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Hydrolytic Enzyme Activities and Protein Pattern of Avocado Fruit Ripened in Air and in Low Oxygen, with and without Ethylene[1]

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ABSTRACT

The effect of 2.5% O_2 atmosphere with and without ethylene on the activities of hydrolytic enzymes associated with cell walls, and total protein profile during ripening of avocado fruits (*Persea americana* Mill., cv Hass) were investigated. The low 2.5% O_2 atmosphere prevented the rise in the activities of cellulase, polygalacturonase, and acid phosphatase in avocado fruits whose ripening was initiated with ethylene. Addition of 100 microliters per liter ethylene to low O_2 atmosphere did not alter these suppressive effects of 2.5% O_2. Furthermore, 2.5% O_2 atmosphere delayed the development of a number of polypeptides that appear during ripening of avocado fruits while at the same time new polypeptides accumulated. The composition of the extraction buffer and its pH greatly affected the recovery of cellulase activity and its total immunoreactive protein.

Softening is a common and very pronounced feature that attends ripening of fleshy fruits. Softening in avocado fruits is accompanied by an extensive breakdown of the cell wall (22), which is associated with an increase in the activities of hydrolytic enzymes such as cellulase, polygalacturonase, and acid phosphatase (3, 21, 23). Information concerning the synthesis and regulation of polygalacturonase and acid phosphatase during avocado ripening is very limited. Although a cardinal role for cellulase enzyme in avocado softening has been suggested (3, 21), elucidation of its role *in vivo* is far from complete (12). However, increase in cellulase activity during ripening of avocado closely correlates with an increase in the levels of cellulase protein and that of cellulase mRNA (9, 28, 29), indicating a pretranslational regulation of cellulase synthesis (9).

It is well established that O_2 levels below those in air retard the rate of ripening, and softening of climacteric fruits (13–15). The precise mode of action of low O_2 in delaying fruit ripening is not clear. However, since O_2 is required for the action of ethylene (8), it is a reasonable assumption that the retarding effects of low O_2 on fruit softening reflect a diminution of ethylene action.

In the present communication we report the effect of 2.5% O_2, with and without ethylene, on both the changes in the activities of the hydrolytic enzymes that may play a cardinal role in cell wall degradation and the steady state protein pattern during ripening.

MATERIALS AND METHODS

Plant Material

Freshly harvested avocado fruits (*Persea americana* Mill., cv Hass) were air shipped overnight from University of California, Riverside, to the laboratory at College Park, MD. Alternatively, preclimacteric avocado fruits were purchased from the local wholesale market. Individual fruits were enclosed in glass jars of appropriate size with minimum void space, and kept at 20°C under a steady flow of 50 to 60 ml·min^{-1} humidified CO_2-free air. The flow rates were selected to ensure that CO_2 accumulation did not exceed 0.3%. Carbon dioxide and ethylene production were measured daily by a gas chromatograph equipped with either thermal conductivity (CO_2) or flame ionization (ethylene) detectors. Initially, CO_2 and ethylene levels were measured to ensure the stage of ripeness of the fruits. Ripening of the fruits was initiated by introducing 10 μl/L ethylene into the air stream and the respiration rates were monitored. When the rate of CO_2 evolution reached 60 to 80 mL/kg^{-1}·h^{-1}, values that were one-half to two-thirds of those at the climacteric peak, the exogenous supply of ethylene was discontinued. At this point, a set of three fruits was transferred to 2.5% O_2. Another set of three fruits was placed in 2.5% O_2 + 100 μL/L ethylene. The fruits incubated in air alone served as the controls. Unless otherwise indicated, fruits remained for 6 d in the modified atmospheres following which they were returned to air for 3 d.

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

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between overt physiological changes, such as respiration, and changes in the activities of enzymes and protein patterns.

Seven samplings taken in the course of the experiment were 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) 2, 4, and 6 d following the transfer of fruit from ethylene atmosphere to modified atmospheres; and (d) 1 and 3 d after returning the fruits from 2.5% O_2 to air. Immediately upon removal, the sampled plugs were dropped into liquid N_2 in a mortar and ground to a fine powder with a pestle. Subsequently, the powder was stored in air-tight vials at −70°C until analyzed.

Preparation of Fruit Extracts for Enzyme Assays

One g frozen fine powder was allowed to thaw on ice in 10 mL of 50 mM Na-acetate (pH 5), containing 5 mM β-mercaptoethanol, 0.4 M NaCl, 0.5 mM PMSF, and 10 μM leupeptin. After homogenizing the mixture for 15 min, the slurry mixture was centrifuged at 20,000×*g* for 20 min, and the supernatant used as the cell-free extract.

Cellulase

Cellulase was assayed at 25°C viscometrically using the method of Almin *et al.* (1). The assay mixture (final volume 5 mL) contained 3 mL of 1.5% (w/v) carboxymethylcellulose in 40 mM Na-acetate (pH 5), 0.1% cycloheximide, 0.1% chloramphenicol, and 2 mL of cell-free extract. All assays were run in duplicate and the results presented are an average of two assays.

Polygalacturonase

PG[3] was assayed viscometrically by the method of Almin *et al.* (1). The assay mixture (final volume of 5 mL) contained 1.5 mL of cell-free extract and 3.5 mL of 1.2% (w/v) Na-polypectate in 40 mM Na-acetate (pH 5.5), containing 0.1% (w/v) cycloheximide and 0.1% (w/v) chloramphenicol. The antibiotics were included in the assay mixture to inhibit microbial growth. PG activity was also determined by measuring spectrophotometrically the rise in the liberated reducing sugars, following the method of Nelson (20). The reaction mixtures contained 0.1 mL of cell-free extract and 0.2 mL of 0.5% (w/v) polygalacturonic acid in 0.1 M Na-acetate (pH 5.5). Cycloheximide and chloramphenicol at final concentrations of 0.1% (v/v), were added as antimicrobial agents. The reaction was carried out at 25°C for 10 min.

Acid Phosphatase

The activity of AP was determined by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate. Five mL of the reaction mixture containing 50 μL cell-free extract, 80 μl of 0.3 $mg \cdot ml^{-1}$ *p*-nitrophenyl phosphate solution, 0.5 μmol Na-acetate (pH 5), were incubated for 10 min in a shaking water bath at 40°C (23). One mL aliquot of the reaction mixture was removed at intervals of 10 min and added to test tubes containing 3 mL of 0.02 N NaOH. The tubes were shaken, and absorbance at 407 nm was measured after 10 min. AP units were expressed in μmol of *p*-nitrophenyl phosphate hydrolyzed $\cdot g^{-1} \cdot h^{-1}$.

[3] Abbreviations: PG, polygalacturonase; AP, acid phosphatase.

Total Protein Extraction and SDS-PAGE

Frozen tissue powder (1 g) was thawed in 4 mL of buffer containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM $NaHCO_3$, 20 mM $MgSO_4$, 10 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 was centrifuged at 20,000×*g* for 20 min. The clear supernatant was used for fractionation by SDS-PAGE using 10 to 18% gradient polyacrylamide gels and the buffer system of Laemmli (17).

Protein samples were prepared for electrophoresis by adding 2 volumes to 1 volume of sample buffer consisting of 3.75 mL of 0.5 M Tris-HCl (pH 6.8), 3 mL of 100% glycerol, 1.5 mL of β-mercaptoethanol, 0.9 g of SDS, and 0.5 mL of 0.04% bromophenol blue (19). Samples were heated at 90°c for 4 min prior to electrophoresis. Following electrophoresis, the gels were stained with 0.05% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol-10% (v/v) acetic acid and destained in 20% (v/v) methanol-7% (v/v) acetic acid. After washing with several changes of distilled H_2O to remove acetic acid, the gels were stained with silver according to Wray *et al.* (30). The gels were then partially destained in Kodak R-46[4] for 15 min and completely destained in 20% (w/v) Na-thiosulfate for 5 min. The destained gels were washed in dH_2O, equilibrated with 50% (v/v) methanol, and then restained with silver (30).

Protein Blotting and Immunodetection

Transfer of electrophoretically resolved proteins on SDS-polyacrylamide gels onto nitrocellulose filters (0.1 μM pore size, Schleicher and Schuell) was carried out essentially as described by Towbin *et al.* (27). The transfer buffer consisted of 25 mM Tris-192 mM glycine, 20% (v/v) methanol, and 0.02% (w/v) SDS. The electroblotting was allowed to take place overnight at 40 V. Detection of proteins on nitrocellulose filters for reactivity with the antiserum to cellulase was carried out as previously described (16). Biotinylated proteins (10) and ^{14}C-mol wt markers (BRL) were used as mol wt standard markers.

Protein Determination

Protein concentration was measured by the method of Bradford (7) using BSA dissolved in the extraction buffer as a standard.

All treatments were run with three replicates and each experiment was repeated at least twice.

RESULTS

Extraction Buffers for Cellulase

Preliminary experiments indicated that extracts of avocado tissue prepared with 40 mM Na-acetate (pH 5), 0.4 M NaCl,

[4] Mention of specific instruments, trade names, or manufacturers is for the purpose of identification and does not imply an endorsement by the U.S. Government.

and 0.25% Triton X-100 according to a previously published method (4) were poorly resolved by SDS-PAGE and the immunoblotting of the cellulase protein was not satisfactory. However, when fruit proteins were extracted in 50 mM Tris-HCl (pH 7.4), containing NaCl and several protease inhibitors, protein resolution on SDS-PAGE and development of immunoblots were greatly improved. The composition of the buffers was varied as described in Table I. The avocado tissue was extracted immediately upon excision from the fruit in order to avoid any postmortem changes that might be influenced by storage of the tissue. The results (Fig. 1) showed that, in general, the extracts prepared with Na-acetate buffer contained fewer polypeptides than those prepared with Tris buffer; particularly, some of the high mol wt polypeptides were missing (*cf.* Fig. 1A with 1B). Inclusion of Triton X-100 in acetate buffer resulted in diminution of several additional bands (Fig. 1A; *cf.* lane 1 with lanes 4, 6, 7, 8). Inclusion of Triton X-100 in the Tris buffer, on the other hand, did not greatly affect the pattern of polypeptides resolved in its absence (Fig. 1B; *cf.* lane 1 with lanes 4, 6, 7, 8). The omission of protease inhibitors, *viz.* PMSF and leupeptin, and the reducing agent β-mercaptoethanol from both extraction buffers also affected the protein profiles on SDS-PAGE (Fig. 1, A and B, see lanes 7 and 8).

Using an antibody to avocado cellulase, we were able to ascertain the effect of several ingredients in the buffers listed in Table I on the amount of extractable cellulase protein. The results (Fig. 2) showed that omission of PMSF, leupeptin, and β-mercaptoethanol from the extraction buffers decreased the level of extractable cellulase protein (Fig. 2, panel A: lanes 7 and 8; panel B: lanes 7 and 8; Fig. 3). The addition of EDTA and $NaHCO_3$ to Tris-buffer enhanced the extractable cellulase protein, while their inclusion in the Na-acetate buffer produced the opposite effect (Fig. 2, panel B: 1, 2, 5; panel A: 1, 2, 5). In general, higher amounts of cellulase protein were extracted at pH 7.4 than at pH 5. The addition of 10% (v/v) glycerol to the acetate buffer (pH 5) raised the amount of extractable cellulase protein to that of Tris buffer (Fig. 3, column 5). Glycerol is known to stabilize the activity of enzymes and the native structure of proteins. Alpi and Beevers (2) stabilized catalase activity by the inclusion of 35% glycerol in the extraction buffer.

The effect of buffer composition on the activity of extractable cellulase was also studied (data not shown). These experiments revealed the following: (a) the pH of the buffer influenced the extractable cellulase activity; (b) addition of EDTA decreased appreciably the extractable cellulase activity in both buffers; however, loss of cellulase protein was observed only when Na-acetate was used; (c) omission of protease inhibitors and β-mercaptoethanol did not influence cellulase activity; and (d) inclusion in the buffers of 0.5% (v/v) Triton X-100 increased the extractable cellulase activity by 1.5-fold.

Although we found that extracts prepared with Tris-HCl (pH 7.4) contained higher amounts of cellulase protein as compared to those prepared with Na-acetate (pH 5) (Fig. 2), surprisingly, cellulase activity was higher when the latter buffer was used (data not shown). As the assay of cellulase activity required pH adjustment of the extract from 7.4 to 5, it was deemed reasonable to investigate the possibility that protein losses might occur during pH adjustment. For this, fresh tissue was extracted with the Tris buffer. The extract was then split into two parts, one was used as the control and the other was adjusted to pH 5. Equal amounts of protein were electrophoresed and immunoblotted. The results (Fig. 2B, lanes 9 and 10) showed no loss of cellulase protein upon pH adjustment. Thus, the reason for the apparent contradictory effects of Tris-HCl on the extractable cellulase protein and activity was not resolved.

In view of the above findings, a medium containing 50 mM Na-acetate (pH 5), 0.2 M NaCl, 0.5 mM PMSF, 10 μM leupeptin, and 5 mM β-mercaptoethanol (acetate buffer) was routinely used to prepare extracts for cellulase activity measurements while another medium containing 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 20 mM $NaHCO_3$, 20 mM $MgSO_4$, 0.5 mM PMSF, 2 mM EDTA, 10 μM leupeptin, 5 mM β-mercaptoethanol, and 10% (v/v) glycerol (Tris buffer) was the choice for quantifying total cellulase protein.

Effect of 2.5% O_2 and 2.5% O_2 + 100 μL/L Ethylene on Enzymic Activities

Figure 4 shows changes in respiration and the activities of cell wall degrading enzymes, *viz.* PG, cellulase, and AP in avocado fruits ripened in air or held in a modified environment of 2.5% O_2 or 2.5% O_2 + 100 μL/L ethylene.

Table I. *Buffer Composition and Molarities Used to Extract Total Protein and Cellulase for Activity Measurements and Immunoblotting*

Medium	Na-Acetate (pH 5)								Tris-HCl (pH 7.4)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
50 mM Na-acetate (pH 5)	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
50 mM Tris-HCl (pH 7.4)	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
0.4 M NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20 mM $MgSO_4$	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
20 mM $NaHCO_3$	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−
0.1 M EDTA	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
5 mM β-mercaptoethanol	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
0.5 mM PMSF	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
10 μM Leupeptin	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
10% Glycerol	+	+	−	−	+	+	+	−	+	+	−	−	+	+	+	−
0.25% Triton X-100	−	−	−	+	−	+	+	+	−	−	−	+	−	+	+	+

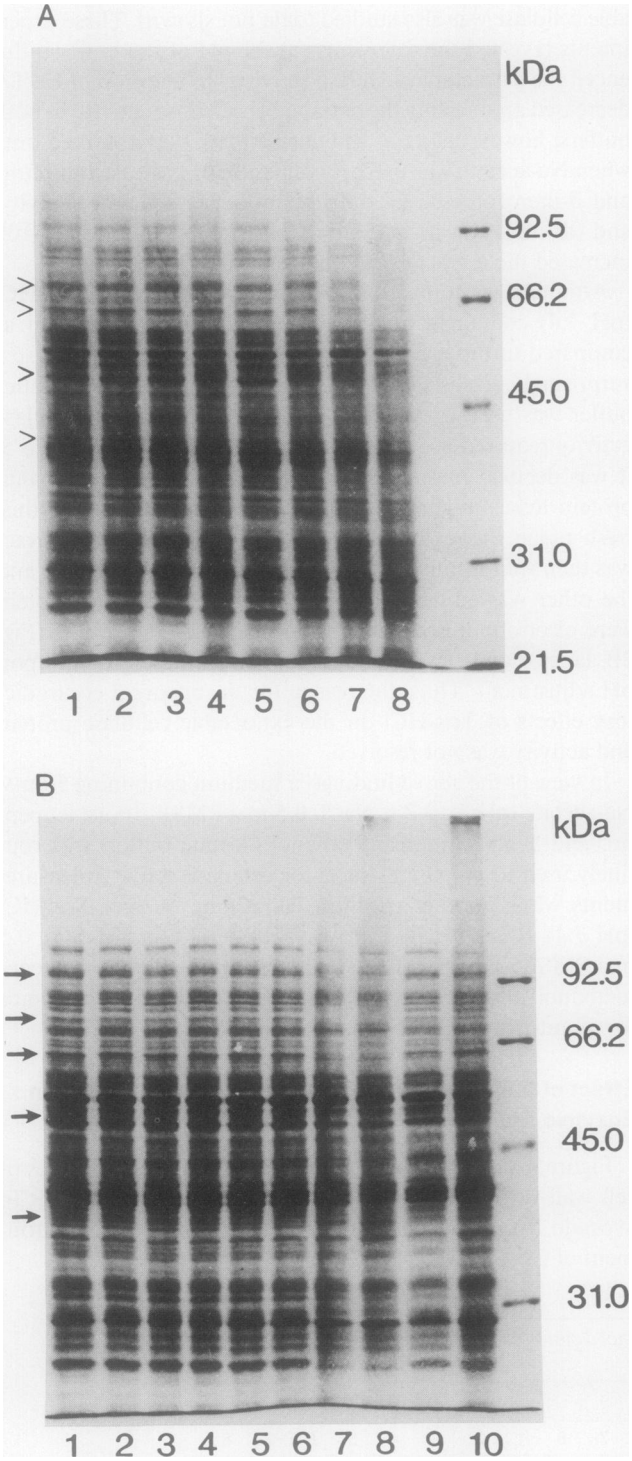

Figure 1. Effect of pH and buffer composition on the recovery of total proteins and their resolution by SDS-PAGE. Panel A, Na-acetate (pH 5); panel B, Tris-HCl (pH 7.4). Lanes 1 to 8 in both panels correspond to respective number of the buffer listed in Table I. Twenty-five μg protein were applied to each lane. Lanes 9B, 10B: effect of pH adjustment from 7.4 to 5. Sample from No. 4B buffer was adjusted to pH 5 with dilute HCl. The control was also diluted, and the protein content was measured. Thirty μg protein from the control (9B) and the sample whose pH was adjusted (10B) were applied and electrophoresed.

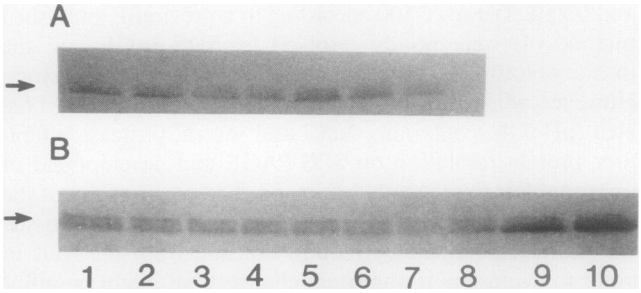

Figure 2. Effect of pH and buffer composition on recovery of cellulase protein. Panel A, Na-acetate (pH 5); panel B, Tris-HCl (pH 7.4). For details see legend to Figure 1.

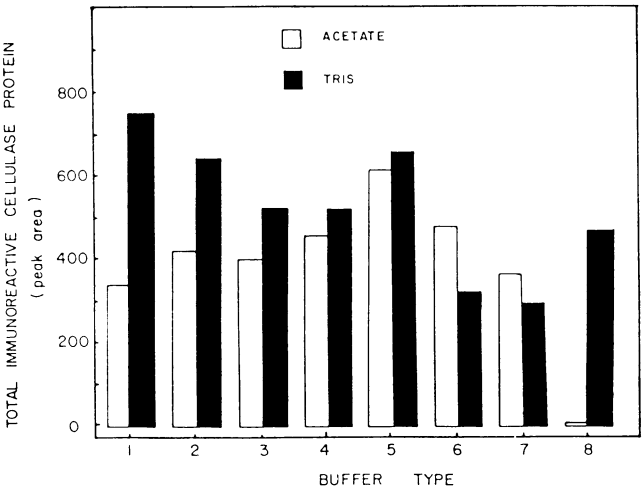

Figure 3. Quantification of cellulase protein extracted with either Na-acetate (pH 5) or Tris-HCl (pH 7.4). The Western blot in Figure 2 was scanned by laser densitometry. Height of band represents peak areas. See legend to Figure 1 for details.

Respiration

Fruits ripened in the humidified air stream after a brief ethylene treatment showed a typical climacteric rise in respiration followed by a decline that coincided with the advanced softening of the fruit (Fig. 4A) as was evidenced by applying a gentle pressure to the fruit held in the palm of the hand. Low O_2 atmospheres with or without ethylene decreased the rate of CO_2 evolution and altered drastically the respiratory drift (Fig. 4A). When fruits held in the controlled atmospheres were transferred to air, respiration rates increased in the first day, declining thereafter (Fig. 4A).

Polygalacturonase

PG increased markedly in fruits ripened in air. The modified atmospheres appreciably slowed down the rate of increase in PG activity (Fig. 4B). In contrast to respiration which showed only a transient increase in fruits returned from low O_2 atmospheres to air, PG activity continuously increased in these fruits (Fig. 4B).

Table II. *Qualitative Changes in Abundant Polypeptides during Avocado Ripening in Air and in Response to 2.5% O_2 with and without 100 μL/L Ethylene*

Band	Mol Wt	Qualitative Change[a]		
		Air	2.5% O_2	2.5% O_2 + C_2H_4
	kD			
a	110.0	I	D	D
b	90.5	I	D	D
c	81.9	F	I	I
d	79.0	I	ND	ND
e	74.0	NC	NC	NC
f	65.9	I	D	D
g	61.0	NC	NC	NC
h	57.0	NC	NC	NC
i	54.5	F	I	I
j	51.5	I	D	D
k	43.8	I	D	D
l	41.6	I	D	D
m	39.5	I	D	D
n	37.7	NC	NC	NC
o	36.5	I	NC	NC
p	35.3	ND	I	I
q	32.3	I	I	I
r	28.8	I	D	D
s	25.7	I	D	D
t	24.7	NC	I	I
u	16.6	NC	NC	NC
v	13.5	I	D	D

[a] I, increase; D, decrease; F, fluctuation; ND, not detectable; NC, no change.

Acid Phosphatase

Low O_2 prevented the normal rise in AP activity seen in fruit ripened in air (Fig. 4D). Ethylene (100 μL/L) addition to 2.5% O_2 atmosphere caused a small, transient increase in AP activity which dropped toward the end of the storage period. Although acid phosphatase activity increased upon returning the fruits from modified atmospheres to air, the total AP activity in the treated fruits did not reach those seen in fruit held in air alone (Fig. 4D).

Cellulase

Cellulase activity peaked late in relation to the climacteric rise in respiration and then declined (Fig. 4C). The suppressive effect of storage in low O_2 on cellulase activity was more dramatic than that on either PG or AP activities. The various enzyme activities were determined in samples taken from the same fruit, so that a direct comparison between the enzyme activities and respiration was possible. Therefore, the slowed rate of ripening in 2.5% O_2 or 2.5% O_2 + 100 μl/L ethylene, as evidenced by lower respiration rates, closely correlated with lower activities of the above mentioned enzymes in the same fruits. Inclusion of 100 μl/L ethylene in 2.5% O_2 tended to produce a modest increase in cellulase activity toward the end of the treatment (Fig. 4C) that, however, was not significantly different from that of 2.5% O_2 alone. The trend of increase in PG and AP activities in fruit returned from modified atmospheres to air was not apparent with cellulase activity.

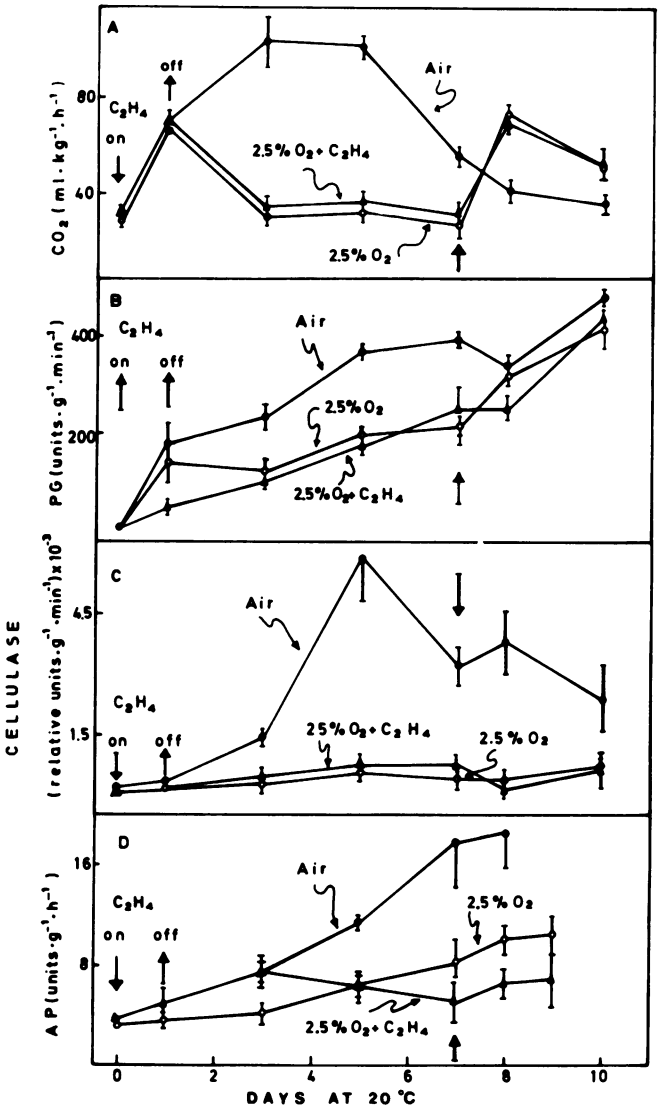

Figure 4. Effect of 2.5% O_2 with and without 100 μL/L ethylene on respiration rates (A) and activities of polygalacturonase (B), acid phosphatase (C), and cellulase (D) of avocado fruits. Ethylene (100 μL/L) was introduced in the air stream passing over the fruits on d 0. Ethylene treatment was stopped on d 1. Single fruits ripened in air or held in 2.5% O_2 with and without 100 μL/L ethylene were sampled on d 3, 5, and 7. Arrows indicate the day of transfer to air of fruits previously held in modified atmospheres. On return to air, these fruits were sampled on d 8 and 10.

The activity of cellulase showed a slight rise only 3 d after the transition to air.

Polypeptide Profile during ripening and in response to low O_2 atmospheres

Figure 4 shows that dynamic changes occur in the polypeptide pattern during the ripening of avocado. Among the polypeptides resolved by SDS-PAGE, changes in 22 polypeptides were followed during fruit ripening in air and under low O_2 treatments (Fig. 5; Table II). Polypeptides a, b, d, j, k, l, m, n, q, r, s, and v accumulated in fruits ripened in the

humidified air stream. The polypeptides a(110 kD), b(90.5 kD), j(51.5 kD), k(43.8 kD), l(41.6 kD), m(39.5 kD), n(37.7 kD), q(32.3 kD), r(28.8 kD), and s(25.7 kD) were present at the early stages of ripening, and their levels increased in the course of ripening in air. Polypeptides c(81.9 kD) and f(65.9 kD) fluctuated, showing initially an increase (by the 3rd d) (c), followed by a decrease on the 4th d (f). On the other hand, polypeptides e(74 kD), g(61 kD), h(57 kD), and u(16.6 kD) remained fairly constant during ripening in air. Polypeptide i(54.5 kD) was visible in preclimacteric fruit but subsequently disappeared.

Low O_2 atmospheres caused a dramatic alteration in the polypeptide profile of the avocado fruit (Fig. 5, Table II). In general, low O_2 prevented both the observed increases in the levels of certain polypeptides and the appearance of new ones. For example, the intensity of the polypeptide bands a, b, d, f, j, k, l, m, r, s, and v (Fig. 5, Table II), which increased in fruit held in air, was diminished in 2.5% O_2-treated fruits. Of these, polypeptide d(79 kD), which appeared after the respiratory climacteric peak in air (Fig. 5A), was not observed in 2.5% O_2-treated fruits. However, upon return of the fruits held in low O_2 atmospheres to air, this protein appeared on d 1 but disappeared by d 3. It is interesting to note that polypeptides f(65.9 kD), k(43.8 kD), m(39.5 kD), and s(25.7 kD), whose levels diminished under 2.5% O_2, were recovered as soon as the fruits were transferred back to air. Another set of polypeptides, *viz.* e(74 kD), g(61 kD), h(57 kD), n(37.7 kD), and v(16.6 kD), did not demonstrate changes within the time frame of the experiment. Polypeptide o(35.5 kD) remained fairly constant under 2.5% O_2 atmosphere, although a slight increase in its level occurred in air.

Low O_2 atmosphere tended to enhance the levels of polypeptides c(81.9 kD), i(54.5 kD), p(35.3 kD), q(32.3 kD), and t(24.7 kD) (Fig. 5). Of these, protein c(81.9 kD), which showed an initial rise with the onset of ripening and a subsequent decrease, increased in response to low O_2 treatment. Polypeptide i(54.5 kD) was present during the first 2 d in air and disappeared later on. This polypeptide showed no detectable changes in response to low O_2 as well as during the first day upon transferring the fruits back to air, but it decreased by the 3rd d. The intensity of polypeptides q(32.3 kD) and t(24.7 kD) was stronger in fruits kept in 2.5% O_2 than in air and polypeptide p(35.3 kD) appeared to be present only in fruits kept in low O_2.

The inclusion of ethylene in the low oxygen treatments did not seem to alter significantly the effects of low O_2 on the protein patterns (Fig. 5), with the possible exception of polypeptides j(51.5 kD) and v(13.5 kD) whose levels may have been enhanced by ethylene.

DISCUSSION

The data demonstrate that ripening of avocado fruit is associated with substantial increases in the activities of polygalacturonase, acid phosphatase and cellulase, consistent with previously published reports (3, 21, 23). Furthermore, it is shown that the development of these enzyme activities is suppressed to different degrees in fruits stored in low O_2 atmospheres. For instance, the suppressive effect of 2.5% O_2 atmosphere on the development of acid phosphatase and cellulase activities were more pronounced than on polygalacturonase activity. Also, upon return of fruits from low O_2 atmosphere to air, the rate and magnitude of increase in polygalacturonase activity far exceeded that seen for either acid phosphatase or cellulase. These data indicate that the regulation of accumulation of these enzymes may have different requirements for O_2 concentration. On the basis of the present data alone, however, it is not possible to assess the apparent affinity for O_2 in each case.

The retarding effect of low O_2 on the enzymic activities seen here may be mediated through a possible decrease in the rate of production of metabolic energy as a result of the decrease in respiration. However, this may not be the case because this decrease in respiration may not reflect a restriction of the electron transport chain, because of the known high affinity for oxygen of the cytochrome oxidase, but rather a diminution of the biological efficacy of ethylene (8). Second, avocado fruits undergo normal ripening in the presence of cyanide (26, 28). Under these conditions, the production of ATP is expected to markedly diminish, suggesting that normal ripening can proceed even when ATP production is limited. Thus, it is likely that low oxygen-mediated repression of the enzymic activities may be a result of its interference with the action of ethylene. In the case of cellulase, this low O_2 effect is possibly manifested at the pretranslational level since the accumulation of cellulase mRNA was suppressed (16).

The effect of the introduction of 100 μl/L ethylene in low O_2 atmosphere on the enzymic activities varied for each enzyme case. A slight initial increase in cellulase activity in response to ethylene seen here is consistent with detection of immunoreactive cellulase and its mRNA transcript in similar samples (16). Ethylene might preferentially induce the expression of cellulase compared to the induction of both PG and AP at low O_2. It seems that a bimolecular reaction, in the catalytic sense, between O_2 and ethylene might influence gene expression during fruit ripening.

Analysis of proteins present in avocado mesocarp at various stages of ripening suggests that both synthesis and degradation of proteins are involved in the ripening process (5). Comparison of polypeptides present in ripened fruit in air with those of the fruit held in either 2.5% O_2 or 2.5% O_2 + 100 μl/L ethylene revealed an evident redirection of protein synthesis in low O_2 atmospheres. Low O_2 not only suppressed the induction of new polypeptides associated with normal ripening but also induced the accumulation of some others (Fig. 5; Table II). It is interesting to note that the 81.9, 54.5, 35.3, and 32.3 kD polypeptides that accumulated in fruit stored in 2.5% O_2 are also common to maize primary roots held under anaerobic conditions (24). These data suggest a similar mechanism of protein induction under hypoxia, although at 2.5% O_2 aerobic respiration seems well sustained in avocados (6). The suppression of a number of other polypeptides in response to low O_2 environment may be a result of either suppression of translation due to the dissociation of polysomes, as was reported for soybeans (18) and maize (25) and/or repression of the expression of mRNA (16, 25), or both. Thus, some of the observed changes in the protein profiles during controlled atmospheres may be related to the mode of

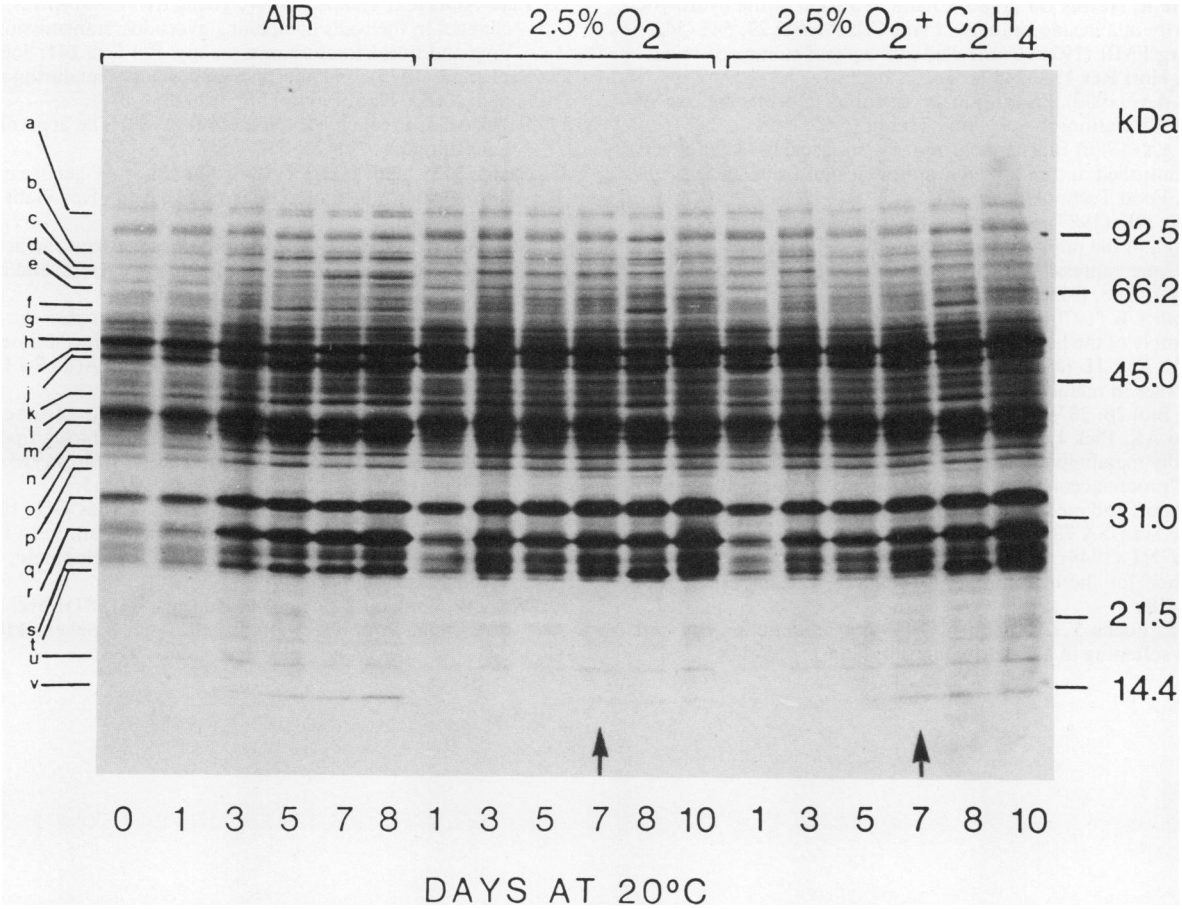

Figure 5. SDS-PAGE of total protein extracted from preclimacteric avocado fruits (d 0), ethylene-treated fruits (d 1), air ripened fruits and those held in 2.5% O_2 or 2.5% O_2 + 100 μL/L ethylene. Avocados stored in low oxygen with and without ethylene for 6 d were transferred to air on d 7 as indicated by arrows. On return to air, these fruits were sampled on d 8 and 10. The gel was stained twice with silver (30).

action of low O_2 in processes other than those directly associated with avocado ripening.

Finally, we have shown that extracts prepared with Tris-buffer had both higher amounts of cellulase protein and a larger number of polypeptides than when acetate-buffer was used. In contrast, higher cellulase activity is recovered with acetate-buffer than Tris-buffer. The fact that omission of protease inhibitors from acetate-buffer did not alter the extractable cellulase activity indicates that the presence of protease inhibitors is more critical during solubilization of samples with SDS and during electrophoresis consistent with observations that proteases can hydrolyze nondiscriminately the denatured proteins (11).

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