

Cellular and Subcellular Localization of Peroxidase Isoenzymes in Plants and Cell Suspension Cultures from *Lupinus polyphyllus*

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Leaves, stems, roots, leaf protoplasts and cell suspension cultures of *Lupinus polyphyllus* and isolated vacuoles were studied for cellular and subcellular localization of peroxidase isoenzymes. Isoelectric focusing revealed 16 peroxidase isoenzymes. The *basic* peroxidase isoenzymes are predominantly localized in the vacuole and, to a minor degree, unbound in the intercellular space. The *acidic* isoenzymes are cell wall-bound in plants and not detectable in suspension-cultured cells. Large amounts (up to 11.0 U/ml) of a single basic isoenzyme are detectable in the spent medium of cell suspension cultures.

Introduction

Plant peroxidases are easy to detect, usually abundant and therefore, have been extensively studied. For the biological function of plant peroxidase isoenzymes (*i.e.* *basic* and *acidic*) several physiological processes have been postulated: *e.g.* metabolism of organic compounds, lignin formation and defence in response to stress [1, 2]. One step towards the elucidation of the role of specific peroxidase isoenzymes is to study their localization and molecular biology.

In plants and cell suspension cultures of tobacco [3] and in *Lupinus albus* [4–6] *acidic* peroxidase isoenzymes are located in the cell wall. These data are in agreement with the possible function of acidic peroxidases in plants: lignification and cross-linking of extensin monomers and of feruloylated polysaccharides [7, 8]. Additionally, in tobacco a high expression of *acidic* peroxidase isoenzymes after wounding and tobacco mosaic virus infection has been reported [9], stressing the role of peroxidase as a defence protein. The *basic* peroxidase isoenzymes of tobacco are localized in the vacuole [3].

Previous studies on cell suspension cultures of *Lupinus polyphyllus* have indicated that the spent

culture medium contains large amounts of hydrolytic and oxidizing enzymes, including peroxidase [10, 11]. Further, the high degradation potential of spent culture medium for secondary metabolites could be responsible for the low product yield often encountered in cell suspension cultures [12, 13].

Although peroxidase isoenzymes were studied in different plant species, an investigation of this enzyme in *Lupinus polyphyllus* is necessary to understand the role of peroxidase in defence and alkaloid metabolism. In this paper we report on the cellular and subcellular localization of peroxidase isoenzymes in *Lupinus polyphyllus*, plants and cell suspension cultures. Different tissues and compartments were analyzed by isoelectric focusing (IEF), followed by *in situ* peroxidase staining.

Materials and Methods

Plants and cell suspension cultures

Lupinus polyphyllus plants were grown in the greenhouse. Cell suspension cultures of *Lupinus polyphyllus* (LpSp3) were derived from stem tissue [14] and maintained routinely in culture through weekly transfer of about 5.0 g fresh weight in 75 ml LS-medium [15].

Preparation of protoplasts from leaf tissue

1.5 g surface sterilized leaf tissue were cut in 5 mm pieces and incubated in 10 ml 0.3 M mannitol, 10 mM CaCl₂, 2.5% cellulysin (Calbiochem, Frankfurt, F.R.G.) and 0.5% pectinase (Roth, Karlsruhe, F.R.G.) and 5 mM 2-(N-morpholino)-ethanesulfonic acid (MES)-KOH (pH 6.0) with moderate shaking at

Abbreviations: DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; ICS, intercellular space; IEF, isoelectric focusing; MES, 2-(N-morpholino)-ethanesulfonic acid; QA, quinolizidine alkaloids; Tris, Tris(hydroxymethyl)-aminomethane.

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room temperature for 16 h. The crude protoplast preparation was filtered through two layers of nylon clothes of 100 μm and 58 μm mesh size and the filtrate was centrifuged at $120\times g$ for 5 min. The resulting pellet was washed twice with 20 ml 0.3 M mannitol and 5 mM MES-KOH (pH 6.0).

Preparation of vacuoles from leaf protoplasts

2×10^6 protoplasts of the pellet obtained above were suspended in 5 ml (40 °C) buffer consisting of 0.1 M mannitol, 10% Ficoll 400, 20 mM EDTA, 2 mM dithiothreitol (DTT) and 10 mM (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)-KOH (pH 8.0). After 20 min at room temperature, the mixture was overlaid with a 5%, 2.5% and 0% Ficoll 400 step gradient, containing 0.4 M mannitol and 10 mM HEPES-KOH (pH 8.0). The vacuoles were collected from the upper interphase after centrifugation in a swing-out rotor at $300\times g$ for 15 min.

Preparation of protoplasts from suspension-cultured cells

1.5 g cells of a 3 day old suspension culture were incubated in 5 ml 0.5 M mannitol, 10 mM CaCl_2 , 0.5% cellulysin, 1.5% cellulase Onozuka R-10 (Serva, Heidelberg, F.R.G.), 1.0% hemicellulose (Sigma, München, F.R.G.) and 5 mM MES-KOH (pH 5.5) with moderate shaking at room temperature for 4 h. The crude protoplast preparation was filtered through two layers of nylon clothes of 100 μm and 58 μm mesh size and the filtrate was centrifuged at $120\times g$ for 5 min. The resulting pellet was washed twice with 20 ml 0.5 M mannitol and 5 mM MES-KOH (pH 5.5).

Preparation of vacuoles from protoplasts of suspension-cultured cells

2×10^6 protoplasts of the pellet obtained above were suspended in 2 ml (40 °C) buffer consisting of 0.4 M mannitol, 5.0 mM EDTA, 2.0 mM DTT, 10.0% Ficoll 400 and 5.0 mM HEPES-KOH (pH 7.8). After 10 min at room temperature, the mixture was overlaid with a 5%, 2.5% and 0% Ficoll 400 step gradient, containing 0.4 M mannitol and 10 mM HEPES-KOH (pH 7.8). The vacuoles were collected from the upper interphase after centrifugation in a swing-out rotor at $300\times g$ for 15 min.

Extraction procedures of peroxidase isoenzymes

6.0 g plant tissue was frozen in liquid nitrogen, grinded in mortar and added to 20 ml 0.1 M Tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 8.0) and 1.0 mM DTT under slight magnetic stirring at room temperature for 10 min. The protoplast- and vacuole-suspensions were frozen in liquid nitrogen and thawed at room temperature. For the extraction of peroxidase isoenzymes from the intercellular space (ICS) 3.0 g intact leaf tissue was totally covered with 15 ml 0.1 M Tris-HCl (pH 8.0) in a dessicator flask. The pressure was lowered to 50 Torr for 5 min and set normal again. The leaf tissue was taken from the buffer, blotted carefully on a filter paper and centrifuged at $500\times g$ for 15 min to give 0.5 ml extract of intercellular space [16]. All crude extracts, including the suspension culture medium, were filtered and desalted *via* NAP-25 (Sephadex G-25) columns (Pharmacia, Freiburg, F.R.G.).

Peroxidase enzyme assay

Peroxidase activity was assayed in a buffer, containing 0.1 M potassium phosphate (pH 7.0), 1.0 mM H_2O_2 and 2.0 mM guaiacol. The increase in absorbance was monitored at 436 nm in a UV-VIS spectrometer (Kontron, F.R.G.).

Isoelectric focusing and in situ peroxidase staining

5.0 μl samples containing 0.05 units peroxidase were subjected to analytical IEF on prefabricated polyacrylamid gels with ampholytes in the pH-range 3.0 to 10.0 (Serva, Heidelberg, F.R.G.). The samples were focused for 2.5 h at 0.03 W cm^{-2} at 10 °C. After focusing the gels were soaked for 5 min in 20 ml 0.1 M potassium phosphate (pH 7.0) and then transferred to 10 ml substrate solution. The peroxidase-specific bands appeared within 15 sec as brown bands. The enzyme reaction was stopped relatively early in order to avoid an over-developing of the basic peroxidase isoenzymes during the *in situ* staining; as a consequence the *acidic* peroxidase isoenzymes, whose activity is much lower than that of the *basic* isoenzymes, are hardly visible on the photographs (Fig. 1–4). The gels were washed several times with water and air-dried. For determination of pH-gradient pI-marker proteins (Serva, Heidelberg, F.R.G.) were focused and visualized by coomassie blue-staining.

Results

Tissue-specific expression and total activities of lupin peroxidase isoenzymes

Partially purified soluble extracts were prepared from leaves, stems, roots and cells and the respective spent medium of a suspension culture of *Lupinus polyphyllus*. Peroxidase isoenzymes were separated by analytical IEF (pH-range 3.0–10.0). By *in situ* staining we could detect at least 16 peroxidase isoenzymes (Fig. 1, Table I). A unique isoenzyme pattern is typical for each of the tissues studied. In all of them the *basic* peroxidase isoenzymes are dominant.

For total peroxidase activities the soluble extracts were analyzed spectroscopically with H₂O₂ and guaiacol as substrates. As shown in Table II, the lowest peroxidase activity of intact plants was found in the ICS of leaf tissue, whereas we found maximal peroxidase activities in suspension-cultured cells and spent culture medium (Table II, Fig. 2). During a culture cycle of 14 days we observed in both, cells and spent medium, first a strong increase of peroxidase activity and then a substantial decrease. Whereas in cells the maximum of peroxidase activity is at the beginning of the growth phase, in the spent medium the maximum is reached three days later,

shortly before the cells enter the stationary growth phase.

Cellular and subcellular localization of peroxidase isoenzymes in leaf tissue and cell suspension culture

The electrophoretic analysis of soluble extracts from whole leaves, ICS, protoplasts and vacuoles of leaf tissue indicates that the *acidic* peroxidase isoenzymes lp 10, lp 14 and lp 15 are cell wall-bound, since they are missing in the peroxidase isoenzyme pattern

Table I. Distribution of peroxidase isoenzymes (lp) in plant and cell suspension cultures of lupin. Soluble extracts of leaf (l), stem (s), root (r), suspension-cultured cells (c) and spent culture medium (m) were analyzed by isoelectric focusing and *in situ* peroxidase staining. The isoelectric points (pI) of peroxidase isoenzymes were determined by pI-marker proteins (see Fig. 1).

Peroxidase isoenzymes	pI-Values	Tissue
lp 1	> 10	l, s, r, m
lp 2	> 10	l, r, c
lp 3	10.0	l, r
lp 4	9.6	l, s, r, c
lp 5	9.3	l, s, r, c
lp 6	8.5	l, s, r, c
lp 7	8.0	l, s, r, c
lp 8	7.3	l, s, r
lp 9	7.0	l, s
lp 10	6.7	l, s, r
lp 11	4.7	r
lp 12	4.4	r
lp 13	4.0	r
lp 14	3.5	l
lp 15	> 3.5	l
lp 16	> 3.5	r

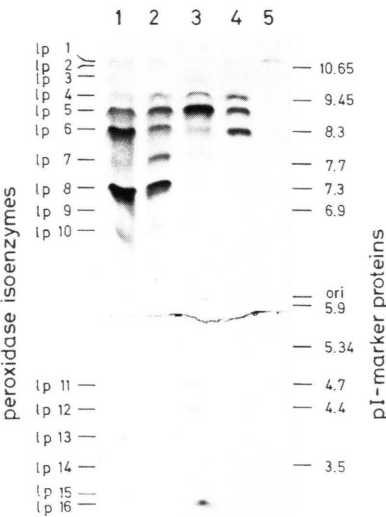


Fig. 1. Analytical isoelectric focusing of soluble extracts from leaf (lane 1), stem (lane 2), root (lane 3), suspension-cultured cells (lane 4) and spent culture medium (lane 5). *In situ* peroxidase staining revealed 16 isoenzymes (lp 1–16). The pH-gradient of the gel was determined by pI-marker proteins.

Table II. Total peroxidase activities of soluble extracts from leaf, stem, root, intercellular space (ICS), suspension-cultured cells and spent culture medium were assayed spectroscopically in a potassium phosphate buffer (pH 7.0) containing H₂O₂ and guaiacol as substrates. The activities were determined as volume units (U) and related to gram fresh weight (f.w.). The activities of suspension-cultured cells and spent culture medium are the maximal activities during a 14 day culture period (see Fig. 2).

Tissues	U/g f.w.
Leaf	20.1
Stem	9.1
Root	37.0
ICS	0.3
Cells	65.3
Medium	56.0 ^a

^a 11.0 U/ml.

of protoplasts (Fig. 3). The *basic* peroxidase isoenzyme pattern of the whole leaf is identically with that of protoplasts except for lp7, which is probably another cell wall-bound isoenzyme. The *basic* isoenzymes lp1, lp2, lp4, lp5, and lp6 are localized in the vacuole. The *basic* isoenzyme lp3 is not detectable in vacuoles, but in cells and protoplasts indicating a intracellular localization. In the ICS only the *basic* isoenzymes lp6 and lp8 were found as unbound peroxidases. *In vivo* stained cross-sections of leaf tissue or of isolated protoplasts and vacuoles confirm that peroxidase isoenzymes are localized mainly in the vacuole and cell wall (data not shown).

For comparison of peroxidase isoenzymes of the intact plant with those of cell suspension cultures sol-

uble extracts of cells, spent medium, protoplasts and vacuoles of a suspension-cultured cells were analyzed on the same gel as the plant extracts (Fig. 4). All peroxidase isoenzymes of the plant cell suspension culture were also detectable in the plant. But we found two surprising differences: first, *acidic* isoenzymes are not detectable and second, we found no difference between the intact cells and the protoplasts. This indicates that *acidic* peroxidase isoenzymes seem to be expressed only in differentiated plant tissues and are located in the cell wall. Thus *acidic* peroxidase isoenzymes could be used as a differentiation marker of regenerating lupin cultures. A further interesting feature of the cell suspension cultures studied is the localization of the most basic per-

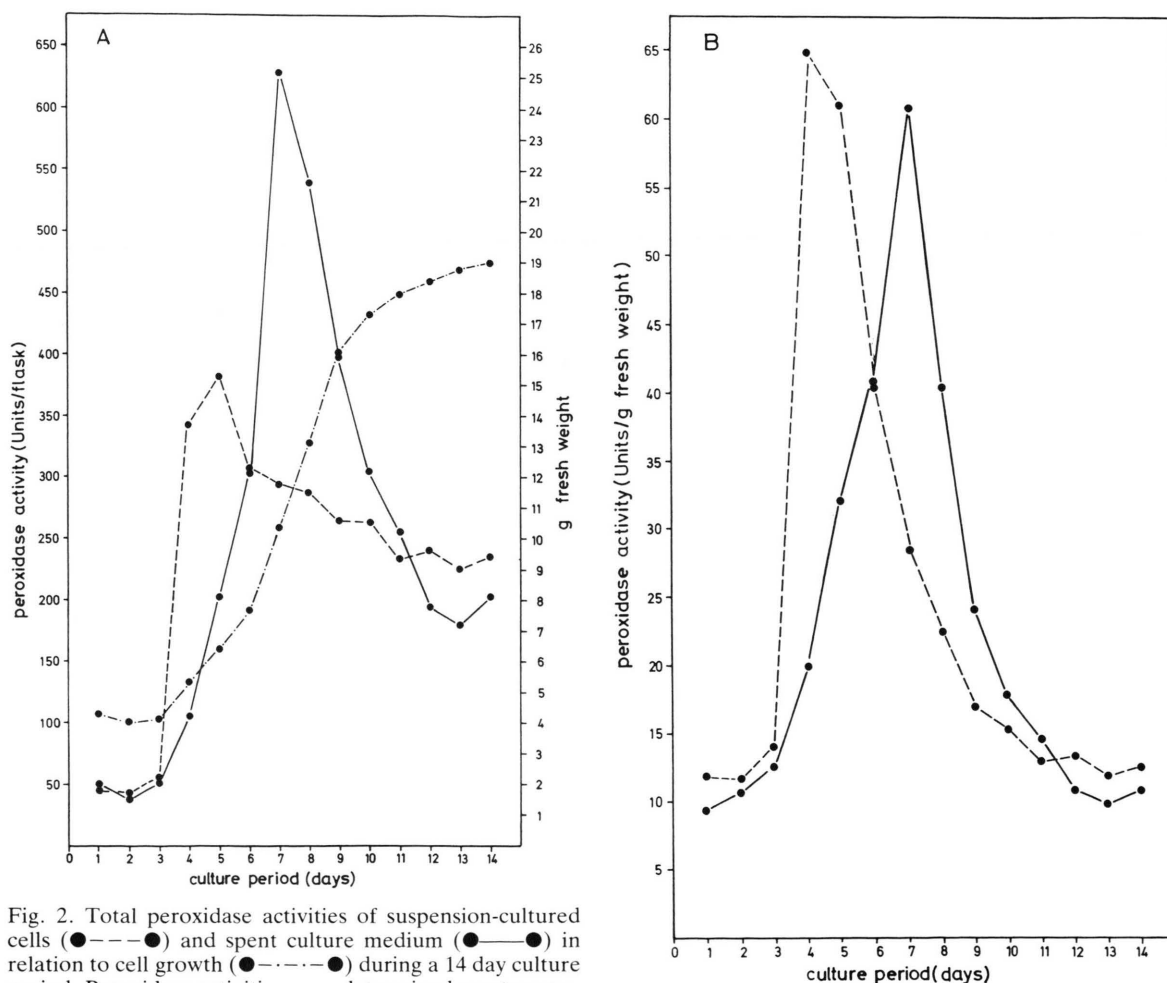


Fig. 2. Total peroxidase activities of suspension-cultured cells (●---●) and spent culture medium (●—●) in relation to cell growth (●---●) during a 14 day culture period. Peroxidase activities were determined spectroscopically as volume units/flask (A) and volume units/g fresh weight of cells (B).

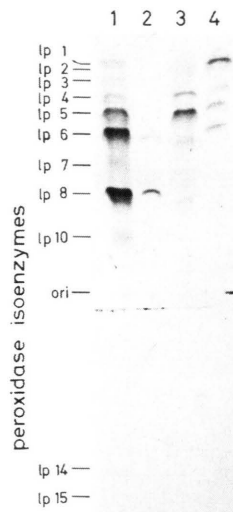


Fig. 3. Analytical isoelectric focusing of soluble extracts from whole leaf (lane 1), intercellular space (lane 2), protoplasts (lane 3) and vacuoles (lane 4) of leaf tissue. *Acidic* peroxidase isoenzymes were only detectable in the whole leaf extract (lane 1). Peroxidase isoenzymes are found mainly in the cell wall (lp7, lp10, lp14, lp15) and the vacuole (lp1, lp2, lp4, lp5, lp6) and to a minor degree inter- (lp6, lp8) and intracellularly (lp3).

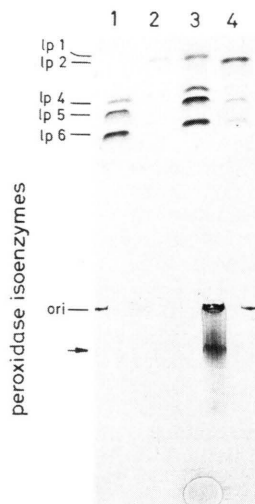


Fig. 4. Analytical isoelectric focusing of soluble extracts from suspension-cultured cells (lane 1), spent culture medium (lane 2), protoplasts (lane 3) and vacuoles (lane 4) of suspension-cultured cells. *Acidic* peroxidase isoenzymes were not detectable. *Basic* peroxidase isoenzymes are found in the vacuole (lp2–6) and the spent medium (lp1). The arrow indicates a peroxidase impurity of the enzyme mixture used for protoplasting of suspension-cultured cells.

oxidase isoenzyme lp1: unbound lp1 exists as the sole peroxidase isoenzyme in large amounts in the spent culture medium (up to 11.0 U/ml), but is not detectable in the vacuoles, however it is present in vacuoles isolated from *L. polyphyllus* leaf protoplasts (Fig. 3).

Discussion

With analytical isoelectric focusing and the high sensitivity of *in situ* peroxidase staining we have used a reproducible and effective method to study the cellular and subcellular localization of peroxidase isoenzymes from *Lupinus polyphyllus*. The number of the detected isoenzymes is similar to tobacco [9] and peanut [17], but the dominance of *basic* isoenzymes is a typical feature of *L. polyphyllus*.

Our data clearly indicate that in *L. polyphyllus* plants *acidic* peroxidases are localized extracellularly, mainly in the cell wall. These results are in agreement with studies on tobacco plants [3] and *Lupinus albus* [5, 6]. If the extracellular localization of *acidic* peroxidase isoenzymes is common in plants, in there a correlation with biological functions? There is evidence that several *acidic* peroxidase isoenzymes are part of the active defence system in plants: 1) *Acidic* peroxidases in tobacco plants are induced after wounding and virus infection [9, 18, 19]. 2) With regard to the unspecificity of peroxidase for the oxidizable substrates [12] another function could be the degradation of pathogen-released toxins. 3) Peroxidases are essential for lignification and extensin-crosslinking [7, 8], a process often associated with wound reaction and the antimicrobial response.

As in tobacco plants [3], the *basic* peroxidase isoenzymes of *L. polyphyllus* plants are localized in the vacuole, except for lp3, which could be present in the cytoplasm and for lp8 in the ICS.

Since unbound peroxidase isoenzymes of ICS are found in low amounts (Table II), they seem to play a minor part in *L. polyphyllus* plants. However, as shown in *Sedum album* leaves [20], unbound intercellular peroxidases are inducible by specific stress situations.

In contrast to *L. polyphyllus* plants and cell suspension cultures of tobacco [3] we found no cell wall-bound or *acidic* peroxidase isoenzymes in suspension-cultured cells of *L. polyphyllus*. This indicates that in *L. polyphyllus* the expression of peroxidase isoenzymes in cell suspension cultures differs from that in plants.

Further, we assume that the high peroxidase activities in cells and spent medium of suspension cultures are due to the permanent stress situation to which the cells are exposed *in vitro*. Surprisingly, we could detect only the most basic peroxidase isoenzyme Ip1 in the spent culture medium. It has been argued that extracellular proteins of cultured cells derive from dead cells. In this case we should expect the whole set of peroxidase isoenzymes, which are present in the cells, also in the medium. For cell cultures of apples it could be shown that the set of isoenzymes of esterase, α -galactosidase, β -glucosidase, phosphatase present in the medium differed substantially from that in cells, indicating that the excretion of proteins into the medium is an active and selective process [21]. This feature supports the idea that the spent culture medium functions as a

“lytic compartment” for suspension-cultured cells [10, 11]. Among others, this result is interesting regarding alkaloid production in cell suspension cultures of *L. polyphyllus* [13]: quinolizidine alkaloids (QA) are present both in the vacuoles and the spent medium of suspension-cultured cells [22]. However, production of QA in cell cultures is low, because of continuous QA-degradation, a process in which the *basic* peroxidase isoenzymes are probably involved (Ip1 in the spent medium, Ip2–6 in the vacuole).

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