Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 298–305
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Purification and characterization of proline/hydroxyproline-rich glycoprotein from pearl millet coleoptiles infected with downy mildew pathogen *Sclerospora graminicola*

Shantharaj Deepak [a,b], Sekhar Shailasree [a], Neerakkal Sujeeth [a], Ramachandra K. Kini [a], Shekar H. Shetty [a], Axel Mithöfer [b,*]

[a] *Department of Studies in Applied Botany and Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India*
[b] *Max-Planck-Institute for Chemical Ecology, Department Bioorganic Chemistry, Hans-Knöll-Straße 8, D-07745 Jena, Germany*

Received 1 September 2006; received in revised form 16 October 2006
Available online 12 December 2006

Abstract

Hydroxyproline-rich glycoproteins (HRGPs) are important plant cell wall structural components, which are also involved in response to pathogen attack. In pearl millet, deposition and cross-linking of HRGPs in plant cell walls was shown to contribute to the formation of resistance barriers against the phytopathogenic oomycete *Sclerospora graminicola*. In the present study, the purification and characterization of HRGPs that accumulated in coleoptiles of pearl millet seedlings in response to *S. graminicola* inoculation has been carried out. Periodic acid Schiff's staining revealed that the purified protein was a glycoprotein. The protein to carbohydrate ratio was determined to be 95.5%:4.5% (w/w). Proline amounted for 20 mol% of the total amino acids as indicated by amino acid composition analysis. The isolated protein had a p*I* of 9.8 and was shown to be composed of subunits of 27, 17, and 14 kDa. Cross reactivity with the monoclonal antibody MAC 265 and the presence of the signature amino acid sequence, PVYK, strongly suggested to classify the purified glycoprotein as a member of the P/HRGPs class. In the presence of horseradish peroxidase and H_2O_2 the purified glycoprotein served as a substrate for oxidative cross-linking processes.

Keywords: Oxidative cross-linking; Proline/hydroxyproline-rich proteins; Pearl millet; *Sclerospora graminicola*

1. Introduction

All plant pathogens interact with plant cell walls, which are highly hydrated and gel-like. At the simplest level the walls provide a physical barrier between pathogens and the protoplast of the plant cells (Vorwerk et al., 2004). Plant cell walls include mainly long cellulose fibrils, hemicellulose, pectin and several structural wall glycoproteins such as hydroxyproline-rich glycoproteins (HRGP) (Lamport, 2001) and glycine-rich proteins (GRPs) (Showalter, 1993; Cassab, 1998; Vorwerk et al., 2004). Several reports have shown that HRGPs are involved in numerous cellular processes of plants including development (Sommer-Knudsen et al., 1998). An overall increase in the level of HRGPs in plant tissues has also been shown to be a result of fungal and bacterial infection or treatment with elicitors obtained from pathogens (Bradley et al., 1992; Brownleader et al., 1995; Kang and Buchenauer, 2003; Shailasree et al., 2004). Cooper and Varner (1984) demonstrated that cell wall HRGPs became insoluble in carrot cell wall mediated by peroxidase and hydrogen peroxide. These changes in properties of the structural proteins are thought to be brought about by cross-linking of their monomeric subunits (Wojtaszek et al., 1995, 1997; Otte and Barz, 2000). The speed at which the cross-linking (insolubilization) of HRGPs occurs in bean and soybean cells after incubation

* Corresponding author. Tel.: +49 3641571263; fax: +49 3641571256.
E-mail address: amithoefer@ice.mpg.de (A. Mithöfer).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.10.024

with fungal elicitors suggests a rapid defense mechanism against pathogen preceding gene expression as these HRGPs are believed to strengthen the cell wall barrier against invading pathogen (Bradley et al., 1992).

The mechanism of cross-linking of the monomeric subunits by H_2O_2 and peroxidase might involve the formation of isodityrosine (IDT) a diphenyl phenolic within and between the HRGP monomers (Fry, 1982). As postulated by Wojtaszek et al. (1995, 1997), after infections oxidative cross-linking of O-glycosylated HRGPs, that depend on a pronounced oxidative burst might occur as well. However, the molecular mechanism for this process is still not known. The whole process strongly decreases solubility of HRGPs thereby they lose their extractability in salts and SDS (Bradley et al., 1992). Finally, this leads to the formation of a cross-linked mesh of defined porosity interpenetrated by cellulose microfibrilar wrap (Epstein and Lamport, 1984).

HRGPs include extensins, arabinogalactan proteins (AGPs), proline/hydroxyproline-rich proteins (P/HR GPs), and solanaceous lectins (Sommer-Knudsen et al., 1998). Among these, the P/HRGPs and extensins are known to be insoluble proteins whereas AGPs are soluble proteins. Although most of the earlier studies on infection-induced accumulation of HRGPs were carried out in dicots, e.g., lettuce against *Pseudomonas syringae* (Bestwick et al., 1995), French bean against *Xanthomonas campestris* (Brown et al., 1998); in recent years few reports have appeared on the accumulation of HRGPs in monocots as a response to infection: wheat against *Fusarium culmorum* (Kang and Buchenauer, 2003) and pearl millet against *Sclerospora graminicola* (Shailasree et al., 2004).

Studies from this latter laboratory have demonstrated the accumulation of HRGPs in pearl millet as a response to infection by downy mildew pathogen *S. graminicola* as early as 2 h post inoculation in the resistant cultivar IP18296, which continued to increase with time recording a maximum accumulation by 9 h (Shailasree et al., 2004). Immunocytochemical studies suggested that these HRGPs are cross-linked in the papillar structure of the resistant pearl millet cultivars upon inoculation with *S. graminicola* (Shailasree et al., 2004).

Our current understanding of the structure of each of the sub-groups of HRGPs is incomplete and thus it is difficult to design definitive experiments to address the question of their function on the molecular level (Sommer-Knudsen et al., 1998). Hence the objective of the present study was directed towards the identification, the purification and characterization of this particular glycoprotein for the elucidation of its role in the pearl millet downy mildew host–pathogen system.

2. Results and discussion

The present study has been undertaken to identify the HRGPs involved in resistance offered by pearl millet to *S. graminicola* infection as previous studies have shown that HRGPs are induced as a response to *S. graminicola* infection in seedlings of the resistant pearl millet cultivar IP18296 (Shailasree et al., 2004). The quantification of HRGPs was determined by monitoring the Hydroxyproline (Hyp) content in the samples as this colorimetric estimation of Hyp is reported to be a sensitive indicator for the presence of HRGPs (Raggi, 2000). Extraction of ionically bound proteins from cell walls is commonly achieved using concentrated salt buffers, especially those containing $CaCl_2$ (Robertson et al., 1997), SDS (Bradley et al., 1992), and highly concentrated lithium chloride for extraction of glycoproteins (Voigt and Frank, 2003). However, these methods did not result in extraction of HRGPs as determined by Hyp content (results not shown). Hence for extraction of cell wall proteins, a method was employed involving treatment combining acid and ethanol. This method is known to solubilize HRGPs that are secreted towards the cell wall and not yet crosslinked. By this procedure most of other proteins are denatured and the preparation is reported to contain basic proteins up to 70% of the proteins extracted (Mellon and Helgeson, 1982). Further purification steps included Sephadex G 200 chromatography, followed by RP-HPLC.

2.1. Purification and biochemical characterization

The Sephadex G200 profile of total cell wall proteins extracted by acid:ethanol treatment showed one major and three minor peaks. Hyp determination of the peaks showed that only fractions 21–28 of the major peak contained HRGPs. These fractions were pooled and were further analyzed by RP-HPLC, identifying a single major peak at 4 min 30 s that contained maximum level of HRGPs as determined by Hyp estimation. These combined steps yielded 100 μg HRGPs out of 1 g fresh weight in purified form as evidenced by results of electrophoresis.

Total cell wall proteins separated by SDS–PAGE revealed several bands ranging from molecular weight 43 to 14 kDa (Shailasree et al., 2004). Here, the crude extract was further purified and the resulting protein was subjected to 1D- and 2D-electrophoresis. When analyzed by 2D gel electrophoresis, the isolated protein was deglycosylated first to avoid protein bands that are too diffuse. The protein had a basic p*I* of 9.8, and was composed of the same three polypeptides that were detected in the crude extract, one major peptide of 17 kDa and two minor peptides of 27 and 14 kDa, which obviously were co-purified (Fig. 1A). To reveal the glycoprotein character of the purified protein, periodic acid-Schiff's (PAS) staining was carried out immediately after both SDS–PAGE and native acidic PAGE electrophoresis. The three bands corresponding to 27, 17, and 14 kDa stained positively with PAS (Fig. 1B) as well as the single protein observed in a native gel (Fig. 1G). In that native acidic (pH 4.2) polyacrylamide gel system, the purified protein migrated as one diffused band not entering the 8% separation gel and thus suggesting a high

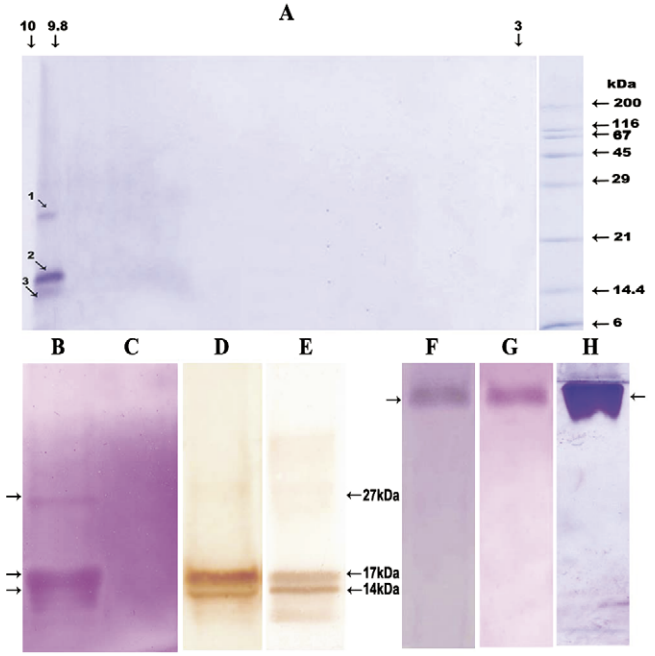

Fig. 1. Analysis of purified HRGP (10 μg) extracted from cell walls of pearl millet coleoptiles by acid ethanol treatment. (A) 2D electrophoresis of deglycosylated HRGP separated on a 3–10 non-linear pH gradient in the first dimension and SDS–PAGE in the second dimension visualized by Coomassie Blue staining. The numbers corresponding to the protein spots were further analyzed by LC MS/MS. (B) Periodic acid Schiff's (PAS) staining of the protein after SDS–PAGE. (C) PAS staining of the deglycosylated protein after SDS–PAGE. (D) Western blot of protein following SDS–PAGE using MAC 265 monoclonal antibody. (E) Western blot of deglycosylated protein following SDS–PAGE using MAC 265 monoclonal antibody. (F) Western blot of protein following native acidic PAGE (pH 4.2) using MAC 265. (G) PAS staining of protein after native acidic PAGE (pH 4.2). (H) Coomassie Blue staining of the protein after SDS–PAGE under non-reducing conditions (without β-mercaptoethanol).

molecular weight. Similarly, Stuart and Varner (1980) demonstrated that soluble extensin from carrot that did not enter polyacrylamide gels containing SDS and/or 8 M urea, was also resolved in acidic polyacrylamide gels. Deglycosylated protein showed no PAS staining (Fig. 1C). Moreover, no clear shift in mobility could be seen because the carbohydrate content in protein was just 4.5%. As the glycoprotein isolated in this study separated into one major 27 kDa and two minor bands of 17 and 14 kDa upon SDS–PAGE it might represent a heteromer composed of a number of polypeptides. Thus, our results are similar to those reported by Brownleader et al. (1995) where purified extensin-1 was resolved by acid urea-PAGE into monomers, dimers and trimers (86, 172, and 258 kDa). Obviously, the purified heteromer is stabilized by mercaptoethanol-sensitive disulfide bridges because in SDS–PAGE without mercaptoethanol treatment no separation into subunits was detected (Fig. 1H).

2.2. Immunological studies

MAC 265 monoclonal antibodies originally generated by Vandenbosch et al. (1989) are directed against a 95 kDa matrix glycoprotein from infection threads present in the pea-Rhizobium symbiosis. The antibody has successfully been used to identify HRGPs in legumes (Millar et al., 1992; Rathbun et al., 2002). Here, Western blotting of purified protein was carried out following both native acidic and SDS–PAGE. The high molecular weight purified HRGP entering the native acidic gel also reacted positively to MAC 265 (Fig. 1F). Under denaturing conditions, three proteins with molecular weight 27, 17, and 14 kDa were detected on the blots by MAC 265. The 17 kDa protein reacted with greater intensity compared to 27 and 14 kDa proteins on the Western blots (Fig. 1D). MAC 265 also recognized the 27, 17, 14 kDa protein component of deglycosylated HRGP under denaturation condition (Fig. 1E). The cross reaction of these polypeptides with this antibody strongly suggests that they all belong to HRGPs.

2.3. Chemical analysis

Further characterization of purified glycoprotein included the compositional analysis. The carbohydrate and protein contents of the purified protein are shown in Table 1. The composition of purified protein was 95.5% (w/w) protein and 4.5% carbohydrate (w/w). The amino acid composition of the protein revealed high amount of proline accounting for 20 mol% of the total amino acid and Hyp accounted for 6.3 mol% of the total amino acids. Significant amounts of lysine, alanine, serine and glycine were also observed.

Table 1
Amino acid and carbohydrate composition of the purified HRGPs

Protein	95.5% w/w
Carbohydrate	4.5% w/w
Amino acids	*mol%*
Asp/Asn	1.52
Glu/Gln	3.90
Ser	5.84
Gly	9.82
His	1.82
Arg	2.58
Thr	1.43
Ala	13.91
Pro	20.01
Tyr	3.95
Val	5.75
Met	1.16
Cys	0.17
Ile	1.59
Leu	2.34
Phe	1.69
Lys	16.05
Hyp	6.32
Carbohydrates	*mol%*
Galactose	63.3
Arabinose	15.2
Mannose	21.3

The total amino acid composition and the carbohydrate compositions of purified HRGPs are indicated. Glu/Gln and Asp/Asn cannot be distinguished after hydrolysis of protein.

Carbohydrate analysis indicated that galactose accounted for up to 63.3 mol%, arabinose 15.2 mol% and mannose 21.5 mol%. It is reported that in the P/HRGPs glycosylation ranges from <3 mol% to approximately 70 mol% of the glycoprotein and the carbohydrate fraction of the P/HRGPs from French bean contains mostly arabinose, galactose and mannose (Sommer-Knudsen et al., 1998).

Shifts in the circular dichroism (CD) spectrum reflect a transition from a structured to a less structured molecule (Ferris et al., 2001) indicating a role for carbohydrate side chains in maintaining the conformation of the molecule (Dey et al., 1997). The native HRGP from pearl millet showed a spectrum minimum of 203 nm which shifted to 201 nm after deglycosylation (Fig. 2). Such shift in the CD spectrum has been reported for glycosylated polyproline II rods, a HRGP from *Chlamydomonas reinhardtii* as well (Ferris et al., 2001).

2.4. Peptide analysis

For further characterization and homology search polypeptides obtained after 2D-electrophoresis were excised, digested with trypsin, and the peptides were subjected to LC/MS/MS. In the 27 kDa polypeptide, two peptide sequences were identified, PVYKYTK and HPPPDLGLSSK, which indicated similarities with hydroxyproline-rich glycoproteins such as the nodule specific hydroxyproline-rich glycoprotein (P/HRGPs) LENOD2 (Accession No. Q06841) from *Lupinus luteus* L., and extensin precursor of *Daucus carota* L. (Accession No. P06599). The spot of 17 kDa also contained the peptide sequence of PVYKYTK and a second sequence, KPPPVGR, which showed similarities to early nodulin proline-rich protein (ENOD2) from *Glycine max* L. (Accession No. P08297) and hydroxyproline-rich glycoproteins such as the nodule specific hydroxyproline-rich glycoprotein (P/HRGPs) LENOD2 (Accession No. Q06841) from *Lupinus luteus* L. The spot at 14 kDa contained the peptide sequence HPPPDLGLSSK found in the 27 kDa polypeptide. The results based on peptide sequence database search identified the purified protein as P/HRGPs, a sub-class of the HRGPs superfamily as they have the sequence PVYK, which is reported to be a signature sequence for P/HRGPs (Datta et al., 1989; Sommer-Knudsen et al., 1998). To our knowledge, this sequence has not been described for other P/HRGPs from monocotyledons before.

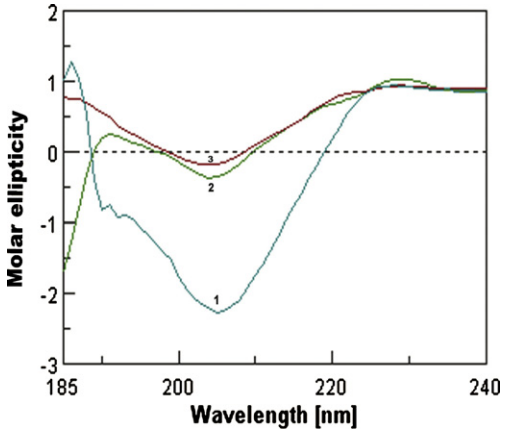

Fig. 2. Circular dichroism spectra of the HRGP module before (2) and after (3) deglycosylation. Spectra of the P/HRGP glycoprotein show a minimum at 203 nm shifting to 201 nm after deglycosylation. A polyproline standard (1) has a maximum at 227 nm and a minimum at 205.

2.5. In vitro cross-linking of HRGP

The mechanism by which HRGP accumulation contributes to disease resistance involves cross-linking between these molecules to form a network for providing anchorage to lignin and create a barrier impenetrable to fungal hyphae. It has been shown that this cross-linking process is mediated by hydrogen peroxide (H_2O_2) and peroxidases (Fry, 1982; Wojtaszek et al., 1997; Otte and Barz, 2000). Thus, cross-linking studies in the presence of H_2O_2 and horseradish peroxidase were attempted in vitro using the purified protein as substrate. The different modified reaction mixtures were separated by SDS–PAGE. The result of PAS staining and immunoblots with MAC 265 showed an additional protein band of 66 kDa only in the sample containing P/HRGP, peroxidase, and H_2O_2 (Fig. 3). The intensities of the other bands, 14, 17, and 27 kDa, in this sample were marginally reduced. These results also confirm the concept that HRGPs can be crosslinked by peroxidase and H_2O_2.

Interestingly, Shailasree et al. (2004) observed three polypeptides (14, 17, and 27 kDa) in the resistant pearl mil-

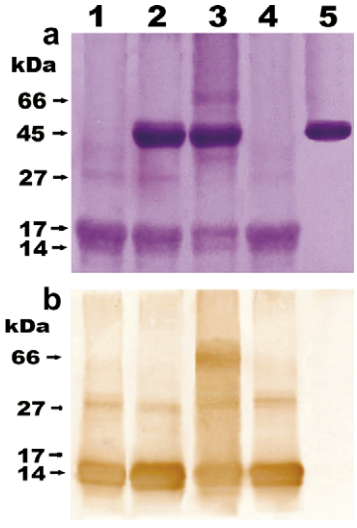

Fig. 3. In vitro studies on oxidative cross-linking of purified P/HGRP by H_2O_2 and horse radish peroxidase on SDS–PAGE followed by (A) PAS staining and (B) immunoblot using MAC 265 monoclonal antibody. Lane 1: 10 μg purified HRGP; lane 2: 10 μg purified HRGP + 1 U HRP; lane 3: 10 μg HRGP + 1 U HRP + 1 μl H_2O_2 (8 mM); lane 4: 10 μg HRGP + 1 μl H_2O_2 (8 mM); lane 5: 1 U HRP.

let cultivar also used in the present study. In a susceptible cultivar only two polypeptides (17 and 27 kDa) were detectable. Moreover, in these susceptible plants structural defense responses like cross-linking were not detected in contrast to the resistant plants. Both these results taken together with the successful in vitro cross-linking shown in Fig. 3 indicate a specific but still unknown role for the 14 kDa protein in the cross-linking process.

3. Conclusion

The HRGP purified in the present study belongs to the P/HRGPs subclass. It cross reacts with MAC 265 monoclonal antibodies and contains the signature amino acid sequence for P/HRGPs, PVYK (Sommer-Knudsen et al., 1998). The expression of P/HRGPs is influenced by factors associated with pathogen infection or environmental stresses such as elicitor treatment and wounding, suggesting that the synthesis of these proteins is sensitive to external stimuli (Cassab, 1998; Sommer-Knudsen et al., 1998). Furthermore, chitin binding P/HRGP from French bean is reported to bind the hyphae of a pathogenic fungus, *Colletotrichum lindemuthianum* in host–microbe interaction (Millar et al., 1992). Knowledge of precise properties of these glycoproteins may be important in order to understand their role in cell wall architecture and also their significance in plant disease resistance when deposited in the wall by cross-linking mechanism. Thus, investigation of the role of P/HRGPs during induction of resistance and their role in containment of the downy mildew pathogen is currently under way.

4. Experimental

4.1. Pathogen and host

Downy mildew pathogen *Sclerospora graminicola* (Sacc) Schroet. isolated from pearl millet (*Pennisetum glaucum* (L.) R. Br.) cv. HB3 and maintained on the same cultivar under greenhouse conditions was used for all inoculation experiments. The pearl millet cultivar IP18296 [highly resistant with 0% disease incidence] was used in the study.

4.2. Inoculation of the plant material

The seeds of IP18296 pearl millet cultivar were surface sterilized in 0.1% sodium hypochlorite solution for 15 min, washed thoroughly with sterile distilled water and germinated on moist filter paper under aseptic conditions at 25 ± 2 °C in dark for two-days. The two-day-old seedlings were dip inoculated with 4×10^4 zoospores ml^{-1} suspension of *S. graminicola* (Safeeulla, 1976). The seedlings were harvested at 9 h after inoculation and coleoptiles were used for cell wall extraction (York et al., 1986).

4.3. Purification of HRGPs

4.3.1. Preparation of cell wall proteins

Cell wall proteins were extracted from coleoptiles of pearl millet seedlings (Shailasree et al., 2004). All procedures were carried out at 4 °C. Coleoptiles from the seedlings were homogenized in 0.5 M potassium phosphate buffer (pH 7.0). Cell walls were repeatedly washed with the same buffer followed by distilled water. The pellet was suspended in three volumes of 1.25 N HCl:absolute alcohol (1:3). After two days, cellular debris was removed by centrifugation and proteins in supernatant were precipitated in 3 vols. of cold acetone and incubated at 4 °C overnight. Precipitated proteins were centrifuged at 10,000*g* for 15 min, acetone was decanted and pellet was air-dried.

4.3.2. Chromatographic analyses

The acetone precipitate was dissolved in 0.05 M sodium acetate buffer (pH 3.5). Proteins (3.2 mg) were loaded onto Sephadex G-200 column (60×1 cm) equilibrated with 0.05 M acetate buffer (pH 5.8) and eluted in the same buffer. Column fractions of 2 ml were collected and protein content in the fractions was monitored at 280 nm. Hyp was estimated in all fractions containing protein (Prockop and Udenfriend, 1960) for the presence of HRGPs. The HRGPs containing peaks were pooled and lyophilized.

HRGPs eluted after gel filtration were load onto RP C-18 (Vydac column, 150×3.9 mm) connected to Shimadzu 24 (LC-6A) HPLC equilibrated with 0.1% v/v trifluroacetic acid (TFA). Protein was eluted using linear gradient of 0.1% v/v TFA in 80% acetonitrile at a flow rate of 0.75 ml/min. Column eluents were monitored for protein at 220 nm.

4.3.3. Deglycosylation of HRGPs

Purified HRGP (100 μg) was deglycosylated using trifluromethane sulphonic acid for 3–4 h on ice as described by Edge et al. (1981). The deglycosylated protein fraction was precipitated using ether and *n*-hexane (9:1, v/v) and subsequently incubating the solution for 1.5 h at −80 °C. After centrifugation (15 min, 5000*g*, 4 °C), the transparent pellet was washed with 95% (v/v) ethanol and dried under vacuum. The deglycosylation of purified HRGP was assessed under 1D PAGE and 2D PAGE.

4.3.4. Electrophoreses

HRGP and deglycosylated HRGP concentration was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard. SDS–PAGE was performed using 12% separating gel. The standard proteins for molecular mass determination obtained from Amersham Biosciences, Sweden, were used. Acidic native PAGE (pH 4.2) was performed cathodically by the method of Reisfeld et al. (1962) in the 8% separating and 5% stacking gel using Pyronin Y as tracking dye. Protein banding pattern was visualized with Coomassie Brilliant Blue R250.

For 2D-electrophoresis, purified deglycosylated protein (50 μg) was redissolved in IEF buffer (0.7 M urea, 2.0 M thiourea, 4% CHAPS, 10 mM DTT, 1.0% v/v carrier ampholytes) pH 3–10 (GE Health Care, Germany). Non-linear, immobilized pH gradient strips (7 cm, pH 3–10) were rehydrated overnight with 300 μl IEF buffer containing the purified protein. Electrofocusing was carried out using Immobiline dry strip aligner Multiphor II at 12 °C, applying the following program: a linear increase from 0.001 kVh for 0.01 h, 2.8 kVh for 1.3 h, and finally 2.2 kVh for 1.05 h. After focusing, the proteins were reduced by incubating the strips with 1% w/v DTT for 10 min and alkylated with 2.5% w/v iodoacetamide in 10 ml of equilibration buffer (6 M urea, 30% w/v glycerol, 2% SDS, and 50 mM Tris–HCl, pH 8.8) for 10 min. The strips were then transferred to 12% SDS–PAGE gels for second dimension electrophoresis, using SDS electrophoresis buffer (250 mM Tris, pH 8.3, 1.92 M glycine and 1% SDS) with 100 V applied for 5 h. The proteins spots were visualized with Coomassie Blue.

4.4. Characterization of HRGP

4.4.1. Glycoprotein detection

Purified HRGP and deglycosylated HRGP as glycoprotein was identified by periodic acid Schiff (PAS) stain. SDS–PAGE and acidic native PAGE gel was soaked in 7.5% acetic acid for 1 h at 25 ± 2 °C and then transferred to 1% w/v aqueous periodic acid and 3% acetic acid. After incubating at 4 °C for 60 min, the gels were washed several times with distilled water. They were placed in Schiff's reagent for 60 min at 4 °C in the dark. Bands developed were recorded in Bioprofile Image Analysis System (Vilber Lourmat, France).

4.4.2. Western blot analysis

Immediately after SDS electrophoresis of glycosylated and deglycosylated HRGP, gels were blotted onto polyvinylidene difluoride (PVDF) (Millipore) membrane using Multiphor II (Pharmacia Biotech, Sweden) electrophoretic transfer apparatus following the manufacturer's protocol. The blots were blocked in 2% fat-free milk in Tris-buffered saline (10 mM Tris–HCl, pH 8.0, containing 150 mM NaCl). The blots were probed with MAC 265, a rat monoclonal antibody raised against a 95 kDa glycoprotein involved in the pea-Rhizobium symbiosis (Vandenbosch et al., 1989). Similarly immediately after native acidic PAGE immunoblot was carried out. Subsequently, the blots were developed as reported previously (Shailasree et al., 2004).

4.4.3. Protein analysis by LC MS/MS

Protein sample spots were excised from Coomassie Blue-stained 2D gel and destained as given below. Each sample was washed 4 cycles with 70 μl of acetonitrile/50 mM ammonium bicarbonate (1/1 v/v) for 20 min each. Second wash was done in 2 cycles with 70 μl 70% acetonitrile for 20 min each. Washing was repeated for complete removal of Coomassie Blue. Gel plugs were washed with 20 mM $NaHCO_3$, pH 7.0, for 2 × 30 min followed by 70 μl of acetonitrile–50 mM ammonium bicarbonate (1/1 v/v) for 20 min each. Tryptic digestion was performed by adding 10 μl of 0.02 μg/μl trypsin in 50 mM NH_4HCO_3 for overnight at 37 °C. Then 15 μl of 50% (v/v) acetonitrile, 0.1% (v/v) trifluroacetic acid (TFA) was added and samples were sonicated for 10 min and diluted by the addition of 10 μl of MilliQ water to sample. The sample was placed at 37 °C for an additional 20 min to reduce the concentration of acetonitrile. μC8 ZipTip columns were equilibrated with 90% acetonitrile/0.1% TFA (v/v) and the equilibrated with 0.1% (v/v) TFA and the samples were loaded onto the column and desalted using a 0.1% TFA solution. Prior to ionization and nano LC-MS/MS analysis, dried peptides were dissolved in 10–15 μl of aqueous 0.1% formic acid for application on a CapLC XE nanoLC system (Waters, USA). A mobile phase flow of 0.1% aqueous formic acid (20 μl/min, 5 min) was used to concentrate and desalt the samples on a 5 mm × 350 μm Symmetry-300 C18 precolumn with 5 μm particle size. Next, the peptides were eluted on a 150 mm × 75 μm NanoEase Atlantis C18 column, particle size 3 μm, and separated with an increasing acetonitrile gradient (in 0.1% aqueous formic acid). Phases A (5% MeCN in 0.1% formic acid) and B (95% MeCN in 0.1% formic acid) were mixed using a gradient program set to 5% phase B in A for 5 min, increased to 40% B in 25 min, and to 60% A in 10 min, and finally increased to 95% B for 4 min with linear gradient rise between each time point. Separated peptides from the NanoLC were directly transferred to the NanoElectroSpray source of a QTOF Ultima tandem mass spectrometer (Waters, USA). A TOF analyzer was used in reflectron mode. De novo sequences of tryptic peptides were achieved using nanoelectrospray ionization-mass spectrometry in combination with nanoflow reversed phase chromatography. MS/MS spectra of sample ions were obtained on a Q-TOF Ultima mass spectrometer. The data were collected by MassLynx v4.0 software. Protein Lynx Browser v2.2 software (Waters, USA) was used for further data processing (deconvolution, baseline subtraction, smoothing). The resultant de novo peptide sequences were subjected to database blast queries, the Swiss-Prot (http://www.expasy.ch) or EMBL (http://www.narrador.embl-heidelberg.de).

4.4.4. Chemical analysis

Purified HRGP was hydrolyzed in 6 N HCl at 110 °C for 18 h and used for carbohydrate estimation by phenol sulfuric acid method (Dubois et al., 1956) using glucose as standard and total protein content using BSA as standard (Lowry et al., 1951).

The amino acid composition was determined by amino acid analyzer (Shimadzu CR4A Chromatopac). The purified HRGP was hydrolyzed in vacuo at 110 °C in constant boiling HCl for 24 h using the Pico-Tag workstation. Amino acid analysis was performed by pre column

derivatization using phenyl isothiocyanate. The phenyl thiocarbamoyl amino acids were analyzed by RP-HPLC (Bidlingmeyer et al., 1984).

Glycosyl composition of purified HRGP was carried out by hydrolysing the sample with 2 N TFA, derivatizing the neutral and amino sugars to alditol acetate and analysis by GC/MS (Merkle and Pope, 1994). GC was performed on an Alltech EC 5 column, 15 m × 0.25 mm, 0.25 μm; Finnigen Tracs MS (70 eV) with 1 μl injection. An injection splitter was used at a ratio of 1:50. The temperature programme started with 190 °C for 2 min, increased to 240 °C at the rate of 10 °C/min and kept temperature at 240 °C for 10 min. Identification of component sugars was based on comparison of the retention times with those of standard sugars (Merkle and Pope, 1994).

4.4.5. Circular dichroism (CD) spectroscopy

Circular dichroism spectra of poly L-proline (30 kDa, Sigma) and the HRGP before and after deglycosylation were carried out using 1 μg/μl purified protein in 10 mM phosphate buffer (pH 7.2) on a Jasco-J810 spectropolarimeter. Spectra were averaged over two scans with a bandwidth of 1 nm, and step resolution was 0.1 nm. All spectra are reported in terms of mean residue ellipticity with the 185 ± 240 nm region using a 1 mm path length.

4.5. In vitro cross-linking studies

Oxidative cross-linking of HGRP by H_2O_2 and horse radish peroxidase was carried out in vitro following the procedure of Otte and Barz (2000). Reaction mixtures were incubated for 30 min at 25 °C. The reaction was performed in 10 mM sodium acetate buffer, pH 3.5, and stopped by the addition of 8 μl SDS–PAGE sample buffer (0.5 M Tris–HCl, pH 6.8, 20% glycerol, 0.02% bromophenol blue + β-mercaptoethanol) followed by incubation at 100 °C. The reaction mixture was analyzed by SDS–PAGE for glycoprotein by PAS staining and Western blot probed with MAC 265.

Acknowledgements

The authors are grateful to the Department of Science and Technology, Government of India and Indian Council of Agricultural Research, New Delhi for financial support, and the DANIDA-ENRECA for the facilities made available for the research programme. The authors thank E.A. Rathbun for providing MAC 265 monoclonal antibody, D. Spiteller for help in carbohydrate analysis, and A. Muck for providing the peptide sequences. S.D. thanks German Academic Exchange Service (DAAD) for financial support to carry out research work at the Max-Planck-Institute for Chemical Ecology, Jena, Germany. S.S. acknowledges the research fellowship received from Council of Scientific and Industrial Research, New Delhi, India.

References

Bestwick, C.S., Bennett, M.H., Mansfield, J.W., 1995. Hrp mutant of *Pseudomonas syringae* pv *phaseolicola* induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in lettuce. Plant Physiol. 108, 503–516.

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., 1984. Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. 336, 93–104.

Bradley, D.J., Kjellborn, P., Lamb, C.J., 1992. Elicitor and wound induced oxidative cross-linking of a proline rich plant cell-wall protein: a novel, rapid defense response. Cell 70, 21–30.

Brown, I., Trethowan, J., Kerry, M., Mansfield, J., Bolwell, G.P., 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and Fench bean mesophyll cells. Plant J. 15, 333–343.

Brownleader, M.D., Ahmed, N., Trevan, M., Chaplin, M.F., Dey, P., 1995. Purification and partial characterization of tomato extensin peroxidase. Plant Physiol. 109, 1115–1123.

Cassab, G.I., 1998. Plant cell wall proteins. Annu. Rev. Plant Phys. Plant Mol. Biol. 49, 281–309.

Cooper, J.B., Varner, J.E., 1984. Cross-linking of soluble extensin in isolated cell walls. Plant Physiol. 76, 414–417.

Datta, K., Schmidt, A., Marcus, A., 1989. Characterization of two soybean repetitive proline rich proteins and a cognate cDNA from germinated axes. Plant Cell 1, 945–952.

Dey, P.M., Brownleader, M.D., Pantelides, A.T., Trevan, M., Smith, J.J., Saddler, G., 1997. Extensin from suspension-cultured potato cells: a hydroxyproline-rich glycoprotein, devoid of agglutinin activity. Planta 202, 179–187.

Dubois, M., Gilles, K.H., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Edge, A.S.B., Faltynek, C.R., Hof, L., Reichert, L.E., Weber, P., 1981. Deglycosylation of glycoproteins by trifluoromethanesulphonic acid. Anal. Biochem. 118, 131–137.

Epstein, L., Lamport, D.T.A., 1984. An intramolecular linkage involving isodityrosine in extensin. Phytochemistry 23, 1241–1246.

Fry, S.C., 1982. Isodityrosine a new cross-linking amino acid from plant cell wall glycoproteins. Biochem. J. 204, 449–455.

Ferris, P.J., Woessner, J.P., Waffenschmidt, S., Kilz, S., Dees, J., Goodenough, U.W., 2001. Glycosylated polyproline II rods with kinks as a structural motif in plant hydroxyproline rich glycoproteins. Biochemistry 40, 2978–2987.

Kang, Z., Buchenauer, H., 2003. Immunocytochemical localization of cell wall-bound thionins and hydroxyproline-rich glycoproteins in *Fusarium culmorum*-infected wheat spikes. J. Phytopathol. 151, 120–129.

Lamport, D.T.A., 2001. Life behind cell walls; paradigm lost, paradigm regained. Cell. Mol. Life Sci. 56, 1363–1385.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mellon, J.E., Helgeson, J.P., 1982. Interaction of hydroxyproline-rich glycoproteins from tobacco callus with potential pathogens. Plant Physiol. 70, 401–405.

Merkle, R.K., Pope, I., 1994. Carbohydrate composition analysis of glycoconjugates by gas-liquid chromatography/ mass spectrometry. Methods Enzymol. 230, 1–15.

Millar, D.J., Slabas, A.R., Sidebottom, C., Smith, C.G., Allen, A.K., Bolwell, G.P., 1992. A major stress inducible Mr-42000 wall glycoprotein of French bean (*Phaseolus vulgaries* L.). Planta 187, 176–184.

Otte, O., Barz, W., 2000. Characterization and oxidative in vitro cross linking of an extensin like protein and a proline-rich protein purified from chick pea cell walls. Phytochemistry 53, 1–5.

Prockop, D.J., Udenfriend, S.A., 1960. Specific method for the analysis of hydroxyproline in tissues and urine. Anal. Biochem. 1, 228–239.

Raggi, V., 2000. Hydroxyproline-rich glycoprotein accumulation in tobacco leaves protected against *Erysiphe cichoracearum* by potato virus Y infection. Plant Pathol. 49, 179–186.

Rathbun, E.A., Naldrett, M.J., Brewin, N.J., 2002. Identification of a family of extensin-like glycoproteins in the lumen of *Rhizobium*-induced infection threads in pea root nodules. Mol. Plant-Microbe Interact. 15, 350–359.

Reisfeld, R.A., Lewis, V.J., Willams, D.E., 1962. Disk electrophoreses of basic proteins and peptides on polyacrylamide gels. Nature 195, 281–283.

Robertson, D., Mitchell, G.P., Gilroy, J.S., Gerrish, C., Bolwell, G.P., Slabas, A.R., 1997. Differential extraction and protein sequencing reveals major differences in patterns of primary cell wall proteins from plants. J. Biol. Chem. 272, 15841–15848.

Safeeulla, K.M., 1976Biology and Control of the Downy Mildew of Pearl Millet, Sorghum and Finger Millet. Wesley Press, Mysore, India.

Shailasree, S., Kini, K.R., Deepak, S., Kumudini, B.S., Shetty, H.S., 2004. Accumulation of hydroxyproline-rich glycoproteins in pearl millet seedlings in response to *Sclerospora graminicola* infection. Plant Sci. 167, 1227–1234.

Showalter, A.M., 1993. Structure and function of plant cell wall proteins. Plant Cell. 5, 9–23.

Sommer-Knudsen, J., Bacic, A., Clarke, A.E., 1998. Hydroxyproline-rich plant glycoproteins. Phytochemistry 47, 483–497.

Stuart, D.A., Varner, J.E., 1980. Purification and characterization of a salt extractable hydroxyproline-rich glycoprotein from aerated carrot discs. Plant Physiol. 66, 787–792.

Vandenbosch, K.A., Bradley, D.J., Knox, J.P., Perotto, S., Butcher, G.W., Brewin, N.J., 1989. Common components of the infection thread matrix and the intercellular space identified by immunocytochemical analysis of pea nodules and uninfected roots. EMBO J. 8, 335–341.

Vorwerk, S., Somerville, S., Somerville, C., 2004. The role of plant cell wall polysaccharide composition in disease resistance. Trends Plant Sci. 9, 203–209.

Voigt, J., Frank, R., 2003. Proteins are constituents of the insoluble glycoprotein frame work of the Chlamydomonas cell wall. Plant Cell 15, 1399–1413.

Wojtaszek, P., Trethowan, J., Bolwell, G.P., 1995. Specificity in the immobilization of cell wall proteins in response to different elicitor molecules in suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). Plant Mol. Biol. 28, 1075–1087.

Wojtaszek, P., Trethowan, J., Bolwell, G.P., 1997. Reconstitution in vitro of the components and conditions required for the oxidative cross linking of extracellular proteins in French bean (*Phaseolus vulgaris* L.). FEBS Lett. 405, 95–98.

York, W.S., Darvill, A.G., McNeil, M., Stevensen, T.T., Albersheim, P., 1986. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. 118, 3–41.