Cytochemical localization of peroxidase activity in the protonema of the moss *Ceratodon purpureus* during differentiation of gametophore buds induced by kinetin

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(Received: November 22, 1975)

Abstract

An increase in the activity of peroxidases under the influence of kinetin has been demonstrated in the protonema of the moss *Ceratodon purpureus*. It was connected above all with initiation and acceleration of the differentiation process of gametophore buds which showed a high activity of these enzymes. The increase in peroxidase activity was also noted in the degenerating intercalary cells which produced the buds.

The colour reaction characteristic for peroxidases was best noticeable in the minute spherical granules (peroxysomes), nucleoli and cell walls.

INTRODUCTION

Peroxidase activity has been demonstrated in many types of plant tissues and organs at various stages of ontogenesis (Jensen, 1955; Van Fleet, 1959; Breidenback and Beevers, 1967; O'Brien, 1967; Khan et al., 1968; Tolbert et al., 1968; Berthier et al., 1970; Müller, 1970; Berger et al., 1971; Hall and Sexton, 1972; Molnar and La Croix, 1972; Goff, 1975). The ubiquitous distribution of these enzymes at the cellular level is shown by their localization in microbodies (Frederick and Newcomb, 1969), Golgi vesicles (Hepler et al., 1972; Nougarede and Lescure, 1970; Hall and Sexton, 1972; Poux, 1969), endoplasmic reticulum (Nougarede and Lescure, 1970), inner membranes of chloroplasts (Hepler et al. 1972), mitochondrial cristae (Hebant and Marty, 1972), ground cytoplasm (Raa, 1973), chromosomes (Raa, 1973), plasmalemma (Hepler et al., 1972) and in vacuole (Poux, 1969; Nougarede and Lescure, 1970; Hall and Sexton, 1972; Hebant and Marty, 1972). Numerous authors detected peroxidases in cell walls (Jansen et al., 1960; Lipetz and Garro, 1965; Hepler et al.,

1972; Halperin and Minocha, 1973). Recently Goff (1975) demonstrated in onion root cells the product of peroxidase enzyme reaction in all the above mentioned structures and additionally in both free and membrane bound ribosomes.

Although peroxidases belong to the earliest identified enzymes, their function is not so far completely understood (Paul 1963; De Jong, 1967). A relationship is supposed between them and phenomena of disease resistance (Kosuge 1969; Severs et al., 1971), cell wall extension (Ridge and Osborne, 1971), lignification (Sandaures et al., 1964; Hepler et al., 1972; Halperin and Minocha, 1973), ethylene biosynthesis (Mapson and Wardale, 1972), transport (De Jong, 1966), IAA oxidation (Siegel and Galston, 1955). By inactivating IAA, peroxidases may also affect cell differentiation (Stoner et al., 1968; Pooviah and Rasmussen, 1973). According to Raa (1972), in this type of control of the differentiation process the isoenzymes bound with definite cell structures, such as ribosomes and chromosomes, can participate. Chromosomal peroxidase, in the opinion of this author, not only is involved in organization of the chromosome, but may also play a role similar to that of histones or function as an allosteric enzyme with IAA as modulator.

Halperin and Minocha (1973) attribute the stimulation of cell wall lignification by cytokinins in callus tissue to the influence of these substances on the synthesis or selective transport of certain peroxidase isoenzymes capable of forming strong covalent bonds in the cell wall where they may play the role of lignine polymerase (Brown, 1969).

The number of isoperoxidases associated with various cell functions may vary (Goff, 1975), and some isoenzymes may be transformed into others (Paul and Stigbrand, 1970).

Schneider and Szweykowska (1974) demonstrated a considerable increase in peroxidase activity and the appearance of a new isoenzyme in the protonema of *Ceratodon purpureus* after 120 h of incubation in kinetin medium. At the same time the well known cytokinin effect of induction of a large number of gametophore buds appeared in the protonema, so it was possible that a relationship existed between the enhanced activity of the enzyme and the bud-inducing effect.

The present study was undertaken to localize cytochemically the peroxidase activity in the protonema of *Ceratodon purpureus* subjected to kinetin treatment.

MATERIAL AND METHODS

A bacteria-free, vegetatively propagated clone culture of the protonema of *Ceratodon purpureus* (L. ap. Hedw.) Brid. was used in the experiments. The protonema was grown in Erlenmayer flasks on mineral

Figs 1—3. Young gametophore buds on protonema incubated with kinetin. Peroxidase activity visible in cell wall region and nucleoli. Fig. 1. Kinetin, 5 μ M/l after 24 h incubation, Fig. 2. Kinetin, 5 μ M/l, after 48 h incubation, Fig. 3. Kinetin, 1 μ M/l, after 140 h incubation.

Plate II

Figs 4—5. Protonema after 96 h of incubation in control medium (Fig. 4) and with kinetin at 5 μM (Fig. 5). Numerous gametophore buds visible with high peroxidase activity

Fig. 6. Peroxidase activity in basal and apical cells of buds in material incubated with 1 μ M kinetin for 240 h. Fig. 7. Peroxidase activity localized in basal cells of bud and in walls of subapical filamentous cells in material incubated with 1 μ M kinetin for 140 h.

Plate IV

Fig. 8—9. Peroxidase activity in kinetin-induced gametophore bud (Fig. 8) and in a similar bud after control reaction (boiling)

Plate V

Fig. 10. Large gametophore buds with developed leaves after 120 h of incubation with 5 μ M kinetin. Reaction product particularly well visible in meristematic part of bud (apex). Fig. 11. Young gametophore leaf after 120 h of incubation in 5 μ M kinetin medium. Reaction product is visible in minute spherical granules.

Figs 12—14. Apical cells of protonema incubated in kinetin medium. Fig. 12 — soon after division, with visible reaction product in cell wall (arrows), after 24 h of incubation with 5 μ M kinetin. Fig. 13 — with symptoms of degeneration and high peroxidase activity (24 h incubation with 5 μ M kinetin). Fig. 14 — distended and with numerous minute granules showing peroxidase activity (6 h incubation with 1 μ M kinetin)

Plate VII

Figs 15—17. Various stages of lateral branching of protonema incubated in kinetin medium (3 h in 1 μ M; 24 h in 5 μ M; 400 h in 1 μ M kinetin, resp.). High peroxidase activity in the whole cell (Fig. 15 and 16) and particularly in cell wall and nucleolus (Fig. 17).

Fig. 18. Intercalary caulonema cells with lateral branchings. Peroxidase present in the first cells of branchings. Control medium after 6 h incubation

Fig. 19. Peroxidase activity in cell wall of intercalary caulonema cells. Control medium after 6 h incubation.

Figs 20—22. Peroxidase activity in degenerating intercalary cells (Figs 20 and 22) and in functioning cells with well developed chloroplasts (Fig. 21). The material was incubated in 1 μ M kinetin medium and observed after 24 h (Fig. 22), 240 h (Fig. 21) and 400 h (Fig. 20) of incubation.

agar medium (Kofler 1959) covered with cellophane discs, under continuous fluorescent light of ca. 1000 lux and a temp. of ca. 25° C. After 3 weeks of growth, the protonemas (Ca. 2 cm in diameter) were divided into halves and transferred to mineral control solution (composition the same as in stock culture, with the omission of agar) or the same solutions with kinetin (at 1 and 5 μ M, resp.) added. Morphological and cytochemical examinations were made at various times from 3 h to 17 days.

Peroxidase activity was determined in unfixed material by the *p*-phenyldiamine method after Raa (1973) and by the benzidine method after Molnar and La Croix (1972) and Pooviach and Rasmussen (1973). In the benzidine method a 5 per cent ammonium chloride solution was used for stabilization of the blue colour and to prevent its changing to brown. As control served material boiled for 5 min. or incubated without H₂O₂. Both controls showed no trace of reaction.

All the photographs included in the paper show the results with the benzidine method.

RESULTS

In the material studied, particular attention was paid to the gametophore buds induced with cytokinin, and to the apical cells, cells of the lateral branchings and intercalary cells of the protonema.

Gametophore buds. The first bud initials were observed after 24 h of incubation of the protonema with kinetin. They almost whole showed a rather intensive colour indicating peroxidase activity. It was particularly intensive in the cell wall region and in the nucleolus of the considerably enlarged nucleus (Fig. 1). Peroxidase activity in several-cell buds was less intensive. It also appeared mainly in the cell walls and nucleoli (Fig. 2). As the size of the bud increased and the stalk cell elongated, the peroxidase activity shifted towards the upper part of the bud (Fig. 3). After 96 h of incubation with kinetin, numerous buds developed exhibiting high peroxidase activity (Fig. 5). In kinetin-free control medium this activity was associated mainly with cells initiating the lateral branchings (Fig. 4).

In most of the fully developed buds (after 120—240 h of incubation in kinetin medium), high peroxidase activity could be revealed in the basal and apical cells of the bud and in the curved several-cell filaments (Fig. 6—9) growing out from the subapical cells. Fig. 9 shows an example of the boiled control of enzyme estimation and the specificity of the colour reaction.

Gametophore buds at the stage of leaf development showed a particularly high peroxidase activity in the apical meristematic region (Figs. 6, 10). In the young leaf cells peroxidase was localized in minute granules (Fig. 11).

Apical cells of the protonema. Increased peroxidase activity was observed in the area of the newly formed cell walls of the dividing cells (Fig. 12). This was more frequently found in protonema incubated with kinetin, which stimulates divisions of apical cells, than in the protonema from kinetin-free control. On the other hand, as early as after 6 h of incubation in kinetin medium, symptoms of degeneration of a large number of apical cells were observed (Fig. 13, 14). In the degenerating apical cells enzyme activity was localized in spherical granules which were difficult to be identified in the light microscope.

Cells of the lateral protonema branchings. The moss protonema grows not only by meristematic activity of its apical cells, but it also forms numerous lateral branchings. Cells initiating the branchings showed a high peroxidase activity manifested in intensive colouring of their whole contents (Figs. 15, 16). The wall separating the cell initiating a new lateral branch from the intercalary mother cell exhibited a particularly strong reaction. In the somewhat elongated basal cells of a several-cell branching, the staining of the whole protoplasts was usually less intensive (Fig. 17), although sometimes high peroxidase activity in these cells persisted even when the branch filament reached a considerable length (Fig. 18).

No significant differences could be demonstrated in the peroxidase activity of the cells of lateral branches of the protonema incubated with kinetin when compared with kinetin-free control.

Intercalary protonema cells. Enhanced peroxidase activity was found in cells showing some symptoms of degeneration, particularly in those of the caulonema (Figs 19, 20). This was observed in the protonema treated with kinetin as well as in the untreated (control) one, however, the number of degenerating cells was highly increased in kinetin medium, particularly after a longer incubation time (72 h). In chloronema cells, densely packed with chloroplasts, a positive reaction was found in the minute granules close to the plastids (Fig. 21). The coloured granules in degenerating cells were more varied as regards size and shape (Fig. 22) than in the functioning chloronema cells (Fig. 21).

DISCUSSION

The cytological observations here described confirm the results of earlier biochemical studies (Schneider and Szweykowska, 1974) as regards the promotion by kinetin of the peroxidase activity in the moss protonema. The increase in peroxidase activity consisted mainly in the initiation and acceleration of the process of differentiation of gametophore buds which exhibited a high activity of this enzyme. An enhanced peroxidase activity was also found in degenerating intercalary cells producing the buds. Particularly high activity of the enzyme after 96 h of incubation

of the protonema in the kinetin medium resulted from both the large number of gametophore buds and the degenerative phenomena in the intercalary cells from which the buds originated.

The colour reaction characteristic for peroxidases was found in various cell structures. One can assume that the minute spherical granules occurring mainly in the vicinity of chloroplasts are the microbodies (peroxysomes), the presence of which in moss protonema has been revealed earlier (Młodzianowski, 1970). Larger grains, particularly in degenerating cells, may represent some complexes of cytoplasmic structures difficult to be identified in the light microscope. Strong staining of the vacuolized cells of the basal part of the bud and of the filamentous cells in the subapical bud region suggest the occurrence of peroxidases in the vacuoles as has been demonstrated by a number of authors in different material (Hall and Sexton, 1972; Hebant and Marty, 1972; Goff, 1975).

The enhanced activity of the enzyme in newly forming cell walls or in their vicinity suggests that it plays a role in the process of wall formation. Noteworthy is the fact that older walls of some protonema cells also showed an increased reaction for peroxidase. This cannot be referred to a lignification process as cell walls of the moss gametophytes do not contain lignin. Raa (1973) attributed to the appearance of peroxidase in the cell wall a physiological and ecological role consisting in degradation of IAA produced by bacteria. The latter if not decomposed might penetrate into the cells and disturb processes controlled by the endogenous hormone.

The positive reaction in nucleoli, particularly in young bud initials, may be related to the period of particularly high metabolic activity of the cell. This observation agrees well with the concept that peroxidase is associated with ribosomes (Penon et al., 1970, Raa, 1973; Goff, 1975), the subunits of which are produced in the nucleolus. The role of ribosomal peroxidase, however, is not known, although it is suggested that together with IAA it may regulate the translation process (Raa, 1973).

It is difficult at this stage to attribute the new isoenzyme, found by Schneider and Szweykowska (1974) in the *Ceratodon* protonema after 120 h of exposure to kinetin, to any special structure. It may only be supposed that it is associated with young gametophore leaves which form on kinetin medium in large quantities at this time and exhibit peroxidase activity.

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Cytochemiczna lokalizacja aktywności peroksydazy w splątku mchu Ceratodon purpureus podczas różnicowania się pąków gametoforowych indukowanych kinetyną

Streszczenie

Wykazano wzrost aktywności peroksydaz w splątku mchu *Ceratodon purpureus* pod wpływem kinetyny. Był on związany przede wszystkim z zainicjowaniem i przyspieszeniem procesu różnicowania się pąków gametoforowych, które wykazują znaczną aktywność tych enzymów. Zwiększenie aktywności peroksydaz wykazywały również degenerujące komórki interkalarne, które wytworzyły pąki.

Charakterystyczna dla peroksydaz barwna reakcja występowała przede wszystkim w drobnych, kulistych ziarnistościach (peroksysomach), jąderkach i ścianach komórkowych.