH 5.0 containing 2 mmol/l $ZnSO_4$. All incubations were performed at 37°C for 24 hrs. After incubation, proteins were precipitated with acetone and analyzed as described above. Controls without hydrolytic enzymes were incubated and analyzed similarly.

Lectin-affinity blot analysis. Biotin-conjugated lectins of concanavalin A (Con A), *Ulex europaeus* agglutinin I (UEA-I), *Ricinus communis* agglutinin I (RCA-I), *Griffonia simplicifolia* agglutinin II (GSA-II) and wheat germ agglutinin (WGA) (Vector Laboratories, Burlingame, USA) were used. Portions of about 2.5 µg PRX were precipitated with 80% acetone containing 0.5% HCl. This treatment removes the heme group, thereby inactivating the enzyme (Shannon *et al.*, 1966). The protein pellets were collected by centrifugation at 10,000 x g for 10 mins, vacuum dried and dissolved in 10 µl of deionized water. The obtained samples were spotted on a NC membrane using a slot blot apparatus (PR 648, Hoefer Scientific, Inc., San Francisco, USA). After drying, the blots were blocked by immersion for 1 hr in 10 mmol/l Tris-HCl pH 7.4 plus 150 mmol/l NaCl (TBS) spplemented with 1% BSA and further incubated for 16 hrs in 1 ml of TBS containing 1% BSA and biotin-conjugated lectin (10 µg/ml). For the incubation with Con A-biotin, the TBS was supplemented with a mixture of 1 mmol/l $CaCl_2$ and 1 mmol/l $MnCl_2$. Then the blots were rinsed 3 times (10 mins per rinse) with TBS (including $CaCl_2$ and $MnCl_2$ for Con A) and further incubated for 1 hr in a solution containing streptavidin-conjugated horseradish PRX (2 µg/ml, Vector Laboratories, Inc., Burlingame, USA) in TBS. The conjugate solution was subsequently decanted, and the blots were rinsed with TBS 3 times (10 mins each, with gentle shaking prior to decanting). The blots were then developed in a substrate solution (0.05% DAB, 0.03% $CoCl_2$ and 0.03% H_2O_2 in TBS) for 1–5 mins until a black color appeared, rinsed thoroughly with distilled water and air dried. A control incubation was performed either in the presence of 200 mmol/l lectin-specific inhibiting sugars methyl-α-D-mannopyranoside (Con A), α-L-fucose (UEA-I), α-D-galactose (RCA-I), N-acetyl-α-D-glucosamine (GSA-II and WGA) or with the deglycosylated substrate proteins.

Protein cleaving with formic acid. The glycoproteins, isolated as shown above and freed of heme, were incubated in 70% formic acid (0.1 mg protein/ml) at 37°C for 96 hrs according to a modified protocol described by Jaurequi-Adell and Marti (1975). After the incubation, the mixture was neutralized with 10 N NaOH, diluted to a final volume of 2 ml with water and dialyzed for 5 hrs against water. The cleaved products were precipitated with acetone at -20°C overnight and solubilized with the Laemmli's sample buffer at 100°C for 5 mins. The cleaved samples were electrophoresed alongside with the pre-stained standards (See Blue, Novex Inc., San Diego, USA) and blotted as described for Western blot analysis. The proteins blotted on a NC membrane were then immuno-decorated either with anti-PRX or anti-Glyc (βF1) serum.

Videodensitometry. The gels or processed blots were scanned using the HP-ScanJet 3200C color scanner and after the conversion to a grey-scale mode the files were further processed using the Adobe PhotoDeluxe v.2.0 software (Adobe Systems Inc., San José, USA). Linear peptide maps were constructed directly from scanned gels and/or blots using the Phoretix 1D advanced electrophoresis image analysis software v.1.1 (Phoretix International, Newcastle upon Tyne, UK).

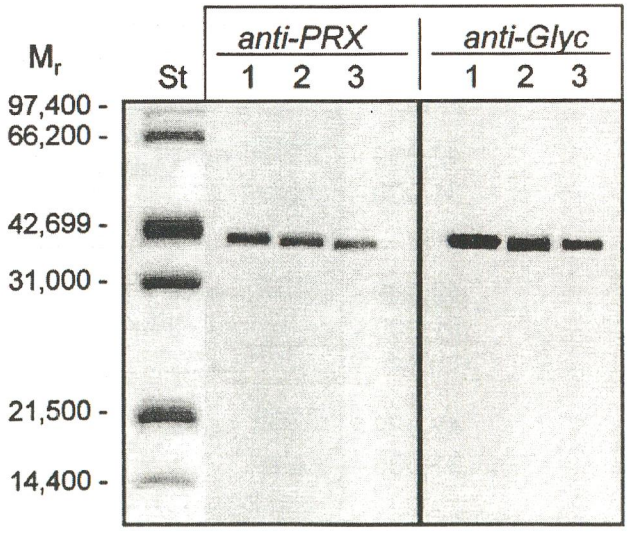

Fig. 1
Glycosylation status of virus-inducible anionic PRX isoforms purified from cucumber cotyledons
Individual purified isoforms of virus-inducible PRX (1 µg per lane) were separated by SDS-PAGE, blotted onto NC membrane, and probed either with the PRX antiserum (at a dilution of 1:100) for 2 hrs or with the βF1 antiserum (anti-Glyc), specific for the xyloseβ1-2 mannose structure, (at a dilution of 1:1000) for 2 hrs. Protein size markers (lane St).

Results

The glycoprotein nature of cucumber virus-inducible PRX

The three molecular forms of cucumber virus-inducible anionic PRX were purified to homogeneity and pure fractions of these three isoforms (PRX1, PRX2, and PRX3) were immunologically demonstrated using the PRX antiserum as shown by the single band in lanes 1, 2 and 3 of Fig. 1. These pure samples were further used in the characterization of the glycosylation status of cucumber virus-inducible PRX. The nature of the carbohydrate chain(s) was analyzed by immunoblot analysis using the βF1 antiserum that specifically recognized the xylose β1→2 mannose structure commonly found in the complex asparagine N-linked glycans of plant glycoproteins. All three virus-inducible PRX isoforms purified from cucumber reacted with this specific antiserum confirming the presence of a complex-type carbohydrate chain(s) attached to the protein (Fig. 1).

Chemical deglycosylation

Purified PRX1, PRX2 and PRX3 were chemically deglycosylated by the treatment with TFMS/anisole and

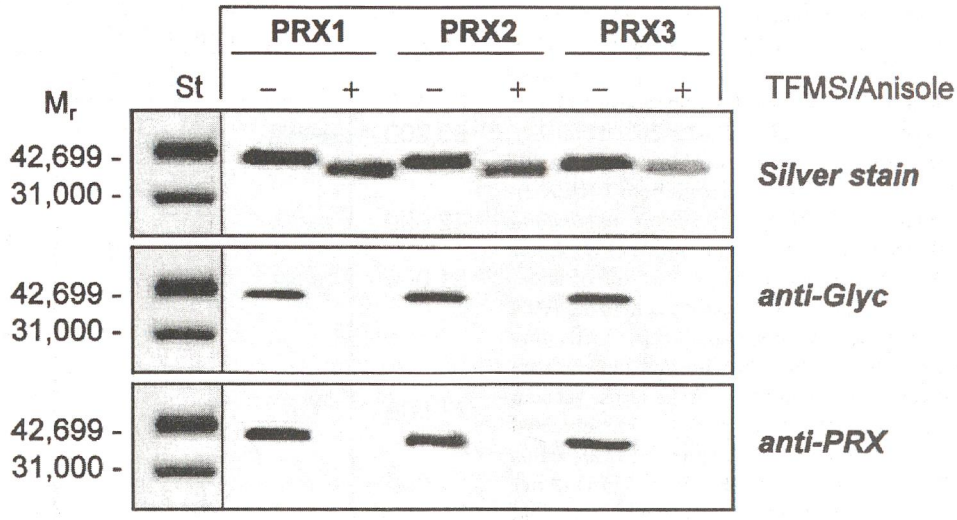

Fig. 2
Chemical deglycosylation and mobility shift analysis of virus-inducible anionic PRX
Purified isoforms PRXs 1, 2 and 3 were chemically deglycosylated by treatment with TFMS/Anisole and together with the native proteins (–) subjected to SDS-PAGE (0.5 μg per lane) and visualized by silver staining. Alternatively, the proteins treated as above were blotted and probed either with the PRX antiserum or the βF1 antiserum (anti-Glyc) for the presence of glycan residues.

together with their native counterparts subjected to SDS-PAGE and visualized by silver staining. Fig. 2 shows that this treatment converted all three isoforms to the products with the same Mr (about 35 K), well below the position of native PRX1 (40.7 K), PRX2 (38.0 K) and PRX3 (37.1 K).

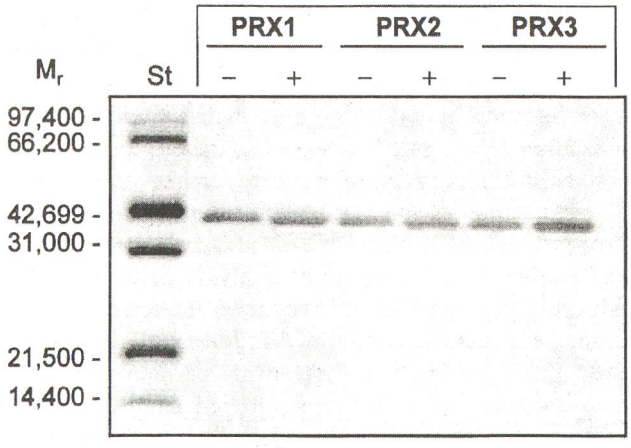

Fig. 3
Enzymatic deglycosylation and mobility shift analysis of virus-inducible anionic PRX
Purified isoforms PRXs 1, 2 and 3 were incubated with (+) or without (–) jack bean α-mannosidase. After the treatment the proteins (0.5 μg per lane) were separated by SDS-PAGE and subjected to immunoblot analysis using the antiserum specific for virus-inducible PRX. Protein size markers (lane St).

Furthermore, the removal of the glycan residue by the treatment with TFMS/anisole was apparently complete since the deglycosylated products were no longer recognized by the xylose-specific antiserum (Fig. 2). The same pattern was observed when the corresponding blot was processed with the PRX antiserum (Fig. 2), indicating that the antiserum specifically recognized antigenic determinant(s) located in the glycosidic side chain(s).

Enzymatic deglycosylation

To avoid the harsh conditions necessary for chemical deglycosylation an enzymatic hydrolysis was attempted, using α-mannosidase. Upon incubation of the native isoforms of virus-inducible anionic PRX with jack bean a-mannosidase (Man1→2,3,6Man) they were all reduced to a single band (Fig. 3). The immunoblot analysis with the PRX antiserum revealed that the reduced form of PRX1 occupied the position corresponding to the non-reduced form of PRX2 and the reduced form of PRX2 occupied the same position as the non-reduced form of PRX3. Moreover, the mobility shift analysis also showed that the treatment of PRXs 1, 2 and 3 with α-mannosidase very slightly reduced the Mr of these isoforms which represented 0.2, 0.1 and 0.2 K for PRXs 1, 2 and 3, respectively. Thus, this result confirmed that the carbohydrate chain(s) contain(s) α-linked mannose in a terminal position but its removal did not substantially abolish the heterogeneity of cucumber virus-inducible PRX.

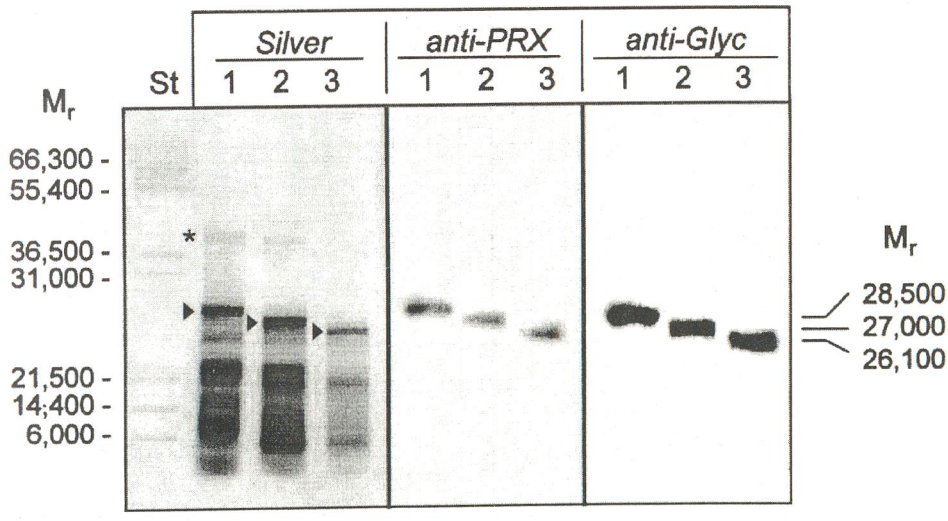

Fig. 4

Cleavage maps and glycopeptide identification after the formic acid treatment

Cleavage products of individual PRX isoforms were separated by SDS-PAGE and visualized by silver staining. The aliquots of the same peptide mixtures were subjected to SDS-PAGE, blotted and probed either with the PRX or βF1 (anti-Glyc) antiserum for the presence of glycan residues. Small triangles denote the position of putative glycopeptides in the peptide maps. Small asterisk denotes the position of intact, uncleaved PRX proteins.

Protein cleavage and identification of a putative glycopeptide

Further analysis of the glycans of the virus-inducible anionic PRX was accomplished by treating purified isoforms with 70% formic acid. The protein products were reisolated, subjected to SDS-PAGE and stained with silver or subjected to immunoblot analysis either with the PRX or βF1 antiserum. Cleavage of PRXs 1, 2 and 3 by formic acid at the Asp-Pro site resulted mainly in 3 stained bands per isoform widely differing in M_r and intensity of staining (Fig. 4). The heterogeneity of resulting peptide maps was further examined after PAGE by densitometric scaning of the bands from this gel. Three major and a few minor peaks were obtained (Fig. 5). The immunoblot analysis (Fig. 4) clearly showed that there was only one glycopeptide per molecule of each isoform and their pattern of distribution over the gel resembled that of non-deglycosylated PRXs 1, 2 and 3 (Fig. 1), suggesting that the three isoforms differed in the degree of glycosylation. Based on the data mentioned above the computer-assisted linear peptide map was constructed for each of the form of PRX (Fig. 6). Since there were differences in particular peptide maps it is evident that the degree of glycosylation is not the only source of the heterogeneity of virus-inducible PRX.

Lectin-affinity analysis

The nature of the carbohydrate chains was further analyzed by lectin-affinity blot analysis using biotin-conjugated lectins that specifically recognize certain sugar configurations. PRXs 1, 2 and 3 reacted only with UEA-I and RCA-I (Fig. 7), indicating that carbohydrate chain(s) contain(s) a fucose that must be linked to the first N-acetylglucosamine residue in the inner core and galactose is present at a terminal position linked to N-acetylglucosamine, respectively. The specificity of this affinity was confirmed by the use of a corresponding inhibitory sugars (α-L-fucose and α-D-galactose). The other lectins tested, Con A, WGA and GSA-II did not show any affinity. Apparently, the lack of affinity for these lectins was due to the fact that PRX carbohydrate chains lack the structural features required for recognition by the lectins. These results suggest that the carbohydrate chain(s) of virus-inducible PRX is (are) not very similar to the small, complex-type carbohydrate chains found in other plant glycoproteins.

Discussion

Our interest in the glycosylation arose initially from the desire to obtain cDNA clones for major cucumber virus-inducible PRX and identify the *in vitro* synthesized product with antibodies against the mature product. Using an immune serum (βF1) specific for xyloseα1-2 mannose structure commonly found in the complex asparagine-linked glycans of plant glycoproteins (Lauriére *et al.*, 1989), this study showed that all 3 isoforms of virus-inducible PRX

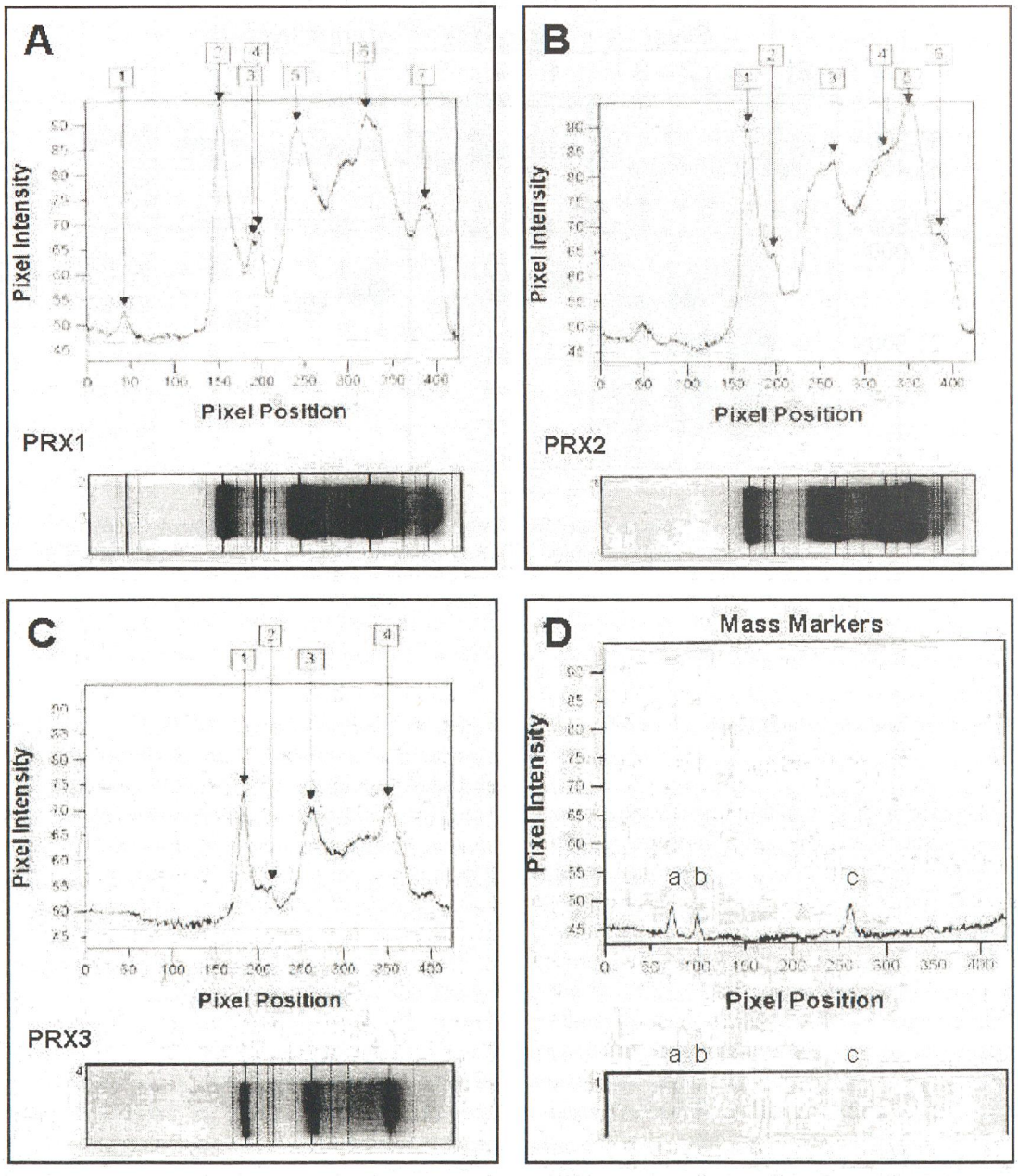

Fig. 5

Densitometric profiles of peptides released from PRXs 1, 2 and 3 isoforms by the formic acid treatment

The purified glycoproteins (100 μg per isoform) were incubated in 70 % formic acid at 37°C for 96 hrs. The cleaved products were separated by SDS-PAGE and silver stained. Computer-assisted peptide profiling and comparative analysis of individual isoforms PRX1 (A), PRX2 (B), and PRX3 (C), and of protein standards (D) were performed.

are glycosylated and contain N-linked bonds between the peptide and the oligosaccharide chain.

Different methods were further used to analyze the nature of the carbohydrate chains of these three electrophoretic forms of major virus-inducible anionic PRX and to test the assumption that its heterogeneity resides in the carbohydrate moiety. Total chemical deglycosylation with TFMS/anisole converted PRXs 1, 2 and 3 to the same-sized products which were no longer recognized by the specific βF1 antiserum. The removal of the heterogeneity by TFMS confirmed that the 3 molecular forms of virus-inducible PRX actually differ in the number and/or length of the glycosidic side chains.

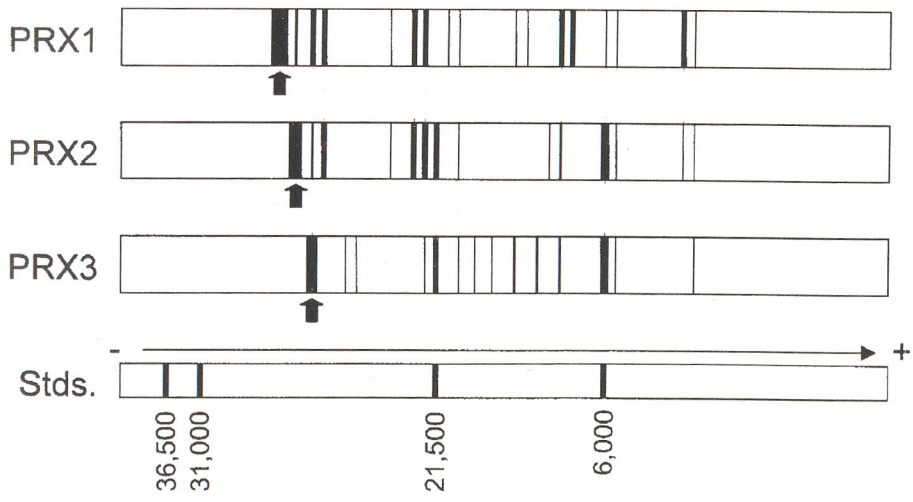

Fig. 6

Linear glycopeptide maps for individual virus-inducible PRX isoforms derived from formic acid cleavage products and densitometric scans
Glycopeptide position of individual PRX isoforms are denoted by solid arrows. Major and minor cleavage sites of isoform PRX1 match those of PRX2 and PRX3 and are visualized as thin dotted vertical lines.

Consequently, immunodetection of the same products with the PRX antiserum clearly demonstrated that the cross-reactivity of the 3 isoenzymes is largely caused by the carbohydrate moieties. This concept is in agreement with the previous findings that in a general protein extract of plants the majority of antibodies is generated against their glycosidic side chains (Evans *et al.*, 1988; Key and Weiler, 1988; Stafstrom and Staehlin, 1988). In this context, however, there is no doubt about it that from the functional point of view the glycosidic chains have besides antigenicity also other functions.

Further analysis of the glycans of the virus-inducible PRX was accomplished by treating purified isoenzymes with jack bean α-mannosidase. Due to a very specific deglycosylation pattern obtained with α-mannosidase, the 3 molecular forms of virus-inducible PRX probably did not differ only in the number of α-linked terminal mannose residues. Two different explanations may be taken into account concerning with the failure of α-mannosidase to remove the heterogeneity of virus-inducible PRX. Firstly, it is tempting to speculate that each of the 3 molecular forms have two or more distinct glycosidic chains linked to a polypeptide backbone. The cell wall β-fructosidase, which contains one high-mannose and two complex oligosaccharide side chains linked to a polypeptide, could be mentioned as an example (Lauriére *et al.*, 1988). Secondly, the removal of only partial heterogeneity may depend on either the accesibility of the carbohydrate chains or the specificity of the α-mannosidase involved, or both. E.g., jack bean α-mannosidase hydrolyses a Man(α-1-3)Man linkage 15-fold more slowly than Man(α-1-2)Man or Man(α-1-6)Man linkages (Montreuil *et al.*, 1986). A similar mechanism of sequential removal of mannose residues from carbohydrate chains of petunia PRX*a* was proposed by Hendriks and Van Loon (1990).

Cleavage of the PRXs 1, 2 and 3 by formic acid at the Asp-Pro site resulted mainly in 3 heavily stained bands per isoform widely differing in M_r and intensity of staining with silver suggesting that there may be multiple glycosylation sites in this PRX. Based on the nucleotide sequence data (Rasmussen *et al.* 1995), cucumber PRX (pCS1 cDNA) has two putative N-glycosylation sites (N-X-S/T), but our immunoblot analysis either with the PRX or Glyc antiserum showed that only one peptide per isoform has attached glycan structures. Furthermore, the wider banding of the largest subunits of PRX1 and PRX2 on the silver stained gel was not due to their heavier glycosylation (Beeley, 1985), because the anti-Glyc immunospecific signal had the same intensity. Therefore, what is the actual glycosylation site and what is the number and composition of the chains associated with virus-inducible anionic PRX remains to be an interesting question.

The lectin-affinity study suggests that, unlike to e.g. petunia PRXa or HRP, mature cucumber PRX contains complex N-linked glycans. This represents an evidence that this glycoprotein passes through the Golgi apparatus on its way to the extracellular matrix or space. In this context it is interesting to note that in addition to mannose, fucose and xylose, petunia PRXa also contains glucose, galactose and arabinose (Hendriks and Van Loon, 1990). Similar observations have been reported for plant PRXs by other workers

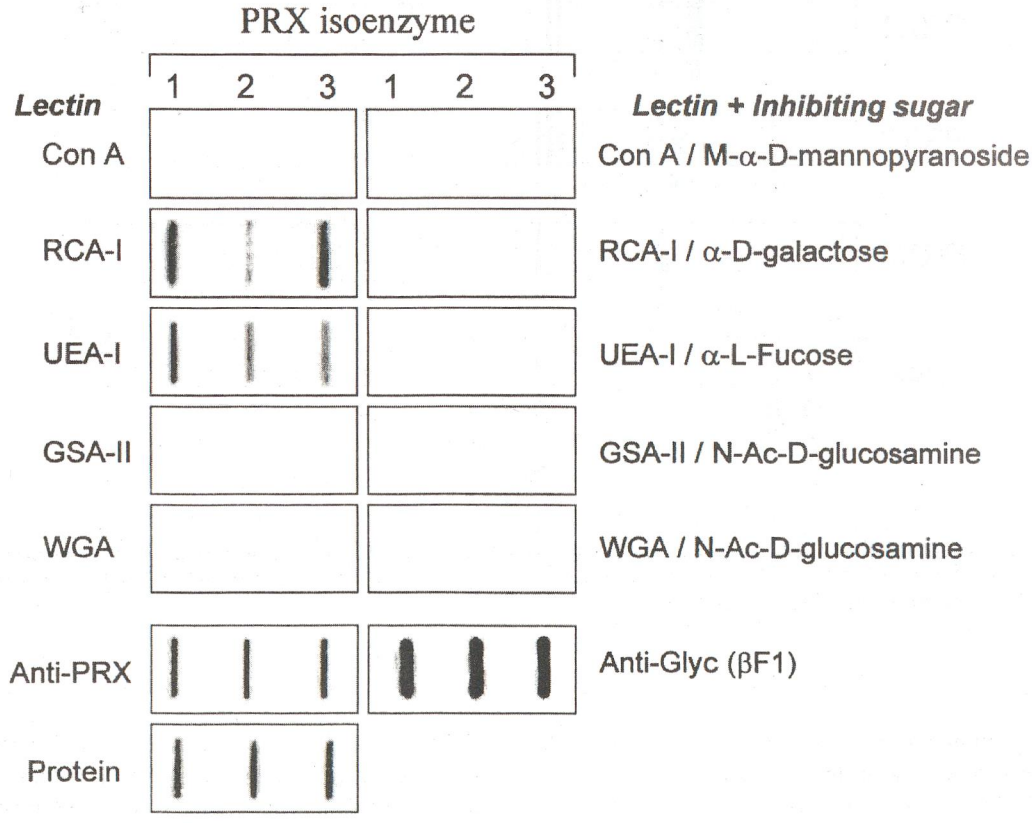

Fig. 7
Screening of lectin specificity of virus-inducible PRX isoforms
Inactivated PRXs 1, 2 and 3 were applied (2.5 μg per slot) onto NC membrane. Each strip was then incubated with one of five different biotinylated lectins. Lectin-affinity was analyzed by staining for HRP activity. Control incubations were performed in the presence of the lectin-specific inhibitory sugars. The presence of intact glycans attached to the tested proteins was confirmed with the PRX or βF1 antisera. Total protein stained with 0.1% Amido Black was used as a loading control.

(Shannon *et al.*, 1966; Choy *et al.*, 1979), but the origin of these sugars is not clear to date.

Further studies are underway to determine the site of each of the glycans of the anionic isoenzymes on their peptide chains. Moreover, the fine structure and sugar composition of the attached glycans will be investigated too.

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