

Suppression of Cellulase and Polygalacturonase and Induction of Alcohol Dehydrogenase Isoenzymes in Avocado Fruit Mesocarp Subjected to Low Oxygen Stress

Angelos K. Kanellis*, Theophanes Solomos, and Kalliopi A. Roubelakis-Angelakis

Institute of Molecular Biology and Biotechnology, P. O. Box 1527, 711 10 Heraklion, FO.R.T.H., Greece (A.K.K.); Department of Horticulture, University of Maryland, College Park, Maryland 20742 (T.S.); and Department of Biology, University of Crete, 714 09 Heraklion, Greece (K.A.R.A.)

ABSTRACT

Expression of polygalacturonase and cellulase, two hydrolytic enzymes of avocado (*Persea americana*, cv Hass) fruit which are synthesized *de novo* during ripening, and alcohol dehydrogenase, a known anaerobic protein, were studied under different O₂ regimes. Low O₂ concentrations (2.5–5.5%) diminished the accumulation of polygalacturonase and cellulase proteins and the expression of their isoenzymes. This pattern of change in cellulase protein was also reflected in the steady-state amount of its mRNA. In contrast, 7.5 and 10% O₂ did not alter the changes observed in fruits ripened in air. On the other hand, alcohol dehydrogenase was induced in 2.5, 3.5, and 5.5% O₂ but not in 7.5 or 10% O₂. The recovery from the hypoxic stress upon returning the fruits back to air for 24 hours, was also a function of O₂ tensions under which the fruits were kept. Thus, the synthesis of polygalacturonase and cellulase was directly related to O₂ levels, while the activity of the isoenzymes of alcohol dehydrogenase was inversely related to O₂ levels. The results indicate that hypoxia exerts both negative and positive effects on the expression of certain genes and that these effects are initiated at the same levels of O₂.

Ripening of climacteric fruits is the final stage of fruit development whose initiation and progress requires the presence of ethylene (5, 30). Avocado ripening is associated with increases in the activities of hydrolytic enzymes such as polygalacturonase and cellulase (1, 15, 19). This increase in activities resulted from the regulated accumulation of their mRNAs (8, 16, 26; GG Laties, personal communication). It is well established that oxygen levels below those in air retard the rate of ripening and softening of climacteric fruits (12, 14, 18, 20). The precise mode of action of low oxygen in delaying fruit ripening is not clear. However, since previous evidence indicates that oxygen is required for the synthesis and the action of ethylene (6), it is a reasonable assumption that the retarding effects of low oxygen on fruit ripening reflect a diminution of ethylene synthesis and action. This is also supported by the fact that a number of metabolic events associated with fruit maturation and ripening which are not regulated by ethylene are also unaffected by low oxygen environments (13).

It should be stressed, however, that the physiological rami-

fications of low oxygen action are broader than fruit ripening. For instance, hypoxic conditions inhibited the accumulation of RNA, protein, and DNA synthesis associated with wounding of potato tubers (7). In a number of plant tissues, anoxia causes marked alterations in the profile of proteins (22–24), stability of mRNA species (11), and gene expression (10, 11, 22, 24).

In view of the above evidence and the limited experimental data concerning the mode of action of low oxygen on fruit ripening, we have initiated studies aimed at investigating the biochemical and molecular aspects of low oxygen action on fruit ripening. Our previous results have shown that transferring initiated avocado fruits to 2.5% oxygen suppresses the activity, immunoreactive protein, and abundance of mRNA of cellulase (15, 16). The above treatment also produces an alteration in the profile of avocado proteins, which involves suppression, enhancement, and the induction of new polypeptides (15).

In the present work, we have attempted to discern at which oxygen concentrations the suppression of specific ripening proteins occurs, and, at the same time, which oxygen levels induce new protein synthesis and especially the expression of the isoenzymes of alcohol dehydrogenase, a known anaerobic protein. The results show that the action of low oxygen on fruit ripening is complex, involving both suppression of enzymes which are induced in the course of ripening and the induction of new ones. Further, the range of oxygen levels which suppress the appearance of ripening enzymes is similar to those that induce the synthesis of the anaerobic isoenzymes of alcohol dehydrogenase.

MATERIALS AND METHODS

Plant Material

Preclimacteric avocado fruits (*Persea americana*, cv Hass) were purchased from the local wholesale market. Individual fruits were enclosed in glass jars of appropriate size with minimum void space and then placed at 20°C under a steady flow of humidified air. The flow rates were selected to ensure that CO₂ accumulation did not exceed 0.3%. Carbon dioxide and ethylene production were measured daily by a gas chromatograph equipped with either thermal conductivity (CO₂) or flame ionization (ethylene) detectors. Initially, CO₂ and ethylene levels were measured to ensure the stage of ripeness

of the fruits. Ripening of the fruits was initiated by introducing 10 $\mu\text{L/L}$ ethylene into the air stream and the respiration rates were monitored. When the rate of CO_2 evolution approached its climacteric peak, the exogenous supply of ethylene was discontinued. At this point, a set of three fruits was transferred to 2.5, 3.5, 5.5, 7.5, and 10% O_2 . The fruits incubated in air alone served as the controls. Fruits remained for 3 d in the modified atmospheres following which they were returned to air for one day.

To avoid the inherent variability in the rate of ripening among individual fruits, plugs of pericarp tissue were removed with a cork borer from the individual fruits at each sampling time. The resulting holes were covered with lanolin and the fruits returned immediately to the respiratory jars. This sampling procedure permitted the monitoring of the physiological, biochemical and molecular changes that occurred in the course of ripening of a single fruit thus generating a better correlation between ripening and underlying molecular events (1, 15, 16).

Four samplings were carried out as follows: (a) preclimacteric stage; (b) upon the discontinuation of exogenous ethylene, which coincided with the transfer of some of the fruits to the modified atmospheres; (c) 3 d following the transfer of fruits from air-ethylene atmosphere to modified atmospheres; and (d) 1 d after returning the fruits from low O_2 to air. Immediately upon removal, the sampled plugs were dropped immediately into liquid N_2 in a mortar and ground to a fine powder with a pestle and lyophilized in a freeze drier. Subsequently, the powder was stored in airtight vials at -70°C until analyzed.

Total Protein Extraction and SDS-PAGE

Frozen lyophilized tissue powder (0.3 g) was thawed in 3 mL of buffer (15) containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM MgSO_4 , 1 mM EDTA, 5 mM β -mercaptoethanol, 0.5 mM PMSF, 10 μM leupeptin, and 10% (v/v) glycerol. The mixture was allowed to stand on ice for 15 min with occasional stirring and then centrifuged at 20,000g for 20 min. The supernatant was passed through Miracloth (Calbiochem) and used for fractionation by SDS-PAGE using 10 to 18% gradient polyacrylamide gels and Laemmli buffer system (17). Following electrophoresis, the gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid and destained in 20% (v/v) methanol and 7% (v/v) acetic acid.

Native Isoelectric Focusing

Native IEF¹ was performed in a vertical minigel system of Bio-Rad as described previously (21). The gels (1.5 mm thick) were prepared from the following mixture: 5.05 mL water, 1.8 mL acrylamide mixture (30% [w/v] acrylamide, 0.8% [w/v] bis-acrylamide), 1.8 mL 50% (v/v) glycerol, and 0.45 mL ampholyte mixture consisting of 3 volumes of pH range 5 to 7 and 1 volume of pH range 4 to 6. Gels were polymerized

with the addition of 40 μL of 10% (w/v) ammonium persulfate and 10 μL TEMED. The cathode solution was 25 mM NaOH and the anode solution 20 mM acetic acid. Protein samples (30 μg) were mixed with 4% (v/v) ampholyte of the same pH range used for the gel preparation and electrophoresed for 1.5 h at 180 V constant and then for an additional 1.5 h at 300 V constant. The temperature of electrophoresis was kept low by holding the mini-gel in ice.

Activity Staining

Following IEF the gels were incubated in 100 mM Na-acetate pH 5 for 10 min. Cellulase and PG activity was stained according to the overlay technique (2). Thin CMC-agarose or pectate-agarose overlay gels for enzyme detection were cast between two glass plates separated by 0.5 mm spacers. On one of these plates, a gel support film for agarose (LKB) was placed so that the agarose overlay was affixed permanently. The agarose solution contained 0.8% (w/v) agarose, 100 mM acetic acid (pH 5), and either 0.1% CMC (w/v) for cellulase or 0.1% (w/v) polygalacturonic acid for PG.

For simultaneous detection of cellulase and PG, IEF gels were sandwiched between a CMC-containing agarose gel-bond and a polygalacturonic acid-containing gel-bond and incubated at 30°C for 1 to 4 h depending on the specific activities of the enzyme(s). After incubation, gels were separated and each one was developed for enzyme activities. To visualize cellulase, agarose-gel-bond sheets were stained in 1% (w/v) Congo red solution for 20 to 30 min and then destained in 1 M NaCl until clear bands (indicating cellulase activity) in red background were obtained. For photographic purposes (better contrast), the agarose overlays were rinsed in 5% acetic acid which turned the red background into dark blue.

To detect PG, agarose-pectic acid overlays were incubated in 1% (w/v) methylene blue for 1 h and destained in water until clear bands were evident in blue background.

ADH: Extraction and PAGE

ADH was extracted from lyophilized frozen tissue (0.3 g) by homogenizing in 100 mM Tris-HCl buffer (pH 8) containing 10 mM DTT, 0.5 mM PMSF, 10 μM leupeptin, and 10% (v/v) glycerol. Native slab PAGE was performed according to Laemmli (17) except that the SDS was omitted and the running buffer was 12.5 mM Tris-96 mM glycine. Gels were stained for ADH activity as previously described (28).

Protein Gel Blotting

Electrophoretic blotting of SDS and IEF gels was carried out in 12.5 mM Tris-96 mM glycine buffer. Immunological detection methods were as described (16). Biotinylated proteins were used as mol wt standard markers (9).

RNA Analysis

Total RNA extraction, electrophoresis, blotting, and hybridization with cellulase probe pAV363 (27) were performed as described elsewhere (16).

¹ Abbreviations: IEF, isoelectric focusing; TEMED, *N,N,N',N'*-tetramethylethylenediamine; CMC, carboxymethylcellulose; PG, polygalacturonase; ADH, alcohol dehydrogenase.

Protein Determination

Protein concentration was measured by the method of Bradford (4) using BSA as a standard.

RESULTS

Respiration

The results of Figure 1 show that fruits ripened in air, after a 24 h ethylene treatment, showed a typical climacteric rise in respiration followed by a decline. Low O₂ atmospheres decreased the rate of CO₂ evolution and this decrease was inversely related to the O₂ concentrations used. Thus, even 10% O₂ produced a decrease in the rate of respiration. The results also indicated that fruits kept in 2.5, 3.5, and 5.5% O₂ sustained aerobic respiration as was evidenced by the constant rate of CO₂ production during the holding period (Fig. 1), which is in agreement with previous results (3, 15). It should be mentioned that increased CO₂ production rates in low O₂ environment is an indication of anaerobic respiration, a phenomenon known as the "Pasteur effect."

Total Proteins

It has been previously shown that 2.5% O₂ not only suppressed the induction of new polypeptides associated with normal ripening but also induced the accumulation of some others (15). Examination of Figure 2 reveals two interesting results. First, the oxygen concentrations which produced the above mentioned changes in polypeptides in avocado fruits were 2.5, 3.5, and 5.5%. For example, the appearance of polypeptides with molecular mass of 32, 28.8, and 14 kD which increased during ripening was suppressed in 2.5, 3.5, and 5.5% O₂ but not in 7.5 or 10% O₂ (Fig. 2). Similarly, the intensity of 54.4 kD, the polypeptide which was present during the first days in air, was stronger in 2.5, 3.5, and 5.5% O₂

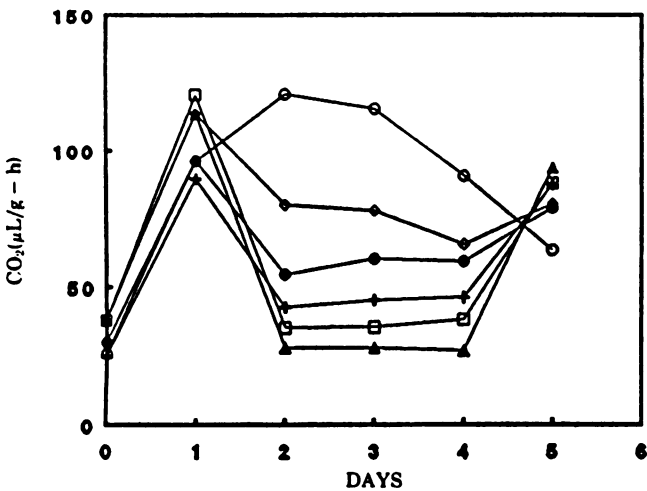


Figure 1. Effect of hypoxia on respiration. Avocado fruits were treated with 10 µL/L ethylene until the respiration rates approached the climacteric peak, and then were transferred to 2.5% O₂ (▲), 3.5% O₂ (□), 5.5% O₂ (+), 7.5% O₂ (●), 10% O₂ (◇), and air (○) for 3 d. On d 4 the fruits were sampled and then transferred back to air for 24 h.

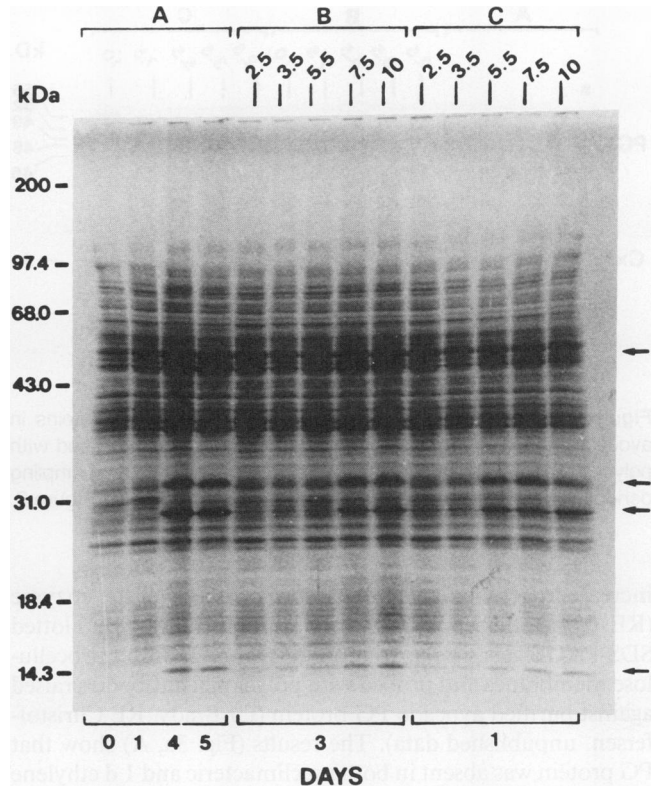


Figure 2. Avocado fruit polypeptide profile changes during ripening in air and under hypoxic conditions. Total proteins were extracted and analyzed by SDS-PAGE from preclimacteric avocado fruits (d 0), ethylene treated fruits (d 1), air ripened fruits (d 4 and d 5) (A) and those held in 2.5, 3.5, 5.5, 7.5, and 10% O₂ for 3 d (B). Panel C shows the fruits which were previously held in low O₂ for 3 d and then were transferred to air and sampled after 1 d.

than in 7.5 and 10% O₂ or air. Second, upon return of fruits to air, the recovery from low O₂ stress is a function of oxygen concentrations. That is, upon transferring the treated fruits for 1 d from low oxygen to air, the accumulation of polypeptides with molecular mass of 31, 28.8, and 14 kD, which was suppressed in 2.5, 3.5, and 5.5% O₂, was commenced only in fruits kept in 5.5% O₂ but not in those held in the other two oxygen concentrations. Furthermore, Figure 2 shows that the pattern of polypeptides of initiated avocado fruits treated with 7.5 or 10% O₂ was similar to that in air, a sign that the "system" is saturated at levels of oxygen at or higher than 7.5% O₂.

Effect of Low O₂ on Total PG and Cellulase Protein

In view of the evidence that low oxygen levels altered the pattern of total polypeptides (Fig. 2) in a manner indicating a relationship between protein synthesis and oxygen concentrations, we focused our study on the effect of low oxygen on the synthesis of specific proteins which were previously shown either to be suppressed (cellulase and PG) (15, 16) or induced (ADH) (11, 22, 24) by low O₂.

It has been previously shown that during avocado ripening there is a dramatic increase in PG activity (1, 15, 19). This

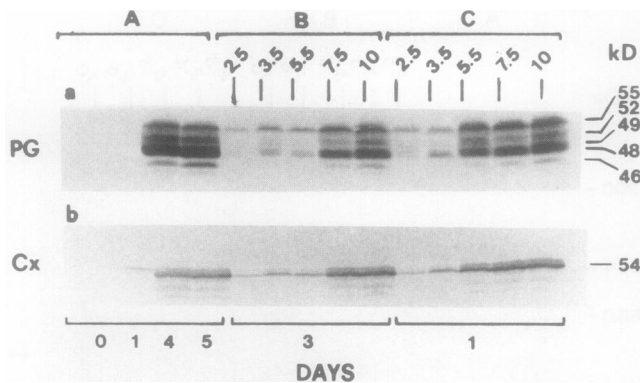


Figure 3. Suppression by low O_2 of PG and cellulase proteins in avocado fruits. Blots of total proteins ($30 \mu\text{g}/\text{lane}$) were probed with polyclonal antibodies raised against PG (a) and cellulase (b). Sampling periods were the same as those described in the legend to Figure 2.

increase in activity is due to *de novo* synthesis of the enzyme (RE Christoffersen, personal communication). We blotted SDS-PAGE gels similar to those of Figure 2, onto nitrocellulose membranes and probed with polyclonal antibodies raised against purified avocado PG protein (CJ Brady, RE Christoffersen, unpublished data). The results (Fig. 3a, A) show that PG protein was absent in both preclimacteric and 1 d ethylene pretreated fruits, whereas it accumulated in large amounts 3 d after the application of ethylene. PG antiserum appears to react with a number of PG polypeptides with a molecular mass of 55, 52, 49, 48, and 46 kD which increase during ripening. The exact relationship of these polypeptides is not known. In contrast to what was observed in PG expression, preclimacteric avocado fruits contained trace amounts of immunoreactive cellulase which increased after 24 h in 10 $\mu\text{L}/\text{L}$ ethylene and reached high levels in fruits ripened in air (Fig. 3b, A), suggesting that the two enzymes, cellulase and

PG, might be under different developmental regulatory control.

The levels of PG and cellulase immunoreactive proteins were dramatically diminished in fruits held in 2.5, 3.5, and 5.5% O_2 , whereas in fruits kept in 7.5 and 10% O_2 , they were slightly lower or similar to those ripened in air (Fig. 3a, B and 3b, B). However, fruits stored in 5.5% O_2 recovered much faster than fruits kept in 2.5 or 3.5% O_2 when transferred to air, as was evident from the immunoreactive protein content of PG and cellulases (Fig. 3a, C and 3b, C). In contrast, PG and cellulase protein exhibited similar levels in the same fruits stored either in 7.5 and 10% O_2 or sampled after 24 h in air, indicating that these oxygen concentrations did not greatly affect the synthesis of these enzymes.

Changes in the Profile of PG and Cellulase Isoenzymes

To evaluate whether low O_2 concentrations differentially suppressed cellulase and PG isoenzymes we stained the isoenzymes of these proteins after native IEF (Figs. 4, 5). It should be mentioned that when total protein extracts were run on native IEF gels in the range of pH between 4 to 7, the PG and cellulase proteins were resolved into 7 and 11 bands, respectively (Figs. 4, 5; our unpublished results). Preclimacteric fruits had no detectable PG isoenzymes (Fig. 4A). However, cellulase isoenzymes from the same fruits were detected by activity staining which is in agreement with the immunoblots (Figs. 3b, A, 5A). It is evident that the isoenzymes of both proteins are developmentally regulated (Figs. 4A, 5A; our unpublished results) with the cationic isoenzymes of both PG and cellulase giving stronger staining later in ripening. Low O_2 concentrations 2.5, 3.5, and 5.5% caused a diminution in the levels of PG and cellulase isoenzymes. It is noteworthy that the cationic isoenzymes of PG and cellulase were hardly detectable in 2.5% O_2 (Figs. 4B, 5B). Figures 4C and 5C demonstrate that the fruits held in modified atmospheres resumed normal ripening as soon as they were transferred to

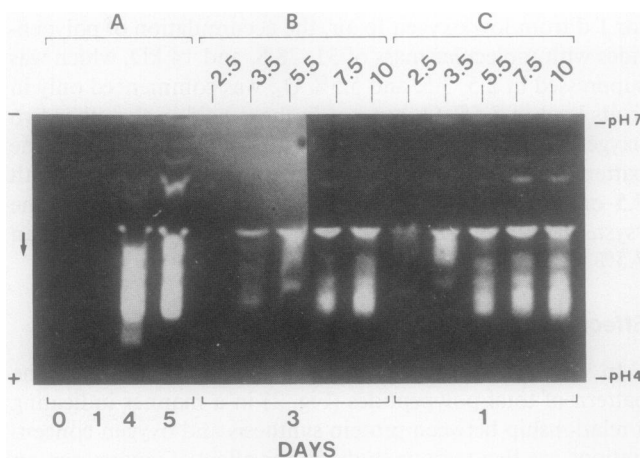


Figure 4. Avocado PG isoenzymes detected by overlay pectate-agarose gel after native-IEF. Total protein extracts were mixed with 4% ampholyte mixture of pH 4 to 6 and pH 5 to 7 in ratio 1:3 and electrofocused for 1.5 h at 180 V and 1.5 h at 300 V. Sampling periods were the same as those described in the legend to Figure 2.

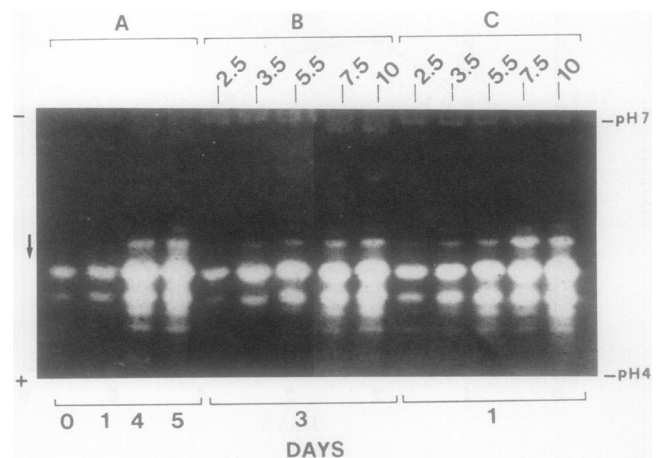


Figure 5. Avocado cellulase isoenzymes detected by overlay CMC-agarose gel after native-IEF. Extracts preparation, IEF conditions, and sampling periods were the same as those described in the legend to Figure 2.

air, as it is indicated by the appearance of all isoenzymes of PG and cellulase.

Suppression of Cellulase mRNA by Low O₂

Fruits held in air (Fig. 6A) showed elevated amounts of cellulase mRNA during ripening which is consistent with previous work (8, 16, 26). Oxygen concentrations 2.5 to 5.5% caused a decrease in cellulase mRNA levels which correlated with the lower amount of cellulase protein and isoenzymes (Figs. 3b, B, 5B, 6B). In contrast, fruits held in 7.5 and 10% O₂ contained amounts of cellulase transcripts similar to those fruits ripened in air. Upon return to air, cellulase mRNA accumulation resembled the pattern shown by its protein and isoenzymes being inversely proportional to O₂ concentrations (Figs. 3b, C, 5C, 6C).

Induction of ADH Activity under Low O₂

The induction of ADH isoenzymes is presented in Figure 7 as a function of oxygen concentrations. Prelimacteric and ripe fruits held in air contained a dominant band indicating that there is a constitutive ADH activity in avocado fruit mesocarp. It is evident that late in ripening and under hypoxia new ADH isoenzymes appeared (Fig. 7). The work by Torres *et al.* (25) indicates that in avocado fruit there are two genes for ADH, Adh1 and Adh2. Therefore, taking into the account that ADH is a dimer, the three bands of the ADH protein pattern seen in late ripening fruit and in 2.5, 3.5, and 5.5% O₂ (Fig. 7B) can be explained as follows: the fastest migrating band is the homodimer Adh2-Adh2, the middle band is the heterodimer Adh1-Adh2, and the slowest migrating band is the homodimer Adh1-Adh1. The oxygen concentrations 7.5 and 10% did not induce new ADH isoenzyme synthesis. Twenty-four hours after aeration, the activity of ADH isoenzymes, though greatly diminished, nevertheless could still be detected on native gels from fruits previously held in 2.5, 3.5, and 5.5% O₂ (Fig. 7C). It is interesting to mention that when

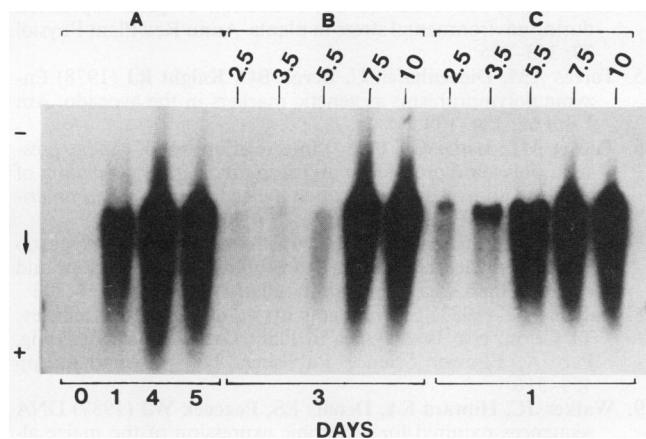


Figure 6. Effect of low O₂ levels on cellulase mRNA in initiated avocado fruits. Total RNA (20 μ g/lane) were fractionated on 1% agarose-formaldehyde gel, blotted on nylon membranes, and probed with pAV363. Sampling periods were the same as those described in the legend to Figure 2.

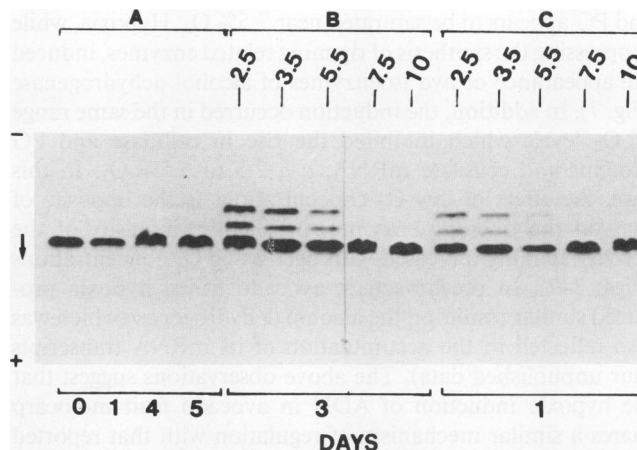


Figure 7. Induction of ADH isoenzymes by hypoxic conditions. Total protein extracts (30 μ g/lane) were electrophoresed under native conditions and then stained for ADH activity. Sampling periods were the same as those described in the legend to Figure 2.

prelimacteric avocado fruits were kept in 0, 1, 2, 3, 5, and 10% O₂ and air for 48 h, ADH mRNA were most abundantly expressed in 0, 1, 2, 3, and 5% O₂ (our unpublished observations) resembling the induction of ADH isoenzymes shown in Figure 7.

DISCUSSION

Ripening of climacteric fruits is a developmentally regulated process which is initiated and regulated by the plant hormone ethylene (5, 30). Fruit ripening is attenuated by temperature, low O₂, and/or high CO₂ concentrations in the ambient atmosphere. In climacteric fruits, low oxygen delays the onset of the climacteric rise in ethylene as well as the rate of the overt changes associated with ripening (12, 14, 18, 20). On the basis of the existing experimental evidence, it is not unequivocally clear whether this effect of low oxygen is exerted on ethylene action (6) or is an indirect result of a general diminution of plant metabolism due to hypoxia. We pointed out in the introduction that the physiological effects of hypoxia on plant tissues are not by any means restricted to the ripening process (7, 10, 22–24).

In our previous studies, we observed that transferring ripening initiated avocado fruits to 2.5% O₂ resulted in suppressing the rise in activity, protein, and mRNA abundance of cellulase, indicating that the low O₂ effect involves events at the mRNA level (15, 16). The present data show that the diminution of the immunoreactive proteins of cellulase and PG as well as the appearance of their isoenzymes occur only as long as the external concentration of O₂ is kept at or below 5.5% (Figs. 3, 4, 5). Further, at least qualitatively, the severity of suppression is inversely related to O₂ levels. In addition, the rise in PG and cellulase proteins, upon transferring the fruits for 24 h from low O₂ to air, is directly related to O₂ concentrations, being higher at 5.5%. This pattern of change in cellulase protein is also reflected in the abundance of its mRNA transcripts (Figs. 3b, 6). Thus, the results suggest that the "system" which is involved in the synthesis of cellulase

and PG appears to be saturated near 7.5% O₂. Hypoxia, while suppressing the synthesis of ripening related enzymes, induced the appearance of two isoenzymes of alcohol dehydrogenase (Fig. 7). In addition, the induction occurred in the same range of O₂ levels which inhibited the rise in cellulase and PG proteins and cellulase mRNA, e.g. 2.5 to 5.5% O₂. In this case, the effect of low O₂ concentrations is the opposite of that on the ripening enzymes, in that the intensity of the activity staining increased with decreasing O₂ concentrations (Figs. 3–7). In preclimacteric avocado fruits, hypoxia produced similar results on the alcohol dehydrogenase which was also reflected in the accumulation of its mRNA transcripts (our unpublished data). The above observations suggest that the hypoxic induction of ADH in avocado fruit mesocarp shares a similar mechanism of regulation with that reported in other plant tissues (7, 22, 24). The present results indicate that hypoxia exerts both positive and negative effects on the expression of certain genes. Further, these effects are initiated at the same levels of O₂ indicating the existence of a system that perceives the concentrations of O₂. In this context, it should be mentioned that genes induced under anaerobic or hypoxic conditions in root tissues, contain conserved nucleotide sequences in the regions from –140 to –60 relative to the transcriptional initiation site which appear to be necessary for anaerobic expression (10, 29). However, it is not known whether the same DNA regulatory sequences or others regulate the suppression of gene expression under hypoxic conditions and whether these sequences exist in fruits. On the basis of the present data alone it is not possible to distinguish whether the effect of O₂ on fruit ripening and induction of anoxic enzymes is the result of a common regulatory mechanism or that the former is due to a direct effect of O₂ on ethylene action.

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