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Purification and Characterization of a Wound-Inducible Cell Wall Cationic Peroxidase from Carrot Roots

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We have isolated a novel cell wall, cationic peroxidase (pI>9.3) from roots of the carrot plant, *Daucus carota*. The purified isozyme, referred to as CP>9.3, has a molecular mass of 45 kilodaltons and an Reinheitzahl value of 2.3. Amino-acid composition analysis and N-terminal sequencing have been performed with CP>9.3. The N-terminal sequence shows no homology to any sequence in the protein and nucleic acid data banks. CP>9.3 activity is induced by wounding in carrot leaves and petioles; this activity is also present in carrot roots but is unaltered by wounding. Enhanced CP>9.3 activity is seen at 12 hr post-wounding and continues for at least 60 hr in leaves and petioles. Based on studies using cycloheximide, early activation of CP>9.3 is not due to *de novo* protein synthesis, but rather to enzyme activation. Temperature and pH optima for CP>9.3 using guaiacol as a substrate have been determined to be 32°C and 4.9.

Peroxidases (donor: H_2O_2 oxidoreductase; E.C.1.11.1.7) are a family of heme-containing isozymes which are distributed throughout the plant kingdom. These isozymes are differentially expressed in various tissues and organs in plants and respond to developmental and environmental cues. Their primary function is to oxidize a variety of hydrogen donors at the expense of hydrogen peroxide. Peroxidase activity in cell walls of plants is presumed to be involved with extensin and proline-rich protein cross-linking (1,2), ligninification (3,4,5,6), suberization (7), disease resistance (8) and wound-healing (9,10). However, the physiological function of individual isozymes is only partially understood and is complicated by the presence of multiple peroxidase isozymes. Our laboratory is interested in characterizing wound-inducible peroxidases from carrot and in elucidating their physiological role. There are a number of peroxidase isozymes in carrot plants. Of these, a cationic peroxidase is found to be wound-inducible in leaves and petioles, whereas, it is constitutively expressed in roots. Here we report the isolation and characterization of this novel, wound-inducible peroxidase with a pI value in excess of 9.3 which is hereafter referred to as CP>9.3.

MATERIALS AND METHODS

Plant material. Carrot plants (*Daucus carota*) were purchased from the grocery store and used for purification of the wound-inducible peroxidase and for all the wounding studies.

Plant manipulations, enzyme activity assay, electrophoresis, and protein determination. For the time course experiment, carrot leaves, petioles and roots were sliced into approximately 10 mm sections and incubated in moist containers for different times following wounding. In the cycloheximide studies, the organ sections were placed in aerated flasks containing 5 μg/mL cycloheximide for 6 hr and then incubated in moist containers (10). Crude peroxidases were extracted with 0.5 M NaCl and dialyzed against distilled water. Protein estimations were done with the Bio-Rad assay

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Abbreviations used: IEF, Isoelectric Focusing; CP>9.3, Cationic peroxidase >9.3; PMFS, Phenylmethylsulfonyl Fluoride; PVP, Polyvinylpyrrolinone; Rz, Reinheitzahl value.

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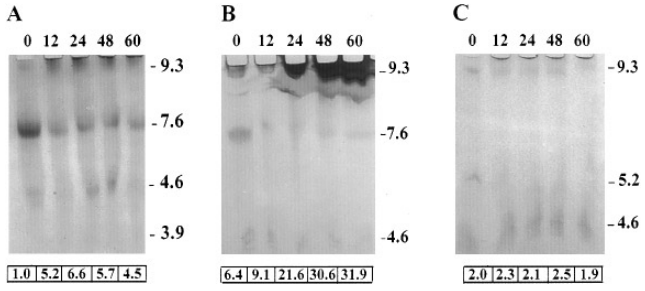

FIG. 1. IEF gels of unwounded and wounded carrot leaves (A), petioles (B), and roots (C) stained for peroxidase activity. Following wounding, this tissue was incubated for 0–60 hrs as indicated above each lane. Salt extracted protein (20 μg) was applied to each lane. The pH of the gel is shown in the right margin for each panel. Quantitation of the wound-inducible peroxidase activity of CP>9.3 in leaves, petioles, and roots by densitometric tracing of the IEF gels is shown below each lane in boxes as relative units with respect to the amount of CP>9.3 at time 0 in leaves which is set to a value of 1.0.

system (11) and peroxidase activity assayed by adding the extract to 2.0 mL of 0.1% guaiacol and 0.03% H_2O_2 in 50 mM potassium acetate, pH 6.0, and measuring the increase in absorbance at 470 nm (12). The crude peroxidase extract (15 μg) was applied to a vertical IEF gel pH 3.5-9 and stained for peroxidase activity with guaiacol. Densitometric tracings of activity stained IEF gels were performed using a Bio-Rad Imaging Densitometer Model GS-670. Units of activity are relative values calculated by measuring peak areas of peroxidase activity in the IEF gel (13).

Enzyme purification. Carrot roots were homogenized in a juicer and the cell wall debris was collected for purification. The fine cell wall pulp was washed with cold distilled water (25-30 L per kg of carrot roots) followed by one wash with cold 100 mM NaCl, pH 6.0. The pulp was then squeezed through four layers of cheese cloth, and the cell wall pulp was used as the source of the cationic peroxidase. The pulp was treated with a minimal volume of 500 mM NaCl, pH 6.0 at 4°C overnight and then squeezed through cheese cloth. The resulting solution was treated with 0.01% insoluble PVP and 0.001% PMSF and then centrifuged at 1000 × g for 20 minutes. The clarified supernatant was dialyzed against distilled water and freeze-dried. The freeze-dried sample was dissolved in 10 mM Tris-HCl, pH 6.2 and passed through a DEAE-macroprep column equilibrated with the same buffer. The void volume fractions contained the cationic peroxidase activity. This was collected and dialyzed against 10 mM KH_2PO_4, pH 7.0 buffer and applied to an hydroxylapatite column equilibrated with the same buffer. Fractions were eluted with step gradients of 40 mM and 100 mM KH_2PO_4, pH 7.0 buffer. Fractions containing peroxidase activity were made 1× in concanavalin-A sepharose binding buffer (1M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 100 mM acetate buffer [pH6.0]) and applied to concanavalin-A sepharose column previously equilibrated with the binding buffer. Peroxidase activity retained on the column was eluted with a linear gradient from 0 to 0.5 M methyl α-D-glucopyranoside in the binding buffer. The fractions containing peroxidase activity were dialyzed and freeze-dried.

Determination of purity of the cationic peroxidase. Purity of the cationic peroxidase was determined by performing a 12% SDS-PAGE and staining it with Coomassie brilliant blue R250 as described by Laemmli (14). Also, purity was analyzed by electrophoresis on horizontal IEF gels pH 3.5-9.5 (LKB, Broma, Sweden) according to the manufacture's recommendations. These gels were Coomassie stained and stained for peroxidase activity (10).

Amino acid composition and N-terminal sequencing of the cationic peroxidase. Amino acid composition analysis was done at The Chemical Instrumentation Center, Ohio State University. For sequencing, the purified peroxidase was electrophoresed on a 12 % SDS-PAGE and then blotted onto a PVDF membrane (Biorad). The coomassie stained protein band was excised and microsequenced by automated Edman degradation on an Applied Biosystems 477A Protein Sequencer at The Research and Technology Center, Ohio University.

RESULTS AND DISCUSSION

Wound-induced expression of the cationic peroxidase. Wounding experiments were performed with carrot leaves, petioles and roots incubated for 0, 12, 24, 48, and 60 hr following wounding. Proteins were extracted with salt from these plant organs, electrophoresed on an IEF gel (pH 3-9.5), and stained for peroxidase activity. For carrot leaves (Fig. 1A), it was observed that on wounding, there is an increase in CP>9.3 activity approximately 12 hr after wounding and this level was maintained for at least 60 hr after wounding. Petioles showed a

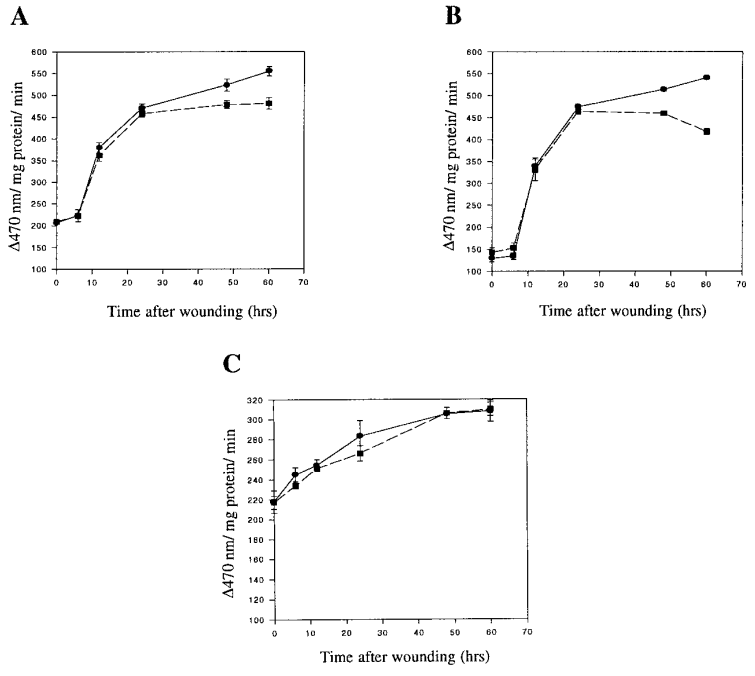

FIG. 2. Total peroxidase activity of carrot leaves (A), petioles (B), and roots (C) following wounding in the presence and absence of cycloheximide. Peroxidase activity (Δ470 nm/mg protein/min) of the different organs was measured at different times following wounding. The dashed line represents peroxidase activity following wounding in the presence of cycloheximide (5 μg/mL) treated organs, whereas the solid line represents the peroxidase activity following wounding in the absence of cycloheximide.

similar pattern (Fig. 1B), but CP>9.3 activity increased much more with wounding than in leaves. In both leaves and petioles, there was a decrease in activity of the pI 7.6 peroxidase isozyme on wounding (Figs. 1A and 1B). In the case of roots (Fig. 1C), it can be seen that CP>9.3 activity is constitutive and was not altered by wounding, but there was a decrease in activity of a pI 5.2 peroxidase isozyme. Since CP>9.3 is expressed in carrot roots even in the absence of wounding, and roots being readily available, we sought to purify the CP>9.3 isozyme from this organ.

Using cycloheximide, it was determined that wound-induced increases in peroxidase activity that were seen within approximately 24 hr after wounding of carrot organs were not due to *de novo* protein synthesis, but rather to enzyme activation. In leaves and petioles, total peroxidase activity increased in response to wounding over time; however, cycloheximide had little effect on this response until approximately 24 hr after wounding (Figs. 2A and 2B respectively). This result indicates that during the first 24 hr after wounding where peroxidase activity increases dramatically, this enhanced activity is not due to *de novo* protein synthesis, but rather to enzyme activation. In roots, total peroxidase activity also increased in response to wounding, but not to as great an extent as seen in leaves and petioles. Moreover, the basal level of peroxidase activity in roots was somewhat higher than in leaves and two times higher than in petioles (Fig. 2C).

Enzyme purification. To determine in which cellular fraction CP>9.3 is found, carrot roots were first homogenized in a juicer, and the homogenate tested for the presence of CP>9.3 on an IEF gel. There was little CP>9.3 activity present, but the major part of CP>9.3 was seen

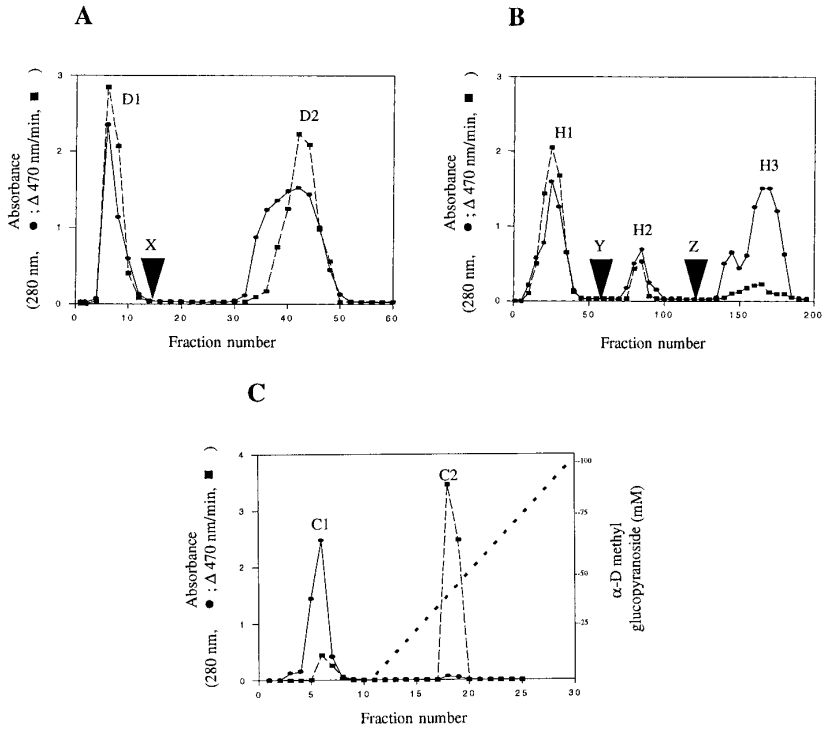

FIG. 3. Chromatography profiles of DEAE-macroprep (A), hydroxylapatite (B), and concanavalin-A sepharose (C) columns used for the purification of CP>9.3. The solid line represents the protein profile, whereas the dashed line indicates peroxidase activity. D1 and D2 are protein and peroxidase activity peaks of the DEAE-macroprep column. H1, H2, and H3 are protein and peroxidase activity peaks of the hydroxylapatite column. C1 and C2 are protein and peroxidase activity peaks of the concanavalin-A sepharose column. X represents the point at which a step gradient was applied to the DEAE-macroprep column (0.5 M NaCl in 10 mM Tris-Cl, pH 6.2). Y and Z represent step gradients (40 and 100 mM potassium phosphate buffer, pH 7.0 respectively) applied on the hydroxylapatite column. A gradient of 0–100 mM α-D methyl glucopyranoside was applied to the concanavalin-A-sepharose column after the void volume was eluted.

to be in the 0.5 M NaCl wash of the cell wall pulp. Therefore, we chose the cell wall pulp as our source of CP>9.3. A 100 mM NaCl wash of the cell wall pulp removed some contaminating protein. Insoluble PVP removed the major portion of the polyphenolics that were generated during extraction from carrot roots. The DEAE-macroprep column was useful in removing the remaining polyphenolics and separating the anionic from the cationic proteins. Most of the peroxidase activity was seen in fractions corresponding to peak D1 (Fig. 3A). Peak D1 also contained the wound-inducible CP>9.3 isozyme of peroxidase as detected on an IEF gel. When D1 was applied to an hydroxylapatite column, peroxidase activity separated into three peaks, H1, H2 and H3 (Fig. 3B). This column was useful in removing a major portion of contaminating proteins. Peak H1 showed the greatest peroxidase activity to protein ratio and, following IEF analysis, was found to contain CP>9.3. After dialysis against water, freeze drying and reconstitution with concanavalin-A sepharose buffer, the H1 fraction was applied to a concanavalin-A sepharose column. Most of the protein eluted in the void volume, however, a small amount of protein having high peroxidase activity bound to the column resin and was eluted with α-D-methyl-glucopyranoside (Fig. 3C).

The purification summary is shown in Table 1. The combination of these purification steps

TABLE 1
Purification Summary for CP>9.3

Fractions	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude salt extract	5000.0	8.18×10^5	163.7	1.0	100.0
DEAE-macroprep (D1)	200.0	2.12×10^5	1059.2	6.5	26.0
Hydroxylapatite (H1)	4.0	4.64×10^4	11692.5	71.4	6.0
Con-A Sepharose (C2)	0.3	2.09×10^4	69507.7	424.7	2.5

[a] A peroxidase activity unit is measured as a change of 1 O.D. at A_{470} nm min^{-1}.

resulted in a 425-fold purification of CP>9.3. To assess the purity of CP>9.3, the concentrated C2 peak sample was analyzed by SDS-PAGE which indicated that the purified enzyme represented a homogenous preparation with a molecular mass of 45 kD (Fig. 4A). As an additional proof of purity, the protein sample (concentrated fraction C2) was electrophoresed on an analytical isoelectric focusing gel and stained for both protein and peroxidase activity. The cationic peroxidase was shown to have a pI value of greater than 9.3. As shown in Fig. 4B, only one coomassie stained band was observed which comigrates with the activity stained band. The Rz value of CP>9.3 was determined to be 2.3 and is also an indication of its purity. The pH and temperature optima were determined to be 4.9 and 32°C respectively using guaiacol as a substrate (data not shown).

Amino acid composition and N-terminal sequence. The amino acid composition data are shown in Table 2. The N-terminal sequence of the cationic peroxidase was determined to be QVVPIASGRGLA. This sequence does not show homology with any sequence in the protein and nucleic acid data banks.

In summary, we have isolated a novel cell wall peroxidase (CP>9.3) from carrot roots that is constitutively expressed in roots and wound-inducible in leaves and petioles. CP>9.3 is expressed in small amounts in healthy leaves and petioles. In this respect it is similar to the anionic peroxidases of tomato and potato which also express wound-inducible peroxidases in unwounded tissue (15). CP>9.3 is also seen in the media of carrot cell suspension cultures (Nair and Showalter, unpublished results). Moreover, given that peroxidase activity is rapidly induced following wounding by a non-*de novo* route, the wound-induced CP>9.3 is apparently

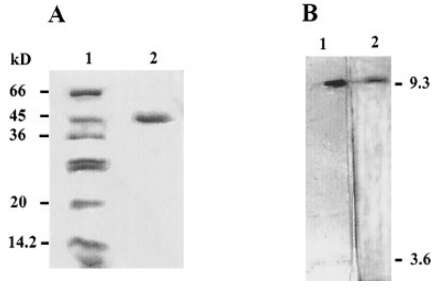

FIG. 4. Determination of molecular mass and purity of CP>9.3. (A) Coomassie stained SDS-PAGE of 2.5 μg of the purified CP>9.3, fraction C2 (lane 2) and molecular mass markers (lane 1). Molecular mass values are shown in the left margin. (B) Analytical IEF gel of the purified CP>9.3 (fraction C2). Duplicate lanes of C2 were electrophoresed and stained for peroxidase activity (lane 1) or coomassie stained (lane 2). pH values are shown in the right margin.

TABLE 2
Amino Acid Composition of CP>9.3

Amino acid	Mol %
Asx	6.5
Glx	3.3
Ser	8.6
Gly	11.9
His	1.0
Arg	7.1
Thr	9.3
Ala	10.5
Pro	7.3
Tyr	2.9
Val	6.3
Met	1.8
Cys	ND[a]
Ile	5.3
Leu	12.2
Phe	5.2
Lys	0.8
Trp	Nd[b]

[a] ND, not determined.
[b] Nd, not detectable.

activated in a manner like some other wound-inducible cationic peroxidases (17,18). Specifically, the enhanced activity of these cationic peroxidases upon wounding appears to be due to the activation of the pre-existing isozyme in the cell wall (19). Indeed, wounding may produce a signal that activates these peroxidases. There is evidence in pea plants that intracellular Ca^{++} levels increase in response to wounding which may in turn induce secretion and/or activation of cell wall peroxidases (20,21). Such activation is in contrast to a number of other peroxidases which are known to be induced upon wounding via *de novo* protein synthesis (10). We are currently interested in determining the physiological functions, precise cellular localization and gene sequence of the CP>9.3 in carrot.

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