

DETECTION OF RIPENING-INDUCED CYTOCHROME P-450 PROTEIN IN VARIOUS VARIETIES OF AVOCADO (*Persea americana*, Mill.)

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The ripe Hass avocado contains one of the highest levels of the cytochrome P450 protein found in the plant kingdom. Therefore it has been used to prepare cytochrome P450 (P450) protein for analysis, and also provided the first cytochrome P450 gene to be cloned and sequenced from a plant source (Bozak et al. 1990). 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. All of this work had been performed with the Hass variety. To determine whether other varieties of avocado grown on either the Cal Poly Pomona campus or at the Pine Tree Research Station (Santa Paula CA) contain similar levels of P450 protein, ripe tissue of the varieties Bacon, Zutano, Macarthur, Pinkerton, Fuerte, and Hass were subjected to Western Blot analysis. We determined that all six varieties contain similar levels of P450 protein, though the Hass variety contains the most. This indicates that CYP71A1 plays a role during ripening common to all these varieties. Therefore, it does not contribute to any characteristic of ripening which is unique to a single variety, such as flavor or skin color changes.

Introduction

High levels of cytochrome P450 protein and activity has been recovered from ripe avocado fruit mesocarp of the Hass variety (Bozak et al., 1992; O'Keefe and Leto, 1989). This is the most commonly grown variety of avocado in America. Of the approximately 170,000 tons of avocado that were harvested in 1996, the Hass variety accounted for 145,000 tons, or about 85% (California Avocado Commission annual report). As the mRNA encoding the cytochrome P450 protein is relatively abundant in ripe avocado, the cDNA was therefore easily isolated and sequenced (Bozak et al. 1990). The nucleotide sequence shared less than 40% homology with any other previously characterized P450 so it was placed in a new gene family, designated *cyp71A1*, and the encoded protein CYP71A1.

The cytochrome P450 protein family contains at least 400 presently characterized members, so is described as a "super-family". P450 protein has been isolated from bacteria, fungi, yeast, insects, mammals and undoubtedly is present in all living organisms. The CYP71A1 protein and *cyp71A1* gene from avocado were the first to be characterized from a plant source (Bozak et al. 1990). Since 1990, at least 30 other plant P450 sequences have appeared in the literature (reviewed in Bolwell et al. 1994).

P450 proteins act as mono-oxygenase enzymes, and the substrate specificity of the CYP71A1 protein has been explored through substrate binding assays using a spectroscopic diagnosis termed a type 1 binding shift. This has revealed that CYP71A1 both in ripe avocado and heterologously expressed in yeast binds most tightly to the monoterpene class of plant compound. This class of compounds comprises the "essential oils" of plants, and often contributes to the flavor of a plant tissue. Further studies to determine the *in vivo* substrate of CYP71A1 through analysis of biochemistry through HPLC analysis have been unsuccessful.

In order to help elucidate the role of CYP71A1 during avocado ripening, it has been assayed for in varieties other than Hass. In particular, we have examined whether P450

protein exists in the ripe mesocarp of six avocado varieties grown on the California State Polytechnic University campus located in Pomona.

This has initiated a collaborative effort between the College of Science and the College of Agriculture typical of current studies in plant physiology/molecular biology. Physiological processes that have been observed for decades are now being dissected and precisely defined through molecular analysis of protein-induced biochemical changes. For instance we now know ripening is a developmental process that occurs through activation of specific genes by the gaseous "ripening" hormone, ethylene (Christoffersen et al. 1984). These specific ripening-induced genes have been cloned and sequenced which has revealed their identity and probable role during ripening. Ripening-induced cellulase and poly-galacturonase are responsible for breaking down tough cell walls which results in fruit "softening". In fruit which get sweeter as they ripen, sugar producing and modifying enzymes are activated at the onset of ripening. The role of the ripening-induced cytochrome P450 from avocado is still undetermined, so this study was initiated to determine if all varieties of avocado contain it in the ripe state, so we could begin correlating its presence or absence with particular oils or flavors unique to each variety.

Materials and Methods

Plant Material Fruit were collected at or near maturity on either the Cal Poly Pomona University campus (Hass, Fuerte, Pinkerton) or at the Pine tree research station (Bacon, Zutano, MacArthur). Fruit was either immediately frozen (unripe) or allowed to ripen at room temperature (ripe). The ripe and unripe avocado mesocarp was cut up into small pieces and frozen by liquid nitrogen. All the fruit was then stored in a -70 degree Celsius freezer until needed for protein isolation procedures.

Microsomal Protein Preparation The method followed is described in O'Keefe and Leto (1989). Approximately 20 grams of avocado mesocarp from each variety were diced into small pieces using a razor blade, placed into a falcon tube and 40mls. of cold microsomal prep. buffer (0.1 M MOPS/NAOH pH 7.0, 0.3 M Sorbitol, 5mM EDTA, 0.1% BSA, 1mM PMSF, 5ug/ml Leupeptin, and 5ug/ml Pepstatin) were added. This was polytroned on the highest setting for about one minute, or until solubilized. The mixture was centrifuged at 20,000g for 30 minutes. The supernatant was transferred to ultracentrifuge tubes and spun at 100,000g for 60 minutes. This fraction contains all microsomal proteins which includes the cytochrome P450 proteins. The pellet was resuspended in 0.5 mls. of resuspension buffer (50% glycerol, and 0.1M MOPS/NAOH pH 7.0) and frozen until analysis.

Immunoblots Protein extracts were separated by 10% SDS - acrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting in transfer buffer (20mM Tris-base, 150mM glycine, 0.1% SDS, and 20% methanol) overnight at 20 Volts. After transfer the nitrocellulose was saturated with 1% bovine serum albumin for 2hr. at room temperature and then incubated for 2 hr. with primary antibody (rabbit anti CYP71A1) diluted to 1:2,000 in 1X TBS and 1% BSA. Excess antibody was washed away with Di H₂O and then 1X TBS and 0.05% Tween 20. The blot was then incubated for two hours with secondary antibody (Goat anti rabbit IgG-peroxidase conjugate) diluted 1:8,000 in 1XTBS and 1% BSA. Excess antibody was washed away with Di H₂O and 1X TBS and 0.05% Tween 20. Visualization of color was carried out using the peroxidase substrate 4-chloro-naphthol for 20 minutes.

Results and Discussion

In order to determine if various varieties of avocado contain ripening-induced P450 protein, the mesocarp tissue was harvested from unripe and ripe fruit. The microsomal fraction

(which contains P450 protein if present) was prepared, and immunoblot analysis to detect P450 protein performed. Analysis of the microsomal fractions of the varieties Bacon, Fuerte, Hass, Macarthur, Pinkerton, and Zutano reveals a 48,000 Dalton cytochrome P450 antigen present in ripe fruit which is absent in unripe fruit of all varieties (Figure 1). Of these varieties, the Pinkerton variety appears to contain the lowest levels (lane 2), and the Hass variety the highest levels (lane 12). The varieties which contain intermediate levels of P450 protein in the ripe state are: Fuerte (lane 4), Bacon (lane 6), Zutano (lane 8), and Macarthur (lane 10). Unripe mesocarp tissue from each variety contains no appreciable amount of P450 (lanes 1,3,5,7,9,11). Therefore, all varieties tested have a ripening-induced P450 that is at least homologous, and may be identical to CYP71A1. The cloning and sequence analysis of the genes encoding these P450 antigens would be necessary to reveal their degree of relatedness to CYP71A1.

The California State University at Pomona campus and Pine Tree Ranch grows at least six varieties of avocado: Bacon, Fuerte, Hass, Macarthur, Pinkerton, and Zutano. The exact inter-relatedness of these six varieties is currently unknown. The Hass variety has been used as a source of the cytochrome P450 gene *cyp71A1* and encoded protein. The CYP71A1 protein plays an undetermined role in modification of the monoterpene content during ripening. A cross-reacting antigen was seen in all five varieties, 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. The *in vivo* role of CYP71A1 is still unknown, but further investigation of the metabolic changes during ripening should lead to a better understanding of the role.

References

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Figure 1. Analysis of cytochrome P450 protein in various avocado varieties.

