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Effects of CO_2 on Cinnamic Acid-4-Hydroxylase in Relation to Phenolic Metabolism in Lettuce Tissue

Jingtair Siriphanich and Adel A. Kader
Department of Pomology, University of California, Davis, CA 95616

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Abstract. **An atmosphere of air + 15% CO_2 prevented the development of cinnamic acid-4-hydroxylase in both lettuce (*Lactuca sativa* L.) and potato (*Solanum tuberosum* L.) tissues. Subsequent removal of CO_2 did not allow the enzyme development to proceed, whereas total phenolic content increased and browning became visible. In addition, CO_2 did not have an inhibitory effect on the enzyme, prepared from potato tissue, per se. Thus, the effects of CO_2 on inhibition of lettuce tissue browning does not appear to involve this enzyme. No tyrosine ammonia lyase activity was found in lettuce tissue.**

CO_2 is known to inhibit browning of physically-damaged plant tissues by inhibiting the activity of polyphenol oxidase (PPO) and lowering the level of phenolic compounds (4, 12). However, the mechanism by which CO_2 reduces phenolic content is not fully understood; it may or may not be related to the browning process. Wounding of plant tissues often causes increases in the activity of various enzymes involved in the phenolic metabolic pathway, e.g., phenylalanine ammonia lyase (PAL), cinnamic acid-4-hydroxylase (CA4H), and hydroxycinnamate CoA ligase, concomitantly with the increase in the phenolic compounds, as a mechanism of self defense against microbial infection (11).

Our previous study (12) on lettuce tissue showed that CO_2 treatment induced PAL activity but, at the same time, CO_2 prevented the production of more phenolic compounds. It is possible that CO_2 might have some influence on the biosynthesis of phenylalanine, the substrate of PAL, as well as on CA4H which catalyses the conversion of transcinnamic acid into p-courmaric acid, the first phenolic compound in the pathway. However, p-coumaric acid also could be formed via the deamination of tyrosine by tyrosine ammonia lyase (TAL).

Few plants contained TAL, and its activity was often low when compared to PAL (6). It is not known whether TAL activity is present in significant quantity in lettuce tissue or if TAL is involved in the phenolic metabolism. This paper reports our investigation of CO_2 effects on the CA4H and TAL enzymes and provide some explanation of how CO_2 might influence phenolic metabolism in wounded plant tissues.

Materials and Methods

Plant material and treatments. Crisphead lettuce tissue used in this study was derived in the same manner as reported previously (12). "White-rose" potatoes were purchased from a local market in Davis, Calif. Potato disks were placed in petri dishes containing moistened filter papers. For the air and CO_2 treatments, lettuce and potato tissues were placed in closed glass containers ventilated with a humidified stream of air or air + 15% CO_2. Light treatment was applied to potato disks by placing the containers under a cool-white fluorescent lamp. Light intensity was set at about 5400 lux, as measured by a photographic light meter, by adjusting the distance between the lamp and the potato disks.

Chemical analysis

CA4H assay. Only fresh tissues were used for CA4H assay according to a slightly modified method of Lamb and Rubery (8). In a cold room (0°C) and using mortar and pestle, 10 g of lettuce or potato tissue were ground with Tris buffer (pH 7.4) containing 2 mM of 2-mercaptoethanol and 1 g polyvinyl polypyrrolidone. The homogenate was centrifuged for 20 min at 10,000× g and the resultant supernatant fraction centrifuged again for 60 min at 100,000× g. The pellet of the 2nd spin was resuspended in 1 ml Tris buffer and used immediately for the enzyme assay. In a total volume of 2.5 ml, the assay mixture contained 125 μmoles Tris buffer, 0.8 μmole trans-cinnamic acid, 1.0 μmole glucose-6-phosphate disodium salt, 0.4 μmole NADP sodium salt, 0.2 unit of yeast glucose-6-phosphate dehydrogenase (G6PDH), and the enzyme preparation (about 0.5 mg protein). The NADPH-generating system first was incubated at 30° for 7 min before the enzyme preparation was added, and the incubation continued for 30 min. The reaction was stopped by adding 0.05 ml concentrated HC1, left for at least 1 min, then made basic by adding 0.1 ml 50% NaOH. The p-coumaric acid produced was detected spectrophotometrically at 340 nm as a consequence of NADP reduction.

Regulation of CO_2 concentration in CA4H assay. Calculated amounts of $NaHCO_3$ (9) were added to the Tris buffer before the assay mixtures were prepared. The mixtures with all components except the enzyme preparation then were bubbled for 60 min at 30°C with different concentrations of CO_2, at a constant O_2 concentration of 16%. After the enzyme preparation was added to the mixture, the test tubes were capped and incubated for another 30 min. The tubes were reopened to allow the addition of HC1 to stop the reaction. By doing this, CO_2 concentration in the gas phase of the tubes at the end of the incubation decreased by about 10% of the intended concentration for the 20% CO_2 treatment and 2% to 3% for the other treatments. However, the total amount of CO_2 (CO_2 and bicarbonate) lost from the liquid mixture was negligible.

TAL assay. To make the enzyme preparation for TAL assay, acetone powder prepared from lettuce tissue was dissolved in

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Tris buffer, stirred for 10 min, and filtered through filter paper. TAL activity was measured spectrophotometrically at 290 nm according to the method of Havir et al. (7) at pH range from 7 to 9.

Detection of trans-cinnamic acid. A modified method of Billett et al. (3) was used. Frozen lettuce tissue was boiled with 80% ethanol. The extract was evaporated under vacuum to near complete dryness; 1 ml of water was added to the residue which was acidified with a drop of 5 N HC1, and extracted twice with 10 ml ether. The ether extract was evaporated with N_2 to dryness, and the residue was dissolved in 0.25 ml absolute ethanol. A 10 µl aliquot was spotted on silica gel impregnated glass fiber sheet and developed with benzene + acetic acid + water (6+7+3) solvent. UV light was used to detect the trans-cinnamic acid. A trans-cinnamic acid standard was found to separate well and to have distinct color from other phenolic compounds found in lettuce tissue.

Results and Discussion

In the control lettuce tissue, CA4H gradually increased almost 2 times by the end of the experiment (Fig. 1). In contrast, CA4H remained stable throughout the storage period of the CO_2-treated tissue. When lettuce tissue was transferred from CO_2 to air, no change in the activity of CA4H was found after 2 days. The low activity of CA4H found in lettuce approached the limits of detection, and the results were error prone. Therefore, potato tissue, which was reported to have high CA4H activity in response to wounding and light treatment (5, 8) was used to confirm the effect of CO_2. In control potato tissue, CA4H increased to about 5 times the original level after 2 days at 5°C (Fig. 2). Under CO_2 the increase in CA4H activity was much smaller. The activity of CA4H declined after 2 days in both treatments under continuous exposure to light. The removal of CO_2 had no apparent effect on the enzyme activity. The same responses also were found in potato tissue when the experiment was done at 10°, but the decline in CA4H activity was accelerated (data not shown). From both the lettuce and potato experiments it was confirmed that CO_2 could maintain a low level of CA4H activity in wounded plant tissues.

Since p-coumaric acid also can be produced through the deamination of tyrosine by the enzyme TAL, activity of this enzyme was tested, but only trace TAL activity was found in lettuce tissue between pH 7 and 9, whether or not the tissue was treated with CO_2. This pathway of p-coumaric acid production therefore was ruled out.

The previous findings showed that CO_2 could lower the pro-

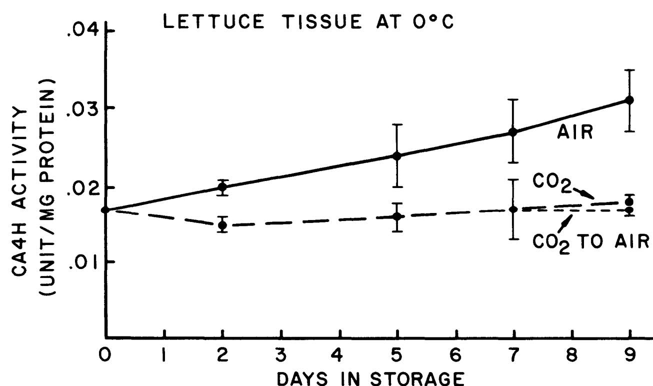

Fig. 1. Cinnamic acid-4-hydroxylase activity of lettuce tissue kept in air or air + 15% CO_2. Bars represent SD.

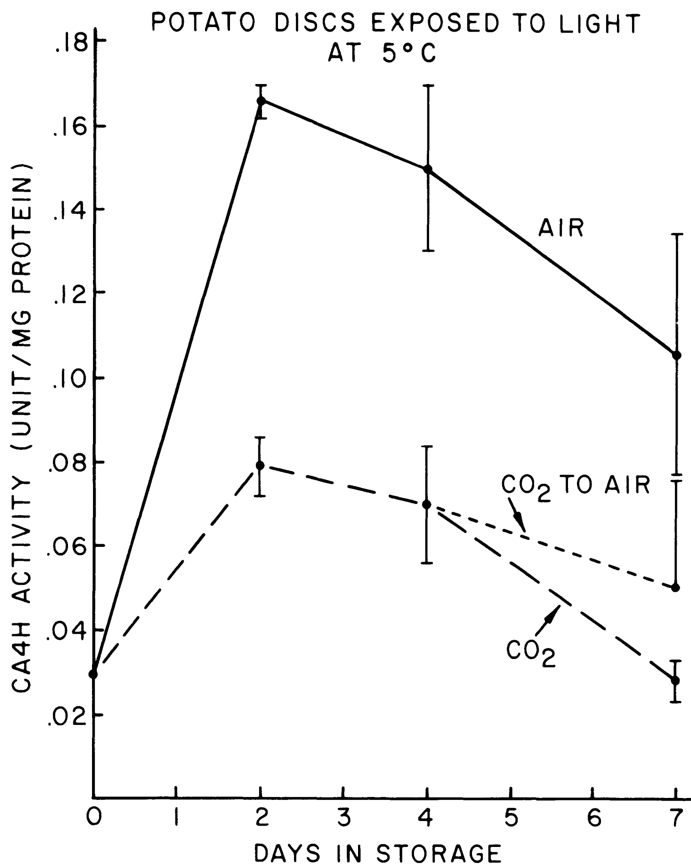

Fig. 2. Cinnamic acid-4-hydroxylase activity of potato disks kept in air or air + 15% CO_2. Bars represent SD.

duction of phenolic compounds when compared to control tissue. However, it did not explain how CO_2 could induce PAL activity without any increase in total phenolic content, which we reported previously (12). There are many possible explanations: CA4H might be inhibited by CO_2; phenylalanine, the substrate of PAL, might be limited in this system; the phenolics might be turning over to other compounds; or the total phenolics determination method used might not be specific enough to show the difference. Nevertheless, direct effects of CO_2 on CA4H were investigated. Figure 3A shows that between 0.03% and 20% CO_2 in air, CO_2 had no apparent effect on the enzyme prepared from potato. CA4H activity, at different CO_2 and $NaHCO_3$ combinations, followed closely the activity when NaCl was present in the assay mixtures in the same concentrations as the $NaHCO_3$ used with CO_2 treatments. The lower (although not always significant) CA4H activity in CO_2 treatments was probably due to the reduced O_2 concentration, i.e., 16% vs. 21% in NaCl treatment.

Since CA4H is a membrane-bound, mixed-function oxidase which requires NADPH specifically as an electron donor, one can speculate how CO_2 might influence the enzyme indirectly. CO_2 might have some effect on the membrane which, in turn, affects enzyme function. Another possibility is that CO_2 might inhibit the production of NADPH. Consequently, not only CA4H but also other systems that require NADPH might be blocked. There are reports (1, 2) which show that 30 mM bicarbonate competitively inhibits 22% of glucose-6-phosphate dehydrogenase (G6PDH) activity in yeast. If this also were the case in plant tissue, the pentose phosphate pathway, which is a source of NADPH as well as erythrose-4-phosphate (the precursor of

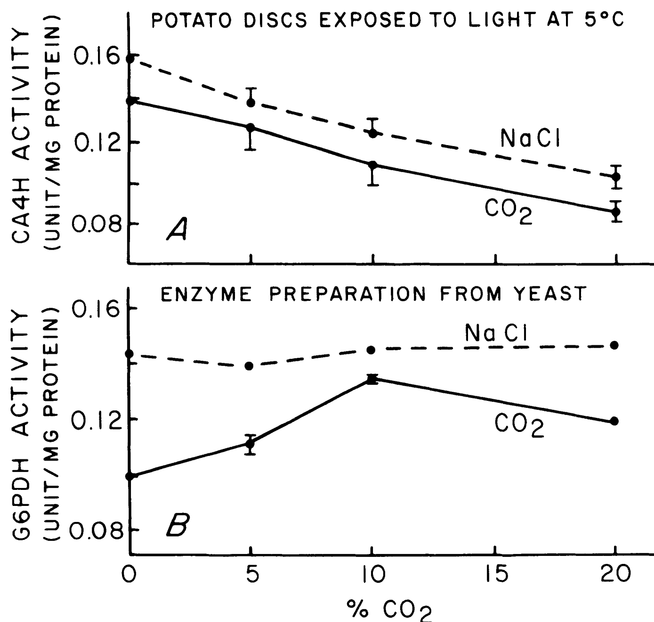

Fig. 3. Effect of CO_2 on: **(A)** cinnamic acid-4-hydroxylase activity in potato tissue, and **(B)** glucose-6-phosphate dehydrogenase (extracted from yeast) activity. Bars represent SD.

phenylalanine and other aromatic compounds), might be blocked. Thus, the observed effect of CO_2 on increased PAL activity without increased phenolics content (12) could be explained on the basis of unavailability of substrate to form additional phenolics. This possibility was checked in the NADPH-generating system used in the CA4H assay. The procedure was similar to that used for CA4H, except that only G6PDH from yeast and its substrate and cofactor were present. The reaction was determined by observing the production of NADPH at 340 nm within the linear range (0–3 min). CO_2 (with bicarbonate concentration in the enzyme assay mixture up to 290 mM) had no inhibitory effect; in fact, CO_2 showed some stimulation to the enzyme (Fig. 3B). It should be noted, however, that in the work by Anderson et al. (1) the assay was done at pH 8.0 vs. 7.4 in our experiment.

In order to confirm that CO_2 did not have inhibitory effects on CA4H directly or indirectly, the possible accumulation of trans-cinnamic acid was investigated in lettuce tissue. Using a thin-layer chromatography technique, spots were observed in the regions of a caffeic acid, chlorogenic acid, and other unidentified compounds. However, no trans-cinnamic acid was found in either the control or in the CO_2-treated tissue (data not shown).

In our previous report (12), it was shown that CO_2 could prevent browning by both lowering the level of total phenolic compounds and inhibiting PPO. Here, we have shown that CO_2 did not have any inhibitory effect on CA4H but prevented the increase in its activity. Furthermore, when CO_2 was removed, no change in CA4H activity was found while browning could proceed. Therefore, it can be concluded that the CO_2 effect on CA4H does not appear to be involved in the browning process. However, by preventing the increase in CA4H activity, CO_2 could impede the defense mechanism of wounded plant tissue. This is because lignin, which is produced by plants in their healing process, has a common pathway of synthesis with the phenolic compounds responsible for browning. Elevated CO_2, especially above 10%, has been shown to reduce suberization and prevent periderm formation in potato tubers (10, 13).

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