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ISOENZYME CHANGES DURING THE GROWTH CYCLE OF PAUL'S SCARLET ROSE CELL SUSPENSIONS

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Abstract—A preliminary study has been made of the development of malate dehydrogenase, glutamate dehydrogenase, esterase and peroxidase activities during the growth cycle of Paul's Scarlet rose cells in batch propagated suspension cultures. These cells, which show no tendency to differentiate in culture, show quantitative changes in isoenzymes during the growth cycle. Qualitative changes were also observed, particularly in the case of peroxidase.

INTRODUCTION

Many studies have been made in an attempt to correlate changes in multiple or iso-enzyme systems with specific developmental events [1–7]. Using plant tissue cultures it should be possible to separate those enzyme changes related to the normal cell cycle from those which are necessary for, or arise from, differentiation at the cellular and multicellular levels.

From previous work [8] it is known that in Paul's Scarlet rose (PSR) cell suspension cultures no changes in the external cell morphology are observed during the growth cycle even though considerable changes in the internal morphology occur. Thus, any isoenzyme changes observed will result solely from the transition of the non-growing cell to the growing and dividing state. Moreover, environmental variations can be strictly controlled and the possibility of contribution to enzyme activity from bacteria is excluded.

The present report assesses the variation of four isoenzyme systems during the growth cycle of PSR cells.

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RESULTS AND DISCUSSION

The data presented are taken from one of three growth cycles where all the isoenzyme systems were examined simultaneously. All three growth cycles produced identical results with regard to number of bands, their relative intensities and their order of appearance. Since a constant amount of protein was applied to each gel at all sampling periods and the incubations were carried out under uniform conditions, the total activity observed for a particular enzyme, as indicated by the intensity of stain production, is probably related to the specific activity of the enzyme and can be compared with the activity of the same enzyme observed at other sampling periods.

Under the culture conditions used four phases can be recognized in the growth cycle of PSR cell suspensions. These phases, lag phase, exponential phase, post-exponential phase and stationary phase are summarized in Fig. 1. Since the cultures were propagated using a ratio of 12 ml inoculum (stationary phase cells) to 60 ml fresh medium all parameters can be expected to undergo a sixfold increase by the end of the growth cycle. This was found to be true not only for fr. wt but also for the total alkali soluble protein and total tris buffer soluble protein (Fig. 1).

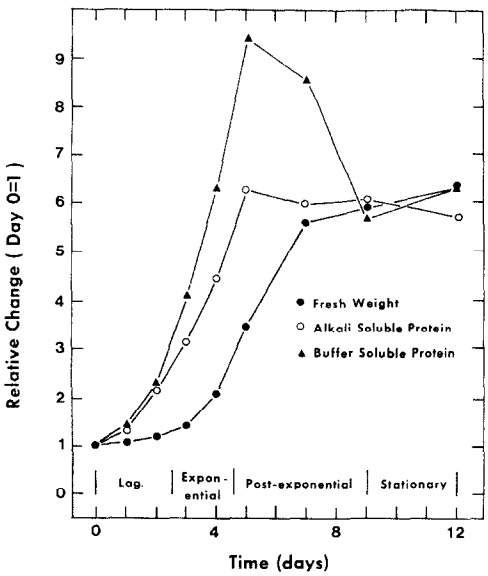

Fig. 1. Relative changes in fr. wt, total buffer soluble protein, and total alkali soluble protein during the growth cycle of PSR suspension culture. Absolute values for the day 0 samples (inocula) are: fr. wt, 1·95 g; total buffer soluble protein, 2·1 mg; total alkali soluble protein, 4·4 mg.

Total alkali soluble protein reached a sixfold maximum by the end of exponential phase and thereafter remained at a fairly constant level. Although the absolute amounts of buffer soluble protein were always lower than those for total alkali soluble protein they showed a much larger relative change, achieving an approximately tenfold increase by the end of exponential phase. A subsequent fall in buffer soluble protein is observed, so that the anticipated sixfold level is reached by the end of the post-exponential phase. With the exception of glutamate dehydrogenase activity, all the isoenzyme systems examined show a loss of activity on a unit protein basis and therefore on a per culture basis after the termination of the exponential phase. As would be expected from a comparison of activity on a per unit protein basis for the duration of a growth cycle, the activities observed during stationary phase are similar to those observed during early lag phase.

Glutamate dehydrogenase

For the greater part of the growth cycle, four very fine, but well resolved, slow-migrating bands of glutamate dehydrogenase activity (L-glutamate: NAD oxidoreductase, deaminating; EC 1.4.1.2:GDH) were observed (Fig. 2). Maximum total activity per unit protein was found during the late lag and early exponential phases of growth (days 2 and 3), which corresponds with a high rate of nitrate uptake by the cell cultures [8]. Activity fell rapidly between days 3 and 4 and by late exponential phase (days 4 and 5) a low total activity was observed. Also, on days 4 and 5 only 3 bands of activity were observed, the slowest migrating band not being detected even when the sample loading was increased. At all stages of the growth cycle band IV showed the lowest activity. This pattern of change of total GDH activity in relation to nitrate utilization and protein synthesis is consistent with the role of plant GDH in the synthesis of amino acids from inorganic nitrogen sources.

Changes in total GDH activity per unit fr. wt have been estimated during the growth cycle of *Nicotiana* suspension cultures [9]. A high level of activity was observed at the beginning of the exponential phase, and activity fell rapidly at first then more slowly at the end of the exponential

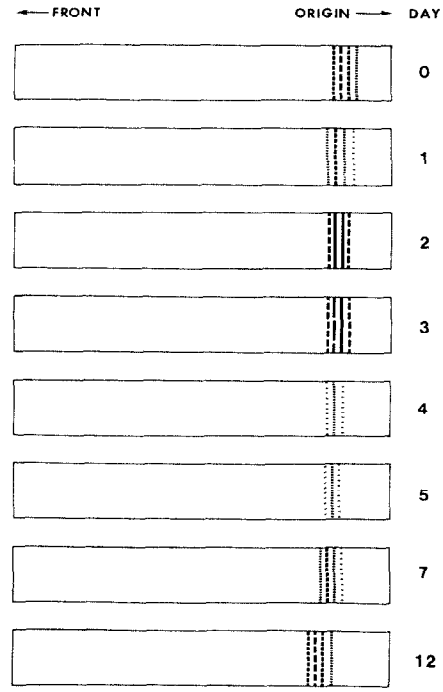

Fig. 2. Changes in glutamate dehydrogenase isoenzymes during the growth cycle of rose cell suspensions. The relative activities of the component isoenzymes are indicated by the intensities of the lines.

phase. Since the initial GDH level and the protein concentrations were not given it is difficult to relate these results to the present observations.

Although GDH plays a primary role in plant metabolism there is considerable variation in the number of isoenzymes reported for a variety of plants at the same developmental stage [10] or the same plant at different developmental stages [3, 11]. By comparison, the present study of the change from the non-growing state to the growing state shows a relatively stable spectrum of activity.

Malate dehydrogenase

Three major bands of malate dehydrogenase activity (L-malate: NAD oxidoreductase; EC 1.1.1.37:MDH) were observed at all stages of the growth cycle with the fastest band appearing to be composite at most sampling periods (Fig. 3). A rapid rise to maximum total activity occurred by early exponential phase (day 3) and this high activity was maintained for the duration of the exponential phase (days 3, 4 and 5). Studies with *Nicotiana* cell suspensions [9] have shown a rapid rise in total MDH per cell to a maximum by mid-exponential phase followed by a very rapid fall during the remainder of exponential growth.

Many species contain at least two separable MDH activities, one in the cytoplasmic fraction and one in the particulate fraction of the cell [1]. Recent studies indicate that the particulate fraction contains more than one isoenzyme, each being characteristic for the organelle of the particulate fraction in which it is found [12–16]. Many studies have shown a single MDH isoenzyme in the cytoplasmic fraction. However, it has been reported that the cytoplasm of *Euglena* [15] contains three MDH isoenzymes which remain closely associated after electrophoretic separation, while the cytoplasm of spinach cotyledons [14] contains 2 MDH isoenzymes and these also remain closely associated after separation by both starch gel electrophoresis and DEAE cellulose column chromatography. Plant cytoplasmic MDH isoenzymes generally exhibit the highest activity and migrate furthest towards the anode [17]. Thus, in the present study, the intensely staining frontal band is probably the cytoplasmic component and its composite nature may result from 2 or 3 overlapping isoenzymes.

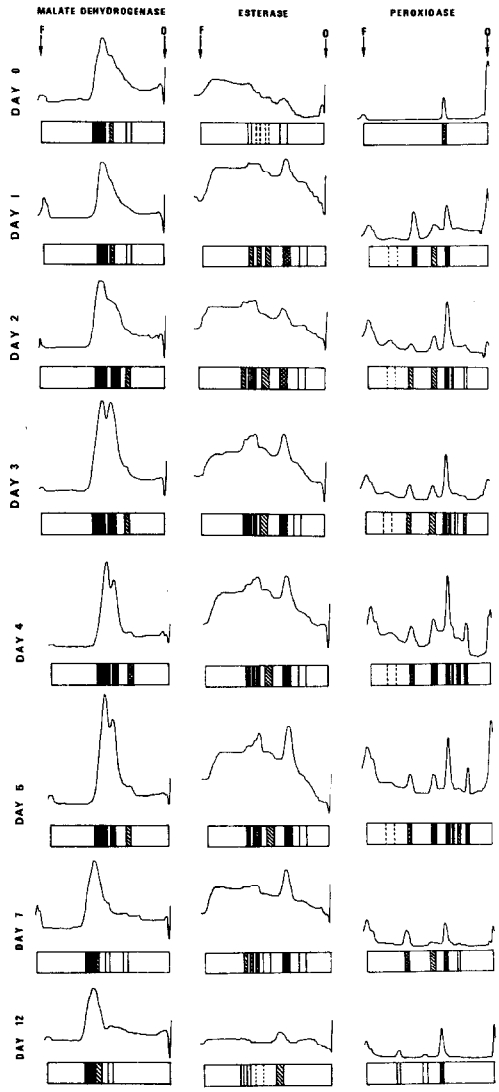

Fig. 3. Changes in malate dehydrogenase, esterase and peroxidase activity during the growth cycle of rose cell suspensions. The relative activities of the component isoenzymes of each isoenzyme series are indicated by the intensities of shading.

Variation have been found in the total number of MDH isoenzymes when different plants, different tissues of the same plant, or tissues at different developmental stages are compared. However, in pea [18] and spinach [14] each tissue examined contained the same number of isoenzymes. Furthermore, there is no change in the number of MDH isoenzymes during cotton leaf development [17] and the failure to detect certain isoenzymes may be the result of comparing samples with different total MDH activity. In our studies with

PSR we have observed a constant number of MDH isoenzymes throughout the growth cycle, but the relative contribution of individual bands to the total activity appears to vary. For example, the intermediate band which showed the largest relative change in activity, had an activity equal to that of the major component of the fast band on day 3, while at all other sampling periods it had a lower relative activity. By contrast, the slowest band had a peak activity on day 4.

Esterase

Gels incubated with the appropriate substrate for the detection of non-specific esterase activity (carboxylic ester hydrolase; EC 3.1.1.1) all showed a rather high level of background staining (Fig. 3). Nevertheless, up to 6 bands of activity were clearly visible with band V being the most prominent. Four bands of activity were apparent in the inoculum sample (day 0). An additional slow-migrating band (band VI) and a fast-migrating band (band I) appeared during days 1 and 2 respectively. There were considerable considerable quantitative changes in the activities of the individual bands during the first 24 hr of growth. Thereafter all bands showed a steady increase in activity during exponential phase to a maximum on day 5. At the end of the growth cycle esterase activity was similar to that observed in the inoculum.

Plant and animal esterases have been extensively studied but very little is known of their substrates and functions *in vivo* [1, 19–23]. Recent studies have examined the changes in esterase activity during the early development of a number of plants [2–4, 24]. Mäkinen [2] has suggested that there is a correlation between esterase activity and mitotic frequency in onion root tips and that this activity is not necessarily dependent on cell division but may be prerequisite for it. We have observed a similar correlation in PSR; a large increase in esterase activity occurs during late lag and early exponential phases and then falls rapidly as cell civision ceases at the end of exponential phase.

Significant organ-specific qualitative differences occur in esterase zymograms from the organs of developing seedlings [2, 3]. However, a study of esterase activity during the development of broad bean roots [24] showed only large quantitative differences between the zymograms of the cell division, enlargement and maturation zones. Results with PSR also show that that quantitative rather than qualitative changes are more important during development. These quantitative changes, which reflect the relative importance of the metabolic pathway in which a particular isoenzyme functions at different stages of the growth cycle, were consistent from one growth cycle to the next.

Peroxidase

PSR cultures exhibited large qualitative differences in peroxidase activity donor: H_2O_2 oxidoreductase; EC 1.11.1.7) during their growth cycle (Fig. 3). Thus, the inoculum (day 0) showed only one band of activity which remained the major band of activity throughout the growth cycle whereas by day 3 a maximum of 7 peroxidases were observed. Although these bands persisted through days 4 and 5, the total peroxidase activity per unit protein was apparent on day 4. Striking quantitative changes in peroxidase activity were also seen during the growth cycle. For example, band II developed rapidly on day 1 but during the subsequent 24 hr it declined in activity. Similarly, band VII, the least mobile band, had high activity on day 4 and 5 whereas on day 3 it had a low activity and could not be detected at all on day 7.

Although the intracellular peroxidase of a number of plant tissue cultures has been examined from a variety of viewpoints [9, 25–31], the changes in individual isoenzymes in relation to development has not been examined. Total guaiacolperoxidase activity has been measured at 4 stages of the growth cycle of *Acer* cell suspension cultures [25] and it was found that stationary phase cells had the highest activity on both a per cell and a per unit protein basis. In PSR cultures the reverse appears to be true, so that the highest levels of benzidine-peroxidase are observed during mid-exponential phase and fall rapidly as stationary phase is approached. This difference may reflect the difference in growth patterns between PSR [8] and *Acer* [32]. In *Acer* cell suspensions a separate phase of cell expansion occurs after the phase of exponential cell elongation whereas in PSR cell expansion accompanies cell division during the exponential phase. Thus, con-

siderable morphological change is still occurring in *Acer* cells after cell division has ceased while in rose cells morphological changes cease with cell division. A correlation between peroxidase activity and morphogenesis has been observed in peanut cell suspensions [26, 27]. Peanut cultures when fractionated into cell masses of different sizes showed increased levels of peroxidase and increased morphological change with increasing size of the cell mass. Similarly, studies with whole plants or plant organs have shown both qualitative and quantitative increases in peroxidase with increasing morphological development [33–36].

The observation that peroxidases from a single plant vary both qualitatively and quantitatively when different organs are compared has been challenged by Rücker and Radola [30]. Using isoelectric focusing they found no qualitative differences amongst the peroxidase isoenzyme complement of two *Nicotiana* callus cultures and the stem and leaves regenerated from one of the callus types. They suggested that the non-occurrence of some bands of activity is a problem of resolution rather than a case of absence. The qualitative variation we observed for PSR peroxidase could reflect such a lack of resolution at certain sampling periods. However, these qualitative changes and also the quantitative changes were reproducible over a number of growth cycles, indicating an ordered sequence of biochemical development. During the post-exponential phase the isoenzyme pattern returns to that observed during the lag phase.

Peroxidase activity is apparently intimately associated with IAA oxidase activity [37–40], polyphenol oxidase activity [41, 42] or both [43, 44]. Some attempts have been made to distinguish more clearly between these activities [45, 46]. In PSR certain peroxidases predominate at specific times during the growth cycle and a study of the properties of the individual bands should clarify the role of peroxidase in development.

EXPERIMENTAL

Tissue and media. Culture methods and media used for the propagation PSR cell suspensions have been described [8]. All cultures used in the present study were initiated by inoculating 60 ml of fresh MX_1 medium with 12 ml of a 14-day-old stock suspension culture. Stock suspension cultures were maintained by transfer every 14 days of 6 ml of 14-day-old suspension into 90 ml of fresh medium. The experimental cultures were sampled at various times during the 12 day growth cycle. Total alkali soluble protein was extracted and estimated as described previously [8].

Preparation of tissue extracts. The extraction buffer used was tris-Pi, 59 mM; pH 6·9, containing 5 mM cysteine HCl and 0·5 mM EDTA (2 Na). Polyclar AT was purified [47] and the dry powder equilibrated with the extraction buffer for 24 hr before use, excess buffer was removed from the Polyclar AT by centrifugation in filtration tubes. The cells were separated from the medium, washed with 10 ml H_2O, washed $\times 2$ with 10 ml of the extraction buffer and their fr. wt determined using filtration tubes [8]. Tissue samples (5 g fr. wt) were homogenized with 10 ml of the extraction buffer, and 1·5 g (wet wt) of equilibrated Polyclar AT were stirred into the homogenate. After standing 10 min the Polyclar AT-homogenate slurry was filtered through cheese-cloth and the filtrate centrifuged at 15000 *g* for 20 min at 4°. The clear supernatant (5 ml) was added to 1 g of dry G-25 Sephadex and equilibrated for 30 min. The supernatant, concentrated *ca* $\times 2$, was collected and a second $\times 2$ concentration effected by the addition of dry G-25 Sephadex in the ratio of 100 mg per 500 μl of supernatant, followed by a further 30 min equilibration period. The protein content of the concentrated supernatant was determined spectrophotometrically [48] and diluted to a concentration of 200 μg of protein per ml. Before applying to the gels, the samples were mixed 1:1 with tris–Pi buffer, 59 mM; pH 6·9, containing 50% sucrose.

Electrophoretic separation. The anionic disc electrophoresis method was modified from ref [49]. The gels were cast in the upright position and the samples were layered onto the surface of the large pore gel, under the upper electrode buffer as a 25% sucrose soln at the rate of 200 μl per tube (100 μg protein per tube). 2 mA per tube was applied until the marker band had migrated 5 mm into the large pore gel. 3·25 mA per tube was then applied until the electrophoresis was terminated when the frontal band had migrated 3 cm into the small pore gel.

Detection of isoenzymes. The composition of the incubation mixtures used to display MDH, GDH, esterase, and peroxidase isoenzymes were: L-Glutamate dehydrogenase: 50 mM glutamic acid (Na) in tris–HCl buffer, 40 mM; pH 7·4, containing 0·1 mg/ml phenazinemethosulphate, 0·5 mg/ml nitro blue tetrazolium, and 0·3 mg/ml NAD. L-Malate dehydrogenase: as for GDH but 25 mM malic acid (1 Na) as substrate. Esterase: Tris–HCl buffer, 8 mM; pH 7·4, containing 0·2 mg/ml 1-naphthylacetate and 0·5 mg/ml Fast Blue RR. Peroxidase: 1 ml of 3% H_2O_2 was added per 100 ml of a stock soln of 7% HOAc containing 16 g of NaOAc and saturated with EDTA and benzidine 2 HCl.

All substrate mixtures were prepared immediately before use and incubations were performed at 35° in the dark.

Following incubation the gels were scanned in a Joyce Loebl Chromoscan using transmitted light and a modified gel chamber [50]. Isoenzyme distribution maps were prepared by reference to the densitometric traces and from observation of the actual gel. For each enzyme activity the bands are numbered in order of decreasing mobility.

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