ISOPEROXIDASES — EARLY INDICATORS OF SOMATIC EMBRYOID DIFFERENTIATION IN PUMPKIN TISSUE

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Total peroxidase activity was determined in several callus lines of the pumpkin and electrophoretic patterns of their isoenzymes were obtained. The highest enzyme activity was determined in the callus lines DE and NA3, which grow in the presence of growth regulators and show high capacity for embryoid formation. Peroxidase activity is considerably lower in the habituated tissue (line Z3ab7) but the increase in enzyme activity was observed prior to embryoid appearance. Various callus lines differ from one another with the respect to their electrophoretic isoenzyme patterns. Although these electrophoretic patterns are influenced by growth regulators, the patterns still reflect the developmental stage of the culture.

Introduction

The enzyme peroxidase catalyzes the oxidation of a variety of substrates during which the reduction of hydrogen peroxide takes place (Betz 1974). It is well known that many enzymes appear in multiple molecular forms, like isoenzymes. Most of these varieties have a genetic basis, which makes isoenzymes ideal natural indicators in biological research (Scandalios and Sorenson 1977). Isoperoxidases represent the enzymatic complex involved in the metabolism of auxins and the synthesis of lignin. Their role in the regulation of growth and differentiation is also known (Siegel and Galston 1967, Wolter and Gordon 1975, Kochba et al. 1977, Thorpe et al. 1978a,b, Rücker and Markotai 1978, van Huystee 1980).
The second author developed a number of pumpkin callus lines which display their rich morphogenetic potentials, above all a high capacity for the genesis of somatic embryoids (Jelask a 1972, 1974, 1977, 1980, Jelenčič 1980). In the present work we have tried to find suitable indicators of metabolic changes which precede the genesis of somatic embryoids, by using the system of peroxidase isoenzymes. We have also tried to detect the differences among the callus lines considering both enzyme activity and the electrophoretic isoenzyme patterns.

Material and Methods

In our experiments the following lines of the pumpkin callus tissue were used: DE, DE₄, NA₃, ₂₅h/T. In contrast to the original cultures which had been grown on semisolid medium, in these experiments the tissues were grown on agitated liquid MS—medium (Murashige and Skoog 1962), with the addition of plant hormones (lines DE, DE₄, NA₃), or without them as the habituated tissue (line ₂₅b/T). Under our culture conditions (12 hours light, 3000 Lux, 25° C, shaking frequency of 90 r. p. m.) each passage lasted 8 days.

For enzyme extraction Tris buffer (phosphoric acid 0.26 mol with respect to Tris), pH 6.9 was used. The volume of 2 ml buffer was used for 0.2 g of tissue. The tissue was homogenized by hand in a mortar with the addition of up to 5% polyvinylpyrrolidone. The whole procedure was carried out at temperatures of 273—277 K (0°—4° C). The homogenate was centrifuged for 20 minutes at 18000 g, and the supernatant was used as crude extract.

The peroxidase activity was measured with the test solution prepared after Siegel and Gals tone (1967). For each 3 ml of this solution 0.01 ml of extract was added, which was previously diluted in the case of too high activity. Absorption was measured by a spectrophotometer (Perkin Elmer 550), at 470 nm. Enzyme activity is expressed as possible absorption (A) per minute per 1 g of fresh tissue, or as specific activity in relation to the content of soluble proteins per 1 g of fresh tissue. Soluble proteins were quantitatively determined in extracts by the method of Bradford (1976).

The polyacrylamide gel system No. 1 was used for electrophoretic separation towards the anode, and No. 5 for separation towards the cathode (Maurer 1968).

The gels were stained by enzymatic reaction in the test solution already mentioned (5·10⁻⁹ mol guayacol, 5·10⁻⁶ mol H₂O₂ in 2·10⁻¹ mol phosphate buffer, pH 5.8).

Results

Our experiments show that the callus lines studied differ somewhat among themselves in their content of soluble proteins (Table 1). These differences, however, vary only within the narrow range of 2 mg per gram of fresh tissue. Repeated measurements of peroxidase activity indicate differences among the lines studied. The highest enzyme activity was found in lines DE and NA₃, which at the same time displayed the most pronounced capacity for embryogenesis, and grew in the presence of plant hormones. The enzyme activity was significantly lower, however, in the embryoids isolated from the callus line DE (Tab. 1).
Table 1. Soluble protein content and peroxidase activity in pumpkin callus lines DE1 - isolated embryoids, DE2 - callus, Zsb/T1 - nonembryogenic culture, Zsb/T2 embryogenic culture

<table>
<thead>
<tr>
<th>Callus line</th>
<th>Growth regulator</th>
<th>Protein content (mg / g fr. weight) a</th>
<th>Peroxidase activity (A470 min⁻¹ / g⁻¹) b</th>
<th>Specific peroxidase activity b/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE1</td>
<td>2,4-D (1.35 \cdot 10^{-6} mol)</td>
<td>8.0</td>
<td>592.0</td>
<td>74.0</td>
</tr>
<tr>
<td>DE2</td>
<td>2,4-D (4.5 \cdot 10^{-6} mol)</td>
<td>7.2</td>
<td>2016.0</td>
<td>280.0</td>
</tr>
<tr>
<td>DE3</td>
<td>NAA (5.37 \cdot 10^{-6} mol) adenin sulfat (10^{-6} mol)</td>
<td>6.2</td>
<td>1720.0</td>
<td>277.4</td>
</tr>
<tr>
<td>Zsb/T1</td>
<td>0</td>
<td>6.8</td>
<td>98.7</td>
<td>14.51</td>
</tr>
<tr>
<td>Zsb/T2</td>
<td>0</td>
<td>7.1</td>
<td>296.0</td>
<td>41.66</td>
</tr>
</tbody>
</table>

The habituated tissue showed the lowest enzyme activity. This tissue had a slightly lowered potential for embryogenesis under conditions of our experiments. We were unable to determine which external factor stimulated embryogenesis, but the increase in peroxidase activity was determined immediately prior to the appearance of embryoids (Table 1).

Electrophoretic patterns of peroxidase isoenzymes show differences among callus lines. The line DE4 (Fig. 1 a) is characterized by the well developed capacity for embryogenesis, but their embryoids are mostly restricted in the initial stages of development. The electrophoretic pattern of peroxidase isoenzymes shows three bands in the anodic group and two bands in the cathodic group of isoenzymes (Fig. 2 b i c).

The line DE (8 year old culture, Jelaška 1980) is morphologically somewhat different from DE4 (2 year old culture), although it grows also in the presence of 2,4-D, but at its higher concentration (Fig. 2 a). This is a very loose tissue showing many separated cells dispersed in the growth medium. The appearing embryoids develop from the initial globular stage to a heartlike one, and finally to the torpedo stage (Fig. 3 a). In this line three bands were present in the anodic group of isoenzymes, and only one with slow relative mobility in the cathodic group (Fig. 2 b i c). The isoenzyme pattern of the isolated embryoids is slightly different (Fig. 3 b i c): one of the two anodic groups moves relatively faster, which happens also with the only band in the cathodic group of isoenzymes.

The line NA3 (Fig. 4 a) shows a very high embryogenic potential, so that the culture consists mainly of embryoids in all the different developmental stages, of malformed plantlets, and of relatively scarce undifferentiated tissue. In spite of the very high enzyme activity detected, only two faint isoenzyme groups are seen on the anodic side (Fig. 4 b), and only one with slow mobility on the cathodic side. This isoenzyme group is probably responsible for most of the enzyme activity (Fig. 4 c).

The callus line Zsb/T (grown on auxin free medium) forms very compact tissue aggregates, and there are no loose cells dispersed in the
nutrient medium (Fig. 5 a). Some cultures within this line display a reduced potential for embryogenesis which is accompanied by some morphological changes (the compactness of the tissue is reduced, Fig. 6 a). So we could within the same line differentiate embryogenic from the nonembryogenic cultures. These two types of culture differ in their electrophoretic patterns of peroxidase isoenzymes. The anodic group of isoenzymes displays four bands in the nonembryogenic callus (Fig. 5 b), while in the embryogenic callus this isoenzyme group displays five bands (Fig. 6 b). Differences between the cathodic groups of isoenzymes are even more pronounced, so that in the embryogenic cultures there are four bands, and in the nonembryogenic one there are only two strong bands and a faint one (Figs. 5 c and 6 c).

Discussion

Considering a fact that significantly lower enzyme activity of peroxidase was detected in embryoids isolated from the DE line (specific activity 53.3) as compared to the callus tissue of the same line (specific activity 280.0) we can conclude that high enzyme activity is characteristic of the very undifferentiated callus, in which embryoids are to be developed only in the next developmental step. Low peroxidase activity was also found in embryos isolated from seeds of wheat (Gaspar et al. 1977) and in pine embryos (Pinus nigra, our preliminary results). It is known that 2,4-D influences the activity and the pattern of peroxidase isoenzymes as well as that of the IAA oxidases. That influence is dependent on 2,4-D concentration (de Fooz et al. 1966, Lee 1972 a, b, Paul and Ohrman 1980). The differences between the DE1 and DE callus lines may be explained in a similar way.

In the habituated tissue the rise in enzyme activity preceded the appearance of embryoids in the culture. This finding is in full agreement with Kochba's results for Citrus sinensis (Kochba et al. 1977).

Although the physiological role of peroxidases is not completely understood, because of the great number of substrates upon which they act, there is a number of papers which point out the importance of peroxidase isoenzymes as indicators of metabolic changes in tissues undergoing differentiation, during stress, or under the conditions of exogenously applied hormones (Thorpe and Gaspar 1978, Sheoran and Garg 1979, Thomas and Delincee 1979, Cairns et al. 1980). On the basis of our experiments we are aware that callus lines of pumpkin can produce the pattern of peroxidase isoenzymes,

Fig. 1—6. Pumpkin callus tissue and electrophoretic isoperoxidase pattern (b — separation towards anode, c — separation towards cathode).

1. a — Callus line DE1. Undifferentiated callus clumps and embryoids.
2. a — Line DE with embryoids in globular and heart stages.
3. a — From line DE isolated embryoids in different developmental stages.
4. a — Line NAa. Embryoids with green leaf-like cotyledones.
5. a — Line Ze8T. Typical compact tissue clumps.
6. a — The same line as 5.a with embryoids in early developmental stage. (k = callus, e = embryoids)
which — in spite of not being so clear and so rich as the pattern found in the tissue of tobacco plant (Mäder and Bopp 1976, Mäder 1976, Nessel and Mäder 1977, Mäder et al. 1980) — can be used as indicators of the changes taking place in the tissue undergoing differentiation. We have noticed that exogenously applied growth regulators influence the peroxidase isoenzyme pattern. This phenomenon is not surprising because the response of a tissue to added plant hormones could be effected through synthesis of RNA and proteins, including enzymes.

The differences in isoenzyme patterns observed between the embryogenic and nonembryogenic cultures, within the same line of habituated tissue (Z_{sh}/Z) are in favour of the fact that peroxidases play a role in the metabolism of endogenous hormones (Raa 1971, Elkinawy and Raa 1973).

**Conclusion**

On the basis of our experiments the following conclusions may be drawn:

1. High enzyme activity in cultures (DE and NA₃) is correlated with the incidence of embryogenesis.

2. The increase in enzyme activity measured at an as yet undifferentiated stage of culture, with no visible phenotypic changes whatsoever, predicts the appearance of embryoids in the habituated tissue.

3. There are differences among the callus lines with respect to their electrophoretic isoenzyme patterns. Growth regulators influence the isoenzyme pattern, but this pattern is also reflection of the specific developmental stage of the culture.

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**SAŽETAK**

IZOPEROKSIDAZE — RANI POKAZATELJI DIFERENCIJACije SOMATSKIH EMBRIIOIDA U TKIVU BUNDEVE

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