Subcellular Localization of a Basic Peroxidase Isoenzyme in Crisphead Lettuce

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Abstract. The subcellular localization of a basic peroxidase (EC 1.11.1.7) isoenzyme in crisphead lettuce (*Lactuca sativa* L.) leaves was studied through subcellular fractionation and protoplast and vacuole isolation. This isoenzyme is mainly located in soluble fractions. Studies using protoplast isolation and vacuole purification indicated that the soluble basic peroxidase isoenzyme is found in the vacuolar sap, probably in equilibrium with the same isoenzyme attached to tonoplast membranes.

Russet spotting is a postharvest disorder that can develop during transport and storage of crisphead lettuce (Ke and Saltveit, 1989). This disorder is characterized by the appearance of many small brown spots along both sides of the midrib, spots that can spread over the leaf blade (Link and Gardner, 1919). Peroxidase (EC 1.11.1.7) is an enzyme that may be crucial in this postharvest disorder due to its known participation in oxidative metabolism during plant storage (Frenkel, 1979) and its role in indole-3-acetic acid (IAA) homeostasis through catabolism (Ros Barceló and Muñoz, 1992). Ke and Saltveit (1988) reported that applying IAA greatly reduced russet spotting and IAA oxidase activities and that lignifying russet spotting-affected cells resulted in cell-wall thickening.

IAA oxidase activity resides mainly in peroxidase isoenzymes of the most basic isoelectric point (pI) (Ros Barceló and Muñoz, 1992), and the expression of these isoenzymes is largely regulated by IAA concentrations in plant tissues (Ferrer et al., 1991). These basic peroxidase isoenzymes also play a role in flavonol turnover (Morales et al., 1993b) and lignin biosynthesis (Morales et al., 1993a). We studied the subcellular localization of a basic peroxidase isoenzymes in 1-month-old crisphead lettuce leaves, in which peroxidase polymorphism is mainly restricted to a sole gene product.

Materials and Methods

Chemicals. Caylase was purchased from Cayla (Toulouse, France), pectinase from Serva (Heidelberg, Germany), and pectolyase Y-23 from Seishin Pharmaceutical Co. (Tokyo). All other chemicals were obtained from Sigma Chemical Co. (Madrid, Spain).

Plant material. 'Nordic Grande' crisphead lettuce seeds were sown in vermiculite in plastic trays. The trays were placed in a growth chamber with an 18-h light and 6-h dark photoperiod at 25°C. Light was provided at $\approx 80 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ by cool-white fluorescent tubes. Water and fertilizers (crone medium) (Hewitt, 1966) were periodically supplied during the 1-month growing period.

Tissue and peroxidase preparation. Ten grams of leaf tissue was homogenized at 4°C with a mortar and pestle in 10 ml of 0.25 M sucrose, 1.0 mM Mg acetate, and 50 mM tris-HCl buffer, pH 7.5. The homogenate was immediately squeezed through one layer of cellulose gauze (0.2-mm mesh) and centrifuged at $1000\times g$ for 5

min. Soluble, membrane, and cell-wall fractions were prepared as described by Ros Barceló et al. (1989).

Ionic bound cell-wall peroxidases were removed from the purified cell-wall pellet according to the method of Ros Barceló et al. (1987). Membrane-bound peroxidase was solubilized as described by Ros Barceló et al. (1988). All peroxidase fractions were dialyzed overnight against 50 mM tris-HCl buffer, pH 7.5.

Mesophyll protoplast isolation. The lower epidermis of 1.0 g of leaf tissue was removed with tweezers and immersed in 20 mM tris-mes buffer, pH 5.5, containing 1.0 mM CaCl_2 , 1.0% (w/v) bovine serum albumin (BSA), and 0.65 M sorbitol to prevent tissue dehydration. The leaves were incubated in a maceration medium (25 ml) containing 1% (w/v) caylase, 0.02% (w/v) pectolyase, 0.2% (w/v) pectinase, 1.0% (w/v) BSA, 1.0 mM CaCl_2 , and 0.65 M sorbitol in 20 mM tris-mes, pH 5.5, at room temperature for 45 min in darkness with occasional stirring. The released protoplasts were filtered through nylon gauze (40- μm mesh), centrifuged at $100\times g$ for 6 min, and washed three times by resuspension in a washing buffer containing 1.0 mM CaCl_2 and 0.65 M sorbitol in 20 mM tris-mes, pH 7.3. The green precipitate was recovered by suction and viewed under a light microscope (400 $\times$) to check protoplast integrity and purity. To determine enzyme activities, mesophyll protoplasts were broken by freezing and thawing, then incubated in 1 M KCl for 30 min and centrifuged at $15,000\times g$ for 15 min. The supernatant was desalted by dialysis.

Vacuole isolation. Vacuoles were prepared from mesophyll protoplasts according to the method of Renaudin et al. (1986) but with minor modifications: 0.5 ml of protoplast suspension was added to 0.88 ml of 10 mM tris-mes, pH 7.3, containing 7.9 mM Na_2EDTA . After 10 min at 4°C and gentle shaking, the suspension was quickly mixed with 5.0 ml of 5 mM Na_2EDTA , 0.36 M sorbitol, and 8.5% (w/v) nycodenz [5-(N-2,3-dihydroxypropylacetamido)-2,4,6-triiodo- N,N'-bis(2,3-dihydroxypropyl)isophthalamide] in 10 mM tris-mes, pH 7.3. Mesophyll vacuoles were purified by flotation and recovered in the uppermost layer (0.7 ml) after centrifugation at $160\times g$ for 6 min at 4°C. Vacuole integrity was checked by staining with 1% (w/v) neutral red in 0.65 M sorbitol and 10 mM tris-mes, pH 7.3. To determine enzyme activities, mesophyll vacuoles were broken by freezing and thawing, incubated with 1 M KCl for 30 min, and centrifuged at $15,000\times g$ for 5 min. The supernatant was desalted by dialysis.

Enzyme activity measurement. Peroxidase activity was determined at 25°C with 4-methoxy- α -naphthol as the substrate according to Ferrer et al. (1990). α -Mannosidase (EC 3.2.1.24), a vacuolar sap marker, was measured as reported by Ros Barceló et al. (1991). Enzyme activities, expressed as moles per second ($1 \text{ mol}\cdot\text{s}^{-1} = 1 \text{ katal}$) for all the assayed enzymes.

Isoelectric focusing and isoenzyme staining. Isoelectric focus-

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Fig. 1. (a) Peroxidase isoenzyme patterns of soluble (lane 1), membrane-bound (lane 2), and ionically bound cell wall (lane 3) peroxidase activity extracted from crisphead lettuce leaves. (b) Isoenzyme patterns of lettuce peroxidase activity of soluble fractions (lane 1) and of mesophyll protoplasts (lane 2). (c) Isoenzyme patterns of peroxidase activity in the starting mesophyll protoplasts (lane 1) and vacuoles (lane 2) of lettuce leaves.

ing and peroxidase isoenzyme staining with 4-methoxy- α -naphthol were carried out according to Calderón et al. (1990) and Ferrer et al. (1990), respectively.

Results and Discussion

Subcellular fractionation of crisphead lettuce leaves revealed that most of the peroxidase activity measured, with 4-methoxy- α -naphthol as the substrate, was localized in the soluble (nonsedimentable) fraction ($27.68 \text{ nkat} \cdot \text{g}^{-1}$ fresh weight, 91.87%); the remaining activity was associated ionically with cell-wall ($0.07 \text{ nkat} \cdot \text{g}^{-1}$ fresh weight, 0.24%) and the membrane fractions ($2.37 \text{ nkat} \cdot \text{g}^{-1}$ fresh weight, 7.89%).

The isoenzyme patterns of soluble, membrane-bound and ionically bound cell-wall peroxidases were analyzed by protein isoelectric focusing in 3.5 to 10.0 pH gradients and staining with 4-methoxy- α -naphthol as the substrate. Staining revealed the presence of only one strongly basic ($\text{pI} > 9.0$, cationic) peroxidase isoenzyme in all subcellular fractions (Fig. 1a). No acidic (anionic, $\text{pI} < 7.0$) peroxidase isoenzymes were present in any subcellular fractions.

Since the soluble fraction contained the most peroxidase activity ($\approx 92\%$) and basic peroxidase isoenzyme, this fraction was used for

Fig. 2. (a) High magnification view of a crisphead lettuce mesophyll protoplast (bar = $15 \mu\text{m}$). (b) Vacuoles (v) found in the uppermost layer of the nycodenz gradients used for vacuole purification by flotation showing trace contamination by chloroplasts (arrowheads) (bar = $20 \mu\text{m}$). (c) Light micrograph of a lettuce vacuole stained with neutral red (bar = $20 \mu\text{m}$).

further studies concerning subcellular localization of peroxidase isoenzyme in lettuce leaves. The nature of intracellular (protoplast) peroxidase activity in lettuce leaves was established by protoplast isolation. Under a light microscope, lettuce protoplasts appeared as spherical naked cells with no apparent membrane damage or disintegration of the internal structure (Fig. 2a). After isoelectric focusing, the peroxidase activity associated with mesophyll protoplasts revealed the presence of a single basic peroxidase isoenzyme (Fig. 1b, lane 2).

To clarify the localization within the protoplast of this strong basic peroxidase isoenzyme, vacuoles were prepared from mesophyll protoplasts through osmotic shock and purified in a nycodenz gradient by flotation. Vacuole purity was checked by light microscopy, and the final vacuole preparations showed no protoplasts and only slight chloroplast contamination (Fig. 2b, arrowheads). High magnification revealed that no membranes adhered to the tonoplast. Vacuole integrity was also checked by staining with neutral red. The results (red-pigmented vacuoles, Fig. 2c) showed that isolated vacuoles retained the H^+ -transmembrane gradient responsible for the accumulation of neutral red.

Peroxidase compartmentalization in lettuce vacuoles was calculated assuming α -mannosidase to be 100% vacuolar (Wagner, 1985). Using this marker, we found that only 28% of mesophyll protoplast peroxidase was vacuolar. However, this value should be regarded with caution because, although mean values for mesophyll vacuolar α -mannosidase are near 100%, they can fluctuate from 47% to 130% (Wagner, 1985).

The isoenzyme patterns of vacuolar peroxidase activity (Fig. 1c, lane 2) were similar to those found in the starting protoplasts (Fig. 1c, lane 1), a result suggesting that the mesophyll protoplast basic peroxidase isoenzyme was partly located in the vacuolar sap.

Likewise, the presence of the basic peroxidase isoenzyme in the vacuolar sap (Fig. 1c, lane 2) and in the fraction ionically bound to membranes (Fig. 1a, lane 2) suggests that this isoenzyme may move freely in the vacuolar sap, probably in equilibrium with the enzyme that is ionically bound to the tonoplast. As has been previously suggested for its homologous basic peroxidase isoenzyme in other plant species, this equilibrium may be controlled by Ca^{2+} (García-Florenciano et al., 1991 and Ros Barceló et al., 1991).

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