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BIOCHEMICAL MECHANISM FOR THE INHIBITION OF PHENYLALANINE AMMONIA-LYASE INDUCTION IN THE ABSENCE OF OXYGEN IN POTATO TUBER TISSUE

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato parenchyma; phenylalanine ammonia-lyase; requirement for O_2; inhibition by cinnamic acid.

Abstract—Induction of phenylalanine ammonia-lyase (PAL) in excised potato parenchyma tissue in the presence of light displayed a rigid requirement for oxygen. Air (21% O_2) gave maximum induction and decreasing the level of oxygen had a parallel effect on the appearance of PAL. Below 5% O_2 no induction was observed. Induction in pure oxygen was at the same level as in air. The development of PAL activity in the light-exposed tissue was controlled by the metabolites of the phenylpropanoid pathway. Of these, cinnamic acid showed maximum inhibitory effect followed by *p*-coumaric acid; caffeic acid and chlorogenic acid correspondingly showed lesser inhibition. Deprivation of oxygen in the incubation atmosphere caused accumulation of cinnamic acid, greatly over the steady state level, and as a consequence PAL activity was inhibited. Admission of oxygen into the medium enabled the accumulated cinnamic acid to metabolize at a faster rate, thereby enhancing the synthesis of PAL. Inhibition of PAL protein synthesis either under anaerobic conditions or by an exogenous supply of cinnamic acid was proved by determining the enzyme activity, as well as [^{14}C]leucine incorporation into the enzyme protein, after affinity chromatography on phenylalanine-conjugated Sepharose-4B.

INTRODUCTION

The requirement for oxygen for the development of phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) is an essential feature of the induction process in plant tissues. Enhancement in PAL activity is not observed when tissue is submerged under water or kept under N_2. In citrus fruit peel, Rivo *et al.* [1] observed 70% inhibition of induction of PAL by dipping the tissues in water. The important function of air in attaining maximum synthesis of PAL has been attributed to the removal of some phenylpropanoid intermediates by oxidation, the presence of which may repress the enzyme activity [2]. The need for oxygen for induction is also evident from the studies of Goldstein *et al.* [3]. They have demonstrated that the extent of development of PAL activity is dependent upon the thickness of the tissue. Thin and outermost tissue showed maximum induction. A recent study by Shirsat and Nair [4] gave a clear-cut demonstration of the absolute requirement of oxygen for induction process by two stimuli, namely, γ-ray irradiation and cutting and exposure to light, in potato tubers. Submersion of the tissue in water in this case abolished the induction of PAL and this effect was reversed by vigorous passing of air into the suspension medium. This oxygen-dependent enhancement of PAL was demonstrated to be due to a *de novo* synthesis of enzyme protein.

Durst [5] has suggested that deprivation of oxygen to the tissue caused accumulation of cinnamic acid which is known to control the synthesis of PAL [6]. Although there are a number of studies that show that O_2 is required for PAL synthesis, the exact nature of the inhibition is not well understood. In these studies we have attempted to establish that the effect of oxygen deficiency is exerted through the inhibition of cinnamic acid 4-hydroxylase, which in turn increases the cinnamic acid level, thereby retarding the synthesis of PAL enzyme protein.

RESULTS

Oxygen concentration in the atmosphere has a profound effect on the development of PAL (Table 1). Maximum induction was observed in air, i.e. 21% oxygen. Reduction in the oxygen level in the air mixture caused a corresponding decrease in enzyme synthesis. 5% oxygen was found to be limiting for the synthesis of PAL. Compared to air, incubation in pure oxygen did not show much increase in the induction of PAL activity.

Similarly, partial exposure of the tissue in air and the rest of the incubation period in water and vice versa considerably affected enzyme synthesis. Either keeping the tissue in air initially for a period and then transferring to H_2O for the rest of the period, or the reverse, showed almost the same amount of inhibition. For example, when tissue was kept in air for 6 hr followed by 18 hr in water, or 18 hr in water followed by 6 hr incubation in air, the extent of inhibition was about 70–80%. It was found, also, that tissue kept 24 hr in water, if then exposed to air for a further 24 hr, had only one-third capacity to synthesize the enzyme. This suggests that some factors required for the synthesis of the enzyme are lost during incubation in water.

Increase in PAL activity in potato tubers in turn causes an accumulation of the metabolic products leading to the synthesis of chlorogenic acid and lignin precursors. While

Table 1. Induction of PAL in potato parenchyma tissue under different gaseous atmospheres

Atmosphere	PAL activity (units/g tissue/hr)
Air (21% O_2)	420 ± 30
Nitrogen	6 ± 1
Carbon dioxide	9 ± 1
Pure oxygen	455 ± 45
18% O_2	368 ± 8
15% O_2	200 ± 8
10% O_2	108 ± 12
5% O_2	25 ± 5
5% O_2	10

Parenchyma tissue slices (5 g) were placed in a Petri dish and kept in a desiccator under different gaseous atmospheres in 5–6 klx light at 25°. For details, see Experimental.

phenylalanine itself, even at a very high concentration, did not show any inhibitory effect, 3 mM cinnamic acid inhibits the appearance of PAL by 80%, and 3 mM *p*-coumaric acid by 78%: caffeic and chlorogenic acids gave about 60% [13]. The inhibition offered by these products of the biosynthetic pathway gives a clue to the mechanism of inhibition of PAL under reduced oxygen concentrations. When the O_2 supply is limited, cinnamic acid 4-hydroxylase, a mixed-function oxidase, cannot function efficiently, thereby allowing the accumulation of cinnamic acid in the system. When [^{14}C]phenylalanine was supplied to tissues under anaerobic conditions [^{14}C]cinnamic acid accumulated due to the residual PAL activity (Fig. 1). In order to prove that the product accumulated was cinnamic acid, an experiment was conducted with 50 g tissue and 400 μCi of [U-^{14}C]phenylalanine under anaerobic conditions. After 24 hr incubation the product was isolated without addition of any carrier cinnamic acid and purified by PC. The UV spectrum of the product was compared with an authentic sample of cinnamic acid and was found to be identical. The incorporation in this case was 20 × 10^3 cpm/g tissue.

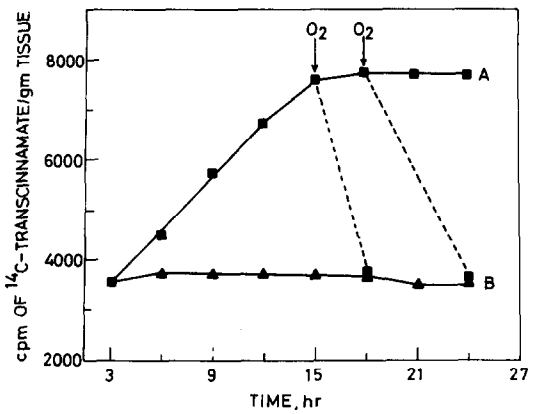

Fig. 1. Endogeneous levels of [^{14}C]cinnamic acid in potato parenchyma tissue incubated in the presence of [U-^{14}C]phenylalanine, in light, under N_2 (curve A) and air (curve B), and the effect of administration of O_2 in anaerobically incubated tissue.

The amount of label in cinnamic acid produced under anaerobic conditions declined sharply on exposure of the tissue to oxygen (Fig. 1), to the steady state level indicating that cinnamate is metabolized again in the system. The removal of cinnamate may in turn activate the synthesis of PAL. But there is no definite proof that these processes are interconnected.

We attempted to demonstrate the direct effect of cinnamic acid on the synthesis of PAL by first labelling the protein fraction with [^{14}C]leucine and then separating PAL from other proteins by affinity chromatography on a phenylalanine-conjugated Sepharose 4B column. PAL isolated from the affinity chromatography column was homogeneous [7]. The [^{14}C]leucine-labelled PAL formed under O_2 and light and purified by affinity chromatography was subjected to tryptic digestion and the main polypeptide fragments were isolated to ensure that [^{14}C]leucine was distributed among all these peptides. The results show that the radioactivity was distributed in four major peptides. The fourth peptide contained the maximum radioactivity. This peptide was subjected to hydrolysis with 6 N HCl and the hydrolysate was chromatographed again. It contained three major amino acids, lysine, arginine and leucine, and the amount of radioactivity applied on the paper was recovered completely from leucine. These studies gave evidence for the *de novo* synthesis of PAL enzyme under O_2 and light. Data given in Table 2 show that if incubation of the tuber tissue with [^{14}C]leucine is carried out under water or under N_2 which gave no enzyme increase, there was no incorporation of [^{14}C]leucine into PAL. When air was passed through the medium, activity as well as incorporation of label from amino acid into protein was restored. We have used this system to study the effect of treatment with cinnamic acid at different time intervals from 0 to 15 hr (Table 3). Treatment with 6 mM cinnamic acid at 0 hr neither showed any development of PAL activity nor incorporation of [^{14}C]leucine. Delaying the treatment by 3, 9, 12, 15 and 18 hr showed that the inhibition was observed only up to 6–9 hr. Afterwards the addition of cinnamic acid did not have much effect on the appearance of PAL or the incorporation of [^{14}C]leucine into protein. However, full recovery of the activity was not observed even at 18 hr.

DISCUSSION

The necessity of oxygen for the synthesis of PAL is reported by many workers. The fact that keeping tissues under water inhibits PAL can be explained by noting that the solubility of oxygen in water is 0.69 ml oxygen/100 ml water at 25° compared to 21% oxygen in air. Limiting the supply of oxygen below 5% adversely affected the synthesis of enzyme, which may be attributed to the reduction in the level of ATP required for synthesis of protein. But there are reports that in anaerobiosis the ATP produced mainly through glycolysis is sufficient to maintain the energy demands for synthetic process [8]. Moreover, in excised potato bud tissue submerged in water protein synthesis was not impaired as judged from the incorporation of [^{14}C]leucine into other proteins [9]. These studies suggested that apart from the deficiency in the production of ATP, depletion of oxygen has other effects on the synthesis of PAL.

Table 2. PAL activity and the incorporation of [^{14}C]leucine into PAL enzyme purified by affinity chromatography from the enzyme obtained from tissue incubated under various conditions

Incubation medium	Amount of PAL activity (units) in 2.5 ml fraction from phe-Sepharose 4B column	Total radio-activity in 2.5 ml fraction (cpm)
Air	1160	17000
N_2	0	200
Still H_2O	0	400
Aerated H_2O	1100	19000

See also Experimental.

Table 3. Synthesis of PAL enzyme protein in the presence of cinnamic acid added at various time intervals into medium, assessed by the incorporation of [^{14}C]leucine and development of PAL in enzyme fraction from the affinity chromatography column

Addition of 6 mM trans-cinnamate to medium at time intervals (hr)	PAL activity (units) in 2.5 ml fraction from phe-Sepharose 4B column	Total radioactivity in 2.5 ml fraction (cpm)
No treatment	1088	18000
0	0	180
3	52	1285
6	300	7000
9	400	9500
12	550	11000
15	720	12500
18	950	15000

Excised parenchyma tissues (10 g) were dipped in 2 ml volume of [U-^{14}C]-L-leucine (37.5 μCi) for 1 hr. They were then transferred to the filter paper kept in a Petri dish and incubated in air under 5–6 klx for 22 hr at 25°. Cinnamate solution (6 mM) was poured onto the tissues at different time intervals and incubated for 22 hr. Methods for the isolation of the enzyme by affinity chromatography and determination of (enzyme) activity and radioactivity are described in the Experimental.

The biosynthetic pathway for phenylpropanoid compounds is under regulatory control by a feedback regulation by various metabolites of the pathway from cinnamic acid to chlorogenic acid [10–13]. In this context, the inhibition of PAL synthesis by cinnamic acid is of great importance. But there are reports that even phenylalanine supplied to the tissue exerts a delayed inhibitory effect on PAL synthesis, i.e. when enough PAL is formed there will be the conversion of supplied phenylalanine to cinnamic acid, which will inhibit the further synthesis of PAL [14]. Our studies have shown that phenylalanine did not have any inhibitory effect even at high concentrations. On the other hand, cinnamic acid at 3 mM level inhibited PAL synthesis to about 80%. So the inability of phenylalanine to inhibit PAL synthesis in this system may be explained on the basis that the cinnamic acid produced is converted to other compounds at a faster rate under our experimental conditions. All the other intermediates of the pathway are inhibitory, but to a lesser extent compared to cinnamic acid.

Since PAL is amenable to regulation by the various intermediates of the pathway one could formulate a valid reason for the requirement of oxygen for the development of PAL activity. Oxygen is a substrate for cinnamic acid-4-hydroxylase. Thus by limiting the supply of oxygen, further metabolism of cinnamic acid will be blocked causing the accumulation of cinnamic acid over the steady-state level. The demonstration of the appearance of PAL activity and reduction in the level of labelled cinnamic acid on administration of oxygen to an anaerobic system substantiate this idea. When the oxygen supply was resumed the accumulated cinnamic acid metabolized at a faster rate and the concentration reached the steady-state level, relieving the inhibition of PAL synthesis.

A final answer to the question whether accumulation of cinnamic acid and PAL induction are related comes from the demonstration that the incorporation of [^{14}C]leucine into PAL enzyme protein was decreased in the presence of cinnamic acid when the enzyme activity was also reduced correspondingly. Maximum inhibition was observed when the treatment with cinnamic acid was within 4 hr after cutting. This period is the lag period for the synthesis of PAL during which the synthetic machinery is being activated. Another phase is up to 10 hr where there is appreciable incorporation into the enzyme as well as an increase in activity. After 12 hr there was a marked increase in enzyme synthesis and incorporation of [^{14}C]leucine into protein. However, treatment with cinnamate even at 18 hr showed reduced synthesis of enzyme. These observations suggest that cinnamate may exert its effect at two levels during the synthesis of PAL protein, namely, at the transcriptional and translational level. Durst [5] has suggested, from similar studies on the synthesis of PAL in Jerusalem artichoke tuber tissue, that PAL activity would be regulated by an inactivator whose synthesis is linked to the cinnamate pool size. But until now there has been no evidence for the existence of such a system. On the contrary, it is evidenced from our studies that addition of cinnamate to a system actively synthesizing PAL will retard the synthetic capacity immediately, suggesting that the compound may have a direct effect. Further studies on the effect of cinnamic acid on the synthesis of *m*RNA for PAL and its effect on cell-free synthesis of PAL will throw more light on the mechanism of action. From the foregoing discussion it is clear that the main effect of the reduction of oxygen level on the synthesis of PAL is the enhancement of the cinnamic acid level in the tissue, which in turn regulates the synthesis of PAL enzyme protein.

EXPERIMENTAL

Potatoes (*Solanum tuberosum* cv Madras) used in these studies were obtained from the local market within 2 weeks of harvest. They were stored in the dark at 0–4° and used for experiments within 2 months.

Isolation of PAL enzyme from parenchyma tissue. For the development of PAL activity, 5 g sliced parenchyma tissue were incubated in air at 25° on a moist filter paper on a Petri dish under 5–6 klx white light for 22 hr. The tissues were blotted out with filter paper and ground in a precooled mortar after freezing with liquid N_2. This powder was mixed with 10 ml 0.1 M borate buffer, pH 8.8, containing 10^{-2} M 2-mercaptoethanol. The mass was thoroughly ground and thawed out and the slurry centrifuged at 12000 g for 10 min. To the supernatant 3 vol. of

cold Me_2CO (−30°) was added and the mixture was centrifuged for 5 min at the same speed. The Me_2CO ppt. was dissolved in 5 ml 0.1 M borate buffer, pH 8.8, and used as the enzyme preparation after centrifugation. For the incubation of the tissue at various gaseous atmospheres, pure O_2 and N_2 were mixed in different proportions to obtain the desired percentage of oxygen in the mixture (monitored with a Clark O_2 electrode). The gas mixture was passed through a desiccator where the tissue was kept in a Petri dish under light.

Estimation of accumulated cinnamic acid under anaerobic conditions. Excised parenchyma tissue (5 g) was soaked for 1 hr in 2 ml soln of [U-^{14}C]-L-phenylalanine (sp. act. 189 mCi/mmol) containing 5 μCi of radioactivity. After this, tissue and the medium as a whole transferred onto a filter paper kept in a Petri dish. For the anaerobic experiment the Petri dishes were kept in a desiccator under prepurified N_2 along with alkaline pyrogallol. The samples were withdrawn at regular intervals for processing for the enzyme preparation and isolation of cinnamic acid.

Isolation of cinnamic acid. At the end of incubation period the tissue was thoroughly homogenized in Me_2CO in an omnimixer. The slurry was filtered and the residue was extracted twice with Me_2CO. The Me_2CO extracts were pooled and concd. To the aq. extract 500 μg of carrier cinnamic acid was added and pH was adjusted to 2.0. The aq. phase was extracted × 3 with Et_2O. The Et_2O was extracted with 5% $NaHCO_3$ soln. The bicarbonate layer was again acidified to pH 2.0 and extracted with Et_2O and the Et_2O after drying was evaporated to dryness. The residue was dissolved in MeOH and separated by paper chromatography in C_6H_6–HOAC–H_2O (2:2:1). The spot corresponding to cinnamic acid was detected under UV and cut out and counted using BBOT in toluene as scintillation fluid.

Isolation of [^{14}C]PAL enzyme using affinity chromatography. In this experiment, 10 g of parenchyma tissue was soaked in 37.5 μCi [U-^{14}C]-L-leucine (sp. act. 122 mCi/mmol) in a 2 ml vol. for 1 hr to absorb the label. Afterwards the tissue and the medium were poured onto a filter paper kept in a Petri dish and incubated under light, in air, N_2, submerged under water, and aerated water. After 22 hr incubation the tissue was washed and taken for PAL preparation as described earlier. The Me_2CO ppt. was dissolved in 20 mM NH_4 acetate soln adjusted to pH 6.0 and the pH of the enzyme soln was adjusted to 6.0 with dil. HOAc. This enzyme extract was subjected to affinity chromatography on a phenylalanine-conjugate of Sepharose 4-B as described by Ussuf and Nair [7]. The enzyme was eluted from the column using 20 mM NH_4 acetate buffer, pH 9.3. The enzyme was eluted in a single fraction. Aliquots were taken for radioactivity determination and enzyme assay.

Tryptic digestion of [^{14}C]leucine labelled PAL enzyme. The active enzyme fraction from 10 experiments was pooled and the enzyme was precipitated with the addition of 3 vol. of cold Me_2CO at −30°. The ppt. was dissolved in a minimum amount of 0.05 M Tris–HCl buffer, pH 8.0. Trypsin was added at the ratio 100 mg protein to 1 mg trypsin. The mixture was digested at 37° for 24 hr. After incubation, the pH was adjusted to 2 with HCl and the digest was analysed for the peptide fragments by PC on Whatman No. 1 paper using BuOH–HOAc–H_2O (4:1:5). The spots were detected by spraying with ninhydrin.

Assay of PAL. The enzyme was assayed according to the method of Zucker [10] with some modifications as described by Pendharkar and Nair [15]. One unit of activity is that amount of enzyme which produces 0.01 increase in absorbance at 290 nm (3.3 nmol of cinnamic acid) [16]. Enzyme activity is expressed as units per g of tissue per hr.

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