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Subcellular localization and polymorphism of peroxidase in horse-radish tumour and teratoma tissue

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Abstract

The localization of peroxidase in cells of horse-radish (*Armoracia lapathifolia* Gilib.) tumour and teratoma tissues was studied. Both tissue lines were derived from the same primary crown-gall tumour induced on the leaf fragments by a wild type of *Agrobacterium tumefaciens* B6S3. Enzymatic activity was measured in cell walls, high-density heterogeneous membrane fraction, microsomal and soluble (no particulate) fractions. The subcellular localization of enzymatic activity was distinct for each transformed tissue. Both tumour and teratoma showed similar isoenzyme patterns, but one soluble acidic isoperoxidase could be considered as a marker of cell differentiation.

Additional key words: *Agrobacterium tumefaciens*, *Armoracia lapathifolia*, cell differentiation, tumour growth.

Introduction

Peroxidases (EC 1.11.1.7) constitute a group of glycoproteins whose main function is the oxidation of different substrates at the expense of H_2O_2. They consist of families of homologous isoenzymes which have apparently different substrate specificities, and perform different physiological functions in plant cells. Peroxidases are involved in the regulation of plant cell growth by participating in the oxidative degradation of indole-3-acetic acid (IAA) and in the loss of cell wall plasticity caused by phenolic cross-linking of cell wall polymers (Ros Barceló and Muñoz 1992).

Purified horse-radish peroxidase can oxidise IAA in the presence of phenolic cofactors (Gaspar *et al.* 1982). Basic peroxidases were shown to be the most effective catalyst of IAA oxidation in the absence of added cofactors (Gaspar *et al.*

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1982, Van der Berg *et al.* 1983). Changes in basic isoperoxidases are concomitant with inverse variations in endogenous IAA levels (Gaspar *et al.* 1985). In hypocotyls of legumes, soluble peroxidases were less abundant in fast growing younger cells than in slowly growing older cells (Goldberg *et al.* 1986, 1989, Ferrer *et al.* 1991a), which was to be expected considering the different IAA decarboxylation rates. This and other data support the idea that basic, mainly soluble, isoperoxidases mediate IAA catabolism and could consequently be involved in the control of cell growth.

The extracellular peroxidases are involved in the formation of phenolic bridges between cell wall polymers (extensin monomers and carbohydrate feruloyl residues). Cooper and Varner (1984) and Everdeen *et al.* (1988) showed that the extensin insolubilization was of an enzymatic nature and that this process was inhibited by the L ascorbate and catalase, two well-known non-specific peroxidase blockers. Similarly, sugar beet pectins, artificially feruloylated glucomannans and monocot arabinoxylans gelify when treated with peroxidase and H_2O_2 (Rombouts and Thibault 1986, Biggs and Fry 1987). Acidic peroxidases, which are predominantly localized in the cell wall, seem to be more efficient in cross-linking reactions and in lignin biosynthesis than basic peroxidases (Ferrer *et al.* 1992, Mäder *et al.* 1977, Zheng and Van Huystee 1991).

Although changes in peroxidase activity have been related to many developmental processes, their causal participation in these events remains obscure. However, it seems that some isoperoxidases could be considered as markers in somatic embryogenesis (Fransz *et al.* 1989, Fry 1990, Krsnik-Rasol 1991).

We have studied the subcellular localization and polymorphism of peroxidase in horse-radish tumour and teratoma tissue and the involvement of peroxidases in the control of growth and differentiation in both tumours.

Materials and methods

Tumour induction: Crown-gall tumours were induced according to Horsch (1985) on the leaf fragments of *in vitro* propagated horse-radish (*Armoracia lapathifolia* Gilib.) plants. A wild octopine strain B6S3 of *Agrobacterium tumefaciens* was used for tumour induction. From the same primary crown-gall tumour tissue two tumour tissue lines were established. One was non-morphogenic containing greenish and white tissue zones. The other, teratoma line produced malformed shoots with fleshy leaves without any rooting potential (Krsnik-Rasol 1991, Muraja *et al.* 1994).

Cell fractionation: Tissue was homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M saccharose, 1 mM magnesium acetate and 1 mM 2-mercapto ethanol by means of a *Model 230 Omnimixer* (*Sorvall*, Minnesota, USA) (three times at rate 6 for 3 s, and twice at maximum rate for 8 s). The homogenate was squeezed through a layer of cheesecloth and centrifuged at 800 *g* for 5 min. The pellet was washed by resuspension in 50 mM Tris-HCl buffer (pH 7.5) containing 1 % (m/v) Triton X-100 and further centrifuged at 800 *g* for 5 min. The procedure was repeated twice more with the same solution and three times with the buffer without detergent

(Ros Barceló *et al.* 1987). The last pellet was taken as a purified cell wall fraction. The supernatant of the 800 *g* centrifugation was centrifuged at 3 000 *g* for 15 min and the obtained supernatant was centrifuged at 100 000 *g* for 30 min. The supernatant of the last centrifugation was considered as a soluble (non particulate) fraction while the pellet represented a heterogeneous microsomal fraction.

Protoplast preparation: Tumour (5 g) grown in the dark for 48 h was hand sectioned and incubated for 75 min in 25 cm^3 of 20 mM Tris-Mes buffer (pH 7.5) containing 0.65 M mannitol. The preplasmolyzed tissue was digested in 25 cm^3 of 20 mM Tris-Mes [2-(N-morpholino) ethane-sulfonic acid] buffer (pH 5.5) containing 0.65 M mannitol, 1 % (m/v) BSA, 1 % (m/v) caylase, 0.1 % (m/v) pectolyase, 0.2 % (m/v) pectinase and 3 mM $CaCl_2$ for 2 h in the dark at room temperature and slowly stirred. After filtration through a layer of cheesecloth, the residue was washed by resuspension in 20 mM Tris-Mes buffer (pH 7.0) containing 0.65 M mannitol and centrifuged at 100 *g* for 5 min. Protoplasts were recovered from the surface and their integrity was checked under a light microscope.

Recovery of peroxidase activity from cell walls and membranes: Ionically and covalently bound cell wall peroxidases were extracted according to Ros Barceló *et al.* (1987). The purified cell wall fraction was washed twice by resuspending it in 1 M KCl in 40 mM Tris-HCl buffer (pH 7.0) at 40 °C. The salt-extracted residue was incubated at room temperature for 60 min with an enzyme solution containing 0.5 % (m/v) pectinase and 1 % (m/v) cellulase in 0.1 M Tris-acetate buffer (pH 6.0). Membrane bound peroxidases were solubilized by incubation of 100 000 *g* and 3 000 *g* pellets with 1M KCl for 60 min and further centrifugation at 100 000 *g* for 30 min and at 3 000 *g* for 15 min, respectively. Prior to enzymatic activity measurements and isoelectric focusing, all the extracts were dialysed overnight against 50 mM Tris-HCl buffer (pH 7.5).

Enzymatic activity measurements and isoelectric focusing of isoperoxidases: Peroxidase activity was measured according to Ferrer *et al.* (1991b) using 4-methoxy-α-naphtol as substrate and expressed in nkat per g of fresh tissue. Isoelectric focusing on polyacrylamide gels (3.5 - 10 pH gradients) and staining of isoperoxidase bands with 4-methoxy-α-naphtol was carried out as previously described (Ferrer *et al.* 1991b).

Chemicals: Caylase was purchased from *Cayla* (Toulouse, France), pectinase and cellulase from *Serva* (Heidelberg, Germany), and pectolyase Y-23 from *Seishin Pharmaceutical Co.* (Tokyo, Japan). All other chemical used in this work were of the highest purity available.

Results and discussion

During a subculture the tumour enters its exponential phase of growth earlier than the teratoma (Fig. 1) and it has a higher growth rate, which is in accordance with the fact

that the tumour predominantly consists of cells with high mitotic activity, while the teratoma is partially formed of differentiated cells with limited growth potential.

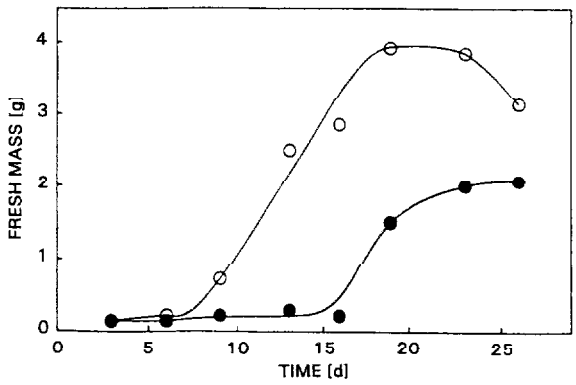

Fig. 1. Growth curves of tumour (*open circles*) and teratoma (*closed circles*) during subculture.

On the 16th day of subculture (when the teratoma was still in the initial phase of growth and the tumour in its exponential phase of growth) and on the 20th day (when the tumour entered a stationary phase and the teratoma was still growing, although at a lower rate) peroxidase activity was localized in different subcellular fractions (Table 1). As repeated assays showed, the microsomal and cell wall bound

Table 1. Peroxidase activity [nkat g^{-1}(f. m.)] in the different subcellular fractions during subculture. The variation coefficients were within 5 - 10 %.

Day	Tissues	Cell walls	Chloroplasts	Microsomes	Soluble	Total	Cell wall [%]
16th	tumour	48.6	53.5	102.5	266.3	470.9	10.3
	teratoma	41.3	51.5	81.3	578.0	752.1	5.5
20th	tumour	55.5	25.7	71.6	54.8	207.6	28.0
	teratoma	25.2	32.2	43.2	152.0	252.6	10.0

peroxidase activity was higher in the tumour than in teratoma tissue. This result could be related to well established intracellular pathway of protein secretion through the rough endoplasmic reticulum (rER), Golgi system and secretion vesicles to plasmalemma. Electron microscopy studies showed that the rER was more abundant in tumour than in teratoma cells (Muraja *et at.* 1994). Peroxidase activities of the high-density (3 000 *g* pelletable) heterogeneous membranous fraction, containing chloroplasts, were rather similar in both tissues. The peroxidase activity found in this chloroplast fraction is probably related to the oxidation of ascorbic acid, thus contributing to the detoxification of oxygen reduced species generated under high irradiance and low rates of CO_2 fixation (Groden and Beck 1979). The tumours studied were grown on a nutrient medium containing saccharose as a carbon source

and under these culture conditions toxic oxygen reduced species may accumulate and be removed by peroxidase. This peroxidase activity could also correspond to a previously described enzymatic activity associated with a similar membranous fraction from lupin hypocotyls (Ros Barceló *et al.* 1988).

Soluble peroxidase activity was considerably higher in the teratoma than in the tumour tissue (Table 1). It decreased between the 16th and 20th day of the subculture in both tissues although to a greater degree in the teratoma.

The involvement of the soluble peroxidase fraction in the cell growth control is not easy to understand. This enzymatic activity is mainly intracellular and obviously cannot be involved in the stiffening of the cell walls. Contradictory results concerning the involvement of protoplast peroxidases in the regulation of the IAA level have been reported. Thus although an inverse correlation between the growth potential and the soluble peroxidase content has been established (Goldberg *et al.* 1986, 1989, Ferrer *et al.* 1991a), other results indicate the poor ability of this peroxidase fraction to catalyze the hormone oxidation. The intracellular peroxidase is mainly localized in the vacuole, and involved in oxidative processes of the secondary metabolites (polyphenols and alkaloids) (Pedreño *et al.* 1993). As polyphenols have also been described as strong inhibitors of IAA oxidation (García-Florenciano *et al.* 1991), protoplast peroxidase would not be involved in the *in vivo* control of the level of IAA. In grapevine protoplasts, a decarboxylative pathway of hormone is not functional, thus restricting the hormone catabolism to the cell walls (García-Florenciano *et al.* 1992).

The total peroxidase activity of both tissue lines decreases with time while the cell wall peroxidase (as percentage of the total activity) increases (Table 1). The peroxidase-catalysed cross-linking of the cell wall polymers contributes to a mechanical restriction of cell growth. From this consideration, it is possible that a low percentage of extracellular peroxidase favours cell growth because such growth is not mechanically restricted. A decrease in growth rate in the stationary phase of the culture (Fig. 1) is caused by extracellular peroxidases (mainly of a basic nature) which catalyze a cross-linking of phenolics of the cell wall polymers.

Although acidic isoperoxidases have been considered as the main candidates to catalyse the reactions which cause the loss of extensibility of cell walls (Ferrer *et al.* 1992, Mäder *et al.* 1977, Zheng and Van Huystee 1991), the basic isoperoxidases are also able to catalyse these reactions. Thus, basic isoperoxidases from lupin hypocotyls catalyse the oxidation of coniferyl alcohol, one of the processes which contribute to wall stiffening (Ferrer *et al.* 1992). Moreover, the cross-linking reactions of the cell wall polymers could be associated with the IAA oxidation, as was demonstrated *in vitro* for coniferyl alcohol oxidation (Ferrer *et al.* 1990), thus decreasing the IAA content of the tumours and, consequently, their growth rates.

To determine the relationship between cell differentiation and peroxidase pattern, both cell wall bound and soluble proteins were analyzed by isoelectric focusing. In the cell wall bound fraction, only basic isoperoxidases were detected showing no differences in isoperoxidase patterns between tumour and teratoma (Fig. 2*A*). The basic isoperoxidases of the soluble fraction coincided with those of the cell walls except for one well-focused isoenzyme (Fig. 2*B*, band *b*), which was present in

soluble fraction only. The acidic isoperoxidase in soluble fraction (Fig. 2*B*, band *a*) was characteristic of teratoma tissue, and of greenish but not white tumour zones.

To ascertain the intracellular localization of the soluble isoperoxidases, soluble and protoplasts proteins (recovered from the supernatant of the centrifugation at 13 000 *g* for 5 min of the protoplast which had been broken by freezing and thawing)

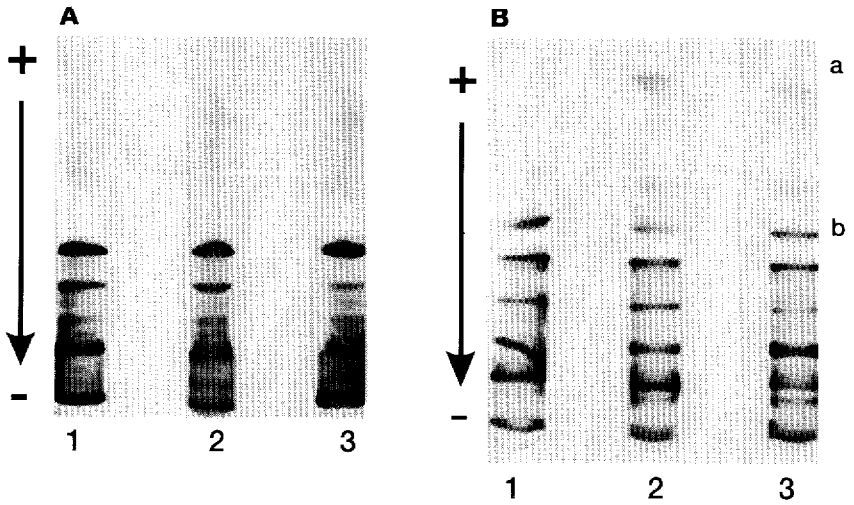

Fig. 2. Staining of cell wall bound (*A*) and soluble (*B*) isoperoxidases separated by isoelectric focusing in 3.5 - 10 pH gradients with 4-methoxy-α-naphtol from white and greenish zones of tumour tissue (lanes 1 and 2, respectively) and teratoma (lane 3).

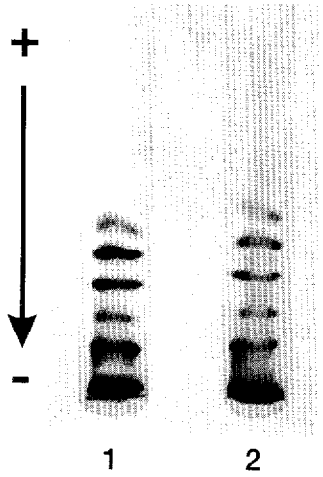

Fig. 3. Staining of soluble isoperoxidases from white zone (lane 1) and protoplasts (lane 2) of tumour separated by isoelectric focusing in 3.5 - 10 pH gradients with 4-methoxy-α-naphtol.

from white zone of tumour were submitted to isoelectric focusing. The coincidence of the isoenzyme patterns of both protein fractions (Fig. 3) demonstrated that the isoperoxidases recovered in the supernatant of the centrifugation at 100 000 *g* are intracellular proteins and are therefore not localized in the apoplastic space.

The intracellular acidic isoperoxidase is specifically expressed in the teratoma and in the greenish zone of the tumour and it was a marker of greening. The greening of the tumour is probably a part of cell differentiation process that precedes morphogenesis of the shoots. Chlorophyll synthesis coincides by chloroplast formation. Thus, a certain degree of differentiation occurs in the tumour. By means of electron microscopy (Muraja *et al.* 1994) tumour plastids were classified as amylochloroplasts. They contained more starch and less thylakoid than whose of the teratoma shoots.

In conclusion, peroxidase activity was localized in soluble, high-density (3 000 *g* pelletable), microsomal and cell wall fractions of horse-radish tumour and teratoma tissue lines. A percentage of cell wall bound peroxidase (only basic isoenzymes) in relation to total activity increased during subculture. This increase was accompanied by a decrease in growth rate indicating that cell wall peroxidase is involved in growth, either by catalysis of cell wall stiffening reactions or by IAA oxidation. In both tumour and teratoma tissues mainly basic isoperoxidases were expressed. One soluble acidic isoperoxidase was found to be specific for green tumour and teratoma tissue and could be considered as a marker of cell differentiation.

References

Biggs, K.J., Fry S.C.: Phenolic cross-linking in the cell wall. - In: Cosgrove, D.J, Knievel, D.P. (ed.): Physiology of Cell Expansion During Plant Growth. Pp. 46-57. American Society of Plant Physiologists, Rockville 1987.

Cooper, B., Varner, J.E.: Cross-linking of soluble extensin in isolated cell walls. - Plant Physiol. **76**: 414-417, 1984.

Everdeen, D.S.I., Kiefer, S., Willard, J.J., Muldoon, E.P., Dey, P.M., Li, X.B., Lamport, D.T.A.: Enzyme cross-linkage of monomeric extensin precursors. - Plant Physiol. **87**: 616-621, 1988.

Ferrer, M.A., Pedreño, M.A., Muñoz, R., Ros Barceló, A.: Oxidation of coniferyl alcohol by cell wall peroxidases at the expense of the indole-3-acetic acid and O_2. A model for the lignification of cell walls in the absence of H_2O_2. - FEBS Lett. **276**: 127-130, 1990.

Ferrer, M.A., Pedreño, M.A., Muñoz, R., Ros Barceló, A.: Soluble peroxidase gradients in lupin hypocotyl and the control of polarly transported indole3-yl acetic acid. - J. Plant Growth Regul. **10**: 139-146, 1991a.

Ferrer, M.A., Calderón, A.A., Muñoz, R., Ros Barceló, A.: 4-methoxy-α-naftol as a specific substrate for kinetic, zymographic and cytochemical studies on plant peroxidase activities. - Phytochem. Anal. **1**: 63-69, 1991b.

Ferrer, M.A., Pedreño, M.A., Ros Barceló A., Muñoz, R.: The cell wall localization of two strongly basic isoperoxidases in etiolated hypocotyls and its significance in coniferyl alcohol oxidation and indole-3-acetic acid catabolism. - J. Plant Physiol. **139**: 611-616, 1992.

Fransz, P.F., De Ruijter, N.C.A., Schel, J.H.N.: Isozymes as biochemical and cytochemical markers in embryogenic callus culture of maize (*Zea mays* L.). - Plant Cell Rep. **8**: 67-70, 1989.

Fry, S.C.: Roles of the primary cell walls in morphogenesis. - In: Nijkamp, H.J.J, Van der Plas, L.H.W., Van Aartrick, J. (ed.): Progress in Plant, Cellular and Molecular Biology. Pp. 504-513. Kluwer Academic Publishers, Dordrecht 1990.

García-Florenciano, E., Calderón, A.A., Pedreño, M.A., Muñoz, R., Ros Barceló, A.: The vacuolar localization of basic isoperoxidases in grapevine cell cultures and its significance in indole-3 acetic acid catabolism. - Plant Growth Regul. **10**: 125-138, 1991.

García-Florenciano, E., Calderón, A.A., Muñoz, R., Ros Barceló, A.: The decarboxylative pathway of indole-3-acetic acid is not functional in grapevine protoplasts. - J. exp. Bot. **43**: 715-721, 1992.

Gaspar, T., Penel, C., Greppin, H.: Peroxidases 1970-80. A Survey of Their Biochemical and Physiological Roles in Higher Plants. - University of Geneva, Geneva 1982.

Gaspar, T., Penel, C., Castillo, F.J., Greppin, H.: A two step control of basic and acidic peroxidases and its significance for growth and development. - Physiol. Plant. **64**: 418-423, 1985.

Goldberg, R., Imberty, A., Chu-Ba, J.: Development of isoperoxidases along the growth gradient in the mung bean hypocotyl. - Phytochemistry **25**: 2171-2174, 1986.

Goldberg, R., Kevers, C., Gaspar, T.: Guaiacol and ascorbate peroxidase compartmentation and gradient along the mung bean hypocotyl. - Biochem. Physiol. Pflanz. **184**: 155-161, 1989.

Groden, D., Beck, E.: H_2O_2 destruction by ascorbate dependent systems from chloroplasts. - Biochim. biophys. Acta **546**: 426-435, 1979.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T.: A simple and general method for transferring genes into plants. - Science **227**: 1229-1231, 1985.

Krsnik-Rasol, M.: Peroxidase as a developmental marker in plant tissue culture. - Int. J. Dev. Biol. **35**: 259-263, 1991.

Mäder, M., Nessel, A., Bopp, M.: On the physiological significance of the isoenzyme groups of of peroxidase from tobacco demonstrated by biochemical properties. II. pH optima, Michaelis constants and maximal oxidation rates. - Z. Pflanzenphysiol. **82**: 247-260, 1977.

Muraja, J., Peškan, T., Lorković, Z., Krsnik-Rasol, M.: Ultrastructure and protein pattern of horse radish (*Armoracia lapathifolia* Gilib.) crown-gall tissue. - Acta bot. croat. **53**: 1-6, 1994.

Pedreño, M.A., Bernal, M.A., Calderón, A.A., Ferrer, M.A., López-Serrano, M., Merino de Cáceres, F., Muñoz, R., Ros Barceló A.: A general pattern for peroxidase isoenzyme localization and function in *Vitaceae*, *Solanaceae* and *Leguminoseae*. - In: Welinder, K.J., Rasmussen, S.K., Penel, C., Greppin, H. (ed.): Plant Peroxidases: Biochemistry and Physiology. Pp. 307-314. University of Geneva, Geneva 1993.

Rombouts, F.M., Thibault, J.F.: Sugar beet pectins: chemical structure and gelation through oxidative coupling. - In: Fishman, L.M., Jen, J.J. (ed.): Chemistry and Function of Pectins. Pp. 49-60. American Chemical Society, Washington 1986.

Ros Barceló, A., Muñoz, R., Sabater, F.: Lupin peroxidases. I. Isolation and characterization of cell wall bound peroxidase activity. - Physiol. Plant. **71**: 448-454, 1987.

Ros Barceló, A., Muñoz, R., Sabater, F.: Lupin peroxidases III. Subcellular localization of membrane-bound acidic isoperoxidases. - Plant Physiol. Biochem. **26**: 575-583, 1988.

Ros Barceló, A., Muñoz, R.: Peroxidases: Their role in the control of plant cell growth. - In: Penel, C., Gaspar, T,. Greppin, H. (ed.): Plant Peroxidases 1980-1990. Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects. Pp. 71-80. University of Geneva, Geneva 1992.

Van der Berg, B.M., Chibbar, R.N., Van Huystee, R.B.: A comparative study of a cationic peroxidase from peanut and a anionic peroxidase from Petunia. - Plant Cell Rep. **2**: 304-307, 1983.

Zheng, X., Van Huystee, R.B.: Oxidation of tyrosine by peroxidase isoenzymes derived from peanut suspension culture medium and by isolated cell walls. - Plant Cell Tissue Organ. Cult. **25**: 35-43, 1991.