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Characterization and oxidative in vitro cross-linking of an extensin-like protein and a proline-rich protein purified from chickpea cell walls

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Abstract

Two cell wall proteins from chickpea, known to be rapidly insolubilised by an elicitor-stimulated oxidative burst in-vivo, were purified from suspension cells. N-terminal protein sequencing revealed them as a proline-rich protein and an extensin-like protein. Oxidative cross-linking could be modelled in an in vitro system utilising horseradish peroxidase, H_2O_2 and the substrate proteins.

Keywords: *Cicer arietinum*; Fabaceae; Chickpea; Cell wall; Oxidative cross-linking; Oxidative burst; Extensin; Proline-rich proteins

1. Introduction

Extensins (hydroxyproline-rich glycoproteins, HRGP), proline-rich proteins (PRPs) and glycine-rich proteins (GRPs) are structural proteins of the primary plant cell wall. With the exception of GRPs, hydroxyproline (Hyp) is abundant in these protein classes and therefore they are designated as Hyp-rich proteins (Showalter, 1993; Sommer-Knudsen, Bacic & Clarke, 1998). Such proteins are thought to play a role in modification of cell wall properties, as strengthening in order to restrict cell extension in the developmental cessation of growth and wall penetration by pathogens. Hyp-rich proteins are secreted into the apoplast where they become ionically bound to acidic polysaccharides (Fry, 1986). Their solubility is strongly decreased during cell development (Smith, Muldoon & Lamport, 1984) or by external stimuli, e.g. wounding or infection (Brisson, Tenhaken & Lamb, 1994). The application of elicitors to plant cells leads within a few minutes to a state in which these proteins lose their extractability in salts and SDS, presumably through the formation of covalent cross-links (Wojtaszek, Trethowan & Bolwell, 1995; Otte & Barz, 1996; Brady, Sadler & Fry, 1996; Brady & Fry, 1997). Although the mechanism of the covalent cross-link is unknown, it seems very likely that a peroxidase-catalysed oxidative coupling of the phenolic rings of Tyr-residues mediates the insolubilisation of the cell wall proteins. Beside isodityrosine (IDT), the trimeric and the tetrameric Tyr-derivates, di-IDT (Brady et al., 1996) and pulcherosine (Brady, Sadler & Fry, 1998), respectively, may form both intra-polypeptide loops (Epstein & Lamport, 1984) and inter-polypeptide bridges (Biggs & Fry, 1990). Earlier studies have shown that structural proteins like collagens (Labella, Waycole & Queen, 1968) and non-structural proteins like galactose oxidase (Tressel & Kosman, 1980) can be cross-linked by the action of peroxidase and H_2O_2 in vitro.

The challenge of plant cells with H_2O_2 had been shown to trigger protein insolubilisation, whereas catalase or peroxidase (POD)-inhibitors as well as reducing

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Table 1
N-terminal amino acid sequences of the purified chickpea proteins p190 and p60 in comparison with sequences from (hydroxy-)proline-rich proteins of other plant species[a]

Protein	Plant species	Sequence	Reference
p190	***Cicer arietinum***	NRHDSYSOOOOOOLV	This work
Extensin	*Phaseolus vulgaris*	NHYSYSSOOOOOVV	Wojtaszek et al. (1995)
HRGP	*Phaseolus vulgaris*	DMYLPOVOOOOV	Wojtaszek et al. (1995)
Extensin	*Vigna unguiculata*	ADAYTHSPPPPPPYV	Arsenijevic-Maksimovic, Broughton and Krause (1995)
Extensin	*Nicotiana tabacum*	PPAKQPSPPPPPPV	de S. Goldman, Pezzotti, Seurinck and Mariani (1992)
Extensin	*Sorghum vulgare*	VSHTPSSPPPPPPP	Raz, Crétin, Puigdomènech and Martínez-Izquierdo (1991)
p60	***Cicer arietinum***	NYEKPOEYNPOIIYHPOE	This work
PRP1	*Glycine max*	DYEKPPIYKPPV-YTPPV	Hong, Nagao and Key (1987)
PRP2	*Glycine max*	NYENPPVYKPPTEKPPVY	Datta, Schmidt and Marcus (1989), Hong, Nagao and Key (1990)
Nodulin	*Pisum sativum*	PHEKPPHENPPPVYKPPY	van de Wiel et al. (1990)
Nodulin	*Glycine max*	PIEKPPTYEPPPFYKPPY	Franssen et al. (1987)
Nodulin	*Medicago sativa*	PHEKPPOVKPPSEYQPPH	Dickstein, Bisseling, Reinhold and Ausubel (1988)
PRP	*Cicer arietinum*	PVEKPPVYKPPIEKPPVY	Munoz, Labrador and Dopico (unpublished)
PRP3	*Glycine max*	PIKKPPVYKPPV-YKPPV	Hong, Nagao and Key, 1990
Extensin	*Nicotiana tabacum*	FSPPPPAYSPPPTYSPPP	Keller and Lamb (1989)

[a] p190 and p60 were finally separated by SDS-PAGE, blotted onto PVDF, stained with Coomassie Brilliant Blue and excised. Protein bands were subjected to amino acid sequencing on an ABi 477A protein sequencer. Sequence homology searches were carried out via electronic mail servers Fasta 3.0-SwissProt Pearson and Lipman (1988) and PIR Protein 51.0 Altschul, Gish, Miller, Myers and Lipman (1990). Underlined codes represent amino acid positions, which are homologous to the corresponding chickpea protein. Note that hydroxyproline (O) is indicated only in the case of amino acid studies; amino acid sequences deduced from DNA sequences cannot distinguish proline from hydroxyproline. Gaps ("-") were inserted to maximise alignment.

agents readily block the elicitor- and H_2O_2-triggered process (Otte & Barz, 1996). Studies in a range of organisms including animals (sea urchin (Shapiro, 1991)), algae (*Chlamydomonas* (Waffenschmidt, Woessner, Beer & Goodenough, 1993)) and plants (soybean (Brisson et al., 1994), french bean (Wojtaszek et al., 1995), chickpea (Otte & Barz, 1996)) indicated the possible involvement of the rapid elicitor- and infection-stimulated production of reactive oxygen species (oxidative burst) in oxidative cross-linking of extracellular proteins. In case of chickpea we could recently show that the H_2O_2 from an elicitor-stimulated oxidative burst directly drives the synchronous insolubilisation of both a putative extensin (p190) and a PRP (p60[1]) in the walls of cultured cells (Otte & Barz, 1996). Cell wall preparations from such cells with endogenous peroxidase activities were also used as a model for the H_2O_2-triggered insolubilisation of the cell wall proteins (Otte, 1998).

The present work shows that these proteins indeed represent members of the PRP and extensin families and that their N-termini contain sequence motifs probably important for the formation of Tyr-bridges. It is demonstrated that H_2O_2 and POD activity are sufficient to trigger the oxidative cross-linking of both purified proteins in vitro.

[1] In reference Datta, Schmidt and Marcus (1989), the p60 was still termed, "p80". "p60" is a correction according to a more accurate determination of its M_r (ca 60,000) by electrophoretic mobility (compare Fig. 1).

2. Results and discussion

2.1. Purification of wall proteins

The chickpea cell cultures were pretreated with 1 mM salicylhydroxamic acid (SHAM) for 20 min before use in the purification process (as described in detail in, Section 3), because SHAM acts as a potent inhibitor of elicitor-stimulated cell wall protein insolubilisation in vivo (Otte & Barz, 1996). $CaCl_2$ readily eluted ionically bound proteins from washed cell wall-rich material. The result was a substantial enrichment of both previously characterised chickpea wall proteins p190 and p60 (Otte & Barz, 1996) relative to their low quantitative presence in a crude total cell extract. The increasing concentration of both proteins during the purification process was proven by their increasing signal intensity in western blot analysis using the antisera anti-p33 and anti-gE1 (Otte & Barz, 1996), generated against a soybean PRP and a carrot extensin, respectively. Proteins in the $CaCl_2$ extract were further fractionated by ammonium sulfate precipitation in which both the p190 and the p60 precipitated almost completely between 20 to 45% saturation. This protein fraction was chromatographically separated using a Mono S-cation exchange column and a NaCl gradient for elution. p190 and p60 were eluted between a NaCl concentration range of 50–300 mM NaCl. Those eluates, which contained almost exclusively p190 and p60, were directly subjected to gel filtration chromatography (Superdex 75). p190 and p60 could not be separ-

ated from each other by gelfiltration despite their different apparent M_r. However, fractions were obtained which only contained both cell wall proteins and were totally free from other proteins.

2.2. The N-terminal sequences of p190 and p60

p190 and p60 were finally separated by SDS-PAGE, blotted onto PVDF, and subjected to N-terminal protein sequencing. The sequences obtained for both proteins were compared with partly homologous proteins from several plant species (Table 1).

The p190 was clearly identified as a member of the extensin family. The most striking property of the p190-N-terminus is a block of six Hyp. The Hyp-rich extensins from dicots are rich in Hyp, Ser, Val, Tyr, Lys, and His and carry the characteristic repetitive pentapeptide Ser-Hyp_4, the latter often as extensive repetitive sequences (Showalter, 1993). The chickpea p190-sequence contains the diagnostic pentapeptide and with the exception of Lys all abundant amino acids characteristic for extensins. The N-terminus shows high sequence similarity to an extensin and a HRGP from *Phaseolus vulgaris*. A Ser-Pro/Hyp_{6-8}-motif appears in sequences deduced from DNAs from such different plant species as *Vigna unguiculata*, *Nicotiana tabacum*, and *Sorghum vulgare* (Table 1). It was not necessary to perform a deglycosylation of this protein prior to sequencing, since arabinose side chains (Hyp-oligoarabinosides) appeared to be completely hydrolysed during the Edman degradation (Wojtaszek et al., 1995).

The p60 was shown to be a PRP with high similarity to nodule-specific PRPs. PRP genes are characterized by repetitive Pro–Pro-pairs, which appear within larger repetitive sequences (Showalter, 1993). PRPs are rich in Pro and Hyp in equimolar amounts (Kleis-San Francisco & Tierney, 1990). Data from amino acid sequence analyses, which are rare compared with data of nucleotide sequences, show hydroxylation patterns, in which Hyp appears exclusively in the second position of the repetitive Pro–Hyp- pairs (Wojtaszek et al., 1995; Averyhart-Fullard, Datta & Marcus, 1988; Kleis-San Francisco & Tierney, 1990). Further abundant amino acids are Val, Tyr, Lys and in nodulin-PRPs Glu and His. The 18-amino acid-N-terminus of the chickpea p60 contains three repetitive Pro-Hyp-pairs and with the exception of His, all typical abundant amino acids, including Tyr and Glu, three times each (Table 1). The first, second, and most third Pro–Pro/Hyp repeats all proteins compared with the p60 which appear in regular intervals. All sequences with the exception of the last two of the second block, carry a common Glu–Lys–Pro–Hyp-motif (EKPP/O, Table 1). While the tripeptides Lys–Pro–Hyp (Wojtaszek et al., 1995; Kleis-San Francisco & Tierney, 1990) and Asn–Pro–Hyp (Averyhart-Fullard et al., 1988) within the p60-N-terminus have been described, the His–Pro–Hyp sequence represents, according to our knowledge, a new hydroxylation pattern.

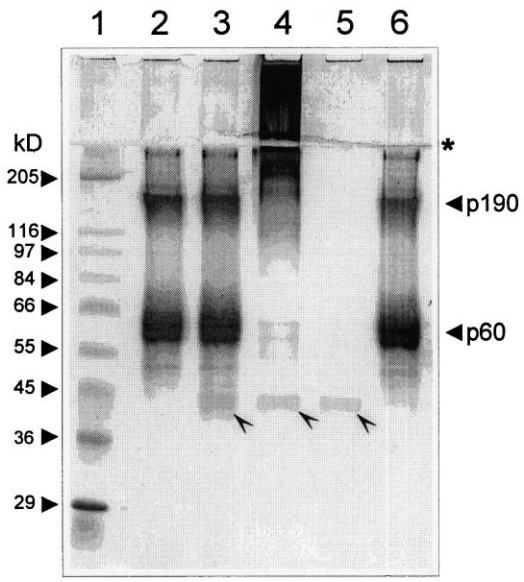

Fig. 1. Evidence for in vitro cross-linking of the cell wall structural proteins p190 and p60. SDS-PAGE analysis of various protein reaction mixtures (see Section 3): p190/p60 + H_2O (lane 2); p190/p60 + horseradish peroxidase + H_2O (lane 3); p190/p60 + horseradish peroxidase + H_2O_2 (lane 4); horseradish peroxidase + H_2O (lane 5); p190/p60 + H_2O + H_2O_2 (lane 6). The stained bands of the horseradish peroxidase (40 kDa) are marked with arrowheads. M_r standards were separated in lane 1. The asterisk at the right indicates the border between stacking and separation gel.

In common with the two HRGPs from *Phaseolus vulgaris*, the chickpea p190 contains Tyr in its N-terminus (Table 1), which is necessary for insolubilisation caused by the putative intermolecular Tyr-couplings. It is likely that Tyr is abundant in these three elicitor-stimulated insolubilised proteins, because of its putative Tyr–Ser–Hyp_4 repeats. The abundance of Tyr in the PRPs is also shown in their N-terminal sequences (Table 1). The positions 2, 8, and 14 of the three Tyr in the p60-N-terminus turn out to be highly conserved within the compared PRP-like sequences (Table 1). The relatively high Tyr-content of these cell wall proteins is most probably a prerequisite for the (pathogen-stimulated) formation of IDT-, pulcherosine-, and di-IDT-cross-links, which strengthen the plant cell wall. The evolutionarily well conserved Tyr-pattern seems to emphasize this biological function.

2.3. In vitro cross-linking of the purified wall proteins

The purified proteins in an aqueous solution were used in a simple cross-linking model reaction in the presence of H_2O_2 and horseradish peroxidase (HRP).

Different modified reaction mixtures were analysed by SDS-PAGE after incubation at 25°C for 30 min (Fig. 1). In contrast to the analysis of the aqueous solution of p190 and p60 (control; Fig. 1, lane 2), both protein bands disappeared from their characteristic positions in the gel as a result of a joint mobility shift (Fig. 1, lane 4). A new high-M_r complex appeared as a broad band, spanning the stacking gel and the bordering part of the separation gel. The density of the HRP band in this complete mixture (Fig. 1, lane 4) remained unchanged during the reaction, when compared with the gel analysis of the same amount of HRP (Fig. 1, lane 5). No high-Mr product appeared when either H_2O_2 or HRP were replaced by water (Fig. 1, lane 3, 6). The mobility shift can be explained as a result of intermolecular peroxidative cross-linking of p190 and p60. Whether only homo- or also heteropolymers, or both, result from this process, could not be shown in this experiment with unresolved p190 and p60, because they could not be separated except by SDS-PAGE. The addition of H_2O_2 to intact chickpea cells causes an almost immediate insolubilisation of both proteins (Otte & Barz, 1996). The cell's own peroxidase activity, which is necessary for the oxidative cross-linking in vivo, could now easily be substituted by HRP in vitro, indicating that specific, ''extensin-PODs'' as suggested by other authors (Brownleader, Ahmed, Trevan, Chaplin & Dey, 1995; Schnabelrauch, Kieliszewski, Upham, Alizedeh & Lamport, 1996), may not be required. The high molecular protein polymers as shown in Fig. 1, lane 4, may represent a material for further elucidation of the insolubilisation process.

3. Experimental

3.1. Material

Cell suspension cultures of chickpea (*Cicer arietinum* L. cv. ILC 3279) were grown as previously described (Kessmann & Barz, 1987). Cells, 3 days after subculturing, were used for experiments. Analytical grade chemicals, H_2O_2, and horse radish peroxidase (EC 1.11.1.7) were purchased from Sigma.

3.2. Extraction and purification of p190 and p80

Cell wall-rich material was isolated as a pellet resulting from homogenisation of cells (w/v = 1:1) in 50 mM HEPES pH 7.0 (buffer A) and subsequent centrifugation (10 min, 27,000 g). The pellet was resuspended (w/v = 1:3) and washed twice with buffer A followed by repeated centrifugation. This material was resuspended in buffer A, now containing 200 mM $CaCl_2$ (w/v = 1:1) and again centrifuged. Proteins in the supernatant were fractionated by ammonium sulfate precipitation. Proteins were separated by centrifugation (20 min, 27,000 g), redissolved in 50 mM Tris/HCl pH 8.0 (buffer B) and desalted via Sephadex G25 (Pharmacia) in buffer B. Protein samples were subjected to cation-exchange chromatography on a fast protein liquid chromatography (FPLC) Mono S HR 5/5 column (Pharmacia; flow rate 0.4 ml min^{-1}) equilibrated with buffer B. The proteins p190 and p80 were eluted by increasing the concentration of NaCl in buffer B from 0 to 500 mM. Fractions containing p190 and p80 were pooled and applied to a FPLC Superdex 75 HiLoad 16/60 gel filtration column (Pharmacia; flow rate 1 ml min^{-1}) equilibrated with 1 M NaCl in buffer B. The p190/p80 pool was desalted, concentrated via lyophilisation, separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), and finally blotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore). N-terminal sequences of Coomassie Brilliant Blue-stained and excised p190- and p80-bands were determined using a gas-phase sequencer (model 477A, Applied Biosystems).

The presence of p190 and p80 in the fractions from each purification step was determined by SDS-PAGE and western blot analysis, using the antisera anti-p33 and anti-gE1 as previously described (Otte & Barz, 1996).

3.3. In vitro cross-linking

The following five mixtures in a 100 mM Tris-HCl (pH 7.0) reaction system were incubated in Eppendorf tubes for 30 min at 25°C prior to gel analysis: (1) 6 l p190/p60-solution plus 2 μl deionisised (*d*) H_2O; (2) 6 μl p190/p60-solution plus 1 μl horseradish peroxidase (HRP) solution plus 1 μl *d*H_2O; (3) 6 μl p190/p60-solution plus 1 μl HRP solution plus 1 μl H_2O_2 solution; (4) 1 μl HRP solution plus 7 μl *d*H_2O; (5) 6 μl p190/p60-solution plus 1 μl *d*H_2O plus 1 μl H_2O_2-solution. p190 and p60 were applied as a concentrated fraction from the gel-chromatography. The horseradish peroxidase (Sigma P-6782) solution contained 1 unit per μl Tris buffer (50 mM, pH 7.0), and the concentration of the H_2O_2 stock added to the mixture was 8 mM. The reactions were stopped by the addition of 8 μl SDS-sample buffer and a subsequent incubation at 100°C. The reaction mixtures were analysed by silverstained SDS-PAGE.

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