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Induction of Cytochrome P-450 Protein in Wounded Avocado Roots(*Persea americana*, Mill)

Eun Young Joo

Dept. of Biology, Kyungsan University, Kyungsan 712-240, Korea

Abstract

The ripe fruit of Hass avocado contains one of the highest levels of cytochrome P-450 protein found in the plant kingdom. To determine whether wounded roots of avocado contain P-450 protein, the roots of avocado were wounded by slicing, and then allowed to incubate in sealed plastic bags, in 0.4M mannitol, and in the solution to make protoplast preparation containing cellulysin and macerase during the specified times. The microsomal proteins were extracted from the samples, separated by SDS-PAGE, and then subjected to Western blot analysis using polyclonal antibodies which are generated against the CYP71A1 protein. Wounded roots in sealed bags produced CYP71A1 within 6 hours after cutting, and those in 0.4M mannitol did not produce CYP71A1 even after 72 hours, but those in the protoplast preparation by cellulysin and macerase induced and produced CYP71A1 was induced in only 24 hours. These results indicate that CYP71A1 plays a role for wound healing for root tissue of avocado, and wound-inducible P-450 protein was not detected in the mannitol solution by preventing a synthesis of ethylene in a liquid state, but the softening of tissues by cellulysin and macerase to make protoplast preparation was involved in an activation of CYP71A1 even in the liquid state.

Key words: cytochrome P-450 protein, avocado, CYP71A1, Western blot analysis

INTRODUCTION

Cytochrome P-450(P450) proteins are important in the oxidative, peroxidative, and reductive metabolisms of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biogenic amines. Many of these enzymes also metabolize a wide range of foreign chemicals including drugs, environmental pollutants, natural plant products, and alcohols. The metabolism of foreign chemicals can frequently produce toxic metabolites, of which some have been implicated as agents that may be responsible for tumor initiation, promotion, and tumor progression(1).

P450 proteins are membrane-bound heme proteins which consist of a protoporphyrin IX and an apoprotein that confers the substrate specificity(2). P450 proteins act as mono-oxygenase enzymes that are involved in a number of biosynthetic and detoxification pathways in plants. Because of their importance in xenobiotic and drug metabolism, these enzymes have been thoroughly studied in mammalian liver(3-7) and insect(8-15) with mechanistic and structural details based to a large extent on studies of the bacterial(*Pseudomonas putida*) camphor hydroxylase system(3).

P450 protein family contains at least 400 presently characterized members, so being described as a "superfamily". P450 protein has been isolated from bacrteria, fungi, yeast, insects and mammals, and undoubtedly is present in almost all living organisms(16). Although considerable progress has been made in characterizing mammalian and bacterial P450 enzymes, much less is known about the plant enzymes. This is due principally to the low amounts of P450 protein present in plant tissue, and apparent liability of the enzyme following detergent solubilization(3). Some plant tissues are particularly enriched in spectrally detectable P450 protein. P450 protein has been purified from tulip(*Tulipa gesneriana*) bulbs(17), and from tubers of Jerusalem artichoke(*Helianthus tuberosus*)(18).

The ripe Hass avocado contains one of the highest levels of the P450 protein. Therefore, it has been used to prepare P450 protein for analysis, and also provided the first P450 gene to be cloned and sequenced from a plant source(19,20). Polyclonal antibodies were generated against the CYP71A1 protein, and used in a western blot analysis of Hass fruit at various stages of ripening. An antigen of approximately 48,000 daltons was absent in unripe fruit, but observed in ripening tissue(16,21). All

of this had been performed with the fruit of avocado.

The ripening of avocado fruit involves a complex set of biochemical and physiological changes which lead to the development of an edible fruit. One aspect of ripening is the softening of mesocarp tissues, a consequence of cell wall degradation. In avocado fruit, the mRNA for cellulase(endo-β-1,4-glucose) has been shown to accumulate during normal ripening(22) or in response to ethylene treatment of unripe fruit(23). Although ethylene is generally recognized as the primary regulatory agent of fruit ripening, the signal transduction pathway, from stimulus recognition to activation of cell and other ripening-associated genes, remains to be elucidated.

This study was initiated to help elucidate the role of CYP71A1 during avocado ripening. It has been assayed for roots other than avocado fruits. In particular, we have examined whether P450 protein exists in wounded and chemical treated root of avocado.

MATERIALS AND METHODS

Plant material

Avocado roots were collected from seedlings of Hass (*Persea americana* Mill.) in the campus of California State Polytechnic Uinversity at Pomona, California, USA. Roots were washed, sliced into 5mm length, frozen immediately in liquid N_2, and stored at -70°C freezer. In order to achieve wounding-promoted induction of P450, slices were placed on wet filter papers in sealed bags at room temperature. At the specified times(0, 3, 6, 24, and 72 hours) after wounding, slices in the bag were frozen immediately in liquid N_2, then stored at -70°C to extract microsomal protein. For further induction of P-450 in liquid state, slices were aged in 0.4M mannitol for the specified times(0, 24, 48, and 72 hours) after wounding, and washed with deionized water three times, then frozen and stored as above. For the protoplast preparation, root slices were treated with 100ml digestion solution containing 0.4M mannitol, 0.5%(w/v) cellulysin(Calbiochem) and 0.5% macerase(Calbiochem) for 24 hours. After digestion, the tissues were rinsed with deionized water three times, and removed water with filter papers. The tissues were frozen in liquid N_2, and used to extract microsomal protein.

Microsomal protein preparation

The micrisomal protein preparation is described by O'Keefe and Leto(24). Approximately 20gms of frozen tissue samples were placed into a tube(Falcon Blue Max 50ml graduated tubes) and 40ml of cold microsomal preparation buffer(0.1M MOPS/NaOH pH 7.0, 0.3M sorbitol, 5mM EDTA, 0.1%(w/v) BSA, 1mM PMSF, 5μg/ml leupeptin, and 5μg/ml pepstatin) were added. This was polytroned on the highest setting for about one minute, or until solubilized. The homogenate was centrifuged at 20,000g for 30min. The supernatant was transferred to ultracentrifuge tubes and spun at 100,000g for 60min. This fraction contains all microsomal proteins which includes the cytochrome P450 proteins. The pellet was resuspended in 0.5ml of resuspension buffer(50% glycerol, and 0.1M MOPS/NaOH pH 7.0) and frozen until analysis.

Immunoblots

Protein concentrations were determined by the Bio-Rad protein assay. Microsomal proteins from roots were subjected to immunoblot analysis following separation by SDS-PAGE on a 10% acrylamide gel(25). After electrophoresis, proteins were transferred to nitrocellulose by electroblotting in transfer buffer(20mM Tris-base, 150mM glycine, 0.1% SDS, and 20% methanol). After transfer, the nitrocellulose was saturated with 1% bovine serum albumin for 2 hours at room temperature and then incubated for 2 hours with primary antiserum(rabbit antiCYP71A1) diluted to 1:2,000 in 1×TBS and 1% BSA. Excess antibody was washed away with deionized water, and then 1×TBS and 0.05% Tween 20. The blot was incubated for 2 hours with secondary antibody, goat anti-rabbit immunoglobin-G-peroxidase conjugate(Bio-Rad), diluted 1:8,000 in 1×TBS and 1% BSA. Excess antibody was washed away with deionized water, 1×TBS and 0.05% Tween 20. Visualization of color was carried out using the peroxidase substrate 4-chloro-1-napthol(Sigma) for 20 minutes.

RESULTS AND DISCUSSION

In order to determine whether the root of avocado contain wounding-induced P450 protein, the root was collected and wounded by slicing with a razor blade and were incubated for 0, 3, 6, 24, and 72 hours, respectively, on wet papers in plastic bags to prevent dessication prior to protein extraction. Microsomal proteins from roots at various stages of wounding were separated by SDS-PAGE and were subjected to Western blot analysis(Fig. 1).

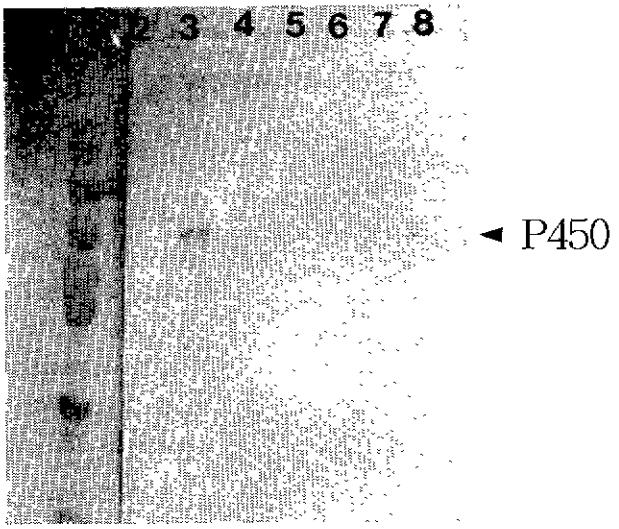

Fig. 1. Western-blot analysis of wounding-induced cytochrome P450 proteins from the root tissues of avocado, *Persea americana*, aged in the plastic bags.
1. Protein molecular weight marker, 2: Dye, 3: Microsomal protein from ripe avocado fruit tissues as a control, 4: Microsomal protein from root tissues aged in the plastic bag for 0hr. after wounding, 5: 3hr. root tissues, 6: 6hr. root tissues, 7: 24hr. root tissues, 8: 72hr. root tissues.

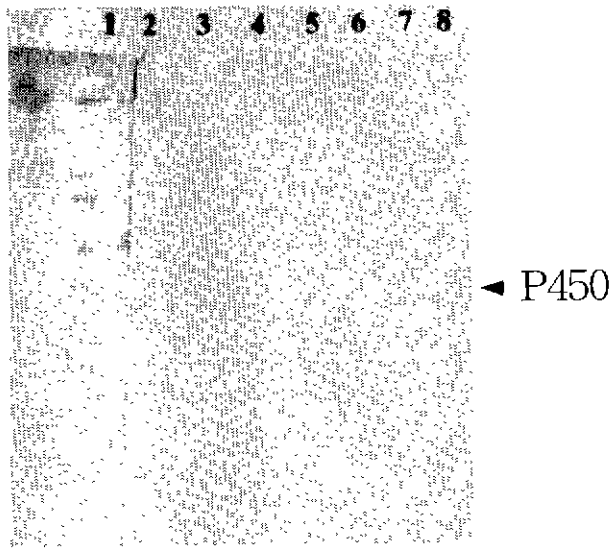

Fig. 2. Western-blot analysis of cytochrome P-450 proteins derived from wounded root tissues of *Persea americana* aged in 0.4M mannitol, and in the solution of protoplast preparation.
1: Protein molecular weight marker, 2: Dye, 3: Microsomal protein from ripe avocado fruit tissue as a control, 4: Microsomal protein from wounded root tissues aged in the mannitol solution for 0hr. after wounding, 5: 24hr. mannitol root tissues, 6: 48hr. mannitol root tissues, 7: 72hr. mannitol root tissues, 8: Microsomal protein from root tissues aged in the solution of protoplast preparation.

High levels of cytochrome P450 protein activity have been recovered from ripen avocado fruit mesocarp of the Hass variety(20,24), and ripe fruit mesocarp of five varieties (Bacon, Zutano, Macarthur, Pinkerton, Fuerte and Hass) contained similar levels of P450 protein, though the Hass variety contained the most(16). The microsomal fraction from ripe fruit of Hass avocado which contains detectable 48kD P450 antigen was loaded in lane 3 and resolved by SDS-PAGE(Fig. 1, lane 3) for a marker of root P450 proteins.

The microsomal fractions from root placed in the bag for 0 hour and 3 hours after wounding contained no detectable 48kD P450 antigens(Fig. 1, lane 4, 5). In addition, the microsomal proteins derived from intact roots were analyzed electrophoretically for a control group, but didn't produce P450 proteins. A 48kD antigen comigrating with purified native avocado P450 starts to accumulate within 6 hours after cutting and gradually increased throughout the 72 hours of wounding in sealed baggies as shown in lane 6, 7 and 8 of Fig. 1. Bozak et al.(20) reported that P450 began to accumulate within 24 hours of propylene treatment and steadily increased until the 4th day of propylene-induced ripening of avocado fruit. Cytochrome P450s are also heavily involved in the response of plants to stress which include wounding, pathogens, elicitors and xenobiotics(2). The CYP71A1 protein plays an undetermined role in modification of the monoterpene content during ripening(2,16). Across-reacting antigen was seen in all five varieties of ripe avocado fruit, indicating a shared biochemical alteration during ripening. This indicates CYP71A1 does not catalyze an enzymatic step unique to a single species, such as that which causes blackening of the skin found only in the Hass variety(16). These results demonstrate that CYP71A1 plays a role in not only ripening of fruit tissues but also wounding of root tissues.

To determine that P450 protein accumulate in response to mannitol treatment of slicing wounded roots, microsomal fractions derived from roots at various stages of aging in 0.4M mannitol solution were also analyzed electrophoretically with microsomal fraction of fruit(lane 3) as a positive control, and were subjected to Western blot analysis(Fig. 2). All of the microsomal fractions from tissues in mannitol did not cause detectable 48kD P450 antigen from the 0 hour to the 72 hours in the solution as shown in lane 4-7 of Fig. 2. This result indicates that

CYP71A1 expression is inhibited in the liquid state by preventing a biosynthesis of gaseous molecules which causes ripening. We now know ripening is a developmental process that occurs through activation of specific genes by gaseous "ripening" hormone, ethylene(16). In fact, CYP 71A1 is induced during increased ethylene biosynthesis triggered by wounding in avocado and in response to ethylene treatment of unripe fruit(23). Therefore ethylene is required to activate CYP71A1 in the root tissue of avocado during wounding.

In order to help elucidate the role of CYP71A1 during wounding in the liquid state, it has been assayed for slicing root which treated with digestion solution containing 0.4M mannitol, 0.5% cellulysin and macerase for 24 hours. Microsomal protein induced P450 protein after only 1 day even in the liquid state as shown in lane 8 of Fig. 2. This suggest that a softening of root tissues by cellulysin and macerase to make protoplast, involved in an activation CYP71A1. In the protoplast preparation. digestion solutions contain the cellulysin and macerase are responsible for breaking down tough cell walls which result in tissue softening. One aspect of ripening is the softening of mesocarp tissues, a consequence of cell wall degradation. It has been observed that protoplast isolated from climacteric fruit of avocado synthesized ethylene at a higher rate than cells obtained from unripe fruit, that a stimulation of ethylene production during protoplast isolation attributes to a protein factor in cellulysin preparation(21). In this experiment, there appears to be an interaction between ripening and softening that affects the synthesis of P450.

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