

Stress-related RNase PR-10c is post-translationally modified by glutathione in birch

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ABSTRACT

The PR-10c (previously termed as Bet v 1-Sc3) protein of birch belongs to the family of intracellular pathogenesis-related proteins. The high-performance liquid chromatography electrospray ionization ion trap mass spectrometry (HPLC-ESI-MS) analysis of PR-10c-His fusion protein, produced in *Escherichia coli*, revealed three major peaks and masses. Enzymatic digestions and HPLC-ESI-MS and matrix assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF-MS) analyses of each fraction indicated that PR-10c-His protein is post-translationally modified by carbamylation and S-glutathiolation. Carbamylation was localized into the N-terminal end of PR-10c-His and does not represent a biologically significant modification. The possible nuclease activity of PR-10c was analysed with S-glutathiolated and reduced fractions of PR-10c-His fusion protein. Both forms of PR-10c-His as well as the dimeric form of the protein possess RNase activity which is capable of digesting different RNA substrates. None of the fractions showed activity against single- or double-stranded DNA. The MALDI-TOF-MS analysis of PR-10c polypeptide extracted from zinc-exposed birch roots showed that the protein is post-translationally modified by glutathione (γ -Glu-Cys-Gly) also *in vivo*. The S-glutathiolated cysteine residue of PR-10c is not conserved among Bet v 1 homologous proteins and is also unique in the PR-10 family. As far as we know this is the first observation of S-glutathiolation in plants, or any post-translational modification in the PR-10 family of proteins.

Key-words: Bet v 1; ESI-MS; MALDI, post-translational modification; ribonuclease; S-thiolation.

INTRODUCTION

Pathogenesis-related (PR) proteins are defined as proteins that are induced by pathological or related conditions in tissues that do not normally express the protein (Van Loon *et al.* 1994). The PR proteins are classified into 14 families (PR1–PR14) on the basis of DNA sequence, or predicted amino acid sequence, serological relationship and/or enzymatic or biological activity (Van Loon *et al.* 1994; Van Loon

& Van Strien 1999). PR proteins are a heterogeneous group of proteins with diverse functions; the only family without a known function or relationship is the PR-1 protein family.

The PR-10 family of intracellular PR proteins is ubiquitous in the plant kingdom, and members of this family have been found in dicotyledonous and monocotyledonous plants (Wen *et al.* 1997). The PR-10 proteins are unrelated to classical PR proteins (PR1–PR5) but are structurally related to tree pollen allergens (Warner, Scott & Draber 1992; Walter *et al.* 1998). The major allergens Api g 1 from celery (Breiteneder *et al.* 1995), Mal d 1 from apple (Vanek-Krebitz *et al.* 1995; Pühringer *et al.* 2000) and Bet v 1 from birch (Breiteneder *et al.* 1989; Swoboda *et al.* 1994) are included in the PR-10 family on the basis of high sequence similarity or stress or pathogen inducibility.

Sequence similarity of PR-10 proteins with ribonucleases from ginseng callus suggests a general function of PR-10 proteins as ribonucleases (Moiseyev *et al.* 1994, 1997). Although Bet v 1 from birch (Swoboda *et al.* 1996; Bufer *et al.* 1996) and LaPR-10 isolated from white lupin roots (Bantignies *et al.* 2000) have been shown to possess RNase activity, the structural homology to the START domain of MLN64 suggests a similar function of Bet v 1 in sterol binding (Tsujishita & Hurley 2000). The experimental evidence of the interaction of PR-10 protein (Pru av 1) with phytosteroids has been reported (Neudecker *et al.* 2001), but the exact function of many PR-10 proteins is still unknown.

The Bet v 1 gene family encodes a group of highly homologous 17 kDa polypeptides. The Bet v 1 isoforms represent the major pollen allergens of white birch (*Betula pendula* Roth.) and are expressed at high levels in mature pollen. Bet v 1-Sc1, -Sc2 and -Sc3 proteins [according to the current nomenclature PR-10a to c (Van Loon *et al.* 1994)] belong to a subset of the Bet v 1 gene family being not, however, constitutively expressed in pollen (Swoboda *et al.* 1995a). The PR-10a and PR-10b proteins are nearly identical and may be allelic variants of the same gene (Swoboda *et al.* 1995a). The PR-10c protein, however, is not classified in the same monophyletic group as are the other members of the Bet v 1 family (Wen *et al.* 1997). Differences in the protein expression level and organ-specific expression profile of Bet v 1 homologous proteins have been suggested (Poupard, Strullu & Simoneau 1998). The PR-10c protein is induced by pathogens (Swoboda *et al.* 1995a), Cu (Utriainen *et al.* 1998), Zn and ozone (Koistinen *et al.* unpublished), but not by wounding, although PR-10a is highly

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wound-inducible (Poupard *et al.* 1998). Furthermore, PR-10a transcripts are reported to be inducible by auxin, while the level of PR-10c transcripts remains unchanged (Poupard *et al.* 2001).

The Bet v 1 family has been extensively studied, and the tertiary structure of variant Bet v 1 ALK 2227 has been determined with X-ray crystallography (Gajhede *et al.* 1996). The structural characterization of Bet v 1 isoforms with liquid chromatography, mass spectrometry and cDNA cloning did not reveal any post-translational modifications, except for the cleavage of the initiating methionine, in either native Bet v 1 or recombinant Bet v 1a (Swoboda *et al.* 1995b). However, such a structural analysis has not been carried out for PR-10c. The aim of the present study was to further characterize the structure and function of the PR-10c protein both *in vitro* and in Zn-exposed plants.

MATERIALS AND METHODS

Expression and purification of PR-10c-His and Bet v 1-His in *Escherichia coli*

A cDNA clone encoding birch PR-10c protein was amplified with PR-10c-specific primers designed to contain cloning sites for *Sa*I and *Pst*I. The resulting fragment was inserted into the expression vector pQE31 (Qiagen, Valencia, CA, USA). The plasmid was transferred into *E. coli* strain M15 [pREP4]. Colonies expressing PR-10c-His protein were screened with the colony-lift procedure using a monoclonal antibody against 6×His Tag (Pharmacia, Uppsala, Sweden). The expression was verified in a small-scale liquid culture, and samples were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Reducing SDS-PAGE was performed according to established procedures using a Bio-Rad Mini-Protean II cell (Bio-Rad, Hercules, CA, USA) and gels consisting of 12% total monomer with 1% cross-linker. A large-scale culture was carried out to produce PR-10c-His (pQE31/PR-10c) and Bet v 1-His (pET28a/Bet v 1). The Bet v 1 construct was the same as described by Spangfort *et al.* (1996) except that 6×His-Tag was used instead of maltose-binding protein. The proteins were purified with Ni-NTA spin kit (Qiagen) under denaturing conditions (elution at pH 4.5) and reversed-phase high-performance liquid chromatography (HPLC) (C4, 214TP54, 5 μ m, 4.6 $\times$ 250 mm, Vydac, Hesperia, CA, USA). Eluent A was 0.1% trifluoroacetic acid, and eluent B was 70% acetonitrile containing 0.1% trifluoroacetic acid. Gradient was: 0 min, B = 20%; 2 min, B = 50%; 20 min, B = 60%; 21 min, B = 100%; 30 min B = 100%; 35 min, B = 20%. Flow rate was 1.0 mL min⁻¹ and UV absorbance was monitored at 214 nm. Protein samples were dried in a speed vac. Reduced and S-glutathiolated forms of PR-10c were prepared by incubating Ni-NTA purified protein with 10 mM dithiothreitol (DTT) or 10 mM oxidized glutathione (GSSG), respectively, for 30 min before HPLC purification. The purity of the fractions was analysed by high-performance liquid chromatography

electrospray ionization ion trap mass spectrometry (HPLC-ESI-MS) and matrix assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF-MS) of trypsin (Promega, Madison, WI, USA) and endoproteinase Glu-C (*Staphylococcus aureus* V8) (Boehringer Mannheim, Germany) digested proteins.

Plant material

The birch clone 142B exposed to 1137 mg Zn kg⁻¹ soil was used to analyse S-glutathiolation of PR-10c *in vivo*. The origin of the clone and details of micropropagation, rooting and Zn exposure have been described previously (Utriainen *et al.* 1997; Kopponen *et al.* 2001).

Protein extraction and two-dimensional electrophoresis

Total protein was extracted from 1 g fresh weight of roots with the phenol partitioning method (Utriainen *et al.* 1998), except that β -mercaptoethanol was replaced by 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma, St. Louis, MO, USA) to prevent unspecific binding of free glutathione during extraction. The protein concentration was analysed after the last washing from the 80% acetone solution using the Bio-Rad Protein Assay Dye reagent (Bio-Rad) and the sample was solubilized in IEF solubilization buffer.

Two-dimensional (2-D) electrophoresis was performed as described by Utriainen *et al.* (1998). The 2-D gels were silver-stained with the mass spectrometry compatible method (Shevchenko *et al.* 1996), except that the gels were fixed in a solution containing 40% ethanol and 5% acetic acid overnight and then washed twice in water for 30 min.

Preparation of samples for mass spectrometry

Enzymatic digestions were performed for HPLC-purified forms of PR-10c-His. The proteins were digested with modified trypsin (Promega) in 25 mM ammonium bicarbonate buffer, pH 8.0, at +37 °C for 16 h, and with endoproteinase Glu-C (Boehringer Mannheim) in 0.1 M ammonium bicarbonate, pH 7.8, at +37 °C for 2 h.

For MALDI-TOF-MS analysis, the PR-10c polypeptide was eluted from 2-D gel using a 1 : 3 : 2 (v/v/v) mixture of formic acid/water/2-propanol (FWI) saturated with α -cyano-4-hydroxycinnamic acid (4HCCA) (Cohen & Chait 1997). The identity of the protein was confirmed from tryptic digestion of polypeptide eluted from identical reference 2-D gel into aqueous solution of formic acid/water/2-propanol (Cohen & Chait 1997).

High-performance liquid chromatography electrospray ionization ion trap mass spectrometry

Positive ion mass spectra were acquired with an LCQ quadrupole ion trap mass spectrometer equipped with an ESI source (Finnigan MAT, San Jose, CA, USA), a Rheos

4000 pump (Flux Instruments, Danderyd, Sweden), Accurate flow splitter (LC Packings, Amsterdam, the Netherlands), and a Rheodyne 7725 injector with a 20- μ L-loop. The tryptic or endoproteinase Glu-C digests (10 μ L) were injected into the reversed-phase column (Vydac C₈, 5 μ m, 0.3 $\times$ 150 mm). The peptides were separated using a gradient from 5 to 70% acetonitrile in 30 min. Eluent A was 0.25% formic acid, and eluent B was 70% acetonitrile containing 0.25% formic acid. The total eluent flow of 4 μ L min⁻¹ was directed into the ESI source. The system was tuned by infusion of peptide MRFA from a T-split to the eluent flow. The spray needle potential was set to 4.20 kV. The stainless steel inlet capillary was heated to 200 °C. The capillary voltage was 10 V and the tube lens offset 30 V.

The full scan mass spectra from m/z 500 to m/z 2000 were measured using 500 ms for collection of the ions in the trap; two microscans were summed. Collision-induced dissociation (CID) MS/MS spectra of the peptides were obtained using the dependent scan mode of the instrument. Full scan MS data were collected until the base peak ion exceeded the preset intensity threshold (2×10^4). This triggers acquisition of a CID MS/MS product ion spectrum of the ion. The collision energy was 35% and isolation width two mass units. Scan range was dependent on the parent mass ion. Mass spectrometry data were analysed with the Xcalibur software (Finnigan).

Matrix assisted laser desorption/ionization – time of flight mass spectrometry

The matrix used for peptide analysis consisted of a saturated solution of α -cyano-4-hydroxycinnamic acid (4HCCA) in 0.1% trifluoroacetic acid:acetonitrile, 2 : 1 (v/v). The experiments were performed using positive ion mode on a time-of-flight mass spectrometer, model Biflex[®] III (Bruker-Franzen Analytik, Bremen, Germany) equipped with multiprobe inlet. The detector signals were transferred to the XACQ program on a SUN workstation (Sun Microsystems Inc., Palo Alto, CA, USA). The saved data were processed with the XMASS 5.0 program (Bruker Daltonics, Bremen, Germany). External calibration for the PR-10c protein, extracted from 2-D gel, was performed with horse heart myoglobin (16951.5 Da) (Boehringer Mannheim) and for peptides with angiotensin (1046.5 Da) and ACTH 18–39 (2465.7 Da).

Isoelectric focusing

Lyophilized proteins were solubilized in IEF solubilization buffer [9.5 M urea, 2% Triton X-100, 0.01% bromophenol blue, 1.6% Bio-Lyte 5/7 ampholyte reagent (Bio-Rad), 0.4% Bio-Lyte 3/10 ampholyte reagent (Bio-Rad)]. Isoelectric focusing was performed in a Protean IIXi cell (Bio-Rad) following the method described by Ploegh (1995). After isoelectric focusing, the gel was fixed with 20% trichloroacetic acid (TCA) for 10 min, rinsed with water for 5 min, and with a solution containing 25% methanol and 10% ace-

tic acid for 5 min. The gel was stained with 0.25% Coomassie Brilliant Blue G-250 (in 9.25% acetic acid, 45% methanol) and destained with a solution containing 25% methanol and 10% acetic acid. The protein bands were excised from the gel, and a protein-free piece of gel was used as a negative control.

Electrophoresis and RNase activity staining

The protein bands excised from IEF gel were equilibrated in 0.5 mL modified SDS-sample buffer [0.065 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% bromophenol blue] for 5 min and subjected to an RNase activity gel assay. SDS-PAGE was performed according to established procedures using a Bio-Rad Mini-Protean II cell (Bio-Rad) and gels consisting of 12% total monomer with 1% cross-linker and 3.0 mg mL⁻¹ RNA (Torula yeast RNA; Sigma). After electrophoresis, the gel was washed, incubated, stained and destained by the method described by Yen & Green (1991), except that the incubation was performed at 53 °C. The incubation temperature was selected based on previous studies indicating that either higher or lower temperatures decrease the activity of most nucleases (Brown & Ho 1986; Yen & Green 1991).

Nucleic acid substrates

RNA was extracted from micropropagated *Betula pendula* clone Wales02 following the method described by Chang, Puryear & Cairney (1993), except that L-spermidine and EDTA were omitted from the extraction buffer.

DNase activity was assayed using pUC19 plasmid as double-stranded DNA substrate either in the super-coiled form or, after digestion with *Hinf*I, in the linearized form. The *Silene* metallothionein (GenBank accession number AF101825) λ DNA was used as a single-stranded DNA template.

RNase and DNase activity assays

HPLC-purified S-glutathiolated and reduced forms of PR-10c-His were solubilized in 10 mM potassium phosphate buffer, pH 7.0. The incubation mixture contained, in final volume of 50 μ L, 5 μ g of RNA from birch and 10 μ g of purified protein. The samples and the control without protein were incubated at 53 °C, and 10 μ L samples were taken from the incubation mixture after 0, 30, 60, 90 and 120 min and kept on ice. Nucleic acids were separated on 1.0% agarose gel containing 0.5 μ g mL⁻¹ ethidium bromide.

DNase activity was assayed using various DNA substrates. Assay mixtures containing 2 μ g of protein and 2 μ g of DNA were incubated at 53 °C for 2 h, and nucleic acids were separated on 1.0% agarose gel containing 0.5 μ g mL⁻¹ ethidium bromide. GeneRuler[®] 1 kb DNA ladder (MBI Fermentas, Vilnius, Lithuania) markers were used as a standard for evaluating the apparent molecular weights.

RESULTS

Analysis of PR-10c-His by reversed-phase HPLC and electrospray ionization mass spectrometry

The Ni-NTA purified PR-10c-His was analysed with reversed phase (RP)-HPLC. By adjusting the gradient, PR-10c-His eluted as four distinct peaks, although peaks three and four were only partially resolved. Analysis of the HPLC fractions with SDS-PAGE and HPLC-ESI-MS indicated that peak 4 was a dimer of PR-10c-His.

The PR-10c-His was further analysed with on-line HPLC-ESI-MS. The MS spectra collected during the elution revealed three major masses: 19808.0, 19851.0 and 20113.0 Da (Fig. 1). These masses corresponded to the HPLC peaks 3, 2 and 1, respectively. The mass of peak 3 corresponded to the predicted mass of PR-10c-His (19807.25), and Δm of +305 Da for peak 1 suggested that the observed modification of the protein is S-glutathiolation. A Δm of +43 Da for peak 2 is consistent with several common modifications including acetylation (+42 Da), carbamylation (+43 Da) and carboxylation (+44 Da). The mass 20156.0 Da corresponded to S-glutathiolated form of PR-10c-His with a Δm of +43 Da.

The Ni-NTA purified Bet v 1-His was also analysed for comparison with RP-HPLC using the same gradient but no post-translational modifications were observed.

Identification and localization of the post-translational modifications of PR-10c-His

The post-translational modifications were confirmed with enzymatic digestion and mass spectrometry. The separated fractions from HPLC were digested with trypsin and endoproteinase Glu-C, and analysed with MALDI-TOF mass spectrometry. The major difference between the HPLC fractions was the appearance of a signal m/z 2162.96 in the tryptic digestion of peak 1 (Fig. 2). This signal corresponded to the peptide 'YCYSIEGGPLGDTLEK' (m/z 1856.89), amino acids 81–97, with S-glutathiolation (Δm of +305.31 Da). Endoproteinase Glu-C digestion revealed the appearance of a signal m/z 2020.68 in the digest of peak 1 (Fig. 3), which corresponded to the peptide 'IDHANFKY-CYSIIE' (m/z 1714.80), amino acids 74–87, with S-glutathiolation (Δm of +305.31 Da). Both peptides contained the only cysteine of PR-10c-His, which strongly suggested that S-glutathiolation was the detected post-translational modification.

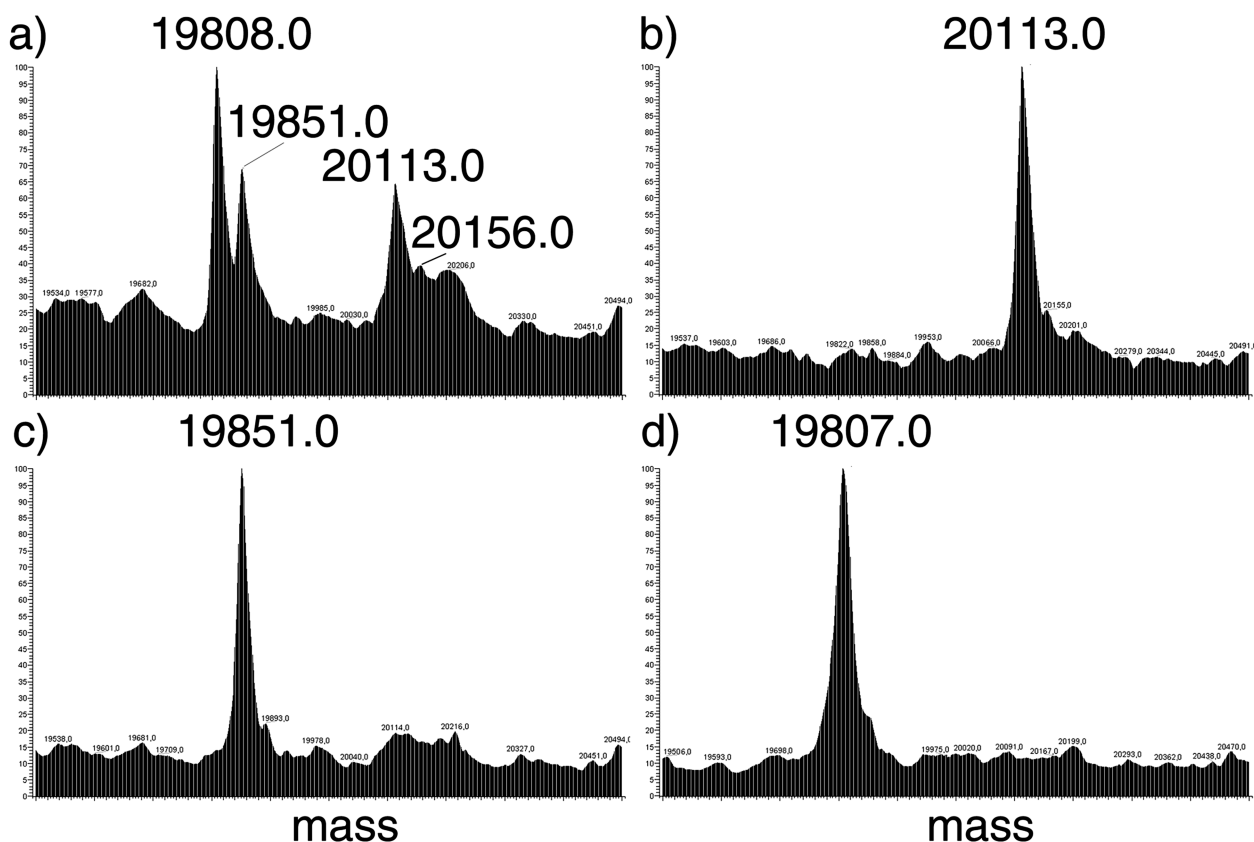


Figure 1. HPLC-ESI-MS analysis of Ni-NTA purified PR-10c-His. (a) A deconvoluted mass spectrum of PR-10c-His; (b) a deconvoluted mass of peak 1 (20113.0 Da) corresponds to the species with Δm , +305 Da relative to PR-10c-His; (c) a deconvoluted mass of peak 2 (19851.0 Da) corresponds to the species with Δm , +43 Da relative to PR-10c-His; (d) a deconvoluted mass of peak 3 (19807.0 Da) corresponds to the calculated mass of PR-10c-His.

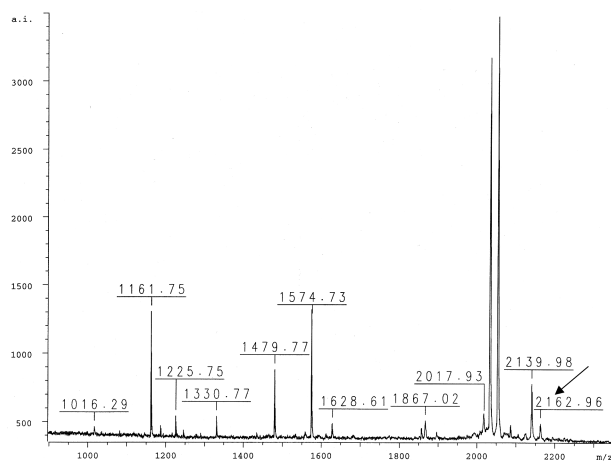


Figure 2. MALDI-TOF mass spectrum of the tryptic peptides from S-glutathiolated PR-10c-His. The signal m/z 2162.96 corresponds to the peptide 'YCYSIEGGPLGDTLEK' (m/z 1856.89) with S-glutathiolation (Δm , +305.31 Da).

The identity of the peptides was confirmed by HPLC-ESI-MS analysis of the trypsin and endoproteinase Glu-C digested fractions. In trypsin digestion, full scan MS-MS spectra for doubly charged signal m/z 1082.50 showed several predicted Y' and B ions of a peptide containing glutathione (mass shift 305.31 Da) in the cysteine residue (Fig. 4). The Y' and B ions with mass shift 305.31 was also detected from full scan MS-MS spectra of doubly charged signal m/z 1011.10 in Glu-C digestion (data not shown).

The presence of a disulphide bond between glutathione and cysteine was also confirmed with HPLC analysis. Treatments with reductant (DTT) or oxidized glutathione (GSSG) decreased or increased the area of peak 1 in HPLC, respectively (data not shown).

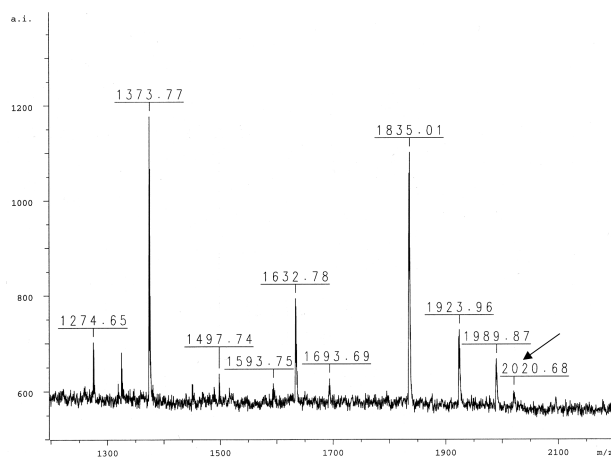


Figure 3. MALDI-TOF mass spectrum of the endoproteinase Glu-C digested S-glutathiolated PR-10c-His. The signal m/z 2020.68 corresponds to the peptide 'IDHANFKYCYSIIE' (m/z 1714.80) with S-glutathiolation (Δm , +305.31 Da).

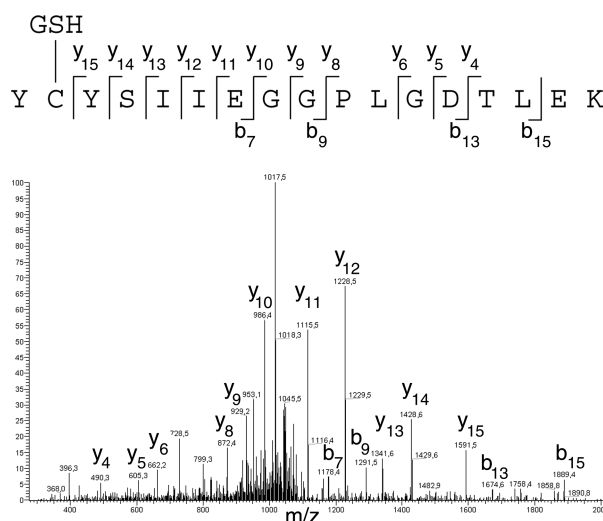


Figure 4. HPLC-ESI-MS-MS spectrum of doubly charged signal m/z 1082.50. The signal corresponds to the cysteine containing fragment 'YCYSIEGGPLGDTLEK' (m/z 1856.89) with a disulphide bond between the cysteine residue and glutathione (m/z 305.31). The theoretical fragmentation of the polypeptide and the corresponding Y' and B ions in the measured spectrum are shown.

A mass difference of 43 Da was shown to be associated with the N-terminal peptide 'MRGSHHHHHHTDPAHSSVPRVDGVFN'Y', amino acids 1–28, of PR-10c-His using endoproteinase Glu-C digestion and HPLC-ESI-MS (data not shown). According to the mass difference and localization, the modification was deduced to be acetylation (+42 Da) or carbamylation (+43 Da). However, *E. coli* is not known to perform post-translational modifications, such as acetylation, common to eukaryotes. Carbamylation of the protein is probably caused by the degradation of urea in buffers used for the purification of PR-10c-His, and does not thus represent a biologically significant modification.

PR-10c is post-translationally modified by glutathione in plant

The possible S-glutathiolation of PR-10c *in vivo* was tested using roots of birch clone 142B exposed to 1137 mg Zn kg^{-1} soil, because the protein is highly expressed in the roots of this clone. The S-glutathiolation of PR-10c *in vivo* was tested by MALDI-TOF analysis of PR-10c eluted from a silver-stained 2-D gel. The PR-10c was slowly crystallised into MALDI-MS matrix solution (Cohen & Chait 1997). The signal m/z 17592.01 and doubly charged signal m/z 8797.64 (17593.28 Da) detected corresponded to the predicted mass of S-glutathiolated PR-10c (17595.88 Da) (Fig. 5). The mass error was less than 0.1%, which is the previously observed accuracy of this extraction method (Cohen & Chait 1997).

Polypeptide identity was confirmed using an identical reference gel and by eluting protein directly into an aqueous solution. Tryptic peptides of the eluted protein were

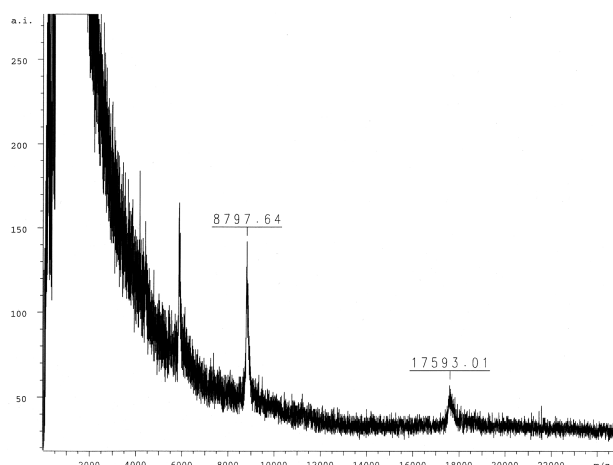


Figure 5. MALDI-TOF mass spectrum of the *in vivo* S-glutathiolated PR-10c protein. The signal m/z 17593.01 and doubly charged signal m/z 8797.64 (17593.28 Da) correspond to the predicted mass of S-glutathiolated PR-10c (17595.88 Da) with the error less than 0.1%.

analysed with HPLC-ESI-MS and two PR-10c-specific peptides (1331.7 and 2140.0 Da) were detected. These results strongly suggest that PR-10c is S-glutathiolated not only in *E. coli* but also *in vivo* in the plant.

Ribonuclease activity of PR-10c-His

Isoelectric focusing was performed to confirm the purity of the HPLC-separated forms of PR-10c-His. Two major bands appeared in all HPLC-purified fractions, although the intensity of the bands varied. The band at the higher and lower pI represented the reduced and S-glutathiolated form of PR-10c-His, respectively. The reduced form of PR-10c-His and the dimer could not be separated from each other with isoelectric focusing, because their pI is the same.

The bands corresponding to the S-glutathiolated and reduced forms of PR-10c-His were excised and subjected to RNase activity gel. A protein-free piece of gel was included

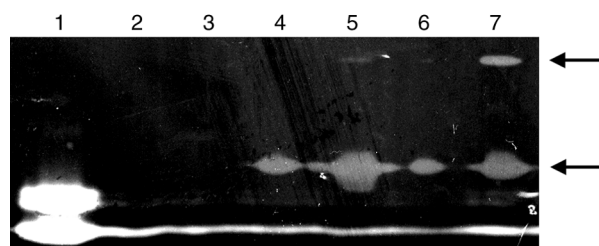


Figure 6. RNase activity gel of PR-10c-His purified by HPLC and separated by isoelectric focusing. Lane 1, 0.1 μ g RNase A (positive control); lane 2, empty; lane 3, protein-free piece of gel (negative control); lanes 4–5, S-glutathiolated PR-10c-His; lanes 6–7, reduced PR-10c-His.

as a negative control and 0.1 μ g of RNase A was included as a positive control. The results indicated that both the S-glutathiolated and the reduced forms of PR-10c-His possess RNase activity (Fig. 6, lanes 4–7). The RNase activity of the PR-10c-His dimer was separately seen in the sample of reduced form of PR-10c-His. The molecular weights of the detected RNases corresponded to the PR-10c-His bands in Coomassie Brilliant Blue-stained reference gel and no contamination was observed in samples (data not shown). There was no detectable RNase activity in the negative control (Fig. 6, lane 3).

Degradation of nucleic acids by PR-10c-His and Bet v 1-His

The reduced and S-glutathiolated forms of PR-10c-His were prepared by incubating the Ni-NTA purified protein with 10 mM DTT and 10 mM GSSG for 30 min, respectively. The proteins were further purified with RP-HPLC and solubilized in 10 mM potassium phosphate buffer. The identity of the two forms of PR-10c-His was confirmed with MALDI-TOF-MS and HPLC-ESI-MS analyses of trypsin and endoproteinase Glu-C digested proteins. The time-course of RNA degradation was assayed by incubating total RNA from birch with PR-10c-His (data not shown). The accumulation of small-molecular-weight products was

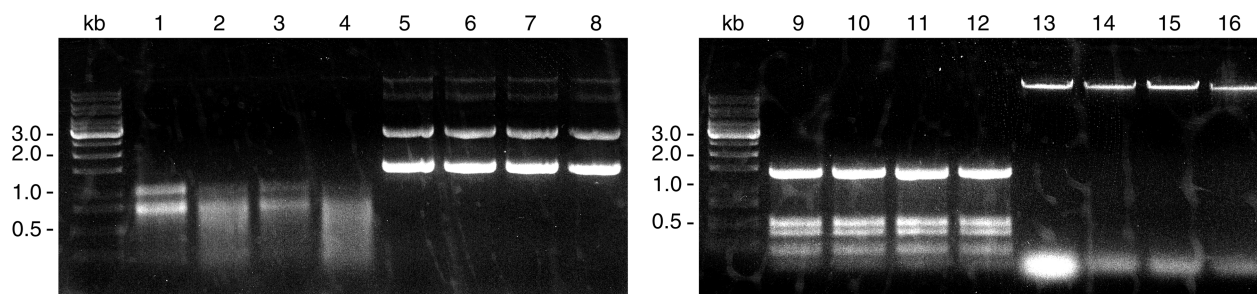


Figure 7. Degradation of nucleic acids by PR-10c-His. The proteins (2 μ g) were incubated for 2 h with different nucleic acid substrates: birch RNA (1 μ g) (lanes 1–4), super-coiled dsDNA (2 μ g) (lanes 5–8), linear dsDNA (2 μ g) (lanes 9–12) and ssDNA (2 μ g) (lanes 13–16). Samples were loaded into 1.0% agarose gel containing 0.5 μ g mL⁻¹ ethidium bromide. The nucleic acid substrates without protein were included as negative controls (lanes 1, 5, 9 and 13). Lanes 2, 6, 10 and 14: S-glutathiolated PR-10c-His; lanes 3, 7, 11 and 15: reduced PR-10c-His; lanes 4, 8, 12 and 16: Bet v 1-His.

observed after 30 min, and partial degradation of 28S rRNA and 18S rRNA was observed after 60 min. The results indicated that the S-glutathiolation does not influence the RNase activity of PR-10c-His protein.

DNase activity of PR-10c-His and Bet v 1-His was assayed by incubating the proteins, isolated as above, with super-coiled or linear dsDNA or ssDNA. Neither Bet v 1-His nor the S-glutathiolated or reduced forms of PR-10c-His showed any activity against the DNA substrates (Fig. 7, lanes 5–8 and 9–16). The RNase activity of the proteins can be seen as partial degradation of birch RNA (Fig. 7, lanes 1–4) and the RNA contamination in single-stranded DNA samples (Fig. 7, lanes 13–16).

DISCUSSION

The PR-10c of birch is a member of a ubiquitous group of intracellular pathogenesis-related proteins. The general function of PR-10 proteins as ribonucleases was deduced and proposed based on sequence similarity with ribonucleases from ginseng callus (Moiseyev *et al.* 1994, 1997). RNase activity of PR-10 proteins has been previously demonstrated for Bet v 1, the major birch pollen allergen (Swoboda *et al.* 1996; Bufe *et al.* 1996) and LaPR-10 from white lupin roots (Bantignies *et al.* 2000). The present results obtained with PR-10c-His produced in *E. coli* indicate that PR-10c is an RNase capable of cleaving RNA from birch and yeast, and that it does not possess DNase activity. These results are plausible because the purity of samples and molecular weight in RNase activity gel was confirmed from reference gel. However, the structural homology of Bet v 1 to the START domain of MLN64, which has been associated with cholesterol transport, suggests a similar function of Bet v 1 and the other members of PR-10 family in sterol binding (Tsujiyama & Hurley 2000). The recent NMR spectroscopy results also suggest the interaction of Pru av 1 with phytosteroids (Neudecker *et al.* 2001) but whether all PR-10 proteins have a similar function is unknown.

Even though PR-10c belongs to the Bet v 1 family, the sequence identities of PR-10c and the different pollen allergens range from 70 to 74% at the amino acid level (Swoboda *et al.* 1995a). However, PR-10c is unique among other Bet v 1 homologous proteins in birch. It has a different expression pattern compared with other members of the Bet v 1 family (Poupard *et al.* 1998) and it does not belong to the same monophyletic group (Wen *et al.* 1997). There is a high homology between PR-10c and Cor a 1-0401 (GenBank accession number AAD48405) from hazelnut (*Corylus avellana*); they have 20 identical amino acids around the cysteine residue (C82) and the whole proteins are 85% identical.

The present results also indicate that PR-10c is post-translationally modified by glutathione both *in vitro* and *in vivo* in plant. Post-translational modifications of the isoforms of Bet v 1 have been studied previously and, except the cleavage of initiating methionine, none were observed (Swoboda *et al.* 1995b). The S-glutathiolated cysteine resi-

due of PR-10c is not conserved among Bet v 1 homologous proteins, suggesting a unique role for S-glutathiolation of PR-10c compared to the other members of Bet v 1 family. There are also other examples of proteins with high sequence homology having different post-translational modifications. Studies on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the yeast *Saccharomyces cerevisiae* indicate that, despite high sequence homology (98% similarity and 96% identity), the different isoenzymes show differential S-glutathiolation in response to oxidative stress (Grant, Quinn & Dawes 1999).

The S-glutathiolation of proteins is currently widely studied in mammalian tissues and cell lines but no such studies have been previously performed in plants. The mixed disulphide formation of proteins with glutathione has been observed in animals (Cappiello *et al.* 1995), cell lines (Figueiredo-Pereira, Yakushin & Cohen 1998) and in *S. cerevisiae* (Grant *et al.* 1999) in response to oxidative stress. S-glutathiolation of proteins may not require enzymatic activity and may occur by the reaction of GSH (reduced glutathione) with partially oxidized reactive protein sulfhydryl groups, or by thiol-disulphide exchange reaction with GSSG (oxidized glutathione) (Thomas, Poland & Honzatko 1995). It should be recognized, however, that glutaredoxin has been more recently reported to catalyse the S-glutathiolation of proteins (Lind *et al.* 1998).

The function of proteins can be regulated through mixed disulphide formation by glutathione. Protein kinase C- α (Ward *et al.* 2000) and creatine kinase (Reddy *et al.* 2000) are inactivated by S-glutathiolation. The GAPDH was shown to be inactivated by S-glutathiolation (Grant *et al.* 1999), in contrast to previous studies that suggested that S-glutathiolation does not affect the dehydrogenase function of GAPDH (Lind *et al.* 1998). Inactivation is primarily due to direct oxidation by hydrogen peroxide. The S-glutathiolation may also positively regulate the function of some proteins. S-glutathiolation activates the phosphatase of carbonic anhydrase III (Cabiscol & Levine 1996), and microsomal glutathione transferase (Dafre, Sies & Akerboom 1996).

The S-glutathiolation of proteins has been associated with buffering of oxidative stress, stabilization of extracellular proteins, protection of critical cysteine residues against irreversible oxidation, and regulation of enzyme activity (Thomas *et al.* 1995; Cotgreave & Gerdes 1998). Furthermore, S-glutathiolation has recently been associated with the regulation of cellular signalling pathways in response to oxidative stress, e.g. the regulation of ubiquitin-proteasome pathway (Klatt & Lamas 2000). The role of glutathione and redox state in the regulation of plant RNase activities has been reported with chloroplast endoproteinase p54. This protein is activated by GSSG and inhibited by GSH *in vitro* (Liere & Link 1997).

Heavy metals can inhibit the activity of several enzymes through reactive thiol groups in mammalian systems (Bandyopadhyay, Chatterjee & Datta 1997; Figueiredo-Pereira *et al.* 1998; Hanahisa & Yamaguchi 1998). Furthermore, heavy metals zinc and cadmium have been reported to

induce oxidative stress and protein-mixed disulphide formation with GSH in neuronal cells (Figueiredo-Pereira *et al.* 1998; Figueiredo-Pereira & Cohen 1999). The present observation that PR-10c is S-glutathiolated *in vivo* in birch roots exposed to zinc suggests a specific role for this protein in response to oxidative stress. We have observed that PR-10c is induced in the leaves and roots of birches in response to oxidative stress (Koistinen *et al.* unpublished). The increasing RNase activity apparently increases the turnover of polynucleotides, and releases nucleotides for the needs of increasing transcription. In consequence, the cell may respond more rapidly to the changed requirements in transcription. Our data demonstrate that, although PR-10c is S-glutathiolated, the RNase activity of the protein remains unchanged. This result is supported by the observation that the disulphide formation between two PR-10c-His proteins does not affect RNase activity. The role of S-glutathiolation of PR-10c could be the protection of the reactive thiol group and RNase activity of the protein against irreversible oxidation and possible inactivation of the protein under heavy metal stress.

The PR-10 family of intracellular pathogenesis-related proteins is an extensively studied group of proteins, due to their close structural relationship with allergens. However, not much attention has been paid so far to the post-translational modifications of these proteins, probably due to the inherent difficulties involved in the study. Although there are several reports on S-glutathiolation in the mammalian tissues and cell lines, the modification of plant proteins is poorly studied. As far as we know, this is the first demonstration of S-glutathiolation, or of any post-translational modification, in the PR-10 proteins.

ACKNOWLEDGMENTS

This work was supported by the Finnish Graduate School in Environmental Science and Technology (ENSTE); by the Academy of Finland, Ecotoxicology Programme (Contract no. 8128), The Research Programme for the Restoration of Boreal Environments (Restore 2000, Contract no. 35555), Finnish Research Programme on Environmental Health, SYTTY (Contract no. 42511); by the European Commission: Phytorehab (Contract No ENV4-CT95-0083) and GMOCARE (Contract No QLK1-1999-00765); and by the Finnish Research Foundation for Natural Resources. The pET28a/Bet v 1 fusion protein was kindly supplied by Dr Spangfort, Denmark (ALK-ABELLO). Professor Howard Davies (Scottish Crop Research Institute) is acknowledged for valuable comments.

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Received 21 September 2001; received in revised form 16 January 2002; accepted for publication 16 January 2002