CHANGES IN IAA OXIDASE AND PEROXIDASE ACTIVITIES AND THEIR ISOZYME PATTERNS DURING THE GROWTH PERIOD OF THE CALLUS FROM TOBACCO PITH

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Abstract: Callus was induced from tobacco pith and subcultured with frequent transfers on Murashige and Skoog modified medium containing 13.5 mg/l IAA and 0.64 mg/l benzyl adenine. The growth curve by measuring the increase of protein content was a typical sigmoid curve for the callus tissue.

The total activities of IAA oxidase and peroxidase were not parallel. IAA oxidase activity was low from 8-26 culture-days and increased after 30 days. The activity of peroxidase decreased slightly at an early stage then increased after 12 days which was earlier than that of IAA oxidase.

Peroxisdae was separated into six isozymes (A, B, E, F, M, N) by disc polyacrylamide gel electrophoresis at 18 days. A correlation was apparent between the relative levels of the peroxidase isozymes and the rate of growth. The development of slower-migrating peroxidase isozyme A and B was accompanied by a rapid growth rate at the log phase. The isoperoxidases of fast-migrating rate (E, F, M, N) were associated with growth retardation.

The zymograph of IAA oxidase after electrophoresis separation was similar to that of peroxidase during the growth period. Peroxidase A, E, F and N possessed IAA oxidase activity, however, isoperoxidase B did not parallel with that of IAA oxidase. Whether these two enzymes were at the same apoenzyme or not was established.

INTRODUCTION

Previous work has shown that the interactions of hormones and isozymes are important in the control of growth and differentiation. There is a mutual interaction between peroxidase and plant growth hormone, IAA. Peroxidase catalyzes the oxidation of IAA[14,15]. Conversely, IAA alters the activity and isozyme number of peroxidase[5,8,10,14,18]. Crystalized peroxidase can also catalyze the oxidation of IAA[12]. The activities of IAA oxidase and peroxidase can not be separated[3,15,16] and they have a similar zymograph after electrophoresis[17].

Galston *et al.*[6] suggested peroxidase and IAA oxidase were at the same apoperoxidase: the peroxidase site requiring heme group, and the IAA oxidase site requiring Mn^{++} and substituted phenol. The oxidation of IAA catalyzed by IAA oxidase is an important event in plant tissue. Basu and Tuli[1,2,19] reported that a product of IAA oxidation in higher plants, 3-methyleneoxindole, might be the active principle in auxin-stimulated responses. But Evans and Ray[5] indicated that this product of IAA oxidation was inactive as a growth promoter. The actual actions of products of IAA oxidation are still unknown.

Lu[11] has separated peroxidase isozymes by starch gel electrophoresis. Because starch gel is not transmitable and has edge effect, so we used "disc" polyacrylamide gel electrophoresis to obtain clear narrow peroxidase bands and IAA oxidase isozymes. We further studied the relation of peroxidase and IAA oxidase to cell growth.

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MATERIALS AND METHODS

Materials.

Callus tissue was originally derived from the pith of tobacco plant (*Nicotiana tobacum* L. cv. Hicks 9) and subcultured for ten generations with frequent transfers on Murashige and Skoog modified medium[18] containing 13.5 mg/l IAA and 0.64 mg/l benzyl adenine. The cultures were grown at 26°C under dark conditions.

Enzyme Extraction.

Two to five grams of the fresh tissue were homogenized with 0.01 M Tris-HCl buffer pH 8.0 (w/v=1/5). The filtrate, after passing a two-layer cheese cloth, was collected and centrifuged at 15,000 g for 30 minutes. The supernatant fluid, which contained both IAA oxidase and peroxidase activities, would be referred to as crude enzyme.

Measurement of the Growth of Callus Cultures.

Growth value was expressed in final protein content divided by initial protein content (Pt/Po). Protein content was determined according to the Lowry method (1951). Equal volume of 10% TCA was added to the crude enzyme extract to precipitate the protein. Then 20 ml 0.1 N NaOH was added to dissolve the collected precipitate. One ml of this solution was added to 5 ml alkaline solution (4% Na_2CO_3: 2% $(CHOH)_2(COONa)_2$: 2% $CuSO_4$=100:1:1), and let stand for ten mininutes then mixed with 0.5 ml Folin-Ciocaeten reagent. Using Spectronic 20 colorimeter the optical density at 660 nm was taken. The standard curve of protein concentration *v. s.* O. D. was estimated with bovin serum albumin.

IAA Oxidase Assay

The enzyme extract, 0.25 ml, was incubated with 8 ml 0.2 M phosphate buffer pH 6.0, 0.5 ml 0.5 M $MnCl_2$, 0.5 ml 10^{-3} M 2, 4-dichlorophenol, 1 ml 0.5 mg/ml IAA in a shaking water bath at 30°C for 20 min. At the start of the reaction and 20 min thereafter 2 ml of this mixture was added to 8 ml Salkowski reagent (conc. H_2SO_4: 0.5 M $FeCl_3$: dist. H_2O=300:15:500) and the optical density at 530 nm was taken after ten minutes. The IAA oxidase activity was expressed as mg of IAA destroyed per mg protein of tissue at 30°C in 20 min.

Peroxidase Assay.

Peroxidase determinations were made by a modification of the procedure of Galston, Lavee and Siegel[65]. The reaction mixture contained 5 ml 0.2 M phosphate buffer (pH 6.0), 0.25 ml 0.02 M pyrogallol and 0.1 ml of enzyme was placed in a colorimeter tube at 30°C water bath; at time zero 0.5 ml 0.1% H_2O_2 was added and the tube rapidly inverted. The increase in absorbance at 470 nm was taken at 20-sec intervals for 3 min in a Bausch and Lomb Spectronic 20 colorimeter. Results are expressed as Δ O. D./mg protein/min.

Electrophoresis.

The gel column was made of 7% (w/v) polyacrylamide in the lower 5.5 cm section and 2.5% polyacrylamide in the upper 1.2 cm section with a diameter of 5 mm. Immediately before electrophoresis, the enzyme extract was mixed with an equal volume of 40% (w/v) sucrose solution and 50 μl of this mixture was loaded on the upper gel. Electrophoresis was carried out by the method of Davis[41]. During electrophoresis the temperature of the buffer system was kept at 4°C and a current of 4 ma per gel was applied until the indicator band of bromophenol blue reached the bottom of the gel.

Peroxidase active bands were identified by flooding the gel for 5 min with benzidine (0.1 g benzidine dissolved in 100 ml 0.1 M acetic acid containing 0.2 ml 35% H_2O_2). After staining, the gels were rinsed in distilled water and stored in 3% acetic acid. The color intensity of the developed bands was measured at 430 nm in a Isco 659 gel scanner and model UA-4 monitor.

The gel was sliced into 2 mm sections for IAA oxidase assay. Each section was extracted separately with soaking solution containing 2 ml 0.2 M phosphate buffer (pH 6.0), 0.125 ml 0.5 M $MnCl_2$, 0.125 ml 10^{-3} M 2, 4-dichlorophenol and 0.25 ml 0.5 mg/ml IAA at 25°C in the dark for 15 hrs. Then the amount of IAA remaining was estimated with Salkowski reagent in the provious method for IAA oxidase assay.

RESULTS

Growth of Tobacco Callus Cultures.

Tobacco callus from stock cultures which had grown on MS-1962 medium[13] containing 13.5 mg/l IAA and 0.64 mg/l benzyl adenine for 40-50 days was loose and yellowish. Growth curve by measuring the increase of protein content was a typical sigmoid curve (Fig. 1). It had 8 days of lag phase and followed by log phase and came into stationary phase at the 30th day after being transfered into a new medium.

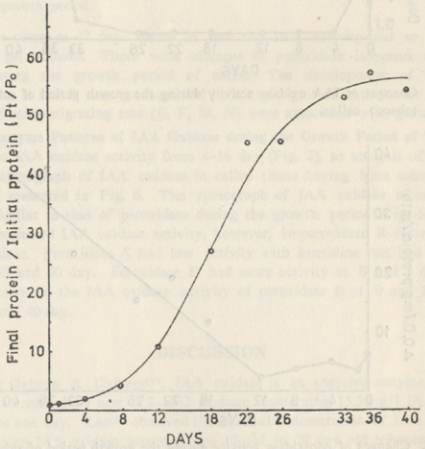

Fig. 1. Growth of 10 th subcultured callus induced from tobacco pith on the medium of Murashige and Skoog (1962) containing 13.5 mg/l IAA and 0.64 mg/l benzyl adenine.

Changes in IAA Oxidase and Peroxidase Activities during the Growth Period of Tobacco Callus.

IAA oxidase increased when callus was transfered on new MS-1962 medium for one day and was very low from 8-26 culture days and increased rapidly after 33 days (Fig. 2).

The activity of peroxidase decreased slightly from 0-12 days then increased after 12 days (Fig. 3). The activities of IAA oxidase and peroxidase were not parallel.

Changes in the Isozyme Patterns of Peroxidase during the Growth Period of Tobacco Callus.

The peroxidase zymogram pattern presented in Fig. 4 showed that four isozymes (E, F,

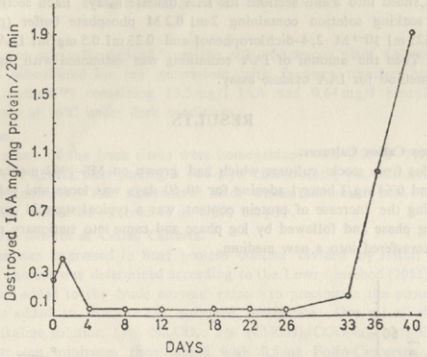

Fig. 2. Changes of IAA oxidase activity during the growth period of subcultured tobacco callus.

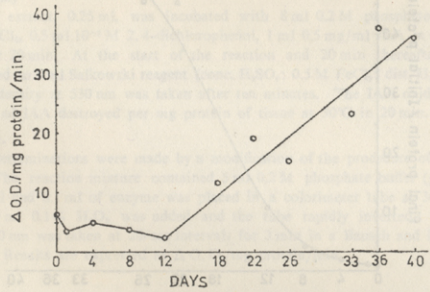

Fig. 3. Changes of peroxidase activity during the growth period of subcultured tobacco callus.

M, N) were present in the tobacco stock callus at the outset of the experiments. Isoperoxidase E had more activity than the others. A new band A appeared at one day after being transfered into new medium. The band M originally present disappeared at 4 day and reappeared at 18 day. Another band B appeared at 8 day and disappeared at 26 day. At 40 day, band A disappeared and a new band C appeared. Isoperoxidase E, F, N persisted in all the time of growth period.

The color intensity of the peroxidase bands shown in Fig. 5 was measured at 430 nm with a Isco 659 gel scanner and model UA-4 monitor. Band E had a boader and higher peak and band F so near to band E sometimes became the shoulder of band E. Band A and B had less

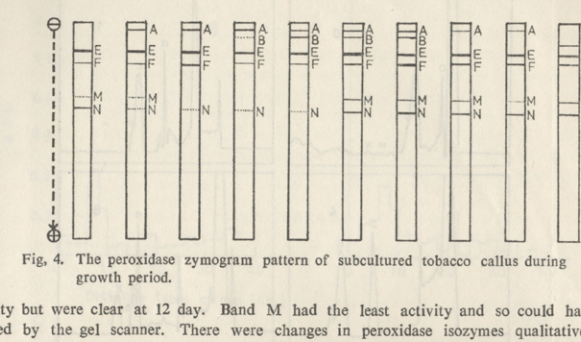

Fig. 4. The peroxidase zymogram pattern of subcultured tobacco callus during growth period.

intensity but were clear at 12 day. Band M had the least activity and so could hardly be detected by the gel scanner. There were changes in peroxidase isozymes qualitatively and quantitatively during the growth period of callus. The development of slower-migrating peroxidase isozyme A and B was accompanied by a rapid growth rate at the log phase. The isoperoxidase of fasting-migrating rate (E, F, M, N) were associated with growth retardation.

Changes in the Isozyme Patterns of IAA Oxidase during the Growth Period of Tobacco Callus.

There was no IAA oxidase activity from 4–16 day (Fig. 2), so analysis of its isozyme was not made. The zymograph of IAA oxidase in callus tissue having been subcultured for 0, 1, 33, 40 days was presented in Fig. 6. The zymograph of IAA oxidase after electrophoresis separation was similar to that of peroxidase during the growth period (Fig. 5, 6). Peroxidase A, E, F and N possessed IAA oxidase activity, however, isoperoxidase B did not parallel with that of IAA oxidase. Peroxidase A had low activity with benzidine but had high activity of IAA oxidase at 33 and 40 day. Peroxidase E had more activity at 0 and 1 day than that at 33 and 40 day, however, the IAA oxidase activity of peroxidase E at 0 and 1 day was lower than that at 33 and 40 day.

DISCUSSION

According to Galston & Dalberg[7], IAA oxidase is an adaptive enzyme, so it increased when callus was transfered on new MS–1962 medium containing 13.5 mg/l IAA and 0.64 mg/l benzyl adenine for one day. Lee[9] observed the optimal concentration of IAA for the increase of two fast-migrating IAA oxidase isozymes was 10 μM in 20 day old tobacco callus but with 5 μM kinetin, the development of these two IAA oxidase isozymes was completely repressed and suggested a multiple control by different types of growth substances. In this experiment, the activity of IAA oxidase was low in callus subcultured on MS medium containing 71 μM IAA and 2.8 μM benzyl adenine from 8–26 day and increased after 33 days. We suggest there is a possible causal relation between IAA oxidase activity and the subsequent growth pattern. The rate of cell division and growth was great at the log phase through the interaction of IAA and cytokinin and a control mechanism existed to repress the IAA oxidase.

New isoperoxidase formed when the total activity of peroxidase increased but the number of isozymes did not increase. There were changes in peroxidase isozymes qualitatively and quantitatively during the growth period. Whether the new isozymes are derived from any preexisting isozyme needs further evidence.

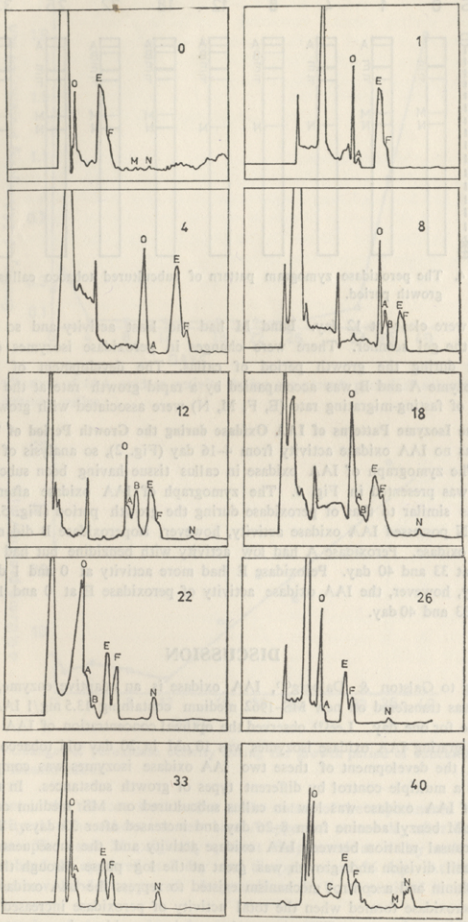

Fig. 5. Peroxidase isozymes were detected at 430 nm with a Isco 659 gel scanner and model UA-4 monitor.

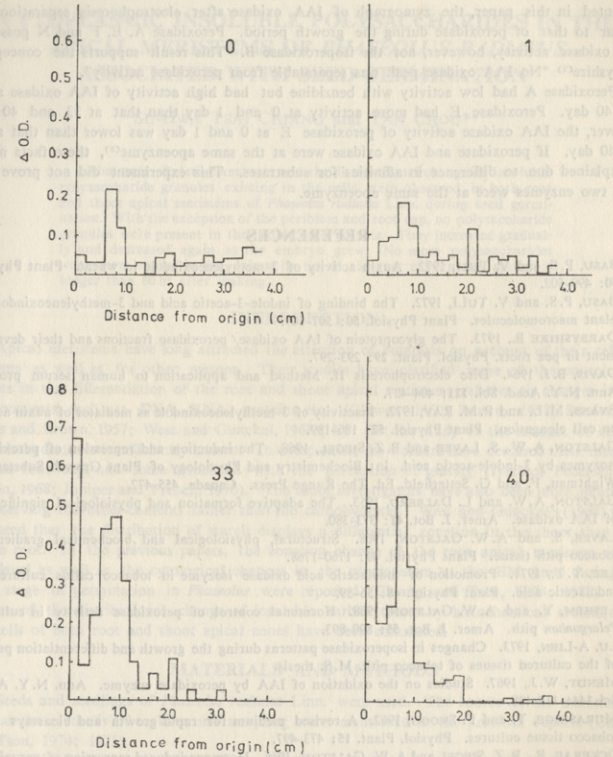

Fig. 6. IAA oxidase zymograph of tobacco callus during growth period.

A correlation was apparent between the relative levels of peroxidase isozymes and the rate of growth. The development of the two slower-migrating peroxidase isozyme A and B was accompanied by a rapid growth rate at the log phase. The isoperoxidases of fast-migrating rate (E, F, M, N) were associated with growth retardation. Perhaps isozymes of peroxidase and IAA oxidase function in different capacities under different conditions and their relative levels regulate the pattern of growth.

The total activities of IAA oxidase and peroxidase were not parallel. IAA oxidase activity was low from 8–26 day but peroxidase activity increased after 12 day. That means callus tissue had peroxidase activity from 12–26 day but contained no IAA oxidase. From the data

presented in this paper, the zymograph of IAA oxidase after electrophoresis separation was similar to that of peroxidase during the growth period. Peroxidase A, E, F and N possessed IAA oxidase activity, however, not the isoperoxidase B. This result supports the concept of Darbyshire[3] "No IAA oxidase peak was separatable from peroxidase activity".

Peroxidase A had low activity with benzidine but had high activity of IAA oxidase at 33 and 40 day. Peroxidase E had more activity at 0 and 1 day than that at 33 and 40 day, however, the IAA oxidase activity of peroxidase E at 0 and 1 day was lower than that at 33 and 40 day. If peroxidase and IAA oxidase were at the same apoenzyme[6], these facts might be explained due to difference in affinities for substrates. This experiment did not prove that these two enzymes were at the same apoenzyme.

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