Plant Cell Reports (1989) 8:471–474

Plant Cell Reports

Sequential release of both basic and acidic isoperoxidases to the media of suspension cultured cells of *Capsicum annuum*

J. Cuenca, E. García-Florenciano, A. Ros Barceló, and R. Muñoz

Department of Plant Biology (Plant Physiology), University of Murcia, Campus de Espinardo, E-30071 Murcia, Spain

Received June 12, 1989/Revised version received August 12, 1989 – Communicated by A. M. Boudet

Summary. The establishment of suspension cell cultures from trimmed cotyledons of pepper (*Capsicum annuum* L.) provides a new experimental system for studying the relationship between release of peroxidase (EC 1.11.1.7) into the free intercellular spaces and plant cell growth. In contrast with several other species, the total peroxidase activity in the medium increased continuously during the post-exponential growth phase of the pepper cell culture, and this was correlated with the growth inhibition of pepper cells cultivated in suspension. The increase in the peroxidase activity in the culture medium was the consequence of a differential release of isoperoxidases, prominently marked by a primary release of basic isoperoxidases, followed by a strong increase in the level of acidic isoperoxidases. Thus, pepper cells cultures constitute a new experimental system for studying the regulation of the sequential release of basic and acidic isoperoxidases, which occurs during the growth cessation of plant cells.

Key words: Acidic isoperoxidases – Basic isoperoxidases – *Capsicum annuum* – Medium – Sequential release.

Introduction

Extracellular peroxidases have been thoroughly investigated during recent years since they are the main enzymes responsible for the cross-linking of the phenolic compounds of the plant cell wall (Biggs and Fry 1987). Peroxidase is a constitutive enzyme of the cell wall and free intercellular spaces (Bolwell 1988), which is apparently regulated in both its catalytic activity and its protein level during well-defined stages of the plant cell differentiation (Bolwell 1988).

Attempts to elucidate the mechanism of regulation of both the catalytic activity and protein level of the free intercellular peroxidase by effectors, have lead to the conclusion that protons may be involved in the regulation *in vivo* of this extracellular enzyme in several ways: a) protons can modulate extracellular peroxidase activity inducing a transition in the kinetic properties of this peroxidase, the enzyme acquiring regulatory properties (Pedreño et al. 1989), and b) protons can modulate the binding of isoperoxidases to cell walls and, therefore, the level of the enzyme in the free intercellular spaces (Ros Barceló et al. 1988). This last fact, together with the calcium-mediated control of the peroxidase secretion (Penel et al. 1984), seem to be the two main factors in controlling the level of peroxidase in the free intercellular spaces.

The release of peroxidase from the cell wall into the free intercellular spaces is partially responsible for the increase in free intercellular peroxidases during the growth cessation of lupin hypocotyl cells (Ros Barceló et al. 1989), and this increase is related, in this organ, to the lignification of xylem cells (Ros Barceló et al. 1989). However, this increase in free intercellular peroxidases during the growth cessation of plant cells has not been generally reported in much simpler experimental systems, such as plant cells cultivated in suspension, although Chibbar et al. (1984) reported significant increases in the free intercellular peroxidases during the growth cycle of the cell line WCA1 of suspension-cultured carrot cells.

Suspension-cultured cells possess several advantages over plant tissues for studying the release of cell wall peroxidases into the free intercellular spaces since, on one hand, the medium of cultured cells may be equated to an enlarged free intercellular space (Van Huystee and Tam 1988) and, on the other hand, because suspension-cultured cells are easily separated from the medium by centrifugation. In this context, we have established the conditions under which the growth of pepper cells cultivated in suspension is actively stimulated so as to provide an experimental system for studying the relationship between release of peroxidases to the medium and plant cell growth. In this way, a two-step sequential release of both basic and acidic isoproxidases was found, and this release was clearly associated with the growth inhibition of pepper cells cultivated in suspension.

Offprint requests to: A. Ros Barceló

Materials and methods

Plant material and culture. Seeds of red pepper (*Capsicum annuum* L. var. Fresno) were sterilized with 20 % (v/v) Domestos sodium hypochlorite for 20 min, and rinsed four times with sterilized water. Seeds were sown aseptically on Murashige and Skoog (1962) basal medium (MSB). Seedlings were allowed to grow under a 14/10 h photoperiod regime of 1200 lux dim fluorescent light at 25 °C. Induction of vigorous friable callus was carried out on the 20-day-old seedlings, starting from trimmed cotyledons with petioles (the distal one-half of the blade was removed), cultured on Murashige and Skoog complete medium (MSC; Gunay and Rao 1978). For this purpose, the MSC medium was supplemented with 3 % (w/v) sucrose, 0.8 % (w/v) Difco-Bacto agar, and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). The medium pH was adjusted to 5.8. Callus cultures were both induced and maintained under a 14/10 h photoperiod regime of 4500 lux fluorescent light at 25 °C, and at a relative humidity of 80-85 %.

Starting from friable callus sub-cultured for 5 months, pepper cells were cultured in suspension on MSC medium, supplemented with 3 % (w/v) sucrose and 1 mg/l 2,4-D, in 500-ml flask, containing 200 ml of medium (pH 5.8), with orbital shaking (105 min^{-1}). Suspension cultured cells were grown for 23 days at 25 °C, and under a 14/10 h photoperiod regime of 2000 lux fluorescent light, at a relative humidity of 80-85 %.

Pepper cells grew in clumps of up to 10 cells. Plant cell culture growth was measured as the packed cell volume, and expressed as a percentage.

Culture samples. Samples (10 ml) for analyses were taken at various days after the beginning of culture. Cultures were initiated by addition of 20 ml cells to 200 ml fresh culture medium. Cell suspensions from two or three flask per time, were pooled and the cells were separated by centrifugation (500 $g_{max.}$ for 5 min). The supernatants ("medium samples") were used without further treatments for peroxidase assay and isoelectrofocusing.

Analytical methods. Peroxidase (EC 1.11.1.7) activity was measured by following the increase in absorbance at 470 nm, using 0.2 mM H_2O_2, and 2.5 mM guaiacol as described earlier (Ros Barceló et al. 1987). Isoelectrofocusing and zymographic stain of peroxidase isoenzymes with benzidine was achieved as described previously (Ros Barceló 1987). The benzidine-stained gels were recorded at 460 nm, using a Joyce-Loebl MKII scanner densitometer. Individual isoenzyme activities were calculated from the integrated recording, on the basis of the total peroxidase activity (peroxidase activity measured using either guaiacol or benzidine leads to similar results) overlayed initially on the polyacrylamide gel.

Protein concentration in the media was determined using the Coomassie G-250 dye binding microassay (Spector 1978), using bovine serum albumin as standard.

Results

The results on changes in the packed cell volume and growth rate during the growth cycle of the pepper cells cultivated in suspension are shown in figure 1. The pepper cells used in this study differ widely in their growth properties from those described for several other species (Kossatz and Van Huystee 1976; Bredemeijer and Burg 1986) and thus, the ratio of the volume of the cells to that of the medium was about 0.1:1 at day 0 (see Methods), and 19:1 at day 22 (Fig. 1). This strong increase in the packed cell volume was not accompanied by loss of viability of the cells since, when tested by methylene blue, all cells were found to be viable during the entire culture period.

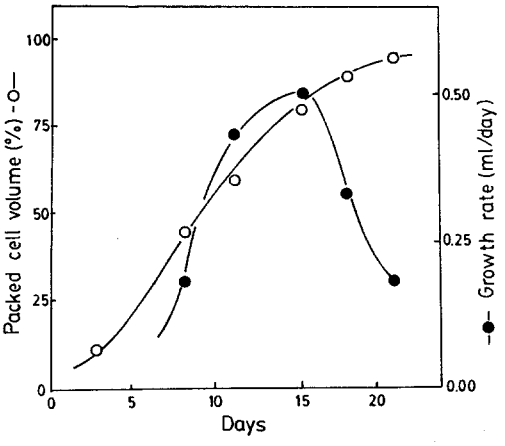

Fig. 1. Changes in the packed cell volume and growth rate, during the growth cycle of pepper cells cultivated in suspension. Data points are the mean of, at least, three independent replicates.

During the exponential growth phase of these pepper cells cultured in suspension, the total peroxidase activity released into the media increased gradually, and reached maximum values at the end of the post-exponential growth phase (Fig. 2). A similar variation was found for the protein released into the media (Fig. 2), although the increase in protein along the growth cycle was less (2-fold) than the increase found for the peroxidase activity (17-fold) (Fig. 2), so the increase in total peroxidase activity on a protein basis (specific activity) increased 8.5-fold during the post-exponential growth phase of these pepper cells cultivated in suspension.

The isoenzyme pattern of the peroxidase activity released into the media after 18 days is shown in Fig. 3. It shows mainly two peroxidase isoenzyme groups, named A_{1-3} and B_{1-6}, which changed both qualitatively and quantitatively during the culture cycle. The most striking changes in the peroxidase isoenzyme patterns took place during the

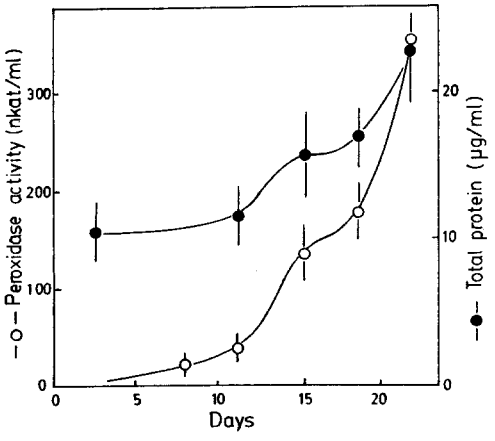

Fig. 2. Changes in the peroxidase activity and protein released into the medium during the growth cycle of pepper cells cultivated in suspension. Aliquots were taken at regular intervals, and tested for peroxidase activity by means of a guaiacol-based kinetic assay. Bars represent SE.

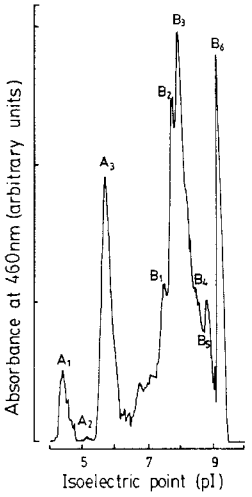

Fig. 3. Isoenzyme pattern of the peroxidase activity released into the medium after 18 days of culture of pepper cells in suspension. Zymographic stain of peroxidase isoenzymes was achieved with benzidine.

exponential (10-15 days of culture) and post-exponential (15-20 days of culture) growth phases (Figs. 4-6).

During the exponential growth phase, the peroxidase isoenzymes of the B group increased in activity, and reached maximum values at day 18 of culture (Figs. 4-5). Subsequently, they decreased again to the level observed at day 11. The pattern of behaviour for all the basic isoperoxidases is similar, however some minor differences are observed. Thus, not all the basic isoperoxidases are released in the same proportion into the culture media, the B_3 isoperoxidase being released in greater amount (Figs. 4-5). On the other hand, the maximal level for the B_{4-5} isoperoxidases in the culture media is reached up to 3 days before (15 days of culture) that for the other basic isoperoxidases, and the level of these isoperoxidases in the media is correlated with the maximal growth rate of the

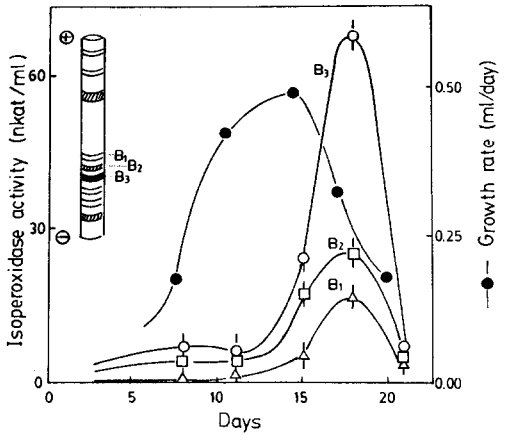

Fig. 4. Changes in the level of the B_1, B_2 and B_3 basic isoperoxidases, and the growth rate, during the growth cycle of pepper cells cultivated in suspension. Isoperoxidase activities were calculated from the integrated recording of the isoperoxidase patterns of the total peroxidase activity found at each age. Typical isoenzyme pattern of medium peroxidase at day 18 of culture. Bars represent SE.

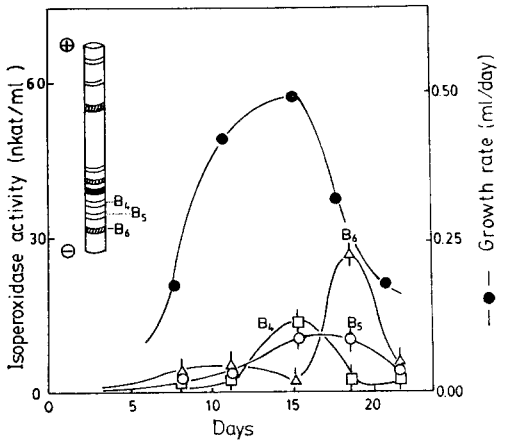

Fig. 5. Changes in the level of the B_4, B_5 and B_6 basic isoperoxidases, and the growth rate, during the growth cycle of pepper cells cultivated in suspension. Isoperoxidase activities were calculated as in figure 4. Bars show SE.

pepper cells cultivated in suspension (Fig. 5).

In contrast to basic isoperoxidases, acidic isoperoxidases are released to the culture media only during the onset of the post-exponential growth phase (Fig. 6), and this occurs in coincidence with the decrease of the level of basic isoperoxidases (Figs. 4-5). Thus, a sequential release of basic and acidic isoperoxidases takes place during the late growth phases of pepper cells cultivated in suspension.

The predominant release of acidic isoperoxidases is the main factor responsible for the strong increase in the level of the total peroxidase activity released to the media at the onset of the stationary phase (Figs. 2 and 6); however, just as for basic isoperoxidases, not all the acidic isoenzymes are released to the media in the same proportion. Thus, the release of the acidic A_3 isoperoxidase is the main factor responsible for the strong increase in the total acidic isoperoxidase activity released to the media, and

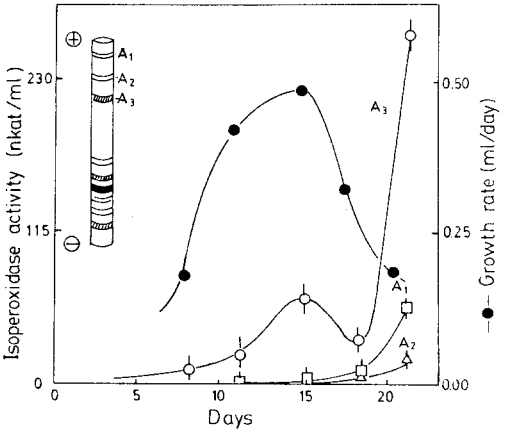

Fig. 6. Changes in the level of the A_1, A_2 and A_3 acidic isoperoxidases, and the growth rate, during the growth cycle of pepper cells cultivated in suspension. Isoperoxidase activities were calculated as in figure 4. Bars show SE. Note the scale expansion of isoperoxidase units in figure 6 over figures 4 and 5.

this release coincided with the growth cessation of the cultured cells.

Discussion

The activity (Fig. 2) and isoenzyme levels (Figs. 4-6) of peroxidase in the medium of cultured suspension cells of pepper changed during the entire growth cycle. In contrast with several other studied species, such as *Arachis* (Kossatz and Van Huystee 1976), *Nicotiana* (Mäder et al. 1981) and *Haplopappus* (Bredemeijer and Burg 1986), the increase in total peroxidase activity in the medium was maintained along the growth cycle, reaching maximum values at the beginning of the stationary phase (Fig. 2). Thus, the increase in the peroxidase activity in the culture medium of pepper cells takes place during both the exponential and the post-exponential growth phases (Fig. 2), this increase being associated with the growth inhibition of the cell culture.

The development of peroxidase activity in the culture medium is the consequence of a differential release of isoperoxidases, prominently marked by a primary release of basic isoperoxidases (Figs. 4-5), and followed by a strong increase in the level of acidic isoperoxidases (Fig. 6).

Such a sequential release of basic and acidic isoperoxidases has been observed frequently by authors studying isoperoxidase changes in relation to external chemical and physical stimuli on physiological process, such as root formation, flower initiation, abscission, and thigmomorphogenesis (Gaspar et al. 1985); however, this is the first report that describes the aforementioned sequential release of isoperoxidases within a basic programme of plant cell development in suspension-cultured cells.

In this sequence of events, Gaspar et al. (1985) have shown that the release of acidic isoperoxidases into the intercellular spaces is the consequence of the ethylene production by the tissues, in response to the applied external stimuli. Thus, ethylene induces the release of acidic isoperoxidases towards the intercellular spaces, acidic isoperoxidases which apparently are involved in catalyzing cell wall cross-linking (Mäder et al. 1980; Biggs and Fry 1987), and so in the stiffening of the cell wall (Taiz 1984).

However, several questions remain to unanswered: a) How and at what level is the sequential release of basic and acidic isoperoxidases regulated?, and b) which is the mechanism which leads to the decrease in the level of basic isoperoxidases, following their release to the media, and prior to the increase in the level of acidic isoperoxidases?

It is evident that the use of these suspension-cultured pepper cells, derived from cotyledons, can help resolve these questions. In this context, several experiments are in progress in our laboratory.

References

Biggs KJ, Fry SC (1987) Phenolic cross-linking in the cell wall. In: Cosgrove DJ, Knievel DP (eds) Physiology of Cell Expansion during Plant Growth. The Amer. Soc. Plant Physiologists, Rockville, p. 46-57.

Bolwell GP (1988) Synthesis of cell wall components: aspects of control. Phytochemistry 27: 1235-1253.

Bredemeijer GMM, Burg HCJ (1986) Peroxidase in suspension cultures of *Haplopappus gracilis*. Acta Bot Neerl 35: 153-159.

Chibbar RN, Cella R, Albani D, Van Huystee RB (1984) The growth of and peroxidase synthesis by two carrot cell lines. J Exp Bot 35: 1846-1852.

Gaspar Th, Penel C, Castillo FJ, Greppin H (1985) A two step control of basic and acidic peroxidases and its significance for growth and development. Physiol Plant 64: 418-423.

Gunay AL, Rao PS (1978) In vitro plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). Pl Sci Letters 11: 365-372.

Kossatz VC, Van Huystee RB (1976) The specific activities of peroxidase and aminolevulinic acid dehydratase during the growth cycle of peanut suspension culture. Can J Bot 54: 2089-2094.

Mäder M, Füssl T, Walker E (1981) On the synthesis of protoheme and peroxidase in suspension cultures of *Nicotiana tabacum*. Z Pflanzenphysiol 104: 207-216.

Mäder M, Ungemach J, Schloss P (1980) The role of peroxidase isoenzyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. Planta 147: 467-470.

Murashige T, Skoog F (1962) A revised medium for rapid growth bioassay with tobacco tissue cultures. Physiol Plant 15: 473-497.

Pedreño MA, Ros Barceló A, Sabater F, Muñoz R (1989) Control by pH of cell wall peroxidase activity involved in lignification. Plant Cell Physiol 30: 237-241.

Penel C, Gaspar Th, Greppin H (1984) Hormonal control of enzyme secretion by plant cells. In: Purohit SS (ed) Hormonal regulation of Plant Growth and Development, vol 1, Agro Botanical Publ., Bikaner, p. 145-168

Ros Barceló A (1987) Quantification of lupin peroxidase isoenzymes by densitometry. Anal Biol Univ Murcia (Ser. Biol. Gen.) 14: 33-38.

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

Ros Barceló A, Pedreño MA, Muñoz R, Sabater F (1988) Lupin peroxidases. II. Binding of acidic isoperoxidases to cell walls. Physiol Plant 73: 238-244.

Ros Barceló A, Pedreño MA, Muñoz R, Sabater F (1989) Physiological significance of the binding of acidic isoperoxidases to cell walls of lupin. Physiol Plant 75: 267-274.

Spector T (1978) Refinement of the Coomassie blue method of protein quantitation. Anal Biochem 86: 142-146.

Taiz L (1984) Plant cell extension: regulation of cell wall mechanical properties. Annu Rev Plant Physiol 35: 585-657.

Van Huystee RB, Tam ASK (1988) Peptides released by peanut cells during growth. J Plant Physiol 133: 645-647.