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CHANGES IN ISOZYMES SHIKIMATE DEHYDROGENASE AND PEROXIDASE DURING GIBBERELLIC ACID INDUCED INDUCTION AND FORMATION OF ANTHERIDIUM IN THE FERN *ANEMIA PHYLLITIDIS*.[1]

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Abstract

Changes in isozymes of shikimate dehydrogenase and peroxidase were studied during the induction and formation of antheridium in *Anemia phyllitidis*. Upto six isozymic forms of shikimate dehydrogenase were detected. Starting from two isozymes in dry spores the number rose to four during germination and six during antheridium formation. Three isozymes of peroxidase were first detected on sixth day of development when antheridia had been formed and spermatogenesis initiated. No qualitative differences in both of these enzymes were found in between vegetative and antheridial prothalli. The observed differences in between two treatments were quantitative, the amount of both enzymes being greater in antheridium bearing prothalli.

Introduction

The fern, *Anemia phyllitidis,* is a very good model for morphogenetic studies as in the single-celled sporeling the antheridial induction and formation can be initiated at any time of experimentation by gibberellic acid treatment (Schraudolf, 1966). Our interest has been to find out specific biochemical markers which are associated with the transition of a vegetative cell into a reproductive cell. So far results on changes in protein biosynthesis during gibberellic acid induced antheridial formation (Iqbal & Schraudolf, 1977) and on the presence of poly (A) RNA in *Anemia* have been reported (Iqbal, 1977). The present study deals with the changes in isozymic forms of two important enzymes, shikimate dehydrogenase and peroxidase during gibberellic acid induced induction and formation of antheridium. The shikimate dehydrogenase is an important enzyme in plants and catalyses *in vivo* the NADPH-linked reduction of dehydroshikimate to shikimate, while peroxidases may have a significant role in regulation of cell growth and differentiation (Galston & Davies, 1969; Obst & Herkin, 1973; Wolter & Gordon, 1975; Kahlem, 1975).

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Material and Methods

Spores of *Anemia phyllitidis* were surface sterilised in 10% (v/v) clorox for 5 minutes and after several rinses in distilled water, were sown on the surface of a mineral salt medium (Mohr, 1956) for normal vegetative growth. For induction and formation of antheridium, spores were sown on mineral salt medium supplemented with gibberellic acid (5×10^{-2} g/l). In each Petri dish (ϕ 10 cm) 20 ml of the nutrient medium and 10 mg dry weight of spores were added. Growth conditions were similar as reported earlier by Schraudolf (1966).

Enzyme extraction:

(a) *Shikimate dehydrogenase* (SDH. E.C.1.1.1.25). Dry spores, vegetative and reproductive prothalli upto 10 days of development were homogenised in the 0.1 M phosphate buffer ($K_2H\ PO_4$ + $K\ H_2\ PO_4$.7 H_2O/glutathione 10^{-3}M, pH 7.5; ratio of buffer to material 4:1). Following homogenisation the tissue fragments were removed from the sample by straining through 4 layers of cheese cloth. The sample was centrifuged at 14000 g for 20 min at 4°C. The supernatant was immediately used or stored at −20°C, until assayed.

Isozymes separation was carried by disc electrophoresis on 7% acrylamide gel (gel system No. 1a, Maurer, 1971). For the visualisation of the isozymes, the gels were stained with tetrazolium salt method (MTT tetrazolium 3-4, 5-Dimethyl Thiozolyl-2-2, 5 diphenyltetrazolium bromide) with shikimic acid as the substrate solution. Without the addition of shikimic acid no colour detection of the isozyme was possible. After 15 minutes staining the gels were transferred to 10% acetic acid to stop the reaction. The gels were scanned in Zeiss PMQ 3 photometer at 578 nm. Representative gels were also photographed.

(b) *Peroxidase* (E.C.1.1.11.1.7). Extractions were carried out as for SDH and isozymes separations were carried out on 15% gels (gel system No. 2, Maurer, 1971). For the detection of the isozymes, the gels were kept in the buffer (0.1 M phosphate buffer, pH 7.0) + guaicol (20 mM as the hydrogen donor) + H_2O_2 (10 mM H_2O_2 standardised against 0.1 N KI) for 30 minutes under dark. Afterwards gels were transferred to 7% acetic acid for 10 minutes to stop the reaction. The gels were scanned at 470 nm in Zeiss PMQ 3 photometer.

Electrophoresis:

During electrophoresis (Shandon-SAE 2761 electrophoresis apparatus) the current was adjusted to 1 mA/gel for the first two minutes and then to 2mA/gel for the rest of the electrophoresis time (about 150 minutes). All separations were carried out at 4°C. A

minimum of 4 electrophoretic runs, using different concentrations of the extract (10-20 μg of protein/10 μl; Lowry *et al,* 1951), were carried out for each treatment in an experiment. All experiments described above have been repeated at least 4 times with essentially identical results. The data from the representative experiments are presented in the figures. Observations were taken daily starting from germination of spores till the antheridium formation and spermatogenesis. The results only from those days where a significant change associated with antheridium formation occurred are reported.

Results

Anemia spores germinate 4 days after sowing in both control and GA_3 treatment. On the sixth day after sowing transition from vegetative apical cell to antheridial cell begins and by the eighth day it is complete and spermatogenesis initiates which is complete by about tenth day. The vegetative sporeling is filamentous, having 2-3 cells on the sixth day and 4-5 cells on eighth day of development (Iqbal & Schraudolf, 1977).

(a) *Shikimate dehydrogenase*: SDH. Upto six isozymic forms of SDH, were found, both in spores grown in normal nutrient medium and that supplemented by GA_3. Two distinct bands I and II with Rf 0.50 and 0.53 were detectable even in dry spores (Fig. 1 A). At the time of germination, i.e. on 4th day two new bands were observed in both treatments at parallel Rf values of 0.45 (band III) and 0.65 (band IV; Fig. 1 B). The bands I and II had Rf values of 0.49 and 0.54, respectively, almost corresponding to the values from dry spores. Isozymes forms of band III and IV appear on *a priori* basis, to be related with the process of germination. On 5th day, development of a new band (band V) below and in close proximity of the II band was initiated (Fig. 1 C) which became quite distinct at the same site and Rf at 6th day of development (Fig. 1 D). A sixth band (band VI) above the third band was also detected at the 6th day; (Fig. 1 D). From 6th day onward these additional bands gradually disappeared. At 7th day only five bands were present and the VI band was no more detectable (Fig. 1 E). Finally at 10th day the original pattern observed at the time of germination, i.e. with four bands was restored (Fig. 1 F).

Resolution of VI band as an independent peak in densitometer was not achieved (Fig. 2 D). This probably is due to the fact that the isozyme VI is not sharply separated from its adjacent lower isozyme III. This is substantiated on the basis that even isozymes I, II and III which at 4th and 5th days were separated in distinct peaks (Fig. 2 B & 2 C) at 6th day of development were separated as shoulders (Fig. 2 D), because they are not completely separated from one another.

In comparison with the control vegetative prothalli no specific independent isozyme during the induction and formation of antheridium was detectable. The observed

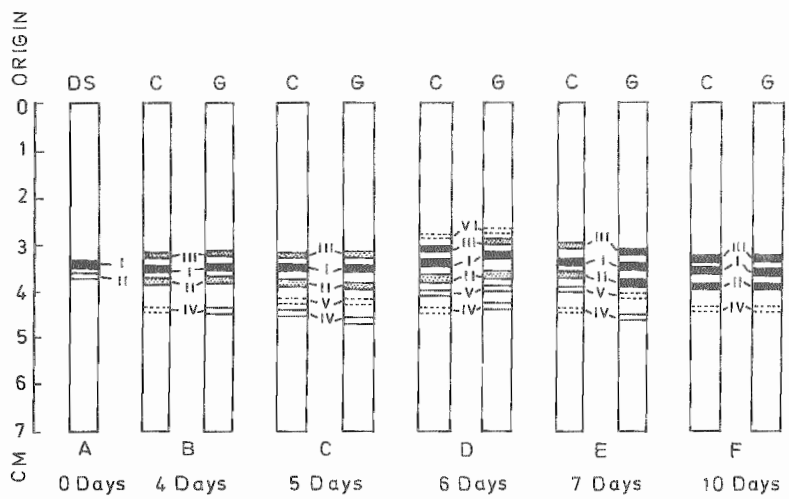


Fig. 1. Electrophoretic separations of isoenzymes of shikimate dehydrogenase at different stages of prothallium and antheridial development.

DS = Dry spores.
C = Control. Prothalli were grown in normal nutrient medium and are vegetative.
G = Prothalli were grown in GA_3 supplemented nutrient medium and are with antheridial development.
A Isoenzyme pattern from dry spores. Only two bands I and II are detectable.
B Isoenzyme pattern from germinated spores at 4th day of sowing. Development of two new bands III and IV is observed.
C At 5th day of sowing, the initiation of band V is detected.
D At 6th day after sowing, two additional bands V and VI are observed.
E At 7th day after sowing, band VI disappeared and only five bands are detected.
F At 10th day after sowing, only four bands corrosponding to 4th day of development are observed.

The isoenzymes are numbered in the sequence they appear during development.

Dense bands =
Less dense bands =
Week bands =
Very weak bands =

differences in the two treatments were quantitative. Amounts of SDH were always higher in reproductive prothalli as compared with the vegetative ones. The differences were clear not only from visual examination of stained gels, but also from densitometric scannings (Fig. 2 A to F).

(b) *Peroxidase.* Peroxidases were not detectable electrophoretically in dry and germinating spores. The detection of peroxidase activity was first observed at 6th day of

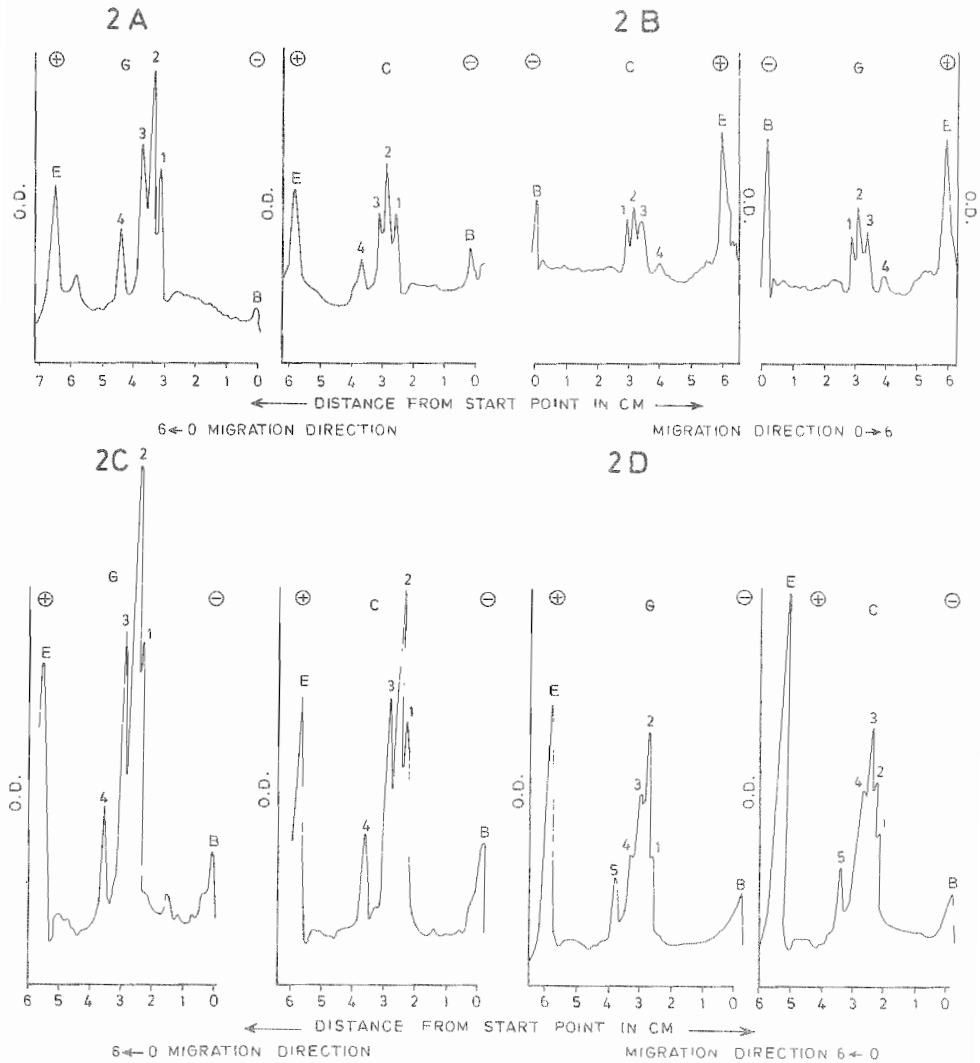
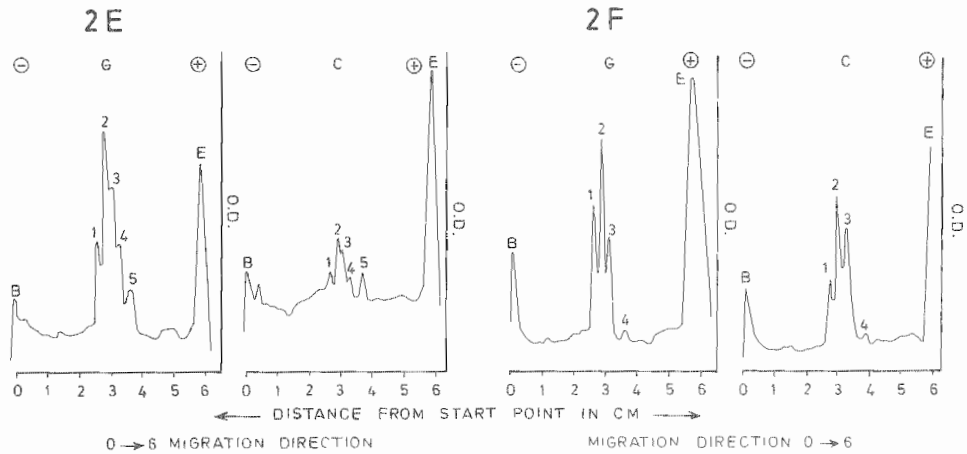


Fig. 2. Spectrophotometric scannings of gels for shikimate dehydrogenase at different stages of the development of vegetative and antheridial prothalli. Gels scanned at 587 nm.

C = Vegetative prothalli were grown in normal nutrient medium.
G = Antheridia bearing prothalli were grown in GA_3 supplemented nutrient medium.

A 3rd day after sowing, four distinct peaks indicating the presence of 4 bands.
B 4th day after sowing, four distinct peaks for 4 bands.
C 5th day after sowing, four peaks, but these peaks are higher in comparison with the peaks of 4th day, an indication of an increased synthesis of SDH.
D 6th day after sowing, five distinct peaks.



E 7th day after sowing, five distinct peaks.
F 10th day after sowing, four peaks comparable to 4th day.

Optical density of all isozymic forms, at all developmental stages is higher in antheridium bearing prothalli as compared with vegetative prothalli.

development when antheridia had been formed. Three multiple molecular forms of peroxidase were detected on the gels (Fig. 3). Sharp quantitative differences between the vegetative and antheridial bearing prothalli were also shown by histo-chemical tests. Histochemical localisation of peroxidase at 6th day was only possible in reproductive prothalli, whereas, in vegetative prothalli tests for peroxidase were negative.

Discussion

It is now well established that the enzymes repertory of an organism undergoes changes during the course of differentiation and development (Scandalios, 1969, 1974). Differential synthesis or activation of enzymes occurs during the differentiation, maturation and senescence of plant tissues, resulting in changes in total activity and isozymic components or both. Although the precise basis for enzyme multiplicity is, in the majority of cases not clear, sequential changes in isozymic forms ultimately reflect the differential expression of genetic information (Scandalios, 1969, 1974; Hammes & Wu, 1971).

The present results show that it may be feasible to use SDH and peroxidase isozyme pattern as predictive indicators of antheridial formation in *Anemia,* as distinct quantitative differences in these two enzymes were observed between vegetative and reproductive prothalli. Shikimate dehydrogenase is an important enzyme in plants and catalyses *in vivo* the NADPH-linked reduction of dehydroshikimate to shikimate. The

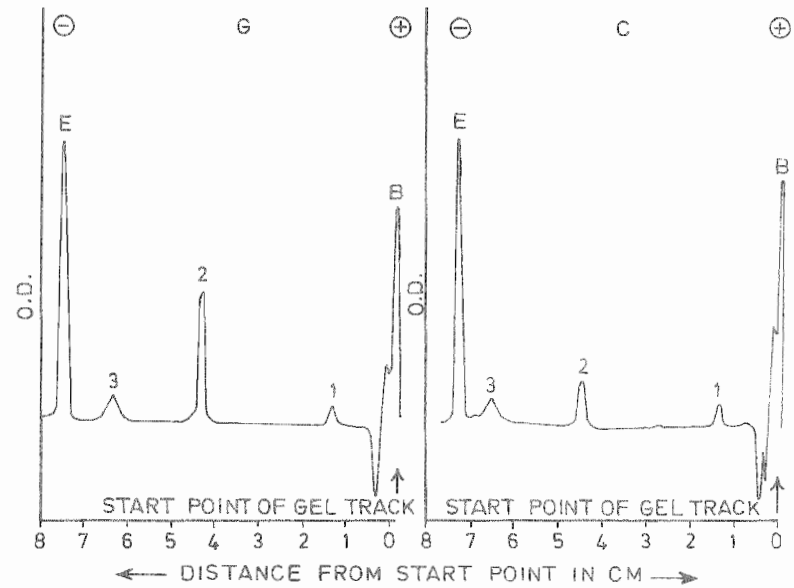


Fig. 3. Spectrophotometric scanning of gels for peroxidase of vegetative and antheridia bearing prothalli at 6th day after sowing.
Gels scanned at 470 nm.

C Vegetative prothalli were grown in normal nutrient medium.
G Antheridia bearing prothalli were grown in GA_3 supplemented nutrient medium.

Three distinct peaks are observable. Optical density of all isozymic forms is higher in antheridium bearing prothalli as compared with vegetative prothalli.

increase of shikimate synthesis during antheridium formation, probably reflects the demand for phenylalanine and tyrosine (shikimate has been shown to be precursor of phenylalanine and tyrosine in higher plants; Fowden, 1967). Together with the other amino acids, these compounds are certainly required to a larger extent during antheridium formation as they serve as substrates for the vigorous protein synthesis.

Peroxidases were not detected during germination and early stages of antheridium formation. Their presence was first noticed when antheridia had formed and spermatogenesis been initiated. Detection of peroxidase activity in *Anemia* seems to be associated with high rate of cell division during antheridium formation and spermatogenesis (De Jong, 1967; Vandern, 1963; Van Fleet, 1959). Another possible relationship between the detection of peroxidase during antheridium formation could be related with the presence of auxins. As auxin itself can either induce or repress the formation of specific isoperoxidase (Ockerese *et al,* 1966; Galston, *et al,* 1968).

The overall picture which emerges from the present study is that GA_3 is a differentiating agent for antheridium formation in *Anemia*. It induces the increased synthesis of SDH and peroxidase alongwith an enhanced synthesis of certain proteins related with antheridial formation (Iqbal & Schraudolf, 1977).

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