

Postharvest Density Changes in Membranes from Ripening Avocado Fruits

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ABSTRACT. An investigation was performed with the goal of identifying, at the biochemical level, organelles that undergo postharvest change during ripening in avocado fruits (*Persea americana* Mill. cv. Hass). Membrane vesicles from mesocarp tissue at defined stages of ripening were fractionated by linear density gradient centrifugation and then assayed for characteristic marker activities. These measurements indicated that the Golgi and plasma membranes increased in buoyant density from 1.11 g-cm⁻³ to 1.14 g-cm⁻³ and from 1.15 g-cm⁻³ to 1.17 g-cm⁻³, respectively, during ripening. The endoplasmic reticulum exhibited a smaller increase in density, while thylakoids and mitochondrial membranes showed no shifts in density during ripening. These membrane changes were apparent at the peak (predicted from measurements of C₂H₄ production) of the respiratory curve that characterizes climacteric fruit ripening and were sustained for at least 6 days past the respiratory peak. Colorimetric lipid analyses of plasma membrane-enriched fractions indicated that the buoyant density increase during ripening was due, in part, to a decrease in the glycolipid : protein ratio. Electrophoretic analyses of fractions containing peak total activities of Golgi, thylakoids, or plasma membrane revealed several changes in the polypeptide banding patterns during ripening.

Avocados provide an excellent system for the study of fruit ripening. Avocados are climacteric fruit that exhibit increased respiratory activity and ripen in response to endogenously produced or exogenously applied C₂H₄ during ripening. The mature avocado fruit starts the ripening process following removal or abscission from the tree.

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Measurement of CO₂ and C₂H₄ production of avocado fruit allows for precise monitoring of developmental changes during fruit ripening (6).

Tucker and Laties (24), using exogenous C₂H₄ to induce avocado ripening, observed a correlation between the first portion of the climacteric respiratory rise and a general increase in polyadenylated mRNA and soluble protein concentrations. The remainder of the respiratory rise was correlated with a general decrease in polyadenylated mRNA and soluble protein concentrations along with specific expression of certain polyadenylated mRNAs and proteins, including cellulase (EC 3.2.1.4). Awad and Young (2) observed that levels of cellulase and polygalacturonase (EC 3.2.1.15) total activity increased during the latter portion of the climacteric rise and reached peak levels a few days following the climacteric respiratory peak, at which time the fruit became soft.

The occurrence of a respiratory climacteric and the enzymatic degradation of cell walls are both events that point to the likelihood of developmental changes in the membranes of ripening avocado fruits. Platt-Aloia and Thomson (20) reported ultrastructural changes in mitochondria (an increase in length of the tubular branching forms was observed) and endoplasmic reticulum (from a sheet or tubular form to a highly vesiculated form) during ripening. In addition, freeze-fracture evidence points to an increase in the number of intramembranous particles per unit surface area of the plasma membrane during ripening (20, 23). Motivated by these previous studies, we undertook a biochemical study of the membrane system of avocado mesocarp tissue. The goal of this work was to identify organelles that undergo changes in density during fruit ripening. The basis for an observed density increase of the plasma membrane during ripening was also examined.

Materials and Methods

FRUIT HARVEST AND RIPENING. 'Hass' avocado fruits were harvested from two trees on the Riverside campus of the Univ. of California at times varying from the beginning of March until early September. Because avocado fruits exhibit variation in the time interval between harvest and the climacteric peak of respiration (28), ripening was monitored for each fruit. The fruits were weighed and placed in individual respiratory chambers at 20°C within 1 hr of harvest. The production of CO₂ and C₂H₄ by each fruit was monitored as described by Eaks (6). Based on these measurements, three stages of ripening fruit were defined: 1) freshly picked fruit (preclimacteric); 2) fruit within 1 day of the climacteric respiratory peak, which occurred \approx 1 day after the peak in C₂H₄ production (climacteric); and 3) fruit at 4 to 6 days past the climacteric peak (postclimacteric).

MEMBRANE ISOLATION. Development of a procedure for isolating membrane fractions from the mesocarp of mature avocado fruit was complicated by the large amount of oil [up to 30% of fresh weight (14)] that is a characteristic constituent of this tissue. The major features of the isolation procedure included an early and relatively vigorous centrifugation step that removed the bulk of the oil as well as any intact chloroplasts and mitochondria. Removal of intact organelles to leave only microsomes derived from membrane fragments was desirable because the principal goal of the project was characterization of membrane density changes during ripening. Density changes observed with intact chloroplasts or mitochondria cannot be unambiguously attributed to membrane changes, since density changes in the chloroplast stroma or the

mitochondrial matrix might also occur during ripening. The second major feature of the isolation procedure was a centrifugation step involving a sucrose density gradient that separated the microsomal membranes on the basis of buoyant density.

The avocado membrane isolation procedure developed was a modification of the procedure described for corn roots (8). Avocado mesocarp was cut into pieces (=10 x 10 x 5 mm) that were transferred to grinding medium {0.25 M sucrose; 3 mM EDTA; 25 mM Tris-Mes [2-(N-morpholino)ethanesulfonic acid] pH 7.4; plus 2.5 mM dithiothreitol added just before use} at 4°C. The volume of grinding medium used was 5 ml per gram of tissue. Fruit slices were homogenized for 2 min in a Waring blender. Preclimacteric fruit homogenate was vacuum-filtered through two layers of filtering cloth (Miracloth; Behring Diagnostics, La Jolla, Calif.) to remove wall debris. Cell wall debris in climacteric and postclimacteric fruit homogenates had become emulsified and, as a result, was not filtered. The filtrate or homogenate was transferred to 250-ml bottles and centrifuged for 60 min at 13,000 x *g*. An aqueous supernatant was present above a pellet of cellular debris, including wall material, nuclei, whole mitochondria, and whole chloroplasts, but below a layer of oily material. This aqueous supernatant was separated and carefully filtered by gravity through two layers of cheesecloth, transferred to 67-ml bottles, and then centrifuged for 1 hr at 80,000 x *g*. The supernatant was discarded, and the pellet resuspended in grinding medium and centrifuged again at 80,000 x *g*. The pellet was resuspended in 2 ml of suspension medium (0.25 M sucrose; 1 mM Tris-Mes, pH 7.4; plus 1 mM dithiothreitol added just before use) and layered on top of a linear (10% to 45%, w/w) sucrose gradient (15). Centrifugation in a swinging bucket rotor was carried out at 82,500 x *g* for 24 hr. The linear gradients were fractionated using a density gradient fractionator at a flow rate of 0.75 ml·min⁻¹ with fractions collected at 2-min intervals.

ENZYME ASSAYS. Vanadate-sensitive adenosine 5'-triphosphatase (V₀⁴-sensitive ATPase, EC 3.6.1.3) was assayed as described in ref. 8. Latent inosine 5'-triphosphatase (IDPase) cytochrome *c* oxidase, and antimycin A-resistant NADH cytochrome *c* reductase activities all were assayed as described in ref. 10.

CHLOROPHYLL DETERMINATION. A 50-μl sample was added to 200 μl of suspension mix in a 1.8-ml microcentrifuge tube. Acetone (1 ml) was added, mixed, and spun for 5 min in a microcentrifuge. Samples were measured on a spectrophotometer for absorbances at 663 nm (*A*₆₆₃) and 645 nm (*A*₆₄₅) against an 80% (v/v) acetone blank. Chlorophyll in micrograms per milliliter was calculated as 8*A*₆₆₃ + 20*A*₆₄₅.

PROTEIN ANALYSIS. The Peterson modification of the Lowry procedure (19) was used for protein determinations.

LIPID ANALYSIS. Membrane samples were extracted in CHCl₃:CH₃OH (2:1) for 1 hr and then mixed with an equal volume of 0.18% (w/v) NaCl. The aqueous phase was removed and then one part CH₃OH and one part 0.18% NaCl were mixed with one part of the organic phase. The aqueous phase was again removed, and the organic phase was concentrated by evaporation under a stream of N₂.

Phospholipids were determined by the method of Bochner and Ames (4), and total sterols were determined by the method of Kates (12). Carbohydrate was determined by the anthrone method (1), with galactose as the standard.

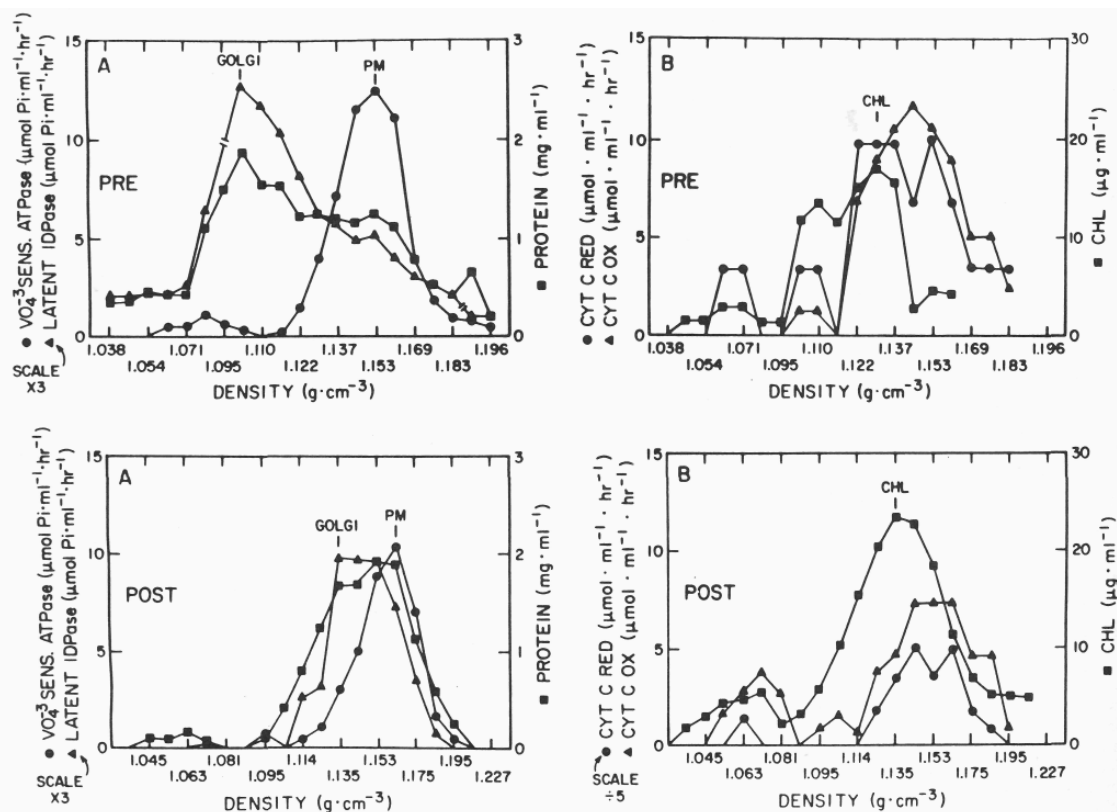


Fig. 1. Marker enzyme and protein determination of linear sucrose gradient fractions from preclimacteric (PRE) and postclimacteric (POST) mesocarp membranes. (A) Protein in mg/ml (■); IDPase (▲), and VO_4^{3-} -sensitive ATPase (●) in $\mu\text{mol Pi/ml per hr}$. (B) Cytochrome *c* oxidase (▲) and antimycin A-resistant NADH cytochrome *c* reductase (●) in $\mu\text{mol cytochrome } c \text{ oxidized or reduced/ml per hr}$; chlorophyll (■) in $\mu\text{g} \cdot \text{ml}^{-1}$. These activities are markers for the Golgi membrane (IDPase), plasma membrane (VO_4^{3-} -sensitive ATPase), mitochondrial inner membrane (cytochrome *c* oxidase), and endoplasmic reticulum (antimycin A-insensitive NADH cytochrome *c* reductase). Points marked GOLGI, CHL, and PM represent fractions used for the electrophoresis in Fig. 2. Data are from a single experiment that was typical of more than three experiments.

PROTEASE ANALYSIS. Agarose gels [1% (w/v) agarose; 0.1 M Tris-Mes, pH 7.5; 200 ($\mu\text{g} \cdot \text{ml}^{-1}$ casein)] were cast 0.75-mm thick onto a plastic support sheet (Gelbond from FMC Corporation, Rockland, Me.) according to the procedure of Gallagher et al. (7). Wells 3 mm in diameter were punched in the agarose layer and then loaded with samples 4 μl in volume and containing either 20 ng trypsin or 2 μg protein from plasma membrane-enriched gradient fractions. After diffusion was allowed to proceed for 24 hr at room temperature, the gels were fixed in 10% (w/v) trichloroacetic acid for 10 min, dehydrated in 95% (v/v) ethanol for 10 min, and then dried while still on the plastic support sheet. Gels were stained overnight in 0.025% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) CH_3OH , and 7% (v/v) acetic acid, and then destained in 40% CH_3OH and 7% acetic acid.

ELECTROPHORESIS. NaDodSO₄/polyacrylamide linear gradient (5% to 20%, w/v) gels were prepared and run as described (9, 13), except the resolving gel buffer pH was changed from 8.8 to 8.4, which increased resolution of the proteins (K.S. Dhugga, personal communication). Gel thickness was 0.75 mm, and loading was 25 μg of protein per lane. Gels were stained with Coomassie Brilliant Blue R-250, as described above.

IMMUNOLOGICAL BLOTTING. Transfer of proteins to nitrocellulose was by the procedure of Burnette (5). Detection with ^{125}I -labeled second antibody was by the procedure of Symington et al. (22).

Results and Discussion

Once avocado fruit mature on the tree, no further phenotypic changes are noticeable until harvest and the subsequent C_2H_4 induced genome expression. During this period between maturation and the onset of the respiratory climacteric, the avocado fruit appears to undergo no further changes in development.

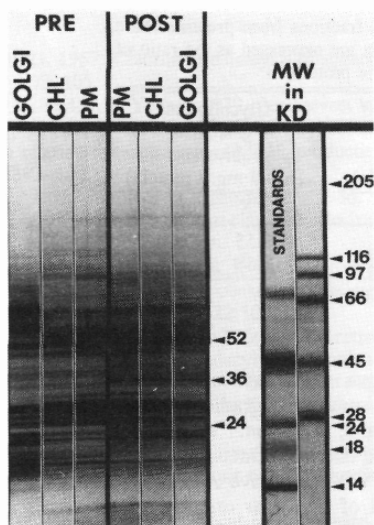


Fig. 2. Electrophoretic analysis of linear sucrose gradient fractions from preclimacteric (PRE) and postclimacteric (POST) avocado fruits. The fractions selected for analysis are marked as golgi (GOLGI), chloroplast thylakoid (CHL), or plasma membrane (PM) in Fig. 1. The gel was stained with Coomassie Brilliant Blue. Known molecular weights (MW) of protein standards are shown in kilodaltons (KD).

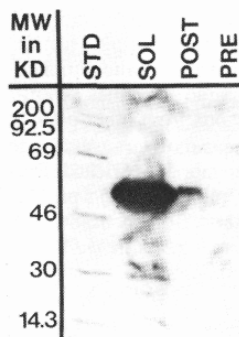


Fig. 3. Immunological blot analysis using anticellulase antibody to mark cellulase protein in electrophoretic separation of polypeptides from plasma membrane-enriched fractions from preclimacteric (PRE) and postclimacteric (POST) fruits. Also shown is a lane (SOL) containing soluble polypeptides from a postclimacteric fruit. The protein load of the SOL lane is substantially greater than the PRE and POST lanes. The known molecular weights (MW) of protein standards (STD) are shown in kilodaltons (KD).

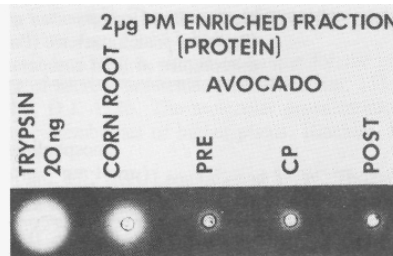


Fig. 4. Assay of protease activity of plasma membrane-enriched fractions. The diffusion plate assay was performed as described by Gallagher et al. (7). Wells (3 mm in diameter \times 0.75 mm) were loaded with 20 ng of trypsin or with 2 μg of protein from plasma membrane (PM) enriched fractions from corn roots or from avocado mesocarp at the preclimacteric (PRE), climacteric (CP), or postclimacteric (POST) stages of ripening. Protease activity is indicated by the appearance of an unstained "halo" around the well.

When membrane vesicles from ripening avocado fruits were separated on linear sucrose density gradients, the banding patterns from climacteric and postclimacteric fruits revealed several buoyant density shifts relative to the banding pattern from preclimacteric fruit (Fig. 1). The most prominent of these changes included a shift in the position of the peak protein concentration from 1.11 to 1.15 $\text{g}\cdot\text{cm}^{-3}$, a shift in the Golgi marker (latent IDPase) from 1.11 to 1.14 $\text{g}\cdot\text{cm}^{-3}$, and a shift in the plasma membrane marker (V_0^{3-} -sensitive ATPase) from 1.15 to 1.17 $\text{g}\cdot\text{cm}^{-3}$. The endoplasmic reticulum marker (antimycin A-insensitive NADH cytochrome *c* reductase, EC 1.6.99.3) showed a slight overall shift to greater buoyant density during ripening, while the mitochondrial inner membrane marker (cytochrome *c* oxidase, EC 1.9.3.1) and thylakoid marker (chlorophyll) both remained at fixed densities.

Table 1. Lipid composition of plasma membrane-enriched fractions from preclimacteric (Pre) and postclimacteric (Post) avocado fruits. The results are expressed as the ratio of micromoles of lipid component per milligram of membrane protein.^z

Stage of ripening	Phospholipid ($\mu\text{mol}\cdot\text{mg}^{-1}$ protein)		Sterol ($\mu\text{mol}\cdot\text{mg}^{-1}$ protein)		Glycolipid as galactose equivalents ($\mu\text{mol}\cdot\text{mg}^{-1}$ protein)	
	\bar{X}	SE	\bar{X}	SE	\bar{X}	SE
	Pre	0.20 \pm 0.02	0.33 \pm 0.05		0.55 \pm 0.04	
Post	0.22 \pm 0.03		0.30 \pm 0.04		0.38 \pm 0.01	

^zEach mean is based on three trials; SE = standard error of the mean.

These increases in membrane density take place by the time the climacteric curve reaches its peak and are then maintained for at least a week (data not shown). Changes in avocado plasma membrane and Golgi membrane density occur during the interval in which cellulase (3) and presumably other wall-degrading enzymes are processed and transported through the endomembrane system. This observation adds weight to the suggestion by Poole et al. (21) that increases in plasma membrane density may be related to increased metabolic activity.

Electrophoretic analyses of polypeptide complement were performed for membrane vesicles from the two organelles, plasma membrane and Golgi, that exhibited the greatest buoyant density shifts during ripening (Fig. 1). This analysis was also performed for the thylakoid fraction, which does not shift during ripening, but is a likely contaminant of the plasma membrane and Golgi fractions. The results for preclimacteric and postclimacteric fruits are compared in Fig. 2, where numerous polypeptide changes are evident. Examples of plasma membrane bands that increase in relative abundance during ripening appear at 36 and 24 kDa. These bands are likely plasma membrane polypeptides, because their relative abundances in the plasma membrane fraction were greater than in the thylakoid and Golgi fractions. Several bands show little, if any, change in relative abundance between plasma membrane, thylakoid, and Golgi fractions. Such bands include a 52-kDa band that appears during ripening and was shown to include cellulase through immunological blot analysis of plasma membrane-enriched fractions with anticellulase antibody (Fig. 3).

Protease activity has been shown to confound the interpretation of electrophoretic results (7). The protease activity (Fig. 4) of avocado plasma membrane fractions corresponding to the region of the gradient at sucrose concentrations > 34% for preclimacteric and 38% for postclimacteric (Fig. 1) was minimal compared to similarly enriched plasma membrane fractions from corn root (2 μg of protein) or to the activity of trypsin (0.02 μg of purified protein). Chymostatin and phenylmethylsulfonyl fluoride, reagents found to inhibit corn root proteases (7), had no detectable effect on the low level of protease activity in the plasma membrane-enriched fractions from avocado (results not shown). No changes in protease activity were detectable during avocado ripening (Fig. 4). This result implies that differential protease activity during ripening was not responsible for the plasma membrane density increase observed.

Further evidence regarding the basis of the observed postharvest changes in plasma membrane density was gathered through experiments on density gradient fractions that were enriched in this membrane. Additional marker analysis data (not shown) demonstrated that these fractions were highly enriched in plasma membrane (VO_4^{3-} -sensitive ATPase specific activity), and depleted in all of the other organelles represented in Fig. 1. A high level of enrichment in the plasma membrane fractions was corroborated by electron microscopy with phosphotungstic acid-chromic acid staining (17) and stereology (26), which indicated 67% to 70% plasma membrane purity in the density gradient fractions from each stage of ripening (data not shown).

The basis of the shift in buoyant density of the plasma membrane during ripening (Fig. 1) was investigated through measurements of the lipid:protein ratio in the plasma membrane-enriched fractions. The results (Table 1) showed that neither the phospholipid nor sterol:protein ratios changed significantly during ripening. The phospholipid (μmol): protein (mg) ratio (≈ 0.2) for the plasma membrane-enriched fractions from avocado was low compared to values reported for plasma membrane-enriched fractions from other plant species (11, 25, 27).

Quantitation of the carbohydrate associated with lipid extracts from the plasma membrane-enriched fractions from avocado showed that the glycolipid (μmol) : protein (mg) ratio fell from 0.55 for preclimacteric fruit to 0.38 for postclimacteric fruit. Since the plasma membrane-enriched fractions contained a measurable amount of chlorophyll, it was important to estimate chloroplast galactolipid (16) contamination in these fractions. This estimate was obtained by measuring the chlorophyll content in the plasma membrane-enriched fractions and then using a galactolipid:chlorophyll ratio of 8 for chlorotic thylakoids (18) to calculate the corresponding level of thylakoid galactolipids. From the results thus obtained, we estimate that only 0.024 (μmol of sugar/mg of protein) of the 0.17 μmol of sugar/ mg of protein decrease between the preclimacteric and postclimacteric plasma membrane fractions (Table 1) could be due to contamination by thylakoid galactolipids. This indicates that the observed increase in buoyant density of the plasma membrane-enriched fractions during ripening (Fig. 1) is due, in part, to a decrease in the glycolipid:protein ratio of this membrane. The lipid data presented are preliminary and will be an interesting topic for further investigation if plasma membrane fractions of higher purity can be isolated.

Membrane density shifts along with changes in polypeptide and lipid patterns during ripening in avocado mesocarp provide additional evidence of the magnitude of developmental change occurring during this period. Further analysis of the avocado membrane system might help to illuminate topics of interest such as the basis of chilling injury, the pathway of site-directed protein transport, and the mechanism of ripening induction.

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