Localization in the Paraveinal Mesophyll Vacuoles

Identification, N-terminal Sequence, Stress-Regulation, and Specific Localization in the Paraveinal Mesophyll Vacuoles

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Wounding of tomato (Lycopersicon esculentum L.) leaves causes systemic induction of a serine-type carboxypeptidase activity. We find this activity to be present in several isoforms. Antibodies raised against the leaf carboxypeptidase inhibited the enzyme activity and the immunoprecipitates were resolved into a 69-kDa polypeptide and a doublet of 35/37-kDa proteins on SDS-PAGE. Immunoblot analysis of the leaf proteins also immunodecorated the 69-kDa and 35/37-kDa proteins. Amino acid sequence analysis of the amino-terminus of the tomato leaf 69-kDa carboxypeptidase showed it to be similar to the barley A-chain carboxypeptidase I [Sorenson et al. (1986) Carlsberg Res. Commun. 51: 475], sharing Ala as the N-terminus and the sequences, AlaProGln and LeuProGlyPhe. Superimposition of a chemical stress (copper treatment) on wounding apparently lowered wound-induced carboxypeptidase activity in the leaf, suggesting that cupric ions might interact with the wound signal. Immunogold electron microscopy indicated that the leaf carboxypeptidase was specifically localized within the inclusions of vacuoles of vascular parenchyma cells. In cupric ion-treated tissues, carboxypeptidase was found redistributed to other parts of the cell, indicating that this treatment, but not wounding, causes general vacuolar membrane damage.

Key words: Carboxypeptidase (immunolocalization) — Ethylene — Immunogold electron microscopy — Oxidative stress — Protein turnover — Senescence.

Nitrogen mobilization and utilization are important components of dynamic cellular metabolism during growth, development and senescence of plant organs (Brady 1987, Brady and Spiers 1991). Protein degradation plays an important role in the plant's overall nitrogen economy, particularly because the availability of nitrogen for plant growth is often limited (Peoples and Dalling 1988). Plants have evolved intricate mechanisms to store, utilize and allocate nitrogen. During leaf expansion, nitrogen is also utilized for the synthesis of chloroplast proteins, two of which are the major products of plastid protein synthesizing machinery, the membrane-bound D1 protein (for a review, see Mattoo et al. 1989) and the stroma-located ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the latter accounting for about 35–50% (w/w) of the mesophyll cell reduced nitrogen (Peoples and Dalling 1988). During the course of senescence, Rubisco and other chloroplast proteins are degraded and the resulting amino acids exported from the senescing leaf for the growth of young leaves. Although significant progress has been made in understanding gene expression during senescence, little is known about turnover of proteins during this process (Mehta et al. 1992).

Most of the studies on plant protein turnover are on the breakdown of storage proteins during maturation and germination of seeds (Preston and Kruger 1976, Wilson 1986) and on the light-mediated degradation of chloroplast membrane proteins (Mattoo et al. 1989). Proteolysis in such cases seems to involve endo- and exo-proteases. Since protein turnover is a norm during senescence and stress (Mehta et al. 1992, 1996), proteases should play a role in these processes. For instance, cysteine proteinases have been implicated in nitrogen mobilization and shown to be up-regulated during senescence of flowers (Jones et al. 1995, Valpuesta et al. 1995), wounding of tobacco (Linthorst et al. 1993), temperature stress of tomato (Schaffer and Fischer 1990), autolysis of Zinnia (Minami and Fukuda 1995) and drought stress of Arabidopsis (Koizumi et al. 1993). Also, the gene encoding leucine aminopeptidase was cloned from tomato and suggested to function as a component in defense response (Pautot et al. 1993). More work is needed to identify and characterize specific proteases that are up or down regulated during growth, development and senescence of plants.

Major exoproteases studied belong to serine-type carboxypeptidases (Mikola and Mikola 1986). A carboxypeptidase activity was shown to increase systemically in distant leaves upon wounding (Walker-Simmons and Ryan 1977). Carboxypeptidases are relatively abundant in most tissues of all higher plants (Winspear et al. 1984, Zuber 1964).
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Wells 1965, Ihle and Dure 1972, Matoba and Doi 1974, Doi et al. 1980, Mikola 1983, Mikola and Mikola 1984). They have been proposed to play an important role, relative to endopeptidases, in the hydrolysis of reserve proteins (Mikola 1983). Because of their presumed association with vacuoles, carboxypeptidases have been implicated in the degradation of reserve proteins which accompanies leaf senescence (Peoples and Dalling 1988) and seed germination (Wells 1965, Ihle and Dure 1972, Mikola and Mikola 1984, 1986). In addition, the rapid and temporal increase in carboxypeptidase activity during tomato fruit ripening and in response to wounding-stress, and its localization within the inclusions of vacuoles in tomato pericarp suggest a role for this protease in protein turnover (Mehta et al. 1996).

To assess the role of carboxypeptidases in plant protein turnover and to provide an insight into their regulation, it is important to isolate, identify and characterize them. We have previously (Mehta and Mattoo 1996) shown that the tomato fruit carboxypeptidase isozymes are monomers or heterodimers of 68 and 43 kDa polypeptides. Here we show that the tomato leaf carboxypeptidase is comprised of a 69-kDa protein and perhaps a 37-kDa polypeptide. We present the N-terminal sequence of the 69-kDa carboxypeptidase, demonstrate that the leaf carboxypeptidase is specifically localized to the vacuoles of paraveinal mesophyll tissue, and that chemical stress induced by treatment with cupric ions (Mattoo et al. 1996, Mehta et al. 1992) superimposed on wounding-stress causes redistribution of the carboxypeptidase protein.

Materials and Methods

Plant material—Tomato (Lycopersicon esculentum cv. 'Pik-Red') plants were grown either in a greenhouse under 16 h daylight and 8 h darkness at temperatures ranging from 25 to 30°C or in the USDA fields at Beltsville, Maryland (U.S.A.). Wounding treatment consisted of cutting leaves into small pieces and incubating them in an aerated solution containing 0.4 M sorbitol and 0.01 M MES-KOH, pH 6.0, under fluorescent white light (6 μmol m⁻² s⁻¹) (Mehta et al. 1991). Samples of leaf segments were removed at various times of incubation and frozen in liquid nitrogen. Some of the tomato leaf segments were incubated in the buffer in the presence or absence of 1 mM CuSO₄ at 30°C or in the USDA fields at Beltsville, Maryland (U.S.A.). Red plants were grown either in a greenhouse under 16 h daylight and 8 h darkness at temperatures ranging from 25 to 30°C or in the USDA fields at Beltsville, Maryland (U.S.A.).

Preparation of cell-free extracts—Total proteins were extracted from intact leaves or leaf segments with 2 vol of extraction buffer (10 mM acetate buffer, pH 5.2, 1 mM EDTA, 0.1 M sucrose, 1 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ pepstatin and 0.1% β-mercaptoethanol) in a Waring commercial blender as before (Mehta and Mattoo 1996). The supplementation with inhibitors of proteases was deemed necessary to prevent any proteolysis during extraction and purification of carboxypeptidase, particularly because a previous study (Walker-Simmons and Ryan 1980) implicated a probable breakdown of the native enzyme by unwanted proteolysis. The homogenate was squeezed through several layers of cheesecloth and filtered through a single layer of Miracloth (Calbiochem, San Diego).

Ammonium sulfate fractionation and dialysis—The crude extract was centrifuged at 5,000 × g for 10 min. Proteins in the supernatant which precipitated between 40 and 80% ammonium sulfate saturation (containing essentially all of the carboxypeptidase activity) were collected by centrifugation at 10,000 × g for 30 min, resuspended and dialyzed against 2 × 4-liter changes of the extraction buffer lacking β-mercaptoethanol. All subsequent purification procedures were conducted at 4°C.

Ion-exchange chromatography—The ammonium-sulfate-precipitated fraction was loaded on a Mono-Q HR 5/5 ion-exchange column (FPLC, Pharmacia) equilibrated with 10 mM Na-acetate, pH 5.6, 1.5 mM EDTA. The column was washed with 10 ml of the equilibration buffer at a flow rate of 0.5 ml min⁻¹ and the bound proteins were eluted with 25 ml of a linear NaCl gradient (0–0.5 M) in the same buffer. Fractions (0.5 ml) were collected and assayed for protein content and carboxypeptidase activity. The fractions containing peak enzyme activity were pooled and concentrated by precipitation with ammonium sulfate to 80% saturation followed by centrifugation at 10,000 × g for 30 min. The precipitates were resuspended in 10 mM acetate buffer, pH 5.2 and dialyzed overnight against the same buffer.

Gel filtration chromatography—The enzymatically active Mono-Q fractions (50–200 μg protein) were pooled and applied to a Superose 12 column (FPLC, Pharmacia) equilibrated with 10 mM sodium acetate, pH 5.2, 1 mM EDTA and 0.2 M sucrose. The proteins were eluted at a flow rate of 0.5 ml min⁻¹. Fractions (1 ml) were collected and assayed for protein content and carboxypeptidase activity. In separate runs, standard proteins were individually gel filtered through the column to calculate Kₐ values. The standards used were blue dextran (Vₐ marker), catalase, aldolase, BSA and chymotrypsinogen (Pharmacia). The location of the standards in the eluted fractions was determined by absorbance at 280 nm.

Ethylene measurement—One g of leaf tissue was cut into segments of about 0.5 cm in diameter and placed in 25-ml Erlenmeyer flasks containing sorbitol-MES buffer, with or without 1 mM CuSO₄. Ethylene released from the leaves was then assayed as described (Mehta et al. 1996).

Determination of carboxypeptidase activity—Carboxypeptidase activity was assayed with the substrate N-carbobenzoxy Z-L-phe-L-ala at 30°C as described previously (Salgo and Feller 1987, Mehta and Mattoo 1996). Z-phe-ala (200 μmol) was dissolved in 2 ml dimethylsulfoxide and made to a final volume of 100 ml with 50 mM sodium acetate, pH 5.0, containing 0.5 mM EDTA (stock substrate solution). The reaction mixture (0.4 ml final volume) containing 0.1 ml substrate, various aliquots (0.7–50 μg) of enzyme protein and the acetate buffer was incubated at 30°C for 1 h. The controls were prepared without the substrate. The reaction was stopped by the addition of 1 ml of freshly prepared trinitrobenzene sulfonic acid (TNBS) solution (10 mg TNBS in 30 ml of 50 mM borate buffer, pH 9.5) and the samples further incubated for 1 h in the dark. The liberated C-terminal amino acid reacts with TNBS to yield a yellow-colored compound which was measured at 405 nm with a multichannel spectrophotometer. The absorbance differences were converted to μmol alanine produced using a calibration curve with L-alanine (up to 50 nmol). One unit of carboxypeptidase activity is expressed as one μmol of L-alanine released per h. Specific activity is given as units per mg protein.

Gel electrophoresis and immunoblotting—Proteins were sepa-
rated by SDS-PAGE according to Laemmli (1970) using 10% polyacrylamide gels overlaid with a 4.5% stacking gel. Samples were prepared for electrophoresis as described (Mattoo et al. 1981). After electrophoresis, the proteins on the gels were either stained with 0.05% (w/v) Coomassie Brilliant Blue R-250 or electrotransferred on to a nitrocellulose membrane (using a Mini Trans-Blot; Bio-Rad) as described by Towbin et al. (1979). The nitrocellulose membrane was processed for immunodetection as described by Cullaham et al. (1989).

Antibody purification—The unfraccionated antiserum to purified leaf carboxypeptidase prepared in mice (Walker-Simmons and Ryan 1980) was a gift from Dr. K. Walker-Simmons. The immunoglobulins (IgG) were precipitated with ammonium sulfate and further fractionated on a protein A Superose HR 10/2 column (FPLC, Pharmacia) according to the manufacturer's instructions. Purified IgGs were stored at −80°C.

Immunoprecipitation of carboxypeptidase activity and proteins—Immunotitration of the tomato leaf carboxypeptidase activity and immunoprecipitation of carboxypeptidase protein from tomato leaf tissue were performed as described by Mehta and Mattoo (1996). A series of 5—25 enzyme units in 250 µl aliquots were incubated with increasing amounts of the anti-carboxypeptidase antibodies diluted in PBS (pH 8.0) for 4—16 h at 4°C. The precipitates obtained were centrifuged at 10,000 × g for 10 min at 4°C. The supernatants were collected and assayed for residual enzyme activity. Control tubes contained preimmune serum instead of the anti-carboxypeptidase antibodies. Protein A-Sepharose CL-4B (50 µl, Pharmacia) was then added to each tube, the tubes centrifuged at 10,000 × g for 5 min and the supernatant assayed for residual enzyme activity.

For immunoprecipitation, ammonium sulfate-sediments were first adjusted to a 25% final concentration of SDS and heated to 100°C for 1 min. After cooling to 4°C, 50 µl of 4 × immunoprecipitation buffer (1 × = 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5 M NaCl, 2% BSA) and 100 µl of Protein A-Sepharose (10% vol/vol in immunoprecipitation buffer) were then added and the mixture incubated for 1 h at 4°C on a nutator. The Sepharose beads were pelleted by centrifugation at 10,000 × g for 15 s. To the supernatant, 1 µl of preimmune serum was added and the mixture further incubated for 1 h at 4°C. Protein A-Sepharose (100 µl) was then added and the mixture further incubated for 1 h. The pellets were collected by centrifugation at 10,000 × g for 5 min and the supernatants were transferred to new tubes. Subsequently, anti-carboxypeptidase antibody (2 µl) was added and immunoprecipitates collected as described above. The immunoprecipitates were washed five times with 1 × immunoprecipitation buffer, twice with 1 M NaCl and once with double distilled water. Finally, 50 µl of sample application buffer (Mattoo et al. 1981) was added to the washed pellet, the mixture heated to 90°C for 3 min followed by brief centrifugation to remove the beads, and the supernatant then analyzed by SDS-PAGE (Laemmli 1970).

Amino acid composition determination and aminoterminal sequencing—Partially purified leaf carboxypeptidase, obtained from ion-exchange column chromatography, was fractionated on 1.5 mm preparative SDS-PAGE mini gels. The fractionated proteins were electrophoretically transferred on to two layers of PVDF membrane (Immobion-P, Millipore) following the protocol of Matsudaira (1987) for N-terminal sequencing. After transfer, the membranes were rinsed with deionized water, stained with 0.1% Coomassie Blue R-250 for 5 min, destained in 50% (v/v) methanol, 10% (v/v) acetic acid, rinsed again in deionized water and air-dried. Individual bands of interest were excised. The N-terminal amino acid sequence of the protein was determined by the Edman degradation procedure using an automated sequencer and the respective PTH-amino acid derivatives identified by reverse phase HPLC analysis at the Biotech Facility, Case Western University Medical Center, Cleveland, Ohio.

Immunogold electron microscopy—Small pieces (2 mm 3) of tomato leaf were fixed in a freshly prepared solution of 0.5% glutaraldehyde and 4% para-formaldehyde in 100 mM sodium phosphate buffer, pH 7.2, at 4°C for 18 h. The fixative was changed twice during this period. Following fixation, the tissue was washed for 1 h in 100 mM phosphate buffer, dehydrated in a cold, graded ethanol series and embedded and polymerized in L. R. White 'Hard' resin at 60°C for 48 h (Warmbadt 1985). Ultrathin sections were cut with a diamond knife on an American optical ultramicrotome and collected on nickel grids for further processing. Nickel grids with attached sections were floated on drops of PBS supplemented with 0.3% (v/v) Tween-20 (PBST) and 1% BSA to prevent non-specific binding of the antibodies. The sections were incubated for 1 h with antibodies against the leaf carboxypeptidase diluted in PBST-BSA, washed with PBST and then labeled for 1 h with the secondary antibodies conjugated to 15 nm colloidal gold (Jansen, Wantage, Oxfordshire, U.K.) diluted 1:40 (v/v) in PBST-BSA. Sections were washed thoroughly in PBS, then in double-distilled water and finally stained with 2% aqueous uranyl acetate for 15 min. Controls with non-immune IgG in place of the primary antisera were run in parallel incubations. Following immunolabeling, the sections were viewed and photographed with a Hitachi HU-11E or H-500 transmission electron microscope.

Results

Tomato leaf carboxypeptidase activity characteristics—Highest specific activity of carboxypeptidase was found in the leaf when different tomato plant tissues were analyzed (Mehta et al. 1996). This activity showed a substrate preference for dipeptides in the order Z-Phe-Leu > Z-Pro-Phe > Z-Phe-Ala > Z-Pro-Leu, which was maximally active in the pH range 5 to 6, had a K_m of 348.2 µM for Z-Phe-Ala, and was inhibited by DFP, PMSF, HgCl_2 and pepstatin but not by β-mercaptoethanol, aprotinin, leupeptin or EDTA (Mehta 1993). These data suggest that tomato leaf contains a Ser-type carboxypeptidase. Attempts to purify the leaf enzyme to homogeneity using ammonium sulfate fractionation, mono-Q chromatography and Superose-12 gel-filtration resulted in very poor recoveries. The enriched carboxypeptidase fractions were comprised of a protein having a molecular weight of 158,000 on gel-filtration (Fig. 1). The gel-filtered fractions containing an active carboxypeptidase were found to contain minuscule amounts of the protein which precluded detection of protein profile by SDS-PAGE. Therefore, alternative, immunological approach was used to further characterize the leaf carboxypeptidase.

Immunocchemical characterization of leaf carboxypeptidase—Immunotitration of the tomato leaf extracts with purified antibody to leaf carboxypeptidase showed that most of the enzyme activity (97%) was precipitated by the antibody (Fig. 2A, closed triangles). Normal rabbit serum did not immunoprecipitate or immunoinhibit the enzyme.
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activity (Fig. 2A, open circles). Immunoprecipitation of carboxypeptidase activity was then carried out by adapting a procedure (Mehta and Mattoo 1996) described in the Materials and Methods section, and the washed immunopellets were analyzed by SDS-PAGE. The polyacrylamide gels were stained with Coomassie Blue to reveal the proteins (Fig. 2B). Two polypeptides of \(M_r \)'s 69-kDa and 37-kDa were immunoprecipitated by the antibody (Fig. 2B, lane 3) but not by the preimmune serum (Fig. 2B, lane 2). The 37-kDa protein migrated as a diffuse band.

Immunoblot analysis (Fig. 3) of the leaf extracts confirmed immunoprecipitation data of Figure 2 and further revealed the possibility that the 37-kDa diffuse band is associated with an additional, 35-kDa polypeptide (Fig. 3). These data suggest that leaf carboxypeptidase activity is associated with the 69-kDa protein and the 37-kDa doublet.

Leaf carboxypeptidase is present in isoforms—The data from the previous section suggested the possibility that leaf carboxypeptidase may exist in several isoforms as also found for the fruit enzyme (Mehta et al. 1996). Therefore, leaf extracts were fractionated on native, non-denaturing gels, immunoblotted and the blots probed with the anti-carboxypeptidase antibody. Indeed, four cross-reactive protein bands, \(L_1\), \(L_2\), \(L_3\) and \(L_4\), were resolved (Fig. 4), suggesting that leaf carboxypeptidase exists in multiple molecular forms. To ascertain the subunit composition of these isoforms, each band from native gels was excised, subjected to SDS-PAGE and immunoblotted. All the isoforms, except the \(L_4\) form, yielded an immunologically cross-reactive polypeptide of 69-kDa (Fig. 4). These results suggest that the leaf carboxypeptidase is mainly comprised of a 69-kDa polypeptide, and that, since in the native form the enzyme shows a molecular mass of 158,000 (Fig. 1), the enzyme is an oligomer. It seemed likely that the 35–37-kDa doublet may represent another isoform not resolved under the present experimental conditions.

Amino acid composition and N-terminal sequence of putative leaf carboxypeptidase—To further differentiate between the 69-kDa protein and the 35–37-kDa doublet (Fig. 3), the amino acid composition of each of the three putative leaf carboxypeptidase proteins was analyzed. The results are presented in Table 1. All three proteins were found enriched in aspartic acid, glutamic acid, serine, glycine, proline, valine and leucine. Although previous amino acid analysis of leaf carboxypeptidase had also revealed higher amounts of aspartic acid and glycine, the preparation analyzed in that publication contained a protein contaminant and the polypeptides were not resolved by gel electrophoresis (Walker-Simmons and Ryan 1980). The 69-kDa protein lacked Phe which was, however, present in significant amounts in the 35/37-kDa doublet. Although the values for different amino acids were similar for the three proteins, there are significant differences.

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<tr>
<th>Amino acid residue</th>
<th>Polypeptide (kDa)</th>
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<tr>
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<td>Asx</td>
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<td>Thr</td>
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<td>Ala</td>
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<td>Leu</td>
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<td>Phe</td>
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<tr>
<td>Trp</td>
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<td>Lys</td>
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Molecular masses of the polypeptides were determined by SDS-PAGE under reducing conditions (see Fig. 3).
N-terminal amino acid sequence analyses of these three proteins revealed that the 37-kDa and 35-kDa proteins were blocked at the N-terminus while the 69-kDa protein was not (Fig. 5). Comparison and alignment of this N-terminal amino acid sequence with other plant proteins showed a high level of identity (59%) with that of barley carboxypeptidase I, A-chain (Sorensen et al. 1986) (Fig. 5, underlined). These data together with those presented above strongly suggest that the 69-kDa tomato leaf protein is a carboxypeptidase.

Effect of copper ions on wound-induced carboxypeptidase activity and ethylene production—Wounding of leaves of a young tomato plant has been shown to elicit a 3-fold increase in carboxypeptidase activity in distant, unwounded leaves (Walker-Simmons and Ryan 1980) as well as in the wounded leaves (Fig. 6). This increase in carboxypeptidase activity in leaf segments occurs as a rapid response to physical wounding. Because wounding and copper treatment have been shown to independently induce senescence syndrome, probably by increasing the production of the plant hormone ethylene (Hyodo 1991, Rushing and Huber 1985), we tested how these two stimuli affect development of carboxypeptidase activity via a via the generation of ethylene production. We questioned whether ethylene could be the signal causing induction of carboxypeptidase. However, when the leaf segments received an additional, chemical stress by cupric ions, the wound induction of carboxypeptidase activity was inhibited (Fig. 6, compare bars 2 and 4; bars 3 and 5). This effect of cupric ions was not reversible within 90 min (Fig. 6, compare bars 3 and 6). These data suggest that cupric ions reduce the wounding effect and inhibit the development of carboxypeptidase activity.

Although suppression of wound-induced carboxypeptidase activity by cupric ions appeared to discount a correlation between ethylene production and increase in carboxypeptidase activity (Fig. 6), we tested how these two stresses given simultaneously affect ethylene production in tomato leaves. The changes in the rate of ethylene production by wounded and copper-treated tomato leaf as a function of time are shown in Fig. 7. Wounding by itself was not very effective in increasing the rate of ethylene production, in contrast to a substantial induction seen in carboxypeptidase activity (Fig. 6). However, leaf segments responded to the presence of copper in the incubation medium with a 3-fold increase in the rate of ethylene production within the
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Fig. 3 SDS-PAGE and immunoblot analysis of soluble leaf proteins. Soluble leaf proteins were fractionated by SDS-PAGE. One part of the gel was stained with Coomassie blue (STAIN), and the remainder electroblotted on to nitrocellulose paper and reacted with antibodies directed against leaf carboxypeptidase (BLOT). Arrows indicate the cross-reactive polypeptides.

The rate of ethylene production in cupric ion-treated tissue remained higher than the controls up to 15 h. This pattern of ethylene production upon wounding and in response to cupric ion-treatment (Fig. 7) is quite different from that observed for carboxypeptidase activity (Fig. 6); therefore, ethylene production and carboxypeptidase activity do not appear to be correlated.

Intracellular localization of carboxypeptidase in tomato leaf—Cell fractionation studies (Bhalla and Dalling 1986) have pointed to the vacuole as the locale for leaf carboxypeptidase from wheat. Direct immunocytochemical evidence has been recently presented showing that the tomato fruit carboxypeptidase is located in the vacuole (Mehta et al. 1996). We were therefore interested in studying the intracellular distribution of carboxypeptidase in a fully-developed tomato leaf using immunogold electron microscopy (Fig. 8). The immunogold label was confined to the electron dense bodies in the vacuoles of the vascular parenchyma cells of veins. Other elements of the vein and ground tissue, e.g., sieve tubes, bundle-sheath cells and mesophyll cells (Fig. 8) were devoid of label. As a control for non-specific binding, non-immune serum was used as a primary antibody. The control IgG was used at the same concentration as the anti-carboxypeptidase antibody and showed very low non-specific background (Fig. 8, inset). It is, therefore, evident that specific labeling of the protein occurs within the vacuoles of vascular parenchymal cells.

Fig. 4 Isozymes of tomato leaf carboxypeptidase and identification of their subunits. Cell extracts from fully-developed leaf were fractionated on native, non-reducing polyacrylamide gels and electrotransferred on to nitrocellulose paper. The immunoblot was then treated with the anti-carboxypeptidase antibodies (A). The cross-reactive leaf proteins bands (L1–L4), as indicated, were then excised, re-electrophoresed in quadruplicate under denaturing conditions (SDS-PAGE) (B) and immunoblotted. Each blot was then developed with the antibodies to leaf carboxypeptidase. Asterisk on the right side of the right panel indicates the 69-kDa polypeptide.

Fig. 5 N-terminal amino acid sequence of the 69-kDa carboxypeptidase from tomato leaf (tCPD-leaf) compared to that of barley carboxypeptidase I, A chain (bCPD-M I, A-chain). The underlined residues denote identical amino acids.

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Fig. 6 Effect of cupric ions on wound-induced carboxypeptidase activity in tomato leaf. Tomato leaf segments were incubated for 0 (bar 1), 1.5 (bar 2) and 3 (bar 3) hours in the absence (bars 1–3) or presence of 1 mM CuSO$_4$ for 1.5 (bar 4) and 3 (bar 5) hours. Duplicate samples shown in bar 4 were washed and then incubated in medium without CuSO$_4$ for additional 1.5 h (bar 6). Cell-free extracts were made and carboxypeptidase activity fractionating at 40–80% ammonium sulfate saturation was assayed (mean values±SD, n=6).

Fig. 7 Effect of wounding in the presence and absence of a chemical stress on ethylene production in tomato leaf. Leaf segments were incubated in the absence (C) or presence (Cu) of 1 mM CuSO$_4$. At the indicated times, ethylene production was measured and quantified (mean values±SD, n=6).

Effect of wounding and copper-treatment on intracellular localization of leaf carboxypeptidase—To determine whether physical (mechanical wounding) or chemical (cupric-ion-treatment) stresses cause a change in carboxypeptidase-protein concentration or its location, tomato leaf segments were incubated with or without cupric ions for 1.5 h, before being processed for immunogold labeling. In Fig. 9, the accumulation of carboxypeptidase protein in tomato leaf upon wounding and copper-stress is shown. A clear increase was seen in the level of immunogold label associated with the inclusion bodies in the vacuoles (B), which is consistent with an increase seen in carboxypeptidase activity in wounded leaf tissue (Walker-Simmons and Ryan 1977, Fig. 5). When leaf tissue was given an additional, oxidative stress (Mehta et al. 1992) by incubating the segments with copper ions, intense immunogold label

Fig. 8 Portions of unwounded tomato leaf tissue from sections treated with anti-carboxypeptidase antibodies. Transections of tomato leaf tissue from fully-expanded leaves were treated first with anti-carboxypeptidase antibody and then with goat anti-rabbit IgG conjugated to 15 nm colloidal gold and processed for immunoelectron microscopy. Immunolabel for carboxypeptidase is located over electron-dense vacuolar material (arrows). A. Portions of spongy mesophyll cells from a fully-expanded leaf. ×6,000. B. Transection of portion of vein of a fully-expanded leaf. ×9,500. Inset in A shows a section using non-immune control IgG. Marker bars = 1.0 μm. Abbreviations: C, chloroplast; IS, intercellular space; M, mitochondrion; N, nucleus; P, plastid; S, sieve-tube member; V, vacuole; VP, vascular parenchyma cell; W, cell wall.
Tomato leaf carboxypeptidase was apparent (Fig. 9). Moreover, the label was now no longer restricted to inclusion bodies in the vacuoles but was distributed throughout the cell. This distribution remained unchanged when the tissue was removed from the copper-containing medium and placed in an isotonic medium for additional 1.5 h (D), data that are consistent with irreversibility of this phenomenon under these conditions. These results indicate that under oxidative stress vacuolar membrane is injured, causing leakage of the vacuolar contents and redistribution of the carboxypeptidase protein throughout the cell.

Discussion

We have demonstrated that the 69-kDa protein identified here is a tomato leaf carboxypeptidase. The antibody immunoprecipitates enzyme activity from cell-free extracts, and cross-reacts with native as well as denatured forms of the enzyme. The N-terminal sequence of the 69-kDa leaf carboxypeptidase has an identity of 59% among 17 residues compared with the barley A chain carboxypeptidase I and shares Ala as the N-terminus and the sequences, Ala-Pro-Gln and Leu-Pro-Gly-Phe. Furthermore, like the barley (Ray 1976) and cotton seed (Ihle and Dure 1972) carboxypeptidases, tomato leaf carboxypeptidase is also rich in aspartic acid and poor in methionine and histidine residues.

We have previously shown that tomato fruit carboxypeptidase constitutes 68-kDa and 43-kDa proteins (Mehta and Mattoo 1996, Mehta et al. 1996) These two proteins are also recognized by the antibody to leaf carboxypeptidase (data not shown) indicating that the fruit and leaf carboxypeptidases share immunological epitopes. The tomato leaf anti-carboxypeptidase antibody also recognized a doublet of 35/37-kDa, which was not found associated with the native carboxypeptidase isoforms analyzed here. However, a 37-kDa protein was previously shown to be a carboxypeptidase (Walker-Simmons and Ryan 1977). The possibility that the 35/37-kDa doublet may represent degradative forms of the 69-kDa carboxypeptidase is not consistent with the observations that the 35/37-kDa proteins are blocked at the N-terminus and contain phenylalanine which is absent in the 69-kDa carboxypeptidase (Table 1).

Fig. 9 Copper-induced oxidative stress and redistribution of carboxypeptidase in tomato leaf. Transsections of tomato leaf tissue from fully-expanded leaves which were unwounded (A), wounded (B), wounded plus copper-treated for 3 h (C), and wounded and treated with copper for 1.5 h and then incubated without copper for 1.5 h (D). Sections were treated with antibodies to carboxypeptidase and goat anti-rabbit IgG conjugated to 15 nm colloidal gold. A, ×10,500; B, ×13,500; C, ×9,500; D, ×13,000. Marker bars=1.0 μm. Abbreviations: ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; P, plastid; S, sieve-tube member; V, vacuole; W, cell wall.
Moreover, several different protease inhibitors were included in the homogenization buffer to prevent any undesired proteolysis during the preparation of cell-free extracts (Mehta and Mattoo 1996). The immunological cross-reactivity would indicate that these proteins share epitopes with the 69-kDa carboxypeptidase and may, in fact, represent the previously identified wound-inducible leaf carboxypeptidase (Walker-Simmons and Ryan 1977).

This study also revealed that the carboxypeptidase activity of tomato leaf responds differently to different stresses. Wounding of the leaf tissue resulted in a sustained increase in carboxypeptidase activity, which is different in the fruit where the wound induction of carboxypeptidase is of a transient nature (Mehta et al. 1996). On the other hand, superimposing a chemical stress (copper treatment) over wounding apparently lowered wound-induced carboxypeptidase activity in both the leaf and the fruit, suggesting that cupric ions might interact with the wound signal.

Another interesting finding is the highly-specific localization of leaf carboxypeptidase within the inclusions of vacuoles of vascular parenchyma cells. By analogy to other proteins with similar localization (Everard et al. 1990), carboxypeptidase may be involved in the degradation of proteins resulting in the mobilization of amino acids and regulation of nitrogen and carbon metabolism. It is also possible that the carboxypeptidase-mediated intracellular protein turnover is designed to supply free amino acids for the synthesis of defense-responsive proteins to help protect the plant from digestive proteinases of plant pests. In this context, it is interesting that the proteinase inhibitors and carboxypeptidase are simultaneously induced in wounded tomato leaf (Walker-Simmons and Ryan 1977). However, the true intracellular substrate(s) for the tomato leaf vacuolar carboxypeptidase remains to be demonstrated.

The results on the redistribution of carboxypeptidase within the cell in cupric ion-treated tissues only during chemical, oxidative stress as opposed to physical wounding indicate that the former treatment causes vascular membrane damage, liberating vacuolar contents into the cytoplasm. This explanation is consistent with earlier reports showing that cupric ions damage membranes and induce leakage of plasma membrane constituents (Loneragan et al. 1981). In contrast to the vacuolar carboxypeptidase, immunolabel of ribulose-1,5-bisphosphate carboxylase, a plastid stroma protein, or that of acyl carrier protein from mitochondria and chloroplasts is not similarly redistributed, in cupric ion-treated leaf tissues (Mehta 1993), suggesting that vacuoles are more susceptible to damage by cupric ions than chloroplast or mitochondrial membranes.

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### References


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