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Characterization of 12-Oxo-Phytodienoic Acid Reductase in Corn

THE JASMONIC ACID PATHWAY

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ABSTRACT

12-Oxo-phytodienoic acid reductase, an enzyme of the biosynthetic pathway that converts linolenic acid to jasmonic acid, has been characterized from the kernel and seedlings of corn (*Zea mays* L.). The molecular weight of the enzyme, estimated by gel filtration, was 54,000. Optimum enzyme activity was observed over a broad pH range, from pH 6.8 to 9.0. The enzyme had a K_m of 190 micromolar for its substrate, 12-oxo-phytodienoic acid. The preferred reductant was NADPH, for which the enzyme exhibited a K_m of 13 micromolar, compared with 4.2 millimolar for NADH. Reductase activity was low in the corn kernel but increased five-fold by the fifth day after germination and then gradually declined.

The conversion of linolenic acid to jasmonic acid by several plant species has recently been demonstrated with *in vivo* experiments (15, 16). The first enzyme of the jasmonic acid pathway, lipoxygenase, has been the subject of several reviews (1, 5, 11). The second enzyme, hydroperoxide cyclase, is present in many plant species and its properties in cell-free extracts have also been reported (12–14, 17). The third enzyme, 12-oxo-phytodienoic acid reductase, has not previously been characterized. Its existence was inferred from the analysis of metabolites produced *in vivo* from 12-oxo-phytodienoic acid in various plant tissues (15, 16). The enzyme, which catalyzes the reduction of a double bond in the cyclopentenone ring of 12-oxo-PDA[1] (Fig. 1), has now been detected in cell-free extracts. This report describes the properties of the reductase enzyme from the corn kernel and corn seedlings.

MATERIALS AND METHODS

Chemicals. Linolenic acid was purchased from NuChek Prep, Inc.,[2] Elysian, MN. Soybean lipoxygenase, NADPH, and NADH were obtained from Sigma Chemical Co. BCA protein assay reagent was a product of Pierce Chemical Co., Rockford, IL. Normal phase TLC plates (Anasil HF) were from Foxboro/Analabs, North Haven, CT, and $LKC_{18}F$ reversed phase TLC plates were from Whatman, Inc., Clifton, NJ.

Plant Materials. An acetone powder was prepared from corn kernels *Zea mays* L., var NK PX443 (Northrup King). First the kernels were rinsed with methanol and acetone to remove fungicide, then ground in a Wiley mill with a 2 mm screen. The resulting meal was ground to a flour in a Quad roller mill. The flour which passed through a 40 mesh sieve was homogenized in cold acetone (−20°C) with a Virtis 45 homogenizer and filtered through a Büchner funnel. Cold diethyl ether (−20°C) was used to wash the powder, and the solvents were then removed under vacuum. Enzymes were extracted from the powder by adding 2 ml of 50 mM K-phosphate (pH 7.2) for each g of powder and incubating the mixture for 30 min on ice. After centrifugation at 17,000*g* for 10 min, the supernatant was decanted and used as the enzyme source. The protein concentration was usually 10 to 12 mg/ml.

For studies of enzyme activities in germinating corn seedlings, the corn kernels were rinsed with methanol and acetone to remove fungicide, then planted between two sheets of moist paper toweling 2 cm from the top. The moist towels were placed on a sheet of waxed paper, rolled up, and placed upright in a beaker of H_2O. The kernels were germinated at 32°C in the light and 28°C in the dark with 16 h light (480 μE m^{-2} s^{-1}, 400–700 nm), followed by 8 h darkness. Seedlings were harvested at various times over a 15-d period. Dry kernels were considered to be day zero and were ground in a coffee grinder prior to enzyme extraction. At each time period 20 seedlings, including roots, shoots, and kernel, were selected for extraction. Plant tissues were frozen in liquid N_2 and ground in a mortar and pestle with 2 ml of 50 mM K-phosphate (pH 7.2) for each gram of kernel or fresh seedlings. After filtration through 4 layers of cheesecloth, the extract was centrifuged for 10 min at 17,000*g* and the supernatant was assayed for enzyme activity. Four reactions were conducted with each supernatant and the results were averaged.

Preparation of Substrates and Internal Standards. The structures of the compounds used as substrates or internal standards in these experiments are shown in Figure 2. The substrate for the 12-oxo-phytodienoic acid reductase assay was 12-oxo-*t*PDA, **2**, a stereoisomer of 12-oxo-*c*PDA, **1**. 12-Oxo-*c*PDA was pre-

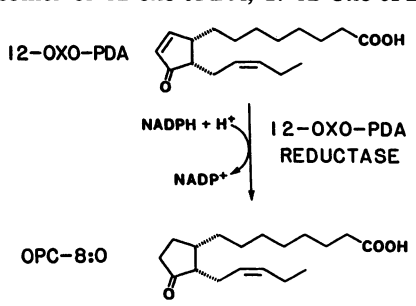

FIG. 1. Reaction catalyzed by 12-oxo-PDA reductase.

[1] Abbreviations: 12-oxo-PDA, 12-oxo-*cis,cis*-10,15-phytodienoic acid; *t*OPC-8:0, (1*R*,2*R*)3-oxo-2-(2′-pentenyl)cyclopentaneoctanoic acid; *t*OPC-10:0, (1*R*,2*R*)3-oxo-2-(2′-pentenyl)cyclopentanedecanoic acid. The designation *t* or *c* indicates the orientation, *trans* or *cis*, of the side chains with respect to the plane of the ring.

[2] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

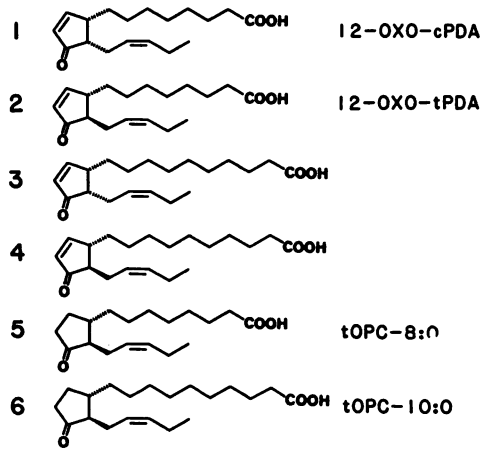

FIG. 2. Structures of compounds used as substrates or internal standards in the corn kernel 12-oxo-PDA reductase or hydroperoxide cyclase assays.

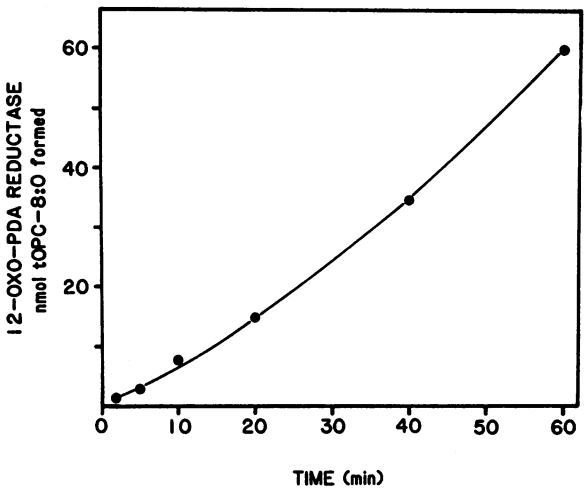

FIG. 3. Formation of *t*OPC-8:0 with time catalyzed by corn kernel 12-oxo-PDA reductase. Reaction conditions were as described in "Materials and Methods."

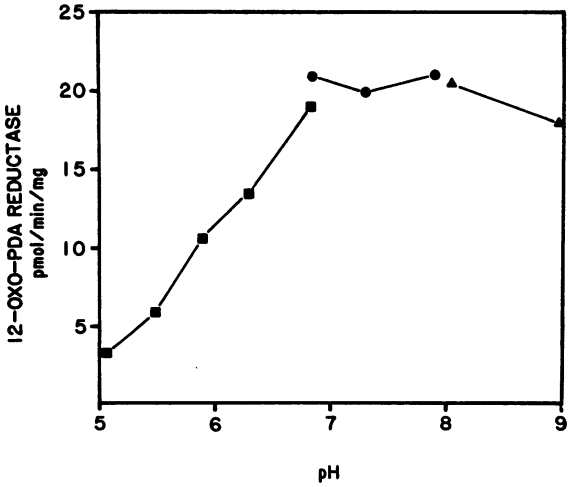

FIG. 4. Activity of corn kernel 12-oxo-PDA reductase as a function of pH. The buffers were 94 mM Mes (■), 125 mM Hepes (●), and 125 mM Tricine (▲). Reaction conditions were as described in "Materials and Methods" except for the change in pH.

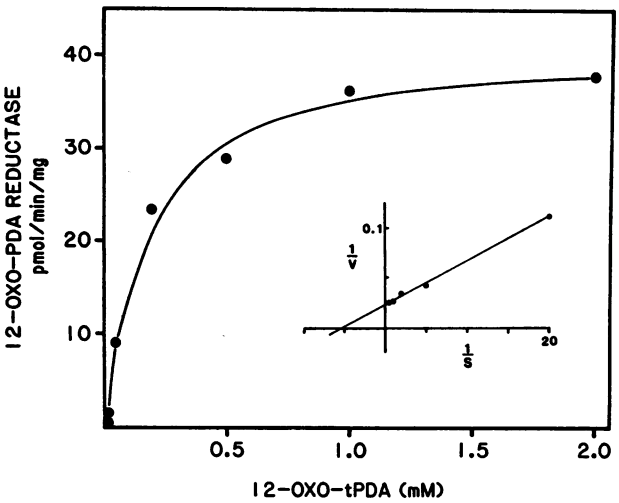

FIG. 5. Effect of increasing the concentration of 12-oxo-*t*PDA on the corn kernel 12-oxo-PDA reductase reaction. Inset shows a Lineweaver-Burk plot. Reaction conditions were as described in "Materials and Methods".

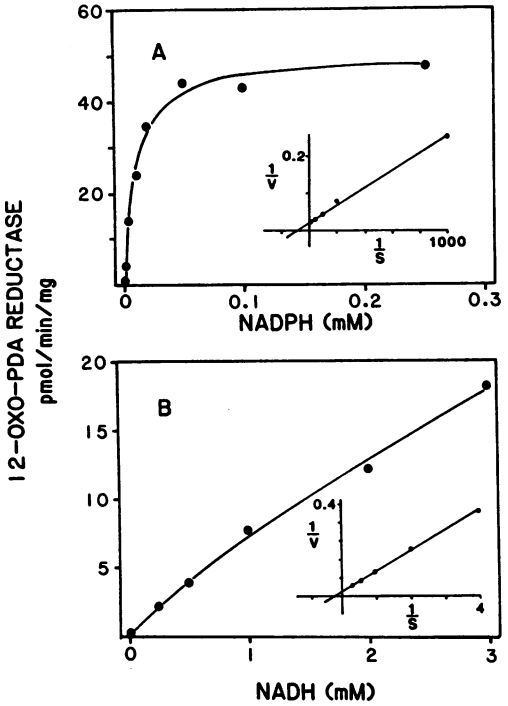

FIG. 6. Effect of increasing the concentration of (A) NADPH or (B) NADH on the corn kernel 12-oxo-PDA reductase reaction. Insets show Lineweaver-Burk plots. Reaction conditions were as described in "Materials and Methods".

pared from linolenic acid and a flaxseed extract according to Zimmerman and Feng (20), and then converted to 12-oxo-*t*PDA by heating without solvent at 150°C for 20 min. The 12-oxo-*t*PDA was purified by three developments in a hexane:diethyl ether:acetic acid (60:40:1, v/v/v) solvent system.

The 20-carbon analog of 12-oxo-*c*PDA, (1*S*,2*S*)3-oxo-2-(2′-pentenyl)cyclopent-4-enedecanoic acid, **3**, was prepared with flaxseed enzymes by the same method as for 12-oxo-*c*PDA above except that 11,14,17-icosatrienoic acid was the substrate. The *trans* isomer (1*S*,2*R*), **4**, was made from the *cis* isomer by thermal isomerization as described above. *t*OPC-8:0, **5**, and *t*OPC-10:0, **6**, were synthesized from 12-oxo-*t*PDA and its 20-carbon analog,

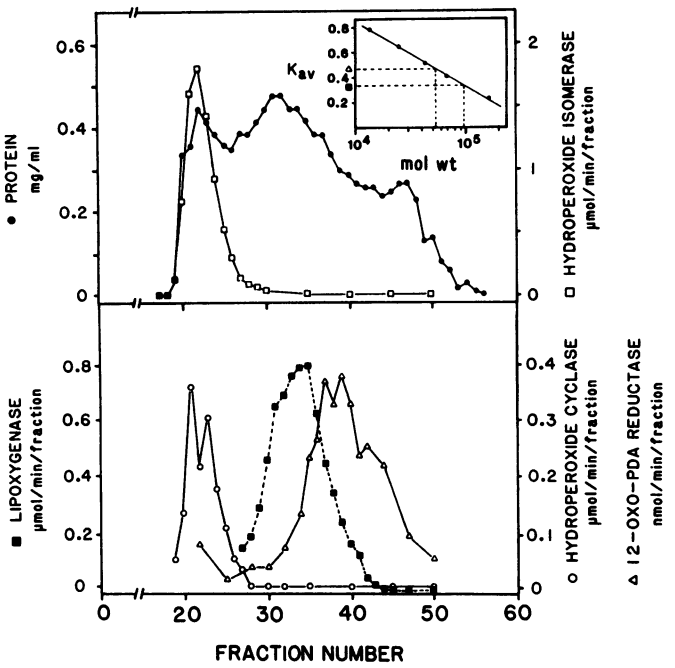

FIG. 7. Separation of corn kernel lipoxygenase (■), hydroperoxide isomerase (□), hydroperoxide cyclase (○), and 12-oxo-PDA reductase (△) on a Sephadex G-200 column (2.6 × 30 cm). Inset, estimation of mol wt by a plot of K_{av} *versus* log mol wt. The 2.8-ml fractions were eluted with 50 mM K-phosphate (pH 7.2).

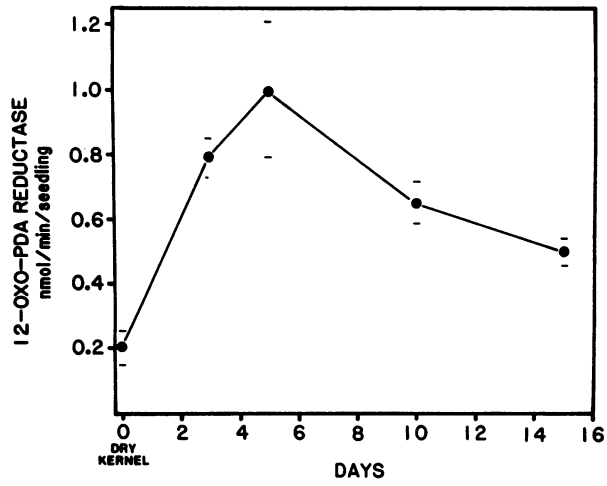

FIG. 8. Effect of age of corn seedlings on the activity of 12-oxo-PDA reductase. Bars represent the standard deviation of the enzyme rates based on four replicate analyses of the same enzyme extract.

4, respectively, by reacting approximately 20 mg of the cyclopentenone compound with about 40 mg of sodium borohydride dissolved in methanol. The resulting ring-saturated, hydroxylated compound was dissolved in 2 ml of acetone and reacted with 0.2 ml of Jones reagent (2.7 M CrO_3 in 4 M H_2SO_4) to convert the hydroxyl group back to an oxo group. The products were purified by TLC with hexane:diethyl ether:acetic acid (60:40:1, v/v/v) and their identities were confirmed by GC-MS (15, 20).

Enzyme Assays. Lipoxygenase was assayed spectrophotometrically at 234 nm in 50 mM K-phosphate (pH 6.0) by the method of Surrey (10). Hydroperoxide isomerase was measured in 50 mM K-phosphate (pH 6.5) as described previously by measuring the decrease in *A* at 234 nm caused by the disappearance of the conjugated 13-hydroperoxylinolenic acid (19).

Hydroperoxide cyclase was assayed by measuring the amount of 12-oxo-*c*PDA formed from 13-hydroperoxylinolenic acid. The substrate was prepared by reacting 1 ml of 8 mM linolenic acid-Tween 20 solution (10) with 2 mg of soybean lipoxygenase in 32 ml of 0.6 mM borate buffer (pH 9). The cyclase reaction mixture contained 8.3 ml of 50 mM K-phosphate (pH 6.5), 1.5 ml of 13-hydroperoxylinolenic acid substrate solution, and 0.2 ml of enzyme solution. After 1 min the reaction was stopped by the addition of 1 ml of 1 M citric acid, and then 10 nmol (2.94 μg) of *t*OPC-8:0 was added as the internal standard. The mixture was passed through a C_{18} silica extraction column and the products were eluted with diethyl ether. The concentrated diethyl ether phase was applied to reversed phase $LKC_{18}F$ TLC plates and developed in an acetonitrile:water:acetic acid (95:5:1, v/v/v) solvent system. Products cochromatographing with 12-oxo-*c*PDA and the *t*OPC-8:0 standards were removed, eluted with diethyl ether, and the methyl esters prepared with diazomethane. The sample was analyzed with a Hewelett-Packard gas chromatograph operating in the split mode with a 25 m × 0.22 mm i.d., cross-linked 50% phenylmethylsilicone fused silicone column (Hewlett-Packard). The temperature was programmed from 200 to 270°C at 10°C/min.

12-Oxo-PDA reductase was assayed by measuring the production of *t*OPC-8:0 from 12-oxo-*t*PDA. The standard reductase reaction mixture contained 50 mM K-phosphate (pH 7.2), 1 mM 12-oxo-*t*PDA, 1 mM NADPH, and enzyme in a final volume of 0.8 ml. The volume of enzyme solution was usually 0.4 to 0.5 ml. 12-Oxo-*t*PDA was used as the substrate rather than the natural substrate, 12-oxo-*c*PDA, because of the tendency of the latter to isomerize to the *trans* form during the analysis. The reaction was terminated after 40 min by extracting the products with 3 ml of methanol:chloroform (2:1, v/v) (2) containing 15 nmol (4.83 μg) of *t*OPC-10:0 as an internal standard. After the addition of 1 ml of chloroform and 1 ml of water, the chloroform phase was removed and concentrated. The sample was applied to TLC plates and developed four times in hexane:diethyl ether:acetic acid (60:40:1, v/v/v). The products which comigrated with *t*OPC-8:0 and *t*OPC-10:0 standards were removed and eluted with diethyl ether. After formation of the methyl esters with diazomethane, the sample was analyzed by GC with the column described above. The column was operated in the splitless mode and the temperature was programmed from 90 to 270°C at 10°C/min.

Protein Assay. Protein was assayed with Pierce BCA protein assay reagent, with BSA as the standard.

Molecular Weight Estimation by Gel Filtration. The mol wt of corn kernel lipoxygenase, hydroperoxide cyclase, hydroperoxide isomerase, and 12-oxo-PDA reductase were estimated by gel filtration on a 2.6 × 30-cm column of Sephadex G-200. The corn kernel extract was eluted with 50 mM K-phosphate (pH 7.2) at a flow rate of 38 ml/h, and 2.8-ml fractions were collected. Proteins used as standards for mol wt markers were ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), BSA (67,000), and aldolase (158,000).

RESULTS

Time Course Study of the 12-Oxo-PDA Reductase Reaction. Figure 3 shows the formation of *t*OPC-8:0 from 12-oxo-*t*PDA over a period of 60 min. Except for a slight lag during the first 10 min, the reaction rate was linear during the period measured. For routine reductase assays, a 40-min reaction time was chosen.

Effect of pH on the 12-Oxo-PDA Reductase Reaction. 12-Oxo-PDA reductase had a broad pH optimum from pH 6.8 to 8.0 (Fig. 4). For the pH study, the enzyme was extracted from corn kernel acetone powder with 20 mM Mes buffer (pH 6.5). The reaction mixture buffers used in the pH study were: 94 mM Mes buffer from pH 5.0 to 6.8, 125 mM Hepes buffer from pH 6.8 to 7.9, and 125 mM Tricine buffer from pH 8.0 to 9.0. A pH

of 7.2 was chosen for routine assays of the enzyme.

K_m of 12-Oxo-tPDA. The effect of increasing the concentration of 12-oxo-*t*PDA is shown in Figure 5. A typical substrate saturation curve was observed, and a Lineweaver-Burk plot showed a straight line (inset). The value of K_m, however, was determined by a direct linear plot (4), which gave a K_m value of 190 μM for 12-oxo-*t*PDA.

K_m of NADPH and NADH. NADPH was the preferred reductant in the 12-oxo-PDA reductase reaction (Fig. 6A). NADH could act as a reductant, but at a much slower rate (Fig. 6B). Typical substrate saturation curves and Lineweaver-Burk plots were observed for both substrates. The K_m for NADPH, determined by the direct linear plot method, was 13 μM, and the K_m for NADH was 4.2 mM.

Molecular Weight Estimation of Enzymes by Gel Filtration. The separation and mol wt estimation of enzymes by gel filtration chromatography on a Sephadex G-200 column is shown in Figure 7. The mol wt of corn kernel lipoxygenase was estimated at 97,000. This value is consistent with the mol wt of plant lipoxygenases reported from other sources, most of which have mol wt near 100,000 (11). The mol wt of 12-oxo-PDA reductase was approximately 54,000.

Hydroperoxide cyclase and hydroperoxide isomerase eluted together in the void volume on the Sephadex G-200 column, indicating a mol wt in excess of 250,000. An attempt to further separate a partially purified cyclase/isomerase preparation by ion-exchange chromatography on a DEAE-Sepharose CL-6B column also resulted in overlapping activities when eluted with a linear NaCl gradient (data not shown). Earlier results with these enzymes from cotton (13) and flaxseed (B. A. Vick, D. C. Zimmerman, unpublished results) were the same. We have never been able to separate these two enzyme activities. Although hydroperoxide cyclase and hydroperoxide isomerase activities appear to be associated with the same protein, more stringent separation attempts, such as gel electrophoresis or isoelectric focusing, are required before such a conclusion can be drawn.

Developmental Changes in 12-Oxo-PDA Reductase Activity. When 12-oxo-PDA reductase was assayed in corn seedlings over a period of 15 d after germination, a striking change in the level of activity of the enzyme was observed (Fig. 8). There was a moderate amount of the enzyme in the ungerminated kernel, but by the 5th d of germination the activity of reductase in the whole seedling, including kernel, had increased 5-fold. Some of the increased reductase activity was due to newly synthesized enzyme, because separate measurements on the new shoots alone indicated that they had an active reductase. After 5 d the activity of the enzyme declined slowly. Similar changes were observed in an earlier report on the activities of lipoxygenase and hydroperoxide cyclase in corn seedlings (14).

DISCUSSION

Desaturation reactions which introduce a double bond between two singly bonded carbon atoms are widespread in prokaryotic and eukaryotic metabolism. Fewer examples can be cited in which hydrogen atoms are added to double bonds of carbon atoms. Three such reactions will be noted here for comparison with the 12-oxo-PDA reductase reaction.

Enoyl-ACP reductase is an enzyme of fatty acid synthesis that catalyzes the reduction of a *trans*-2 double bond of an acyl-ACP molecule. In plants, two forms of the enzyme occur. Some species utilize an enzyme specific for NADH as the hydrogen donor, while others possess an enzyme requiring the presence of both NADH and NADPH (9). 15-Ketoprostaglandin Δ^{13}-reductase catalyzes the reduction of the *trans*-13 double bond of 15-oxo-prostaglandin $F_{2\alpha}$ during the catabolism of prostaglandin $F_{2\alpha}$ in the lung. The enzyme from bovine lung can use either NADH (K_m = 91 μM) or NADPH (K_m = 8 μM) (6) while the human placental enzyme utilizes only NADH (8). Several reductases are active in steroid metabolism. For example, testosterone 5α-reductase reduces the Δ^4 double bond of testosterone to the more potent 5α-dihydrotestosterone. The enzyme from human hyperplastic prostate tissue requires NADPH as the reductant and has a K_m of 1.5 μM for NADPH (7). Similarly, 12-oxo-PDA reductase from corn kernels is most active with NADPH as the hydrogen donor, although NADH is also active at high concentrations. The low activity of the reductase enzyme in comparison with lipoxygenase or hydroperoxide cyclase suggests that this enzyme may be a rate limiting step which determines the amount of 12-oxo-phytodienoic acid that is converted to jasmonic acid.

The physiological function of jasmonic acid is not yet established, although several investigators have shown that it promotes senescence in plants (3, 18). However, our experience with corn (Fig. 8) and other plants (13, 14) indicates that the enzymes of the pathway usually have their highest activity in young, actively growing plant tissues. We believe that jasmonic acid has another, more subtle role in metabolism unrelated to senescence. The plant senescence which is observed after exposure to jasmonic acid may only simulate the consequence of uncontrolled jasmonic acid biosynthesis.

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