

Phenoloxidase activity in *Daucus carota* is restricted to embryogenic cultures

Irene Carlberg, Kenneth Söderhäll and Tage Eriksson

Institute of Physiological Botany, University of Uppsala, Box 540, S-751 21 Uppsala, Sweden

Received 15 May 1985

The enzyme phenoloxidase is present in plants as a proenzyme, which is activated by Ca^{2+} or Mn^{2+} at millimolar concentrations, or by trypsin at lower Ca^{2+} concentrations. In *Daucus carota* cell cultures this proenzyme was shown to be present in the embryogenic strains, whereas the non-embryogenic strains lacked detectable phenoloxidase activity.

Daucus carota *Embryogenesis* *Marker protein* *Phenoloxidase* *Prophenoloxidase*

1. INTRODUCTION

Since the discovery of somatic embryogenesis in carrot cell cultures [1,2] this species has been used as a model for studying embryogenesis in higher plants. While earlier reports describe the external factors affecting embryo formation [3,4], recent studies have focused on biochemical changes occurring during the differentiation process [5–8], but the regulation of embryo formation remains largely unknown.

Sung and Okimoto [9] have detected 2 polypeptides which are synthesized during embryogenesis, and since these polypeptides are not detectable during callus growth, they may possibly be used as markers for the developmental process, as well as the ability to inactivate cycloheximide [10]. We have recently shown that embryogenic carrot strains had much lower proteolytic activity than a non-embryogenic strain, and during embryo formation 3 specific peptidases were detected in the differentiated cell aggregates but not in the undifferentiated [11].

Abbreviations: DFP, diisopropylphosphorofluoridate; 2,4-D, 2,4-dichlorophenoxyacetic acid; L-dopa, L-dihydroxyphenylalanine; PO, phenoloxidase; proPO, prophenoloxidase

Recently, we have shown that the enzyme phenoloxidase is present in plants as a proenzyme which can become activated by millimolar concentrations of Ca^{2+} or Mn^{2+} or by trypsin at lower Ca^{2+} concentrations [12]. Here we show that phenoloxidase (PO) activity in carrot cell cultures is restricted to embryogenic strains, and may therefore be used as a possible marker for embryogenic potential in carrot cell cultures.

2. MATERIALS AND METHODS

2.1. Plant materials

Two non-embryogenic strains (Dc7² and Dc14) and 3 embryogenic strains (Dc8307, Dc8310 and Dc8404) were cultured as described [11]. Three days after subculturing embryo formation was induced by washing the cells 3 times in 2,4-D-free culture medium and then incubating ~1 g (fresh wt) of the cells in 15 ml of this medium in 9 cm Petri dishes. The cultures were kept in darkness at 25°C and after 7 days embryo formation was determined by counting 300–400 cell aggregates in each Petri dish. The embryogenic capacity is expressed as percentage of the cell aggregates which had developed into globular, heart- or torpedo-shaped embryos after 7 days.

2.2. Preparation of prophenoloxidase

After culturing for 3 days in 2,4-D-containing medium the cells were used for prophenoloxidase (proPO) preparations as described [12]. Briefly, the cells were collected by centrifugation ($1000 \times g$, 5 min) and the cell pellet was washed once in 0.01 M sodium cacodylate buffer (pH 7.0), containing 1.5 M NaCl and 20 mM EDTA (= buffer A), and then homogenized in this buffer with a glass piston homogenizer (1 g fresh wt in 2 ml buffer A). The homogenate was centrifuged ($3000 \times g$, 20 min, 4°C), the resulting supernatant was extensively dialyzed against 0.01 M sodium cacodylate buffer, pH 7.0 (= buffer B), and after centrifugation ($10000 \times g$, 20 min, 4°C) the resulting supernatant was used as source of proPO. The protein contents in the preparations were adjusted to approx. 0.8 mg/ml.

2.3. Assay for phenoloxidase activity

PO activity was assayed in a reaction mixture containing either 75 μl proPO preparation, 25 μl of 90 mM CaCl_2 (or buffer B for the controls), 25 μl buffer B and 25 μl L-dopa; or 75 μl proPO preparation, 25 μl of 1 mg/ml trypsin (EC 3.4.21.4, Sigma) in buffer B and 25 μl of 9 mM CaCl_2 preincubated for 10 min at 22°C prior to addition of 25 μl L-dopa. After 5–10 min at 22°C the absorbance at 490 nm was determined and the enzyme activity is expressed as units, where one unit is the amount of enzyme giving an increase in absorbance at 490 nm of 0.001/min.

The presence of proPO in the $3000 \times g$ pellet was determined after suspending the pellet in buffer B, followed by dialyzing this suspension against buffer B, before assay of PO activity.

To establish whether the non-embryogenic cell strains contained any inhibitors of proPO activation or PO activity the following experiments were performed: 100 μl of the embryogenic proPO preparation was incubated for 10 min with 100, 200, 300 or 900 μl of the non-embryogenic cell extract or buffer B for the controls, and then 75 μl of this preincubation mixture was assayed for PO activity as described above.

2.4. Protein determinations

Protein was determined as described by Lowry et al. [13] using bovine serum albumin (Sigma) as standard.

3. RESULTS AND DISCUSSION

ProPO could be isolated from the embryogenic carrot strains and PO activity was induced by addition of either 15 mM CaCl_2 or with trypsin at 1.5 mM CaCl_2 (table 1, [12]). In contrast, no PO activity could be detected in the non-embryogenic strains using these treatments (table 1). In its active form carrot PO tends to aggregate and most of the activity is present in the $3000 \times g$ pellet, whereas the inactive proenzyme is soluble [12]. Therefore, we assayed PO activity of the $3000 \times g$ pellet obtained after homogenizing the non-embryogenic

Table 1
Phenoloxidase activity in different strains of *D. carota* after activation with 15 mM CaCl_2 or with trypsin at 1.5 mM CaCl_2

Treatment	Phenoloxidase activity (units \cdot mg protein $^{-1}$)				
	Non-embryogenic		Embryogenic		
	Dc7 ²	Dc14	Dc8307	Dc8310	Dc8404
Control	0	0	17 \pm 3	7 \pm 4	2 \pm 1
1.5 mM CaCl_2	0	0	15 \pm 1	8 \pm 2	3 \pm 1
15 mM CaCl_2	0	0	295 \pm 32	329 \pm 61	421 \pm 36
Trypsin + 1.5 mM CaCl_2	0	0	324 \pm 12	221 \pm 24	362 \pm 51
Embryogenic capacity	0	0	19%	12%	31%

Mean value \pm SD of 4 experiments

cell strains, to examine whether proPO was activated in these strains and associated with the cell pellet. However, no PO activity could be detected in the $3000 \times g$ pellet.

As shown in table 2, addition of the non-embryogenic cell extract could not decrease the PO activity from the embryogenic strains, neither when the activity was induced by 15 mM CaCl_2 , nor with trypsin at 1.5 mM CaCl_2 . These results indicate that the non-embryogenic cells did not contain any factor which prevented proPO activation in the embryogenic cells, or any inhibitor of PO activity.

Embryogenic strains of *D. carota* generally lose their capacity for embryo formation during prolonged culture in the presence of 2,4-D [14,15] and fig.1 shows that concomitant with the decline in embryogenic capacity, PO activity is decreased. This indicates that the presence of this enzyme is associated with embryogenesis in carrot cell cultures.

The biochemical mechanism of proPO activation in plants has not yet been elucidated, but since the activation could be partially inhibited by the serine protease inhibitors DFP or benzamidine [12], it may involve a proteolytic process, as is the case for arthropod proPO [16–18]. One possibility for the lack of detectable PO activity in the non-embryogenic strains could be that some activating factor is missing or inactivated during the preparation. Whether this is the case or if the proenzyme

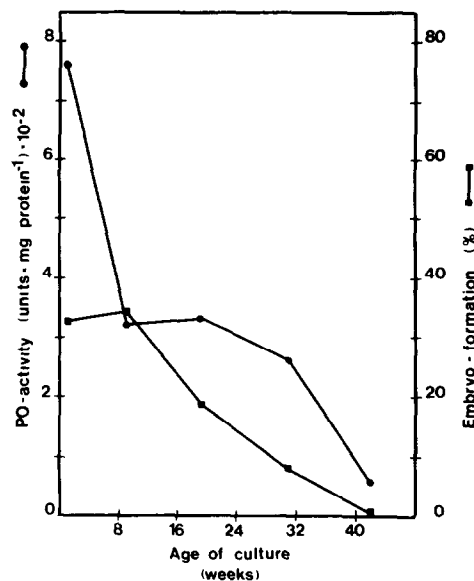


Fig.1. Phenoloxidase activity and embryogenic capacity of a carrot cell culture during 42 weeks. The cells were subcultured every 3rd day. (●) Phenoloxidase activity, (■) embryogenic capacity.

itself is absent from the cells cannot be elucidated until the mechanism for the activation of proPO is known in more detail.

The physiological function of proPO in plants is at present not known, but a role in the regulation of development cannot be excluded, and the results presented here indicate that the presence of proPO may be used as a marker for embryogenic capacity in carrot cell cultures.

Table 2

Effect of non-embryogenic cell extract on the phenoloxidase activity of embryogenic cell extracts after activation by 15 mM CaCl_2 , or with trypsin at 1.5 mM CaCl_2

Preincubation mixture embryogenic extract/ non-embryogenic extract (v/v)	Phenoloxidase activity (% residual activity)	
	15 mM CaCl_2	Trypsin + 1.5 mM CaCl_2
Control	100	100
1:1	110	83
1:2	116	112
1:4	97	93
1:10	105	94

The experiment was repeated 4 times with similar results

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Natural Science Research Council (K.S.) and Swedish Council for Agricultural Research (T.E.).

REFERENCES

- [1] Stewart, F.C., Mapes, M.O. and Smith, J. (1985) *Am. J. Bot.* 72, 693–703.
- [2] Reinert, J. (1958) *Ber. Dtsch. Bot. Ges.* 71, 15.
- [3] Wetherell, D.F. and Dougall, D.K. (1976) *Physiol. Plant.* 37, 97–103.
- [4] Verma, D.C. and Dougall, D.K. (1977) *Plant Physiol.* 59, 81–85.

- [5] Montague, M.J., Armstrong, T.A. and Jaworski, E.G. (1979) *Plant Physiol.* 63, 341–345.
- [6] Fujimara, T., Komamine, A. and Matsumoto, H. (1980) *Physiol. Plant.* 49, 255–260.
- [7] Baker, S.R., Jones, L.H. and Yon, R.J. (1983) *Phytochemistry* 22, 2167–2169.
- [8] Feinberg, A.A., Choi, J.H., Lubich, W.P. and Sung, Z.R. (1984) *Planta* 162, 532–539.
- [9] Sung, Z.R. and Okimoto, R. (1981) *Proc. Natl. Acad. Sci. USA* 78, 3683–3687.
- [10] Sung, Z.R., Lazar, G.B. and Dudits, D. (1981) *Plant Physiol.* 68, 261–264.
- [11] Carlberg, I., Söderhäll, K., Glimelius, K. and Eriksson, T. (1984) *Physiol. Plant.* 62, 458–464.
- [12] Söderhäll, K., Carlberg, I. and Eriksson, T. (1985) *Plant. Physiol.*, in press.
- [13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, K.L. (1951) *J. Biol. Chem.* 193, 265–275.
- [14] Halperin, W. (1966) *Am. J. Bot.* 53, 443–453.
- [15] Smith, S.M. and Street, H.E. (1974) *Ann. Bot.* 38, 223–241.
- [16] Söderhäll, K. (1983) *Comp. Biochem. Physiol.* 74B, 221–224.
- [17] Söderhäll, K. and Häll, L. (1984) *Biochim. Biophys. Acta* 797, 99–104.
- [18] Söderhäll, K. and Smith, V.J. (1985) in: *Hemocytic and Humoral Immunity in Arthropods* (Gupta, A.P., ed.) Wiley, New York, in press.